THE UPPER LIMIT OF PROTEIN THERMOSTABILITY

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ABSTRACT

The upper limit of protein thermostability has been established by examining an assortment of proteins at high temperatures under varying environmental conditions. The general nature of two of the processes known to cause irreversible thermoinactivation of enzymes in aqueous solution (90-100° C) has been quantitatively analyzed. At 100° C and neutral pH, cystine residues undergo destruction via two distinct mechanisms: ß-elimination and disulfide interchange catalyzed by thiols formed during the ß-elimination. The first process is rate-limiting and has a half-life in the range of 1.0 ± 0.4 hrs and 12.4 ± 3.4 hrs at pH 8.0 and 6.0, respectively (and around 6 days at pH 4.0). These half-lives have been found to be remarkably independent of the nature of the protein in over one dozen proteins tested. The thiol-catalyzed disulfide interchange reaction was also quantitatively characterized using mixed disulfides of proteins with glutathione.

A mechanistic description of the irreversible thermoinactivation of immobilized glucose isomerase at the pH-optimum of enzymatic activity (pH 8.0) demonstrated that immobilized enzymes can be successfully analyzed using the same experimental methodologies employed for soluble biocatalysts. Ligands (high fructose corn syrup and the competitive inhibitor xylitol) greatly stabilize the immobilized enzyme at high temperatures. At 90° C in the presence of 2 M xylitol, irreversible inactivation of immobilized glucose isomerase is almost exclusively caused by deamidation of its asparagine and/or glutamine residues. At the temperature close to that of industrial bioreactors (70° C), the time-dependent decay of glucose isomerase activity is brought about by different processes: oxidation of cysteine residues and/or by heat-induced deleterious reactions with high fructose corn syrup.

Three unrelated enzymes (ribonuclease, chymotrypsin, and lysozyme) were shown to display markedly increased thermostability in anhydrous organic solvents compared to aqueous solution. The mechanism of irreversible thermoinactivation in nonaqueous media (110-145° C) for these three enzymes was determined to be heat-induced protein aggregation. By using differential scanning calorimetry, the influence of water content and solvent type (hydrophobic or hydrophilic) on the thermal denaturation of ribonuclease was established. The products of the subsequent heat-induced aggregation were also characterized as being both chemically cross-linked (in part via transamidation and intermolecular disulfide interchange reactions) and physically associated protein oligomers. These findings suggest that the greatly enhanced thermostability of ribonuclease in hydrophobic organic solvents is due to the increased conformational stability of the dehydrated enzyme, and thus demarcates a new upper limit of protein thermostability compared to aqueous solution.

The findings outlined above are analyzed both in terms of enzyme stabilization and the general nature of thermophilicity.

Thesis supervisor: Professor Alexander M. Klibanov

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I. INTRODUCTION

The upper limit of protein thermostability is of interest to biochemists and biochemical engineers alike. Protein thermostability denotes the highest sustainable temperatures of life itself (bacteria from hot springs and hydrothermal vents at temperatures up to 110° C) since the temperature optimum of thermophilic enzymes is frequently at or above the optimum for growth of thermophilic microorganisms (Brock, 1985). Similarly, protein thermostability limits the operating temperature of many industrial enzymatic processes (Klibanov, 1983) such as the production of the sweetener high fructose corn syrup via the hydrolysis (α-amylase, 90-110° C), saccharification (glucoamylase, 50-60° C), and isomerization (glucose isomerase 60-65° C) of starch (Bucke, 1981); detergent and food processing with lipases and proteases at 40-70° C (Ng and Kenealy, 1986; Boyce, 1986); and chemical synthesis and stereoselective resolutions via lipases, proteases and dehydrogenases at 30-60° C (Zaks et al., 1988c). These enzymatic conversions are carried out at elevated temperatures due to enhanced reaction rates, increased solubility of substrates and products, reduced solution viscosity, lessened chance of microbial contamination, and potentially favorable shifts in the thermodynamic equilibrium (Klibanov, 1983).

By understanding why and how proteins lose their biological activity at high temperatures, the biochemist gains insight into the behavior of thermophilic organisms (Zuber, 1978; Brock, 1985, 1986). From the biotechnological viewpoint, a mechanistic understanding of enzyme thermoinactivation is crucial to the biochemical engineer who requires strategies (either conventional chemical and physical methods or protein engineering) to stabilize enzymes when used as practical catalysts at high temperatures (Ahern and Klibanov, 1986, 1988; Klibanov and Ahern, 1987; Mozhaev et al., 1989; Volkin and Klibanov, 1989b).

When exposed to elevated temperatures, all enzymes eventually lose catalytic activity. This thermal inactivation process is classified as either reversible or irreversible depending on whether enzymatic activity is recovered following return to ambient conditions. In accordance

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with the classical work of Lumry and Eyring (1954), the irreversible thermal inactivation of enzymes can be represented by the scheme:

$$N \underset{\longrightarrow}{\overset{K}{\rightleftharpoons}} U \overset{k}{\longrightarrow} I \qquad (1)$$
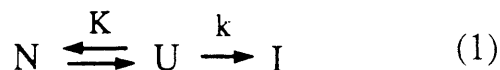
where N is a native, catalytically active enzyme, U is a reversible thermal unfolded enzyme, and I is an irreversibly thermoinactivated enzyme. The first step is the reversible denaturation of an enzyme's native conformation, and K is the equilibrium constant between the N and U forms of the enzyme. Reversible thermal unfolding has been thoroughly studied and its origin and mechanism are well understood (see literature survey). The subsequent irreversible thermoinactivation processes, represented by the rate constant, k, involve both covalent and conformational changes which are specific for individual enzymes. However, recent work in our laboratory using lysozyme (Ahern and Klibanov, 1984), ribonuclease (Zale and Klibanov, 1986), triose phosphate isomerase (Ahern et al., 1987) and microbial α-amylases (Tomazic and Klibanov, 1988a) as model systems, has identified several general, pH-dependent covalent reactions leading to the irreversible thermal inactivation of enzymes at high temperatures (90-100° C): destruction of cystine residues, thiol-catalyzed disulfide interchange, oxidation of cysteine residues, deamidation of asparagine and/or glutamine residues, and hydrolysis of peptide bonds at aspartic acid residues. Because of the general nature of these reactions, they afford the opportunity to define the upper limit of protein thermostability.

The objective of this study is to quantitatively investigate both conformational and covalent thermoinactivation processes in a variety of enzymes under various environmental conditions. By considering the following questions, we aim to more fully understand and quantitatively define the upper limit of protein thermostability:

1. What is the general nature of these degradative covalent reactions? What are the pH dependent rate constants, are these reactions a general phenomenon occurring in all proteins, and do these reactions depend on the amino acid sequence and the tertiary structure of a protein?

2. Do these covalent reactions limit the thermostability of practical, immobilized biocatalysts under operating conditions?

3. Can we define a new upper limit of protein thermostability in nonaqueous environments?

4. By examining the cause(s) and mechanism(s) of irreversible thermal inactivation, what strategies can we develop to stabilize proteins and enzymes at elevated temperatures?

Specifically, we address the first question by identifying and characterizing thermal destructive processes in proteins involving disulfide bonds at 100° C (Volkin and Klibanov, 1987). We examine the second question by ascertaining to what extent these degradative, covalent reactions limit the thermostability of immobilized glucose isomerase in the temperature range of 70-90° C (Volkin and Klibanov, 1989a). We answer the third question by determining how conformational and covalent processes lead to the heat-induced inactivation of enzymes at extreme temperatures (110-145° C) in anhydrous organic solvents. These studies will allow us not only to quantitatively analyze the upper limit of protein thermostability, but to develop strategies to stabilize both practical biocatalysts and model enzymes in either aqueous or nonaqueous environments at elevated temperatures.

II. LITERATURE SURVEY

Environmental conditions can be altered in a variety of ways (temperature, pH, denaturants, salts, organic solvents) to cause protein denaturation and subsequent inactivation (Volkin and Klibanov, 1989b). Perhaps the most extensively examined mode of protein denaturation is reversible thermal unfolding which has been the topic of investigation for almost fifty years (Neurath et al., 1944; Anson, 1945; Putnam, 1954; Kauzman, 1959; Joly, 1965; Tanford, 1968; Timascheff and Fasman, 1969; Tanford, 1970; Pace, 1975; Lapanje, 1978; Privalov, 1979; Pfeil, 1981; Jaenicke, 1981; Creighton, 1983).

2.1 Reversible Thermal Denaturation

The native, catalytically active conformation of an enzyme is maintained by a delicate balance of noncovalent forces: hydrogen bonds, hydrophobic, ionic and Van der Waals interactions. Upon an increase in temperature, heat weakens and disrupts this balance of noncovalent forces (with the exception of the hydrophobic interaction below 60° C; Tanford, 1968) and the protein molecule unfolds. Since the active center of an enzyme consists of amino acid residues brought together via the three dimensional structure, this unfolding event inactivates the enzyme. Upon recooling, the noncovalent bonds reform and the enzyme regains its native, catalytically active conformation because this combination of secondary and tertiary structure is thermodynamically favored. The "thermodynamic hypothesis" states that the native conformation of a protein exists in a given environment such that the free energy of the entire system is minimized; furthermore, this conformation is determined solely by the amino acid sequence. This hypothesis is based on the classical experiments of Anfinsen and coworkers who demonstrated that ribonuclease, once reduced and unfolded in urea, can refold into the native, catalytically active structure by removal of urea and the reoxidation of sulfhydryl groups (for a review, Anfinsen and Scheraga, 1975).

When small, monomeric proteins are reversibly denatured by heat, a two-state phenomenon is commonly observed where only the folded (N) or unfolded (U) states are present. Therefore, at 50% unfolding, enzyme molecules are 50% N and 50% U rather than all molecules being half unfolded. This sharp transition between N and U indicates that denaturation is a cooperative phenomenon in which the disruption of any significant portion of the folded structure leads to the unfolding of the entire molecule.

The temperature induced unfolded state of a protein (U) approaches a random coil configuration but some nonrandom conformations still exist, especially near disulfide bridges (Tanford, 1968). This fact is not surprising since abolishing all favorable intramolecular interactions in a chemically diverse polypeptide chain is most unlikely. Biphasic kinetics has been observed for the reversible thermal unfolding of some proteins, and has been suggested to show the existence of an unfolding intermediate (Mulkerrin and Wetzel, 1989).

Reversible protein denaturation can be observed by many different experimental techniques: increase in solution viscosity, decrease in optical rotation and UV absorbance, loss of enzymatic activity and urea-gradient electrophoresis (Creighton, 1983). In addition, calorimetric measurements of the specific heat capacity changes within a protein solution versus temperature allow not only for the detection of reversible thermal unfolding, but the calculation of thermodynamic parameters which distinguish the folded and unfolded states (Privalov and Khechinashvili, 1974; Pfeil and Privalov, 1976). Using hen-egg white lysozyme as a model system, the thermodynamic parameters of reversible thermal denaturation were quantitatively investigated leading to the following three conclusions: First, the enthalpy change upon unfolding coincides with the enthalpy change calculated via the temperature dependence of the equilibrium constant (the van't Hoff equation), therefore, the simplicity of this process agrees with the two-state hypothesis for single domain proteins. Second, the difference in enthalpy between the folded and unfolded states of the enzyme is proportional to the content of hydrogen bonds above 110° C and the number of the hydrophobic interactions below 110° C. Finally, the free energy difference between the folded and unfolded states is no greater than 10-20 kcal/mole.

As the temperature increases, the enthalpy and entropy vary similarly and compensate each other, so that the net free energy change is a relatively small difference between these two factors (Creighton, 1983). In other words, as the temperature rises, there is a competition between the folded (stabilizing hydrophobic interactions) and unfolded (destabilizing effect of conformational entropy) states of a protein molecule.

Most protein denaturation studies have used small, monomeric models (ribonuclease, lysozyme, bovine pancreatic trypsin inhibitor, and antibody fragments; Anfinsen, 1973) that completely and reversibly unfold. It should be noted that more complicated, multidomain proteins show multistate behavior indicating that domains and subunits unfold separately (Pfeil, 1981).

2.2   Protein Folding

The two-state nature of protein denaturation is a convenient model for thermodynamic analysis at equilibrium, but it does not tell us how refolding occurs or what pathway it follows. This problem is perhaps the most fundamental question in biochemistry today. Current trends in "the protein folding problem" have been recently reviewed (King, 1986; Jaenicke, 1987; Creighton, 1988; King, 1989).

In their classic experiments with ribonuclease, Anfinsen and coworkers showed that despite the many possible combinations of disulfide pairing, not to mention the countless possible arrangements of noncovalent bonds, the reduced and denatured enzyme is able to form all the correct interactions to regain its native, active conformation when reoxidized in the absence of denaturant. Interestingly, when reoxidation is carried out in the presence of denaturant, the resultant enzyme is catalytically inactive and contains many if not all reoxidation products (Anfinsen, 1973). Although all of the catalytic activity is recovered, the process occurs on the time scale of hours in vitro while the in vivo rate of protein synthesis for an enzyme the size of ribonuclease (13,700 kD or 124 amino acids) is on the order of minutes. This discrepancy in

rates led investigators to look for an enzyme which may catalyze refolding in vivo; a disulfide interchange enzyme was subsequently isolated from the endoplasmic reticulum of eukaryotic cells and shown to catalyze the refolding of denatured ribonuclease in vitro (Freedman, 1984).

Many small, monomeric enzymes and proteins have been shown to refold quantitatively from the denatured state (for a review, Baldwin, 1975; Anfinsen and Scheraga; 1975; Creighton, 1978). However, similar to protein denaturation, the refolding of oligomeric enzymes is a more complicated, multistep kinetic process. Jaenicke and coworkers have used NAD-dependent dehydrogenases to examine the folding and association of oligomeric proteins (for reviews, see Jaenicke and Rudolph, 1983; Jaenicke, 1984, 1987). In a typical experiment, these enzymes are denatured (by means of denaturants such as guanidine hydrochloride or at acidic pH) and both the (1) kinetics of refolding and reassociation, and (2) overall yields are then determined either in the presence or absence of stabilizing salts or ligands. The following conclusions were developed based on their work with both monomeric and oligomeric enzymes:

The renaturation kinetics of monomeric octopine dehydrogenase resembles other small, monomeric proteins with rapid formation of a structured intermediate followed by the slow (rate limiting) reshuffling to the native conformation. The subunit refolding of denatured oligomeric dehydrogenases displays similar monomeric behavior with a fast folding step (restoring much of the monomeric secondary structure) and slow reshuffling step to "correctly structured monomers". These monomers then associate further, as discussed below, to native dimers and tetramers or non-native aggregates. For dimeric cytosolic and mitochondrial malate dehydrogenase, the kinetics of association of "correctly structured monomers" to a dimer is either diffusion controlled or less rapid (two orders of magnitude slower), respectively. In the case of tetrameric lactate dehydrogenase (LDH), dimer association is rapid followed by a slower tetramer association with kinetics similar to mitochondrial-malate dehydrogenase. In all cases, although renaturation kinetics vary considerably, the overall yield is consistently 50 to 70%. The formation of incorrect side products (aggregation) competes with the slow, first order renaturation of monomers to "correctly folded monomers". The hypothesis that the competition

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between aggregation and renaturation pathways occurs at this point in the kinetic scheme is supported by experiments which show (1) there is complete reactivation of LDH after denaturation in the presence of structure stabilizing ions, (2) the refolding of dimeric to tetrameric LDH does not result in aggregate formation, and (3) aggregates show quasi-native characteristics as determined by circular dichroism.

Although these experiments give useful information about the in vitro folding of oligomeric enzymes, they do not elucidate in vivo folding pathways. King and coworkers (1986) have used genetic analysis to understand the in vivo folding pathway of the trimeric tail spike endorhamnosidase of the P22 virus. Specifically, their goal is to identify specific amino acid residues which regulate the competition between in vivo kinetic pathways of folded versus aggregated protein. Their system utilizes a temperature sensitive synthesis mutant protein which folds into mature mutant spike protein at 30° C, but aggregates by an off-pathway reaction at 40° C. Most of these protein mutants act by (1) blocking an early step in chain folding (prior to chain association to trimer), and (2) replacing Gly and Thr residues at potential kinetic intermediate protein "turn" sites. These mutants do not affect the native tail spike protein itself, since at 30° C, biosynthesis of the native protein is unaffected. This work shows that specific amino acid residues may promote or hinder the aggregation pathway, thereby implying that aggregation is not a random, but rather a specific process. Therefore, it may be possible to control its formation via genetic engineering.

Current interest in the protein folding problem has led to a keen awareness of the aggregation phenomenon. In addition, biotechnologists have found that during the overexpression of cloned genes in E. coli , there is a concomitant formation of inclusion bodies made up entirely of aggregated recombinant protein (Kane and Hartley, 1988; Mitraki and King, 1989). There are two main advantages in producing an insoluble protein product inside the cell: increased stability towards proteolysis and one-step purification with high yields. Therefore, protein aggregation may be a useful purification technique, but only if renaturation can be achieved. Unlike other causes of protein inactivation, aggregation is not necessarily an

irreversible process. Reactivation of protein aggregates may be possible by breaking up the intermolecular noncovalent interactions via the use of denaturants, and regenerating native disulfide bonds via reduction followed by reoxidation in the absence of denaturant (Zettlmeissl et al., 1979; Rudolph et al., 1979). Environmental conditions are very important (concentration, pH, temperature, additives) to overall recovery yields (Light, 1985).

2.3 Irreversible Thermal Inactivation of Proteins

When a protein solution is heated, rapidly cooled, but catalytic activity is not recovered within a reasonable time, the protein is said to have undergone irreversible thermal inactivation. Failure of the protein molecule to refold appears to contradict the thermodynamic hypothesis unless the protein has undergone a covalent or conformational change which prevents refolding.

Two conformational mechanisms leading to the irreversible thermal inactivation of enzymes have been identified. First of all, there is the previously mentioned protein aggregation phenomenon which is essentially a two step process. Monomolecular conformational changes must initially occur leading to reversible protein denaturation (see above) which exposes the buried, interior hydrophobic amino acid residues to the aqueous solvent. The thermally denatured protein molecules then associate with one another intermolecularly to minimize the unfavorable exposure of hydrophobic amino acid residues to the solvent . The aggregation reaction is therefore a polymolecular process obeying higher order kinetics. Subsequent chemical reactions may also occur, especially intermolecular disulfide crosslinks (Mozhaev and Martinek, 1982).

By working with dilute protein concentrations or by using immobilized enzymes, intermolecular aggregation can be circumvented. In these cases, a second, monomolecular model for conformational irreversible thermal inactivation has been proposed by Klibanov and Mozhaev (1978): at high temperatures, an enzyme loses its native, noncovalent bonds; as the temperature is lowered, non-native interactions may form, which although thermodynamically unfavorable,

remain for purely kinetic reasons. The protein exists in a metastable conformation and cannot spontaneously refold to the native conformation at ambient temperatures. This mechanism is experimentally supported with studies on immobilized trypsin. After heating at high temperatures, the protein solution was rapidly cooled with loss of virtually all of the enzymatic activity. The immobilized enzyme was then completely unfolded and reduced in urea followed by reoxidation of the thiols in the absence of the denaturant; the subsequent recovery of enzymatic activity was nearly complete, and the authors concluded that immobilized trypsin inactivates via incorrect structure formation.

Ahern and Klibanov (1985) and Zale and Klibanov (1986) quantitatively accounted for the processes causing irreversible thermal inactivation of hen egg-white lysozyme at 100° C and bovine pancreatic ribonuclease A at 90° C, respectively. The monomolecular rate constant of irreversible thermal inactivation was determined (protein concentrations were selected so that no aggregation occurred). Reactivation via the method of Klibanov and Mozhaev provided the rate constant of incorrect structure formation, a rather minor process for the above two enzymes. The main inactivation events were identified as pH dependent, covalent changes in the polypeptide molecule: deamidation of asparagine and/or glutamine residues, hydrolysis of the peptide chain at aspartic acid residues, and destruction of cystine residues (Table 1). Zale and Klibanov (1986) further identified the nature of the incorrect structure formation in ribonuclease as thiol-catalyzed interchange of disulfide bonds where the free thiols were generated as a byproduct of cystine destruction.

The relationship between reversible and irreversible thermoinactivation in ribonuclease was investigated quantitatively by Zale and Klibanov (1983). By examining the dependence of k (the rate constant of monomolecular irreversible thermoinactivation; see equation 1) on pH and salts and comparing it quantitatively to the literature values for the dependence of K (the equilibrium constant between native and unfolded enzyme; see equation 1) on the same parameters, the contribution of reversible unfolding and monomolecular irreversible thermal inactivation to the overall rate of the observed thermoinactivation process was determined. At

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Irreversible Thermoinactivation of Two Model Proteins	Rate Constants (hr^{-1})		
	pH 4	pH 6	pH 8
Hen egg white lysozyme, 100° C (Ahern and Klibanov, 1985)			
Directly measured overall process	0.49	4.1	50
Due to individual mechanisms:			
Deamidation of Asn/Gln residues	0.45	4.1	18
Hydrolysis of Asp-X peptide bonds	0.12		
Destruction of cystine residues			6
Formation of incorrect structures			32
Bovine pancreatic ribonuclease, 90° C (Zale and Klibanov, 1986)			
Directly measured overall process	0.13	0.56	23.4
Due to individual mechanisms:			
Deamidation of Asn/Gln residues	0.02	0.15	0.8
Hydrolysis of Asp-X peptide bonds	0.10		
Destruction of cystine residues		0.05	2.8
Formation of incorrect structures [a]		0.31	19.4

Table 1 - The rate constants of irreversible thermoinactivation of lysozyme and ribonuclease: the overall process and contributions of individual mechanisms to thermoinactivation. [a] Shown to be due to thiol-catalyzed disulfide interchange.

temperatures where $K \geq 1$, there is a correlation between the stabilities of an enzyme against reversible unfolding and the overall rate of observed thermoinactivation: the more stable the enzyme is against the former, the more stable it is against the latter . Conversely, at high temperatures where $K \ll 1$, no correlation is seen, and the overall rate of observed thermal inactivation depends solely on monomolecular irreversible thermal inactivation processes.

Tomazic and Klibanov (1988 a,b) further examined the relationship between reversible and irreversible thermoinactivation with studies on the thermostability of several microbial α-amylases. The differences in thermostability between mesophilic versus thermophilic enzyme at 90° C and pH 6.5 (inactivation was due to a monomolecular conformational process with a half-life of 2 and 50 minutes, respectively) were found to be due to additional salt bridges in the thermophilic enzyme which increased the conformational rigidity of the enzyme and thereby reduced the extent of protein denaturation. When this conformational inactivation process was suppressed (via additives or pH changes), α-amylase underwent irreversible thermoinactivation (at 90° C) via deamidation of its asparagine and/or glutamine residues and oxidation of cysteine residues.

2.4 Molecular Mechanisms of Thermophilicity

The denaturation temperature (T_m) of a wide variety of proteins from animals, plants and prokaryotes increases with the adaptation temperature of the source organism (Brock, 1985). Furthermore, the catalytic efficiency (turnover number) of these enzymes under ambient conditions decreases as the adaptation temperature of the organism increases. Both correlations are rationalized in terms of the increased conformational stability of thermophilic enzymes (compared to their mesophilic counterparts) toward reversible unfolding. It is important to re-emphasize that the net stabilization free energy that maintains the native structure of an enzyme is on the order of 10-20 kcal/mole. Therefore, relatively few additional (or strengthened) weak noncovalent interactions (one to two salt bridges, several hydrogen bonds or seven to ten

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methyls within the hydrophobic nucleus) are sufficient to create a thermophilic protein from a mesophilic one. Indeed, the two types of proteins have similar three dimensional structures and differ by only a few minor alterations of the amino acid sequence:

(1) Electrostatic interactions- Perutz (1978) compared the three dimensional X-ray crystal structures of thermophilic and mesophilic ferrodoxins and concluded that the greater heat stability was due to an extra one to two salt bridges within the thermophilic protein.

(2) Hydrophobic interactions- "The greater the hydrophobicity, the greater the thermostability"; this slogan has been proven to be correct in many comparative studies. However, the positioning of an amino acid residue within the three dimensional structure of a protein is a balance between an energy factor (hydrophobic interactions) and a geometric consideration (dense packing; Stellwagen and Wilgus, 1978). Thus, stabilization depends on the number of hydrophobic interactions located within the protein interior and not the total content of hydrophobic amino acid residues.

(3) Binding of metal ions- Additional binding sites for metal ions increases the number of electrostatic interactions which provides a type of chemical crosslinking. For example, thermostable α-amylase and thermolysin from Bacillus sterarothermophilus require Ca^{+2} ions for stability (Ng and Kenealy, 1986). Interestingly, there is a noticeable lack of cystine residues. This deficiency of S-S bonds may be attributable to the absence of an organelle system in prokaryotes (endoplasmic reticulum) or, as discussed in the thesis itself, the heat-sensitive nature of this chemical linkage.

(4) Overall amino acid composition- There are several noticeable differences in the amino acid composition of thermophilic proteins when compared to their mesophilic counterparts. First, thermophilic proteins show an increase in arginine residues at the expense of lysine. Since Arg

and Lys are usually found on the protein surface, this substitution reduces the unfavorable solvent contacts by one methylene group while adding more surface area for charged interactions via the guanidinium moiety. In addition, the pK_a of Arg (~12) is approximately two units higher than Lys (~9.5). Since the pK_a drops as the temperature rises, Arg is better able to maintain salt bridges at elevated temperatures. For example, the thermostability of T_4 lysozyme dramatically decreases (T_m is 10° C lower) by replacing Arg 96 in the native enzyme with His 96 (with an even lower pK_a ~7) in the thermolabile mutant (Grutter et al., 1979). In this case, Nature seems to stabilize existing noncovalent interactions rather than form new ones. Second, polar amino acids, especially Ser and Thr, are found less frequently in thermophilic proteins perhaps enhancing hydrophobic interactions within the protein interior by their absence. Finally, the total number of cysteine residues is reduced or eliminated in thermophilic proteins, however, this observation is difficult to interpret because the total content of cysteine residues in prokaryotic enzymes is, in general, lower than other sources (Fahey et al., 1977).

Protein engineering has recently emerged as a powerful tool to address the most fundamental questions in biochemistry (for reviews, see Smith, 1986; Knowles, 1987; Shaw, 1987) including protein stability (Matthews, 1987a). Both random and site-directed mutagenesis have been used to engineer greater thermostability in enzymes. One of the most popular strategies to increase the thermostability of enzymes against reversible denaturation is to utilize the mechanisms of thermophilicity as described above.

For example, random mutagenesis introduces amino acid substitutions within a protein sequence randomly followed by a screening procedure to identify mutants with favorable properties. This technique has been used to identify mutants with increased thermostability in kanamycin nucleotidyl transferase (Matsumura et al., 1986) and subtilisin BPN' (Rollence et al., 1988). These authors speculate that their stabilization effects are due to the increased conformational rigidity of the mutant proteins by increasing the number of hydrophobic and electrostatic interactions. Imanaka and coworkers (1986) produced mutants of neutral protease

of B. stearothermophilus with increased thermostability by replacing amino acid residues in regions of low homology compared to the mesophilic enzyme.

Site-directed mutagenesis allows for the specific replacement of amino acid residues within a protein sequence, so that specific alterations can be designed a priori and then examined for a predictable change in properties. For example, Matthews and coworkers have enhanced the thermostability of T_4 lysozyme by designing mutants containing sequences that either interact with the α-helix dipoles (Nicholson et al., 1988) or decrease the entropy of the unfolded state (Matthews et al., 1987b). In addition, they have examined the contributions of specific hydrogen bonds to the thermodynamic stability of this enzyme (Alber et al., 1987), and have analyzed why changes in secondary structure (site-directed mutagenesis was used to change a key proline residue in a α-helix) have a surprisingly small effect on the thermal denaturation of the enzyme (Alber and Matthews, 1987; Alber et al., 1988). Other promising and certainly interesting approaches to increase enzyme thermostability via site-directed mutagenesis include the use of linker sequences to fuse two chain molecules into a single polypeptide chain (Kim et al., 1989) and to increase metal ion binding affinities (Pantoliano et al., 1988).

2.5 Thermolabile Amino Acid Sequences

2.5.1 Cystine destruction and thiol-catalyzed disulfide interchange

The destruction of cystine residues is one of the causes of the irreversible thermal inactivation of lysozyme and ribonuclease at pH 8. Destruction of protein bound cystines in strongly alkaline media (pH 12-13) is known to occur via a ß-elimination reaction to yield dehydroalanine and thiocysteine (Nashef et al., 1977; Feeney, 1980; Whitaker and Feeney, 1983). This can be explained by the fact that in the peptide chain, the α-carbon is attached to two electron withdrawing groups (-CONH- and -NHCO-) which make the α-hydrogen relatively labile under alkaline conditions. Although debate continues as to the fate of thiocysteine

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(Florence, 1980), dehydroalanine is known to react with several nucleophilic groups in proteins, especially the ε-amino group of lysine to form lysinoalanine (Bohak, 1964). See Figure 1.

After establishing that cystine residues of ribonuclease underwent ß-elimination at pH 8 and 90° C, Zale and Klibanov (1986) showed that one of the byproducts (free thiols) catalyze thiol-disulfide interchange. Although their kinetic studies clearly demonstrated that the disulfide bonds of ribonuclease had been reshuffled, they could not directly isolate or determine these scrambled species. Historically, Ryle and Sanger (1955) discovered that at neutral and alkaline pH, short peptides can undergo a thiol-catalyzed disulfide interchange reaction at 35° C. Thiols carry out nucleophilic attack on the sulfur atom of a disulfide in the same way as in thiol-disulfide interchange (Cecil and McPhee, 1959; Torchinsky, 1981):

$$R_1\text{-SS-}R_1 + R_2S^- \longleftrightarrow R_1\text{-SS-}R_2 + R_1S^-$$

$$(2)$$

$$R_1\text{-SS-}R_2 + R_2S^- \longleftrightarrow R_2\text{-SS-}R_2 + R_1S^-$$
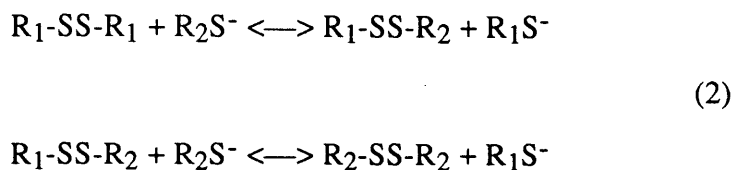
Strategies to design more thermostable enzymes by means of protein engineering have also utilized the introduction of new disulfide bonds (for reviews, see Wetzel, 1987; Creighton, 1988). In particular, cystine residues have been engineered into subtilisin (Wells and Powers, 1986; Pantoliano et al., 1987), dihydrofolate reductase (Villafranca et al., 1983, 1987), T4 lysozyme (Perry and Wetzel, 1984; Wetzel et al., 1988) and λ-repressor (Sauer et al., 1986; Stearman et al., 1988). The results obtained in these systems have been, at best, inconclusive. In the case of subtilisin, very little stabilization against heat-induced autolysis was observed by the addition of an extra disulfide bond, presumably due to the unfavorable geometries caused by the introduction of a new crosslink. Similarly, mixed results have been reported with the other enzymes: λ–repressor showed a slight increase in T_m during reversible thermal denaturation, but no increase in the thermal stability of dihydrofolate reductase was observed. When a disulfide bond was added to T4 lysozyme, the new cross link may have decreased the rate of irreversible

CO
  |
NH
  |
CH-CH$_2$-S-S-CH$_2$-CH   Cystine
  |
CO
  |
NH

OH$^-$

-NH-CH-CO-
    ‖
    CH$_2$

Dehydroalanine

Lysine

Lysinoalanine

S$^{\ominus}$
|
S
|
CH$_2$
|
-CO-NH-CH-CO-NH-

Thiocysteine

Thiol decomposition products including hydrosulfide
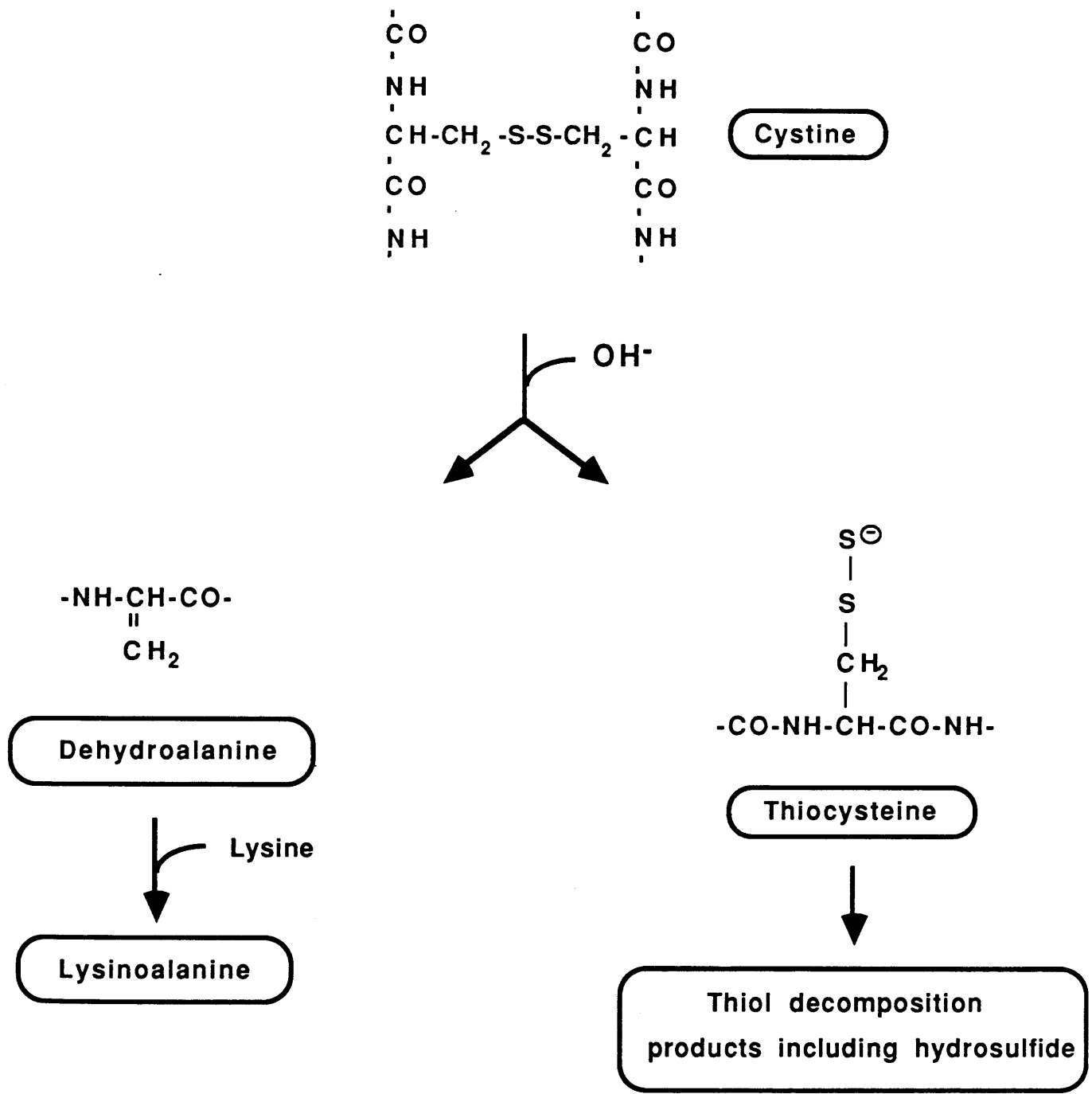
Figure 1- The ß-elimination reaction of cystine residues in proteins.

thermoinactivation by creating more compact solution structures than the unfolded, noncrosslinked molecules (Wetzel et al., 1988).

The information presented in the previous paragraphs concerning the chemical lability of disulfides questions the wisdom of engineering S-S bonds into proteins to stabilize them under extreme conditions such as high temperatures at neutral pH values. In this research project, we will establish the validity and generality of this hypothesis.

2.5.2 Deamidation of asparagine and glutamine residues

The spontaneous, nonenzymatic deamidation of asparagine and glutamine residues is one of the most common post-translational chemical modifications of proteins (Uy and Wold, 1977; Harding, 1985). It has been proposed that deamidation serves as a "biological clock" in both aging and development processes regulating the degradation and clearance of proteins (Robinson and Rudd, 1974). Not only are specific deamidations well-documented in vivo, but there is an intriguing correlation between the in vivo half-lives of proteins and their total amide residue content (McKerrow, 1979). The deamidation of asparagine and/or glutamine residues causes, solely or in part, the irreversible thermal inactivation of lysozyme and ribonuclease at pH 4 to 8.

The earliest research effort aimed specifically at deamidation was carried out by Robinson and coworkers who synthesized pentapeptides of sequence (Gly X_{xx} Asn X_{xx} Gly) or (Gly X_{xx} Gln X_{xx} Gly) and then measured the rate of amide loss under physiological conditions (Robinson and Rudd, 1974). Their rate data showed that (i) both asparagine and glutamine residues deamidate 30 to 2,000 times faster than a simple aliphatic amide; (ii) asparagine deamidates about ten times faster than glutamine on average; and (iii) the rate of deamidation is sequence dependent (Wright and Robinson, 1982). In general, polar, charged or lower steric bulk residues neighboring the asparagine or glutamine residue cause a rate acceleration; however, there are exceptions to this trend.

By synthesizing pentapeptide sequences found in cytochrome C, lysozyme, and aldolase, and measuring their rates of deamidation at 37° C, the sequence-dependent half-lives of deamidation for these enzymes were determined (Robinson and Tedro, 1973; Robinson et al., 1974; McKerrow and Robinson, 1974). By comparing these results with the rate constant of deamidation of the cytochrome C molecule in vitro (Flatmark, 1966), Robinson concluded that the rate of deamidation is governed not only by the amino acid sequence of a protein, but higher-ordered structures as well.

Robinson and coworkers (Robinson and Rudd,1974) assumed that deamidation was a simple hydrolysis reaction and their discussions on the effects of the primary, secondary, and tertiary structure of the polypeptide chain on deamidation involve formation of hydrogen bonds which may accelerate this reaction. However, an alternative mechanism can be formulated to account for the observed deamidation which current experimental evidence strongly favors: the main chain amide acts as a nucleophile attacking the electrophilic asparagine (or glutamine) amide causing ring closure to the imide with subsequent hydrolysis to an α/β aspartyl (or α/γ glutamyl) residue (Wold, 1985), as shown in Figure 2. As a general prediction, the asparagine residue is a somewhat stronger electrophile than glutamine (inductive effect of the electron withdrawing peptide bond is expressed more effectively over a single methylene versus two methylenes) and therefore more susceptible to nucleophilic substitutions.

Historically, three studies in the literature support the imide-formation mechanism. First, it is well known within peptide synthesis circles that peptides of aspartyl esters undergo intramolecular cyclization under basic conditions leading to a cyclic imide derivative (Bodansky and Kwet, 1978). It has been demonstrated that both deamidation of dipeptides of Asn-Gly at room temperature under mildly alkaline conditions (Meinwald et al.,1986) and cleavage of Asn-Gly peptide bonds with hydroxylamine (Bornstein and Balian, 1977) proceed via a cyclic imide intermediate. Second, Buchanan et al. (1962, 1966) and Haley et al. (1967) have noted the formation of β-Asp-Gly sequences in dipeptides from protein digestions. Finally, it has been shown (Yuksel and Gracy, 1986) that the in vitro deamidation of triose phosphate isomerase

-CO-NH-CH-CO-NH-R-
            |
           CH$_2$
            |
           CONH$_2$

Asparagine

↓ NH$_3$

-CO-NH-CH-CO
            | \
            | N-R-
            | /
           CH$_2$- CO

Cyclic imide

H$_2$O

-CO-NH-CH-COO$^\ominus$
            |
           CH$_2$-CO-NH-R-

ß-Aspartic acid

-CO-NH-CH-CO-NH-R-
            |
           CH$_2$
            |
           COO$^\ominus$

Aspartic acid
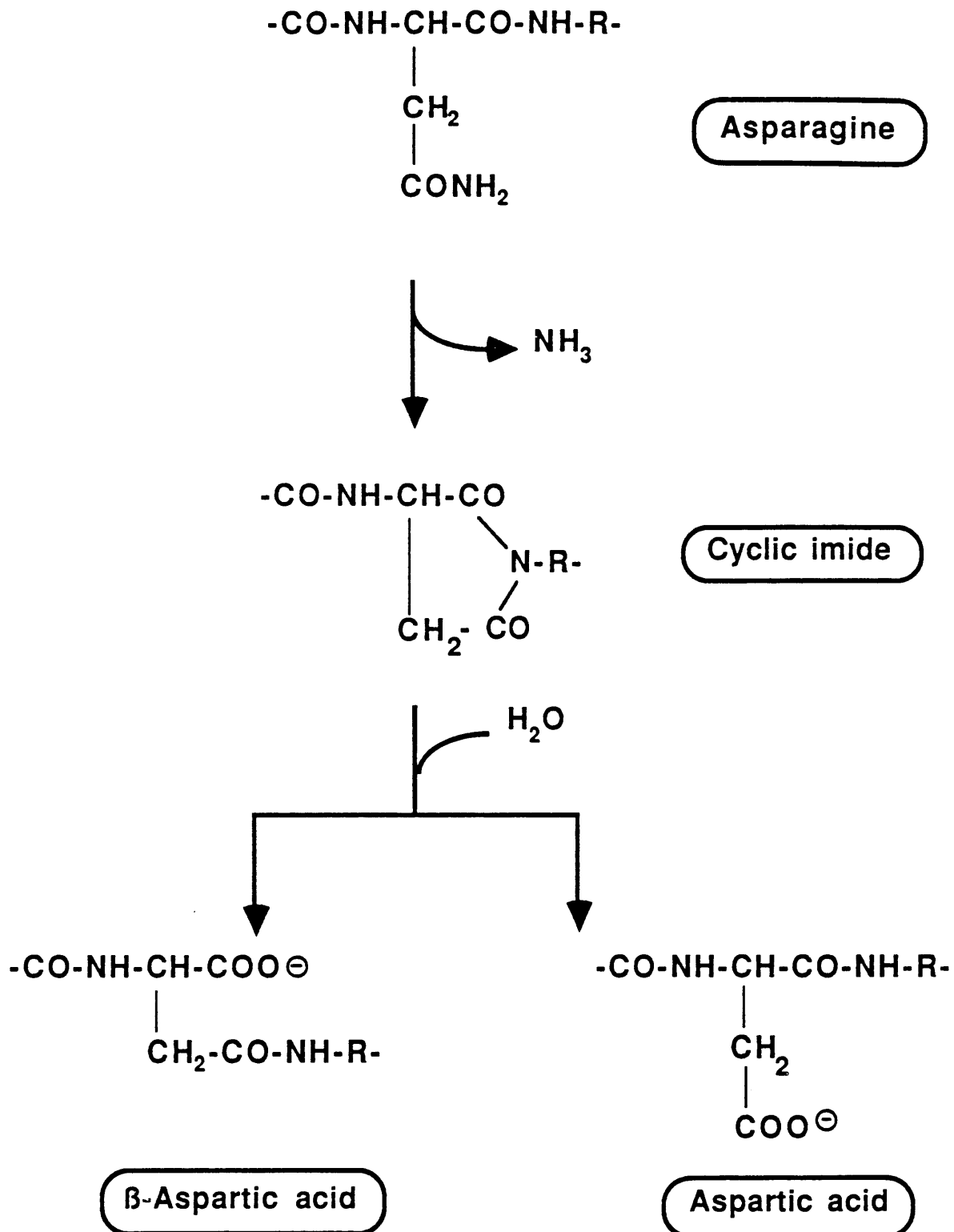
Figure 2 - The deamidation of asparagine (and glutamine) residues in proteins.

under physiological conditions followed a Bronsted plot consistent with a general base catalysis mechanism. In this study, the deamidation of a single Asn at the interface of the dimer was the only sequence examined.

Recent studies (Aswad and Johnson, 1987) reveal that the eukaryotic protein carboxyl methyltransferases (PCMT) exhibits unexpected substrate specificity for sequences containing L-isoaspartyl residues formed in vivo as the byproduct of the deamidation of protein bound asparagine residues. This specificity implies that PCMT may play a key role in the in vivo degradation and repair of proteins damaged by deamidation. Because of these observations, Geiger and Clarke (1987) conducted an in depth investigation into the nature of the deamidation reaction in three hexapeptides containing asparagine residues. Their results confirm the succinimide-intermediate mechanism of deamidation, and they observe both a temperature and sequence dependence to this reaction (Asn-Gly is particularly labile while Asn-Pro and Asn-Leu are more stable presumably due to steric considerations). It is worth mentioning that under strongly acidic conditions deamidation of asparagine and glutamine residues also occurs, but the mechanism and specificity of this process (simple hydrolysis) are biologically unimportant (Venkatesh and Vithayathil, 1984).

The major limitation of the aforementioned deamidation studies is utilization of synthetic peptides to model reactions occurring in folded, biologically active proteins. For example, recent work (Geiger and Clarke, 1987) has shown at an asparagine containing hexapeptide sequence found in triose phosphate isomerase (TIM) deamidates via a cyclic imide intermediate with a measurable half-life under physiological conditions. However, when these results were compared to the rate of deamidation of this hexapeptide within the folded enzyme molecule (Yuksel and Gracy, 1986), the protein-bound asparagine sequence turned out to deamidate over ten times more slowly than the hexapeptide. Clearly, conformational restraints within the protein account for these entirely different rates of deamidation.

By using neutron diffraction techniques to examine time-aged, deamidated protein crystals of trypsin (Kossiakoff, 1988), and kinetic studies on the deamidation of a particularly labile Asn-

Gly sequence in ribonuclease (Creighton, 1989), as well as a protein sequence data base analysis by Clarke (1987), these recent studies have confirmed that a protein's tertiary structure is a principal determinant to protein deamidation under ambient conditions. Moreover, work by Lura and Schirch (1988) has shown that peptide confirmation also affects the deamidation of a Asn residue in tetrapeptides.

Site-directed mutagenesis is now being used to remove labile asparagine residues from recombinant proteins. For example, protein engineering was used to remove an asparagine residue from the dimer interface of triose phosphate isomerase to stabilize the enzyme against irreversible thermoinactivation (Ahern et al., 1987; Casal et al., 1987). In addition, recombinant interleukin-1-α was stabilized against a specific deamidation by the replacement of an asparagine residue with an isosteric serine (Wingfield et al., 1987).

2.5.3 Hydrolysis of peptide bonds at aspartic acid residues

In the case of both ribonuclease and lysozyme, hydrolysis of the polypeptide backbone at aspartic acid residues contributes to the irreversible thermal inactivation of an enzyme under rather acidic conditions such as pH 3 or 4. Such peptide bond cleavage occurs much faster on the C-terminal side of aspartic acid residues than on the N-terminal side (Inglis, 1983), as shown in Figure 3. Tsung and Fraenkel-Conrat (1965) observed that the release of free aspartic acid residues from tobacco mosaic virus protein is a first-order process with a half-life of six hours in 0.03 N HCl at 105° C. Marcus (1985) has shown that the Asp-Pro bond is particularly labile under similar conditions. By studying a series of dipeptides, the undecapeptide physalaemin, and a 63 amino acid CNBr fragment of pig kidney fructose 1,6-biphosphatase, he established a half-life of eleven minutes for the hydrolysis of the Asp-Pro bond in 0.015 N HCl at 110° C, while other Asp-X bonds had half-lives ranging from 84 to over 1400 minutes under the same conditions. The greater lability of this particular bond has been attributed to the more basic

-CO-NH-CH-CO-NH-R-
|
CH_2
|
COOH

Aspartic acid

OH
|
-CO-NH-CH-C $\cdots$ NH-R
| \
| O
| |
CH_2 CO

H_2O

-CO-NH-CH-COOH   + NH_2-R
|
CH_2
|
COOH

Peptide bond clevage
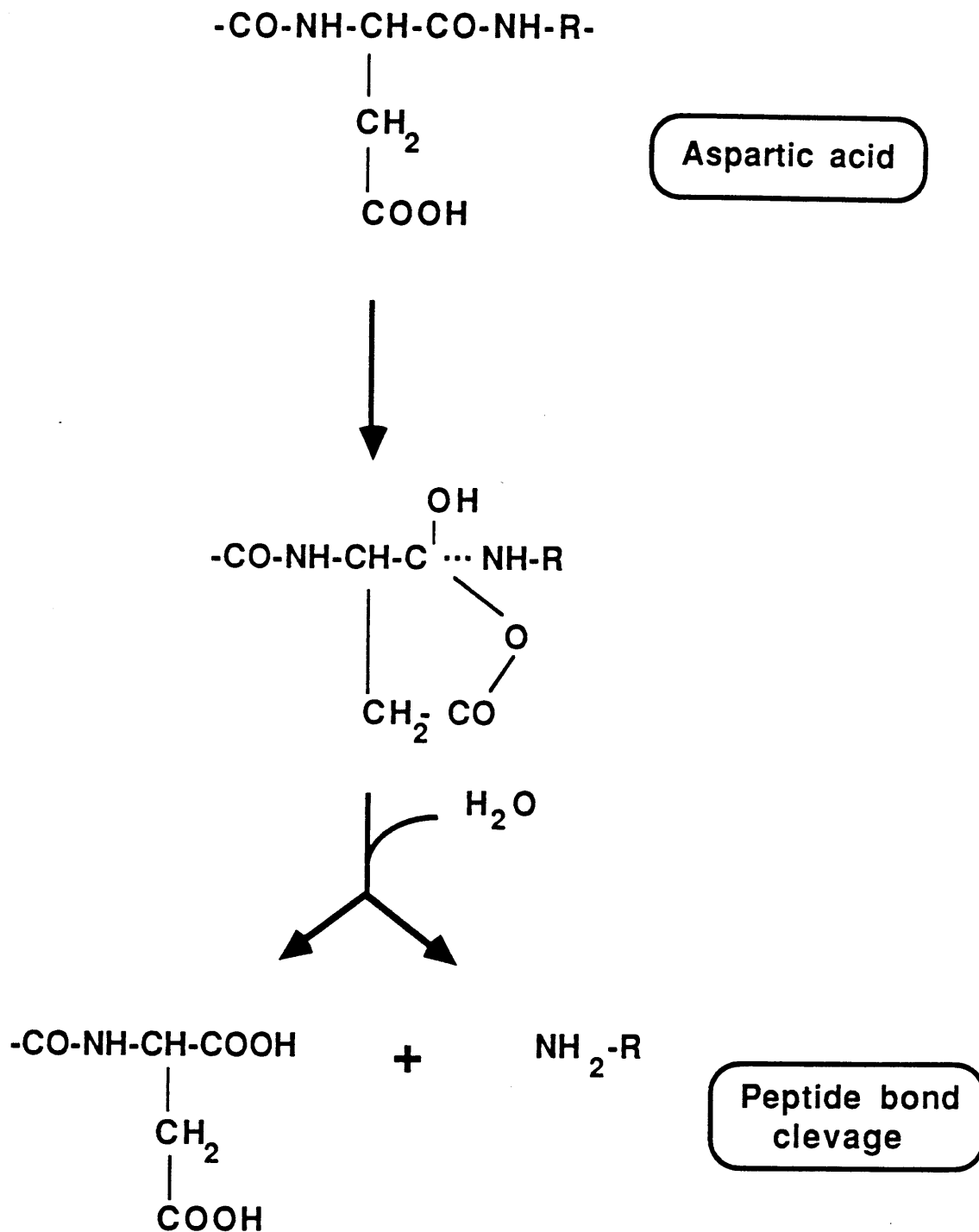
Figure 3 - The hydrolysis of peptide bonds at aspartic acid residues in proteins.

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nature of the proline nitrogen compared to other amino acid residues and to the enhanced α-β isomerization of aspartic acid when linked to proline (Piszkiewicz and Smith, 1970).

2.5.4 Degradative reactions involving cysteine residues

Cysteine residues undergo autooxidation to form either intra or intermolecular disulfide bonds or monomolecular products such as sulfenic acid (Torchinski, 1981). These oxidative reaction are enhanced at higher pH and catalyzed by divalent metal ions especially copper. During their studies on the thermostability of α-amylase, Tomazic and Klibanov (1988) showed that cysteine oxidation by molecular oxygen contributed to the thermal inactivation of the enzyme at 90° C. Similarly, cysteine oxidation caused, in part, the thermal inactivation of T4 lysozyme at 70° C (Perry and Wetzel, 1987).

Cysteine residues can also undergo a heat-induced ß-elimination reaction similar to cystine residues, but at only 3-5% the rate of the disulfide (Whitaker and Feeney, 1983). More importantly, cysteine residues can catalyze disulfide interchange causing the reshuffling of disulfide bonds. An unpaired cysteine residue was shown to interrupt correct disulfide bond pairing in recombinant human fibroblast interferon and interleukin-2. When serine was chosen to replace the free cysteine, the expressed protein formed correct disulfides and was fully active (Mark et al., 1984; Wang et al., 1984). In addition, an unpaired cysteine was also shown to interfere with native disulfide bond formation in T4 lysozyme (Perry and Wetzel, 1986).

2.5.5 Other reactions

The previous sections have described the most commonly observed degradative reactions which occur during the heating of proteins in aqueous solution. However, there are many other less frequently seen reactions which have been identified (Feeney, 1980). For example, hydrogen peroxide can oxidize methionine residues to its sulfoxide counterpart (Torchinsky,

1981). Subtilisin was stabilized against this chemical oxidation by removal of a methionine residue via site-directed mutagenesis (Estell et al., 1985). Racemization of protein-bound amino acids, especially asparagine and serine residues, occurs at elevated temperatures (Steinberg et al., 1984; Bada, 1984). Finally, lysine may react with reducing sugars at elevated temperatures to form a Schiff's base followed by an Amadori rearrangement in a process commonly referred to as a Maillard reaction (Gottschalk, 1972). This browning reaction is commonly seen during the processing of food proteins at elevated temperatures (Feeney, 1980).

2.6 Thermostability of Glucose Isomerase

Glucose isomerase functions naturally as D-xylose isomerase catalyzing the isomerization of xylose to xyulose. This enzyme is widely distributed in microorganisms and plants. Regardless of source, glucose isomerase is a rather acidic protein (pI values from 4-5) with a multimeric structure containing monomers (40 kd) which associate into either dimers or tetramers. The enzyme folds into an eight stranded α/β barrel, similar to triose phosphate isomerase, but contains a large, relatively unstructured loop that forms extensive interactions with the other subunit (Farber and Petsko, 1987b; Glasfeld et al., 1988).

All known D-xylose isomerases can also catalyze the conversion of glucose to fructose with K_m values for glucose 100 fold higher than xylose. In general, all isomerases require the presence of metal ions (Mn^{+2}, Mg^{+2}, Co^{+2}) for their catalytic activity. The pH optimum for enxymatic activity is usually greater than 7. The mechanism of the isomerization reaction is generally believed to involve a cis-enediol intermediate. Both heavy metal ions and sugar alcohols (xylitol) are known inhibitors of the enzyme (Anthrim et al., 1979).

The manufacture of the sweetener high fructose corn syrup (HFCS) using immobilized glucose isomerase is presently the largest commercial enzymatic process with an estimated five billion tons of HFCS produced in 1984 (Jensen and Rugh, 1987). The commercial production of HFCS, outlined in Figure 4, involves the breakdown of a corn starch slurry using α-amylase

and glucoamylase to a dextrose solution. This glucose solution (>95%) is then isomerized to 42% fructose using immobilized glucose isomerase. The high fructose corn syrup solution is subsequently enriched to 55% fructose via chromatography, a necessary step for many food applications (for reviews of the process, and the enzymatic and molecular properties of glucose isomerase, see Anthrim et al., 1979; MacAllister, 1980; Chen, 1980; Barker and Shirley, 1980; Bucke, 1981; Verhoff et al., 1985). Inspection of Figure 4 reveals that all of these enzymatic processes are carried out at elevated temperatures. Advantages of high temperature enzymatic conversions include increased reaction rates, reduced chance of microbial contamination, reduced solution viscosity, increased solubility of substrates and products, and potentially favorable shifts in the thermodynamic equilibrium (Klibanov, 1983).

The thermodynamics of the conversion of aqueous glucose to fructose have been thoroughly investigated (Tewari and Goldberg, 1985), and the proportion of fructose increases as the temperature rises. In fact, if the enzymatic isomerization of glucose could be carried out at 105-110° C, HFCS containing 55% fructose could be directly produced without the subsequent, costly chromatographic enrichment step. However, currently available glucose isomerases are not nearly thermostable enough under these conditions. Most industrially employed glucose isomerases exhibit temperature optima in the range of 80-90° C (in the presence of substrate), but even at these temperatures, insufficient operational stability precludes their use (Hemmingsen, 1980; Jensen and Rugh, 1987).

The enzyme-catalyzed isomerization of glucose into fructose is carried out in industrial bioreactors at 60-65° C where the half-life of the immobilized glucose isomerase is on the order of several weeks (Hemmingsen, 1979; Anthrim et al., 1979). Enzyme stability is affected not only by temperature, but also by pH, oxygen, metal ions, microbial contamination and mechanical problems such as channeling or desorption (Antrim et al., 1979). Many papers, primarily publications about the purification of glucose isomerase from numerous microbial sources, briefly report thermoinactivation experiments as part of the characterization of the enzyme: Danno (1970) from Bacillus coagulans, Chen and Anderson (1979) from Streptomyces

```
        ┌─────────────────────────┐
        │  CORN STARCH SLURRY      │
        │  (35-40% SOLIDS)         │
        └─────────────────────────┘
                   │
                   │          alpha-amylase, 90-110°C
                   │
                   ▼          glucose amylase, 55-60°C
        ┌─────────────────────────┐
        │  DEXTROSE  SOLUTION      │
        └─────────────────────────┘
                   │
                   │
                   │
                   ▼
        ┌─────────────────────────┐
        │  FILTER  AND  DEIONIZE   │
        └─────────────────────────┘
                   │
                   │          glucose isomerase, 60-65°C
                   │
                   ▼
        ┌─────────────────────────┐
        │  HFCS, 42 % FRUCTOSE     │
        └─────────────────────────┘
                   │
                   │          decolorize (carbon)
                   │          chromatography
                   ▼
        ┌─────────────────────────┐
        │  HFCS, 55 % FRUCTOSE     │
        └─────────────────────────┘
```
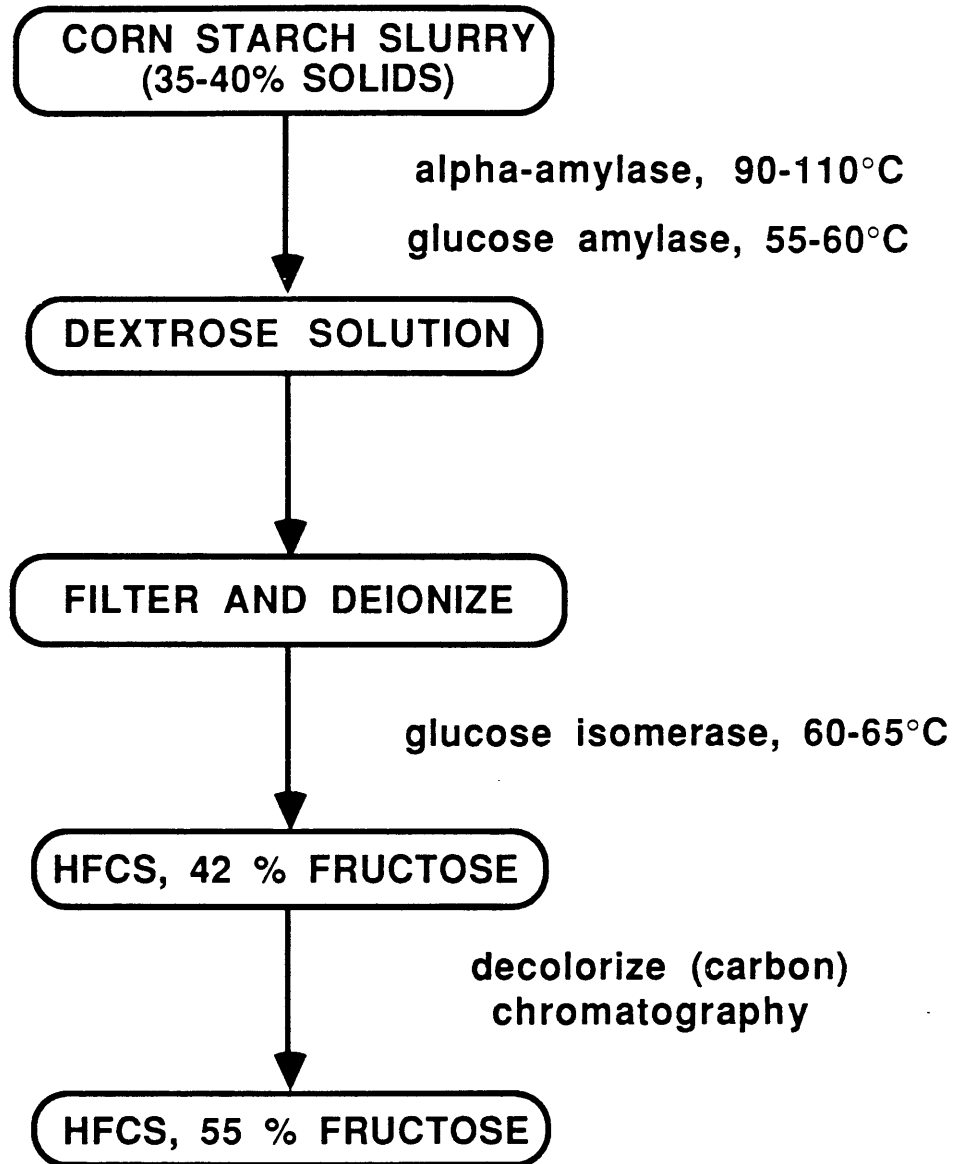
Figure 4 - The commercial, enzymatic production of the sweetener High Fructose Corn Syrup.

<u>flavogriseus</u>, Kasumi <u>et al</u>. (1982) from <u>Streptomyces griseofuscus</u>, and Suekane <u>et al</u>. (1978) from <u>Streptomyces olivochromogenes</u> and <u>Bacillus stearothermophilus</u>. All of these papers describe experiments where the enzyme is simply incubated at various temperatures for a set time period (usually ten minutes), and then assayed for activity. Regardless of the microbial source of glucose isomerase, the enzyme undergoes inactivation between 70-80° C, and some degree of stabilization is observed when heated in the presence of divalent cations such as Mg^{+2}, Co^{+2}, or Mn^{+2}. No mechanistic investigations were attempted in any of these studies.

The thermostability of glucose isomerase in more commercially-oriented environments has been reported by Zittan <u>et al</u>. (1975) and Lee <u>et al</u>. (1976) who examined the behavior of immobilized glucose isomerase in column reactors at various temperatures and substrate concentrations. Verhoff and Goldstein (1982) investigated the role of diffusional resistance limitations, while Chen and Wu (1987) studied the effect of substrate concentration, on the thermal inactivation of immobilized glucose isomerase.

Therefore, despite these numerous studies involving the thermal stability of glucose isomerases from various microorganisms, the exact mechanism of irreversible thermoinactivation (i.e., time-dependent decay of catalytic activity) of this enzyme remains obscure. This lack of fundamental knowledge makes all attempts to stabilize glucose isomerases totally empirical. By determining the cause and mechanism of irreversible thermoinactivation of glucose isomerase, we can develop rational strategies to stabilize this enzyme at elevated temperatures.

2.7 Thermostability of Enzymes in Nonaqueuos Environments

2.7.1 <u>Enzymes in neat organic solvents</u>

The recent discovery that enzymes are catalytically active in organic solvents has greatly expanded the potential for their use as practical catalysts (Klibanov, 1986; Khmelnitsky <u>et al</u>., 1988; Zaks and Russell, 1989; Dordick, 1989; Klibanov, 1989). Perhaps the most neglected

aspect of this emerging technology is the stability of enzymes in nonaqueous media (Aldercreutz and Mattiasson, 1987; Deetz and Rozzell, 1988). It has been reported that enzymes not only function, but display markedly increased thermostability in organic solvents (see below). These observations afford the opportunity to quantitatively investigate the cause and mechanism of this significantly increased thermostability.

Interest in the stability of enzymes suspended in the nonaqueous environment of an organic solvent is not limited to man-made situations. Many enzymes, such as lipases, esterases, dehydrogenases and xenobiotic metabolism enzymes like cytochrome P450, function naturally in hydrophobic environments such as biological interfaces and membranes (Dordick, 1989). Moreover, the "nonaqueous" environment of protein-protein interactions are commonly found whenever two proteins interact, such as the binding of the enzyme trypsin to bovine pancreatic trypsin inhibitor (PTI), the interactions between myosin and actin during muscle contraction, or the specific contacts of an antibody to a protein antigen. As an example of the milieu of protein-protein contacts, when the trypsin-PTI interface is formed, 1,130-1,720 $Å^2$ of accessible surface is removed from contact with water. The residues in the interface closely pack and hydrophobicity is the major factor which stabilizes the association (Chothia and Janin, 1975).

Recent interest in the formation of inclusion bodies during the microbial expression of recombinant proteins, has raised questions about the physical state of densely packed protein aggregates (Mitraki and King, 1989). Naturally formed protein complexes such as collagen involve polypeptide chains which initially associate into triple helices, and then the triple helices form side by side interactions to create higher order structures called fibrils (Creighton, 1983). The stability of collagen matrix against thermal denaturation has been shown to be a function of the amino acid content of the monomers: increasing the total pyrrolidine (proline plus hydroxy-proline) content, raises the T_m from 22 to 52° C (Josse and Harrington, 1963). Although the exact mechanism is unclear, polyproline is known to form a "tight" structure in solution while hydroxyproline is responsible for triple helix formation. Another interesting example of protein-protein stabilization is the trimer of a 666 residue polypeptide chain which forms the tail spike

3 8

endorhamnosidase of P22 (which is 60% beta-sheet). The native protein is extremely thermostable requiring temperatures of 80-90° C for denaturation (King, 1986).

Conventional biochemical wisdom holds that enzymes inactivate when exposed to organic solvents. However, this inactivation occurs via protein denaturation when water-miscible organic solvents are added to aqueous protein solutions (Lapanje, 1978; Volkin and Klibanov, 1989); Conversely, when enzyme powders are dispersed in neat organic solvents, they display markedly enhanced stability, in particular thermostability. For example, Zaks and Klibanov (1984) report that porcine pancreatic lipase inactivates instantly in aqueous buffer at 100° C, but when suspended in 2 M heptanol in tributyrin, the half-life dramatically increases to 12 hours at 100° C. Furthermore, the thermostability of this enzyme correlates directly with the amount of water bound to the enzyme after lyophilization, and to the amount of water added to the system during heating. During their subsequent studies on the enzymatic properties of α-chymotrypsin in various organic solvents, Zaks and Klibanov (1988) report a "pH memory" effect to the thermal inactivation of the enzyme where the pH of the buffer (pH 3 vs. 9) during lyophilization affects its subsequent thermostability (however, this effect may be due to the pH-dependent denaturation of the enzyme before lyophilization).

The thermostability of chymotrypsin strongly depends on the nature of the solvent; the enzyme is more stable in hydrophobic solvents than in hydrophilic ones (Zaks and Klibanov, 1988a). Similar results have been reported by Reslow et al. (1987) for chymotrypsin, Wheeler and Croteau (1986) for the terpene cyclase, Ahern and Klibanov (1986) and Klibanov and Ahern (1987) for lysozyme, and Ayala et al. (1986) for ATPase and cytochrome oxidase. Recent work with mitochondrial F_1-ATPase in toluene-phospholipid-low water systems has shown that as the water content is increased from 0.04% to 2.5%, the enzyme acquires catalytic activity while undergoing rapid thermal denaturation (Garza-Ramos et al., 1989). In addition, the detrimental effects of ligand-induced activation and polyethylene glycol-derivatization on the thermostability of subtilisin Carlsberg in organic solvents have been observed by Russell and Klibanov (1988) and Pasta et al. (1988), respectively. It should be pointed out that in all these studies

thermostability was not the major topic of the paper (no mechanistic studies have been undertaken), but rather reported in order to show the novel properties of these enzymes in organic solvents.

The equivalency of enzyme molecules within the solid particles suspended in organic solvents is an important parameter in order to determine whether all enzyme molecules are exposed to solvent during heating. Sonication of enzyme powders dispersed in organic solvents reduces the average particle size from 270 to 5 μm, as shown by direct microscopic examination (Zaks and Klibanov, 1988 a). Furthermore, the active sites of chymotrypsin and subtilisin were titrated in organic solvents, and up to 70% of the total active sites were shown to be available to the substrates and hence to the organic solvent. The masking of the remaining sites disappeared upon dissolving these enzymes in water, lyophilizing, and then resuspending them in organic solvent (Zaks and Klibanov, 1988 a). In addition, recent work using solid state NMR to examine the structural integrity of α-lytic protease in organic solvents has found no evidence of structural heterogeneity in the enzyme population (Burke et al., 1989; these findings are limited, however, to the active site of the enzyme). Therefore, most, if not all, of the enzyme molecules in organic solvent are accessible to the solvent.

The role of water during enzyme thermal inactivation has been reported not only in these recent studies involving organic solvents. Similar trends have appeared in the literature describing the thermostability of viruses (Grief and Rightsel, 1968), food proteins (Rockland, 1969), enzyme powders (see below), and recently with pharmaceutical proteins (Hageman, 1988). However, the cause and mechanism of these observations remain unclear. Water is an essential reactant in the covalent, degradative reactions causing irreversible thermoinactivation of enzymes in aqueous solution, as well as the solvent which facilitates protein mobility leading to reversible unfolding, incorrect structure formation and aggregation. Therefore, the increased thermostability of enzymes in nonaqueous environments may be explained by resistance to these covalent reactions and/or increased conformational rigidity. Interestingly, Zaks and Klibanov

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(1988b) have recently shown that the conformational flexibility of mushroom polyphenol oxidase in hexyl acetate markedly increases upon hydration of the organic solvent.

2.7.2 Enzyme powders

Rehydration of enzymes from the dry state has been shown to lead to a greater conformational mobility, and at a certain water content, enzymatic activity. Most of the work on water-protein interactions has been conducted with powders or films of model enzymes such as hen egg white lysozyme (Fujita and Noda, 1978; Baker et al., 1983; Rupley et al., 1983; Poole and Finney, 1983; Finney and Poole, 1984; Schinkel et al., 1985; along with general reviews of water-protein interactions by Kuntz and Kauzmann, 1974; Bryan, 1980; Rupley et al., 1980). The protein hydration process for lysozyme is typically broken down into three well-defined categories (Rupley et al., 1983): At a hydration level of 0 to 7% (g/g) H_2O (or 0-60 moles of H_2O per mole of protein), water is bound primarily to charged groups on the protein surface. The mobility of the bound water is approximately 100 times less than bulk water and enzymatic activity is negligible. From 7 to 25% (g/g) H_2O (60-220 moles of H_2O per of mole protein), clusters of water molecules form until most of the protein surface is covered. Protein mobility dramatically increases (as measured by hydrogen exchange) and enzymatic activity becomes detectable at approximately 20% (g/g) H_2O. Finally, as the water content increases up to 35% (g/g) H_2O (220 to 300 moles of H_2O per mole of protein) and above, uncovered surface patches on the protein become hydrated, enzymatic activity approaches one tenth of aqueous solution, protein mobility displays full internal motion and water mobility approaches that of bulk water.

Very little work has been done on the mechanism of thermal inactivation of dry protein powders (Hahn-Hagerdal, 1986). Mullaney (1966) recorded the kinetics of the thermal inactivation of powders of ribonuclease and trypsin (the water content was not measured). It was found that, within experimental error, inactivation could be approximated by first order kinetics between 165-205° C. Carpenter et al. (1962) and Bjarnason and Carpenter (1970)

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examined chemical changes occurring in food and pure proteins, respectively, during heating for 24 hours at 115-145° C. They noted a liberation of ammonia and hydrogen sulfide, as well as a decrease in the lysine and cysteine content. In the latter investigation, they suggest that a transamidation reaction is responsible for some of these chemical changes. In this study, no correlation between these reactions and loss of biological activity was attempted.

More recent studies have used differential scanning calorimetry as a tool to study the temperature-induced conformational changes in the state of a protein (Stein, 1974; Privalov and Potekhin, 1986). Calorimetry has also been used extensively to examine the effects of various environmental factors (additives, pH, ionic strength, etc.) on temperature induced unfolding of biomolecules (Chowdhry and Cole, 1989). This technique measures the excess apparent specific heat of a system in a continuous manner as a function of temperature at a fixed scan rate (°C/ min), i.e., it determines the difference in energy input required (between a protein sample and a reference) to keep the two samples at a constant rate of increasing temperature. When a protein molecule unfolds at its thermal denaturation temperature (T_m), a certain amount of excess energy is required and a thermal transition peak is recorded on the DSC.

Differential scanning calorimetry has been used to measure the effect of hydration on the thermal denaturation of enzyme and protein powders: tropocollagen (Leuscher et al., 1974), ß-lactoglobulin (Ruegg et al., 1975), myoglobin (Hagerdal and Martens, 1976), lysozyme (Fujita and Noda, 1978; 1979), chymotrypsinogen (Fujita and Noda, 1981) and ovalbumin (Fujita and Noda, 1981). The common conclusions from these papers are that : (i) enzyme powders are extremely thermostable in the dry state with T_m values as high as 130° C, (ii) at hydration levels between 5 and 40% water (g/g), the T_m (melting temperature) of the protein decreases, while the net enthalpy change increases with increasing water contents, and (iii) at higher hydration values such as 40 to 300% water (g/g), both of these parameters approach the level obtained for proteins in aqueous solution. Since a protein gains conformational flexibility with increasing water content (Rupley et al., 1983), the thermal stability of a protein decreases (see results and discussion); similarly, the increasing flexibility of the protein molecule allows for a greater extent

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of unfolding and increased number of noncovalent bonds being broken resulting in an increased net enthalpy.

Despite numerous reports of increased thermostability of enzymes in organic solvents, no mechanistic investigation has yet been carried out to determine the processes that cause thermoinactivation. Similarly, reports on the effect of hydration on the denaturation of enzyme powders have not been correlated with either (i) chemical and conformational changes which cause irreversible thermoinactivation or (ii) thermostability in other nonaqueous environments such as anhydrous organic solvents.

III. EXPERIMENTAL SECTION

3.1 Materials

3.1.1 Enzymes and Proteins

All proteins examined for cystine destruction at 100° C were purchased from Sigma Chemical Co.(St. Louis, MO): bovine pancreatic insulin, chymotrypsinogen A, trypsinogen, and ribonuclease A, hen egg-white lysozyme, human transferrin, pepsinogen from porcine stomach mucosa, papain from papaya latex, chicken egg-white conalbumin and ovalbumin, bovine serum albumin, and horseradish peroxidase. We have found all of these proteins to be essentially homogeneous in SDS polyacrylamide gel electrophoresis (see below).

Mixed disulfides of lysozyme (and trypsinogen) with glutathione were prepared according to the general method of Odorzynski and Light (1979): Proteins were dissolved at 10 mg/ml in an aqueous solution containing 8 M urea, 0.2 M ß-mercaptoethanol, and 0.1 M Tris, pH 8.5, under N_2. After incubation overnight at room temperature, the reduced protein was desalted on a Sephadex G-25 column (2.5 x 40 cm) and immediately lyophilized. Protein powder was resuspended at 3 mg/ml in an aqueous solution containing 8 M urea, 0.1 M oxidized glutathione, and 0.1 M Tris, pH 9.5, under N_2 for 18 hrs. The mixed-disulfide was then desalted on a G-25 column and lyophilized.

The hybrid protein containing a mixed disulfide of lysozyme with glutathione (prepared as mentioned above) contained no free sulfhydryl groups, as established by both spectrophotometric titration with Ellman's reagent and treatment with iodoacetic acid, followed by amino acid analysis. Following reduction with sodium borohydride (Brown, 1960), the former method yielded 15.4 moles of thiols/mole of protein; oxidation of the free thiols with performic acid (Odorzynski and Light, 1979), followed by acid hydrolysis and amino acid analysis (see below) of the cysteic acid formed resulted in 15.0-16.3 moles of cysteic acid/mole

of protein. These values are about 90-100% of those predicted by a model in which each of 8 cysteine residues of lysozyme (Imoto et al., 1972) is attached to a glutathione moiety. Isoelectric focusing (see below) of the mixed disulfide revealed a major band and a few minor ones. Hence, the prepared sample, in addition to the main product (lysozyme-glutathione)$_8$, contained some hybrid molecules with fewer glutathione moieties where some of the cysteine residues of lysozyme oxidized to cystines. This conclusion was further confirmed by the number of glutamic acid residues: amino acid analysis (see below) of the hybrid protein yielded 12-12.5 per protein molecule, as compared to the theoretical number 13 (8 from 8 glutathione moieties plus 2 glutamic acid and 3 glutamine residues (Imoto et al., 1972) in a lysozyme molecule). Similar analysis of a mixed disulfide of trypsinogen with glutathione revealed that almost all 12 cysteines formed by reduction of trypsinogen were bonded to glutathione moieties.

Glucose isomerase thermostability studies were done with enzyme (D-xylose ketol isomerase, EC 5.3.1.5) from Streptomyces olivochromogenes which was a generous gift from CPC International's Moffett Technical Center (Summit-Argo, IL). The cell-free enzyme solution obtained was subsequently purified to >95% homogeneity according to a modified procedure of Suekane et al. (1978):

(a) Dialyze 20 ml of cell-free extract (175 mg/ml protein) three times against 0.05 M Tris-HCl, 10 mM MgSO$_4$, pH 7.5 (25° C) (termed buffer A) overnight at 4° C. Final volume is 45 ml.

(b) Add solid ammonium sulfate to 70% saturation. Stir at 4° C overnight, followed by centrifugation and collection of precipitate. Resuspend to 45 ml with buffer A and dialyze three times against buffer A overnight at 4° C. Final volume is 90 ml.

(c) Apply to DEAE-Sephadex A-50 column (4.5 x 20 cm) equilibrated in buffer A at 0.8 ml/min at room temperature. Elute protein with 1 liter of a NaCl gradient (0 to 0.5 M) in buffer A.

(d) Measure protein (OD_{280}) and activity (see next section) and collect peak. Precipitate enzyme by adding solid ammonium sulfate to 70%. Dissolve precipitate in 10 ml buffer A and dialyze three times against buffer A overnight at 4° C. Final volume is 40 ml.

(e) Apply 4 ml to G-150 column (2.5 x 85 cm) equilibrated in buffer A at 0.8 ml/min at room temperature.

(f) Measure protein (OD_{280}) and activity (see next section). By means of OD_{280} analysis on FPLC Superose 12 gel filtration column (0.1 ml/min equilibrated in buffer A), collect fraction of G-150 peak (3/4 total volume) which indicates single, pure peak of enzyme by FPLC gel filtration analysis.

(g) Pool samples and dialyze three times against 55% ammonium sulfate in buffer A overnight at 4° C. Collect crystals, wash with 55% ammonium sulfate in buffer A, then dialyze against buffer A.

Enzyme was >95% pure as shown by FPLC gel filtration chromatography, SDS-PAGE and isoelectric focusing (see below). In addition, the amino acid composition was in good agreement with the (i) calculated value based on the DNA sequence (Farber and Petsko, 1987a) and (ii) the literature value (Suekane et al., 1978).

The purified glucose isomerase was covalently attached to porous glass beads using a modified procedure of Weetall (1976):

(a) Amination of beads - incubate control pore glass beads for 45 min, 100° C in 5% nitric acid followed by a distilled H_2O wash. Add beads (10 g beads to 120 ml solution) to a 10% solution of γ-aminopropyltriethoxysilane in H_2O (pH 3.45 adjusted with 6 N HCl), 75° C, 3 hr with stirring. Wash the beads with 100 ml of H_2O and dry overnight in oven at 100° C.

(b) Enzyme preparation - dialyze enzyme (70 mg per g of beads) three times overnight against 50 mM borate buffer, pH 8.5 containing 10 mM $MgSO_4$ and 1 M xylose (termed buffer B).

(c) Glutaraldehyde coupling - add 125 ml of 2.5% glutaraldehyde solution in 50 mM sodium phosphate buffer, pH 7.0 to 10 g aminated support. Stir for 2 hr at room temperature (beads become pink). Wash extensively with H_2O, then wash with buffer B. Add enzyme to support in smallest possible volume (minimum 1% glucose isomerase), then incubate overnight at room temperature with turning.

(d) Sodium borohydride reduction - wash enzyme-support extensively with 10 mM $MgSO_4$ in H_2O. Add 100 mg $NaBH_4$ to 50 ml of enzyme-support suspension and allow to stand overnight at 4° C (beads become white). Wash extensively with 10 mM $MgSO_4$ in H_2O then store (+ 0.02% sodium azide) at 4° C.

This method afforded 8 to 18 mg of glucose isomerase immobilized on 1 g of the support. The specific activity of the immobilized enzyme was between 80 and 90% of that of its free precursor.

All enzymes used to examine protein thermostability in organic solvents were purchased as dry powders from Sigma Chemical Co: bovine pancreatic ribonuclease A (EC 3.1.27.5) with a specific activity of 95-100 Kunitz units/mg of protein (type X11-A), bovine pancreatic α-chymotrypsin (EC 3.4.21.1) with a specific activity of 58 units/mg protein (Type II), and chicken egg white lysozyme (EC 3.2.1.17) with a specific activity of 49,100 units/mg protein (Grade 1). Ribonuclease was found to be homogeneous as determined by SDS polyacrylamide gel electrophoresis (Laemmli, 1970) and FPLC gel filtration chromatography (see below). In both cases the enzyme migrates at the expected value of 13.7 kD. The amino acid composition of ribonuclease (see below) was in good agreement with the literature data (Smyth et al., 1963). Chymotrypsin and lysozyme were both found to migrate as a single peak at their expected molecular weight values on FPLC gel filtration chromatography.

3.1.2 Reagents

The enzymes yeast hexokinase, Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase, bovine heart lactic dehydrogenase, and bovine liver L-glutamic dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, MO). Controlled pore glass beads (mean pore diameter 2100 Å, surface area 18 m^2/g, α-D(+)-glucose, ß-D(-)-fructose, D-(+)-xylose, NAD^+, NADH, ATP, reduced and oxidized glutathiones, picrylsulfonic acid (TNBS), cytidine 2'-3'-cyclic monophosphate, torula yeast ribonucleic acid, N-benzoyl-L-tyrosine ethyl ester (BTEE) and lyophilized Micrococcus lysodeikticus cells were also obtained from Sigma.

All anhydrous organic solvents, along with xylitol, γ-aminopropyltriethoxysilane and deuterium oxide (D_2O), were acquired from Aldrich Chemical Co.(Milwaukee, WI); the anhydrous solvents were removed from their "sure-seal" containers by use of a 22-gauge stainless steel needle and 5 ml glass syringe as described in the Aldrich packing instructions. The water content of the solvents was determined by Karl Fischer titration as described below. Karl Fischer reagents (hydranal-titrant 5 and hydranal solvent) were acquired from Riedel-de Haen.

Reduced glutathione, [glycine-2-^{3}H]-labelled (1 Ci/mmol), was bought from New England Nuclear; in order to prepare ^{3}H-labelled oxidized glutathione, ^{3}H-labelled reduced glutathione was purified from the stabilizer dithiothreitol via the method of Butler et al. (1976): the glutathione solution was adjusted to pH 2 and extracted three times with a ten-fold excess volume of ethyl acetate. The purified ^{3}H-labelled reduced glutathione was then diluted with its "cold" counterpart and oxidized by bubbling O_2 overnight through its aqueous solution (pH 8) containing 10 μM $CuCl_2$, followed by removal of the Cu^{+2} ions by Chelex ion-exchange chromatography.

High fructose corn syrup (trade name Isoclear 42), containing 71% (w/w) solids and consisting of 42% fructose, 52% glucose and 6% oligosaccharides, was a kind gift from Cargill Co. (Dayton, OH). All other chemicals used in this work were purchased commercially and were of the highest purity available.

3.2 Methods

3.2.1 Enzymatic Assays

Free and immobilized glucose isomerase

Both free and immobilized glucose isomerase were assayed by means of a modified procedure of Visuri and Klibanov (1987): Add enzyme (0.05 mg) to 1 ml of an aqueous solution of 0.1 M fructose, 0.1 M HEPES, and 10 mM $MgSO_4$ (pH 8.0) in 1.5 ml screw-cap glass vials, and shake the solution at 250 rpm and 45° C (under these conditions, the rate of non-enzymatic isomerization of fructose was negligible compared to the enzymatic reaction). Periodically withdraw samples, inactivate the enzyme with 100 µl of 1 N sulfuric acid, neutralize with 167 µl of 1.5 M Tris-base. Assay 800 µl for glucose via the standard hexokinase/glucose-6-phosphate dehydrogenase method (Bergmeyer et al., 1974) by adding 200 µl of a 5X glucose assay solution purchased from Sigma Chemical Co.(glucose HK diagnostic kit). In this assay, glucose is first phosphorylated by ATP in a reaction catalyzed by hexokinase followed by the oxidation of glucose 6-phosphate to 6-phosphogluconate in a reaction catalyzed by glucose 6-phosphate dehydrogenase in the presence of NAD^+. During this oxidation, NAD^+ is reduced to NADH with a consequent increase in absorbance at 340 nm. To determine the amount of glucose formed, a standard curve was prepared by using known concentrations of glucose in the assay solution.

Glucose isomerase activity at different temperatures

Substrate solutions containing 0.1 M fructose, 0.1 M Tris-HCl, and 10 mM $MgSO_4$ were adjusted at room temperature to the pH values that would result (Good et al., 1966; Perrin and Dempsey, 1974) in pH 8.0 at the temperature of the experiment, as determined by our calibration

curves (see Appendix 1). Subsequently, the solutions were preincubated at different temperatures with stirring in the range from 60° to 100° C, followed by addition of immobilized glucose isomerase. Periodically, aliquots were removed, acidified, cooled, and then the initial rates of the enzymatic isomerization of fructose to glucose at each temperature were measured as described above. At temperatures above 80° C, where the rate of the non-enzymatic isomerization became appreciable, it was subtracted from that for the enzymatic reaction.

Ribonuclease

Ribonuclease was primarily assayed according to the procedure of (a) Kunitz (1946), but also by the method of (b) Crook et al. (1960) and (c) Stark and Stein as described by Klee (1966):

(a) 1-5 μg of enzyme was added to 1.5 ml of 1 mg/ml yeast torula RNA in 0.1 M sodium acetate, pH 5.0 at room temperature. The initial rate of hydrolysis was measured by decrease in absorbance at 300 nm over time.

(b) 50-500 μg of enzyme was added to 1.5 ml of a 0.2 mg/ml cytidine 2':3'-cyclic monophosphate in 25 mM phosphate buffer, pH 7.5 at room temperature. The initial rate of hydrolysis was followed by monitoring the change in absorbance at 286 nm as a function of time.

(c) 1-10 μg of enzyme was added to a pH-stat cell containing 3 mg/ml cytidine 2':3'-cyclic monophosphate in 0.3 M NaCl at room temperature with the pH maintained at 7.0 by automatic titration (Radiometer, ABN 80 autoburette) with 20 mM NaOH. The initial rate of hydrolysis was followed by recording the base consumption necessary to maintain pH 7.0 as a function of time.

Chymotrypsin

Chymotrypsin activity was measured according to the method of Hummel (1959): 0.75 ml of 80 mM Tris-HCl pH 7.8, containing 100 mM $CaCl_2$ was added to 0.70 ml of 1 mM BTEE in 50% (w/w) methanol/water at room temperature. Then 1-5 µg of chymotrypsin was added, and the increase in absorbance at 256 nm resulting from the hydrolysis of the benzoyl-L-tyrosine ethyl ester was recorded.

Lysozyme

Lysozyme activity was measured according to the method of Shugar (1952): 1-5 µg enzyme was added at room temperature to 0.33 mg/ml suspension of Micrococcus lysodeikticus cells in 100 mM potassium phosphate buffer, pH 7.0. The decrease in turbidity was recorded over time at 420 nm.

3.2.2 Analytical Determinations

Protein

Protein concentrations were determined by either absorbance at 280 nm (Fasman, 1976) or the method of Lowry et al. (1951): 0-50 µg of RNase is added to 2 ml of a solution containing 0.02% sodium tartrate, 0.01% copper sulfate in a 2% sodium carbonate and 0.1 N NaOH solution. After 10 minutes, 200 µl of a 1:1 Folin reagent: H_2O (Sigma) solution is added and the absorbance of the mixture at 700 nm is recorded after 30 minutes. Standard curves were prepared using known amounts of enzyme.

In the glucose isomerase studies, protein (both free and immobilized) concentrations were determined by HPLC amino acid analysis following complete acid hydrolysis as described below. The amino acid composition of glucose isomerase was in good agreement with the literature value (Suekane et al., 1978).

Free Sulfhydryl Groups

Free sulfhydryl groups were determined with 5,5'-dithiobis-2-nitrobenzoic acid (DNTB or Ellman's reagent) according to the procedure of Riddles et al. (1983). One hundred microliters of a 10 mM DNTB solution in 0.1 M aqueous phosphate buffer, pH 7.2, containing 1 mM EDTA was added to 1 ml of a protein solution plus 2 ml of 1 M Tris buffer pH 8, containing 1 mM EDTA and the mixture was incubated at room temperature for 10 minutes. Absorbance at 412 nm was compared to a standard curve prepared using cysteine. Assay was shown to be unaffected by the presence of 10 μM Cu^{+2}.

Free sulfhydryl groups in glucose isomerase were determined colorimetrically with Ellman's reagent or by reduction and carboxymethylation of the enzyme in aqueous solutions of 6 M guanidine-HCl, followed by dialysis, acid hydrolysis, and amino acid analysis. For immobilized glucose isomerase, the Ellman's reagent titration (one-half volumes) was done with 2.5 mg glucose isomerase with shaking at 250 rpm.

Hydrosulfide

Hydrosulfide (HS^-) determination was based on the method outlined by Rabinowitz (1978): One milliliter samples were removed from a solution of the enzyme that was undergoing thermoinactivation and immediately added to 0.65 ml zinc acetate solution (2% in water) on ice. Fifty microliters of 12% sodium hydroxide solution was then added, and the samples were removed from the ice bath. Dimethylphenylenediamine dihydrochloride (0.25 ml of a 0.1%

solution in 5.5 N HCl) and FeCl$_2$ (50 ml, 23 mM in 1.2 N HCl) were added, and the tubes allowed to stand at room temperature for 30 minutes. The absorbance of the samples was determined at 670 nm and compared to a standard curve using known concentrations of hydrosulfide. The assay was not affected by the presence of small concentrations of cupric chloride (corresponding to 10 μM in the sample).

Dehydroalanine

The time course of the appearance of dehydroalanine residues was measured by acid-catalyzed conversion to pyruvic acid (Bohak, 1964; Patchornik and Sokolovsky, 1964), followed by enzymatic analysis using lactate dehydrogenase (Shifrin et al., 1959). Samples (one ml) were heated in 3 N HCl for 1 hour at 105° C in sealed ampoules and then neutralized with 0.5 ml of 6 N NaOH. To one ml of neutralized sample, 0.25 ml of 1.5 M Tris-HCl, pH 7.4, and 0.25 ml of 0.5 mg/ml NADH in the same Tris buffer were added along with 25 μl of lactate dehydrogenase. The pyruvate concentration was determined spectrophotometrically by measuring the pyruvate-dependent lactate dehydrogenase-catalyzed oxidation of NADH to NAD+ at 340 nm.

Lysinoalanine

The time course of the appearance of lysinoalanine was measured using conventional amino acid analysis of acid hydrolysates (see below). An amino acid not found in native protein samples was observed to elute immediately before histidine in samples from protein solutions heated at 100° C at pH 8. This is the same position as that reported by Bohak (1964) for lysinoalanine. The identity of the new amino acid was confirmed with an authentic sample of lysinoalanine (Sigma).

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Free Amino Groups

Free amino groups in ribonuclease were measured according to the procedure of Fields (1972): 800 μl of 0.1 M $Na_2B_4O_7$ in 0.1 N NaOH and 20 μl of 1.1 M TNBS (picrylsulfonic acid) were added to 200 μl of an enzyme solution of appropriate dilution. After 5 minutes, 2 ml of a 0.1 M NaH_2PO_4 solution containing 1.5 mM sulfite solution (1.5 ml of 0.1 M Na_2SO_3 in 98.5 ml of 0.1 M NaH_2PO_4) was added. The absorbance was measured at 420 nm with N-acetyl-lysine used as a standard.

In this reaction, sulfite is displaced from the TNBS molecule by an attacking nucleophile, in this case, the free amino groups on proteins.

Dissolved Ammonia

The time course of ammonia evolution during thermoinactivation of glucose isomerase was determined by incubating samples of the enzyme in sealed ampoules at varying temperatures and times. All of the ampoules were cooled to room temperature, opened, and the amount of dissolved ammonia was then measured enzymatically using glutamate dehydrogenase (Kun and Kearney, 1974): 0.75 ml of each sample was combined with 0.30 ml of 0.5 M Tris-HCl, pH 8.0, 50 μl of 8 mM NADH in 1% $NaHCO_3$, 0.15 ml of 0.1 M α-ketoglutarate (pH 7.5) and 20 μl of 14 mg/ml glutamate dehydrogenase. The concentration of ammonia in the sample was reflected in the difference between the initial and final absorbance at 340 nm after a 90 minute incubation, resulting from the oxidation of NADH to NAD^+ coupled with the reductive amination of α-ketoglutarate. Standard curves were prepared using solutions of known amounts of ammonium sulfate.

To determine ammonia evolution during the heating of ribonuclease in organic solvents, enzyme samples (5 mg) were placed into 2 ml of anhydrous nonane in a 10 ml screwcap glass vial which was sealed with teflon tape and a Teflon/silicone disc (Pierce). After heating and

cooling, 5 ml of aqueous buffer was injected into the sample to extract the ammonia. Ammonia concentrations were then determined enzymatically as described above.

Amino Acid Composition

Protein samples were degassed and hydrolyzed in 6 N HCl (Pierce) at 110° C for 24 hours. The amino acid composition of the hydrolysates was determined using reverse phase HPLC with a precolumn derivatization (Fernstrom and Fernstrom, 1981). Amino acids derivatized with o-phthalaldehyde (OPA) were separated on a Waters Associated Microbondapak C_{18} column using a sodium phosphate/acetonitrile gradient. The fluorescent amino acid derivatives were detected using a Waters 420-AC fluorescence detector equipped with a 360 nm excitation filter and a 455 nm emission filter.

Cysteic acid determinations required an adjustment of the above buffer (pH of buffer A was changed to 6) in order to resolve cysteic acid from aspartic acid. Lysinoalanine residues were determined by conventional amino acid analysis with post-column derivatization with ninhydrin (Hare, 1975); authentic samples of lysinoalanine (Sigma) were used as a standard.

Isoelectric Focusing

Non-equilibrium isoelectric focusing was carried out according to the method of O'Farrell et al. (1977) on a Biorad Protean II system. Samples containing approximately 20 μg of protein were focused for 2.5 hours at 200 V in a 1.5 mm 4% polyacrylamide gel containing 8 M urea and 5% Ampholine 3.5-10 (LKB) that had been prefocused for one hour at the same voltage. Gels were soaked in 10% trichloroacetic acid, stained with 0.25% Coomassie Blue R-250 in methanol: acetic acid:water (50:7:43) and then destained with methanol:acetic acid:water (40:10:50).

SDS-PAGE and Gel Densitometry

SDS polyacrylamide gels (1.5 mm), either in the presence or absence of 8 M urea, were electrophoresed at a constant current according to the method of Swank and Munkres (1971) and Laemmli (1970), respectively. The Laemmli gel system was carried out on either a Biorad Protean II or a Pharmacia PhastGel apparatus. SDS-polyacrylamide gels were stained with Coomassie Blue R-250 as described above. Quantification of protein bands (50-100 μg ribonuclease per lane) in the gel was determined by means of a LKB 2202 UltroScan Laser Densitometer.

Reduction and Carboxymethylation of Proteins

Protein samples were reduced by preparing a solution containing 1 to 10 mg/ml protein, 6 M GuHCl, and 10 mg/ml dithiothreitol (DTT) in 0.1 M aqueous phosphate buffer, containing 1 mM EDTA, pH 8 under N_2 and then incubated overnight at room temperature. Carboxy-methylation was carried out according to the method of DiBella and Liener (1969): a 100 fold molar excess (over DTT) of iodoacetic acid neutralized in 2.5 ml 1 N KOH was added to 2 ml of the above protein solution and kept at pH 9.0, 25° C, in the dark for 30 minutes. The protein solution was dialyzed overnight vs. deionized water (or desalted on a G-25 column) followed by lyophilization.

Chemical Modification of Ribonuclease

Ribonuclease was acetylated in the presence of the ligand sodium pyrophosphate as described by Zale and Klibanov (1986): 20 mg of protein was added to 20 ml of 10 mM sodium pyrophosphate, pH 6.0, then 20 μl of acetic anhydride (5 x 4 μl) was slowly added over 30 minutes with the pH maintained at 6.0 by addition of 1 M NaOH. Solution was dialyzed against

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sodium pyrophosphate at pH 6.0 and then three times against distilled water at 4° C overnight, followed by lyophilization. The native enzyme (reduced and carboxymethylated) contained 10.9 free amino groups (100% of the expected 11 free amino groups in ribonuclease), while the chemically acetylated enzyme had 3.5 free amino groups (31%). The modified ribonuclease retained 60-70% of specific activity compared to untreated enzyme.

Lyophilization

For studies on enzyme thermostability in organic solvents, enzyme powders were dissolved in distilled water (4 mg/ml) and the pH of the solution was adjusted by automatic addition of 10 mM NaOH on a Radiometer (ABN 80 autoburette) pH stat under N_2. Samples were then snap-frozen with liquid N_2 and lyophilized for at least 3 days on a Labconco freeze dryer at <10 microns Hg vacuum.

Hydration of Ribonuclease Powder

Water content of ribonuclease was adjusted by incubation of the lyophilized enzyme (see above) for one week at room temperature in a desiccator (under vacuum) filled with various saturated salt solutions (potassium chloride, potassium nitrate, potassium sulfate) to give a variety of A_w values (0.84, 0.94, 0.97; Greenspan, 1977) or kept dry under phosphorus pentoxide. Actual water content of enzyme powder was determined by Karl Fischer titration (see below).

Gel Filtration Chromatography

For quantitative analysis of aggregation due to heating in organic solvents, protein samples (1 mg/ml) were injected onto a Pharmacia FPLC Superose 12 column equilibrated in 10

mM phosphate buffer (pH 7.2) with 0.15 M NaCl at a flow rate 0.1 ml/min. Peak areas were determined by weight.

Reactivation of "Irreversibly" Thermoinactivated Ribonuclease

Reactivation of thermoinactivated enzyme was carried out as described by Zale and Klibanov (1986): the enzyme powder was dissolved (after thermoinactivation) in 9 M urea (or 6 M guanidine hydrochloride) containing 70 mM dithiothreitol, 0.1 M Tris and 2 mM EDTA at pH 8.5. After reduction, the enzyme was desalted on a G-25 column equilibrated with 0.1 M acetic acid. Samples were then reoxidized for 18 hours by the addition (1:1) of a 1.0 mM of both reduced and oxidized glutathione in 2 mM EDTA, 2 M Tris buffer at pH 9.0. Recovery of specific activity (over 95% with untreated ribonuclease) was measured using the enzyme assay described above.

Karl Fischer Titration for Water Content Determinations

The water content of both organic solvents and enzyme powders were determined by titration via the Karl Fischer method (Laitinen and Harris, 1975; Connors, 1988). Titration was carried out with Hydranal reagents (Riedel-de Haen) on a Mettler DL18 titrator. The reaction of water with the Karl Fischer reagent is commonly written as follows although the chemistry is certainly more complicated than indicated (Connors, 1988):

$$(1) \quad C_5H_5 N{\cdot}I_2 + C_5H_5 N{\cdot}SO_2 + C_5H_5 N + H_2O \longrightarrow$$
$$2 C_5H_5 NH^+ I^- + C_5H_5 N{\cdot}SO_3$$

$$(2) \quad C_5H_5 N{\cdot}SO_3 + CH_3OH \longrightarrow C_5H_5 NH^+ CH_3 SO_4^-$$

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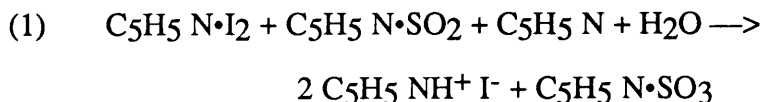
Detection is carried out by electrometric titration using an amperometric technique with two platinum electrodes (Connors, 1988).

The water content of enzyme powders in organic solvents was measured using a modified procedure of Zaks and Klibanov (1988): 15 mg of enzyme powder is added to 5 ml of organic solvent (water content of each is measured before the experiment) in a preweighed centrifuge tube. After sonication and a ten minute incubation, the mixture is centrifuged and the solvent is removed (water content of solvent is determined). The weight of the remaining solvent saturated protein powder is determined via difference by reweighing the centrifuge tube. Five ml of anhydrous dimethylsulfoxide (water content already determined) is added to dissolve the wet powder and the resulting water content of the solution is then determined. A water balance is calculated on the entire system, as described by Zaks and Klibanov (1988), in order to determine the water content of the enzyme powder in organic solvent.

Differential Scanning Calorimetry

The thermal denaturation of RNase in organic solvents and as a dry powder was measured with a Perkin Elmer DSC 7 differential scanning calorimeter. Samples of enzyme powder (1-5 mg) were (i) added directly or (ii) incubated in an organic solvent, centrifuged and the bulk solvent was removed; the powder (either dry or solvent saturated) was then placed into aluminum pans and sealed. An empty aluminum pan was sealed and used as a reference sample. The scanning range was 25-130° C at a heating rate of 10° C min^{-1}.

3.2.3 Kinetic Studies

Heat-induced ß-elimination of cystine residues

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Heat-induced ß-elimination of cystine residues was measured on the basis of disappearance of potential sulfhydryl groups. Protein solutions (10-50 µM) in an aqueous buffer of a given pH (in the presence or absence of a denaturant) were placed in test tubes, sealed with serum stoppers, and incubated in a thermostatically controlled glycerol bath at 100° C. Reduction of cystine to cysteine residues was done according to a modified procedure of Brown (1960): One-half milliliter of 30 mg/ml sodium borohydride and one drop of antifoam A (Sigma) were added to 2 ml of a protein solution (pH adjusted to 8) and incubated at 50° C for 60 minutes. After cooling on ice, 0.5 ml of 3 N acetic acid was added and after 1 hour analyzed for free thiols as outlined below. ß-Elimination of disulfides is known (Whitaker and Feeney, 1983) to generate free sulfhydryls which distort the kinetics of the disappearance of potential SH groups, especially at high conversions of the ß-elimination reaction. To account for this, in separate experiments the borohydride was omitted during the measurements, the time course of accumulation of free thiols was determined, and subsequently subtracted from the data obtained with $NaBH_4$ present (see above) to yield the net time course of the heat-induced cystine destruction.

Heat induced thiol-catalyzed disulfide interchange of disulfide bonds

The time course of reshuffling of disulfide bonds in mixed disulfides of trypsinogen and lysozyme with glutathione was followed by two independent methods. (i) Protein solutions (30 mM) at pH 6 or 8, containing 6 M guanidine hydrochloride and 1 mM EDTA, were heated at 100° C as described above, then cooled, acidified to pH 3, and desalted on a Sephadex G-25 column equilibrated with 0.1 N CH_3COOH. Protein fractions were pooled, and water was evaporated in a speed-vac concentrator. The residue was then dissolved in 6 N hydrochloric acid (Pierce), degassed, placed in sealed ampoules, incubated at 110° C for 24 hours, and then subjected to HPLC amino acid analysis (see below). Each -S-S- interchange results in a loss of one glutathione moiety, i.e., one glutamic acid residue in the subsequent HPLC amino acid

analysis. Hence, the interchange reaction was followed by a reduction in the total number of Glu in the protein fraction. (ii) Protein solutions (30 μM) at pH 6 or 8, containing 6 M guanidine hydrochloride and 1 mM EDTA, were heated at 100° C as described above, cooled, passed through an Amicon ultrafiltration membrane (molecular weight cutoff of 10,000 daltons), reduced with NaBH$_4$ (Brown, 1960), acidified to decompose the reductant, and titrated for free thiols with Ellman's reagent. When ^{3}H-labelled glutathione was used to prepare mixed disulfides with lysozyme and trypsinogen, the concentration of the tripeptide in the low molecular weight fraction passed through the ultrafiltration membrane was measured by its radioactivity.

Alternatively, the disulfide interchange reaction was measured by incorporation of ^{3}H-labelled glutathione into trypsinogen. A buffered aqueous solution (pH 8.0), containing 50 μM trypsinogen and radioactively labelled oxidized glutathione in the presence of 6 M guanidine hydrochloride and 1 mM EDTA, was heated at 100° C as described above. Periodically, samples were withdrawn, cooled, acidified, and subjected to gel permeation chromatography on a Sephadex G-25 column equilibrated with 0.1 M acetate buffer (pH 4.0). The protein fractions were then analyzed for radioactivity in a liquid scintillation counter.

Heat-induced generation of free thiols

Buffered protein solutions (20-200 μM), containing 6 M guanidine hydrochloride and 1 mM EDTA, were heated at 100° C as described above, cooled, and assayed for free sulfhydryl groups with Ellman's reagent. In order to distinguish between low molecular weight and protein-bound thiols, the protein solutions after heating were passed through an Amicon ultrafiltration membrane (10,000 MW cutoff), and then both low and high molecular weight fractions were assayed separately.

Irreversible thermoinactivation of glucose isomerase

Free or immobilized glucose isomerase was placed in a thermoinactivation solution whose pH was adjusted at room temperature to the value resulting in pH 8.0 at the temperature of the thermoinactivation experiment, as determined by our calibration curves (for Tris buffers; see Appendix 1). For pH 9 experiments, the pH of a 0.1 M glycine buffers containing 10 mM $MgSO_4$ and 2 M xylitol was measured at 25° C (8.6, 9.0, 9.5, 10.0, 10.45). After equilibration at 90° C, the pH was remeasured to obtain a calibration curve (7.35, 7.7, 8.5, 8.6, 9.0). For D_2O experiments, the pH of a series of D_2O solutions containing 0.1 M Tris, 10 mM $MgSO_4$ and 2 M xylitol were measured at 25° C (pH values 8.0, 8.59, 8.9, 9.0). After equilibrium at 90° C, the pH of the D_2O solutions were remeasured (pH values 6.65, 7.3, 7.60, 7.75). The pH values were then converted to pD values according to the formula pD = [(meter reading) + 0.4] as described by Schowen (1978) and pD values of 7.05, 7.7, 8.0, 8.15 were obtained for a calibration curve.

Enzyme solutions or suspensions were placed in ampoules, sealed, and then incubated in a thermostatically controlled glycerol bath at a desired temperature. Periodically, samples were removed, cooled (and washed five times with 0.1 M HEPES buffer at pH 8 containing 10 mM $MgSO_4$ in the case of immobilized glucose isomerase), and then assayed for enzymatic activity as described above. In the case of immobilized glucose isomerase, no appreciable leakage (<1%) of the protein from the support was detected (via Lowry assay) during heating. It should be pointed out that the irreversible thermoinactivation of immobilized glucose isomerase has an extremely high activation energy (180 kcal/mol, see text); consequently, even variations as small as 1° C can affect the observed rate constant of inactivation by as much as two fold.

Irreversible thermoinactivation of enzymes in organic solvents

One to two milligrams of enzyme powder, which had been lyophilized from a given pH value as described above, was carefully weighed out and transferred into a glass ampoule. One ml of organic solvent was added, the ampoule was sealed, followed by sonication for 10 seconds. The samples were then heated for various times in a Temp-Blok Module Heater filled with glycerine. After allowing the sample to cool to room temperature, the ampoules were opened and the solvent removed until dryness by evaporation under reduced pressure in a speed-vac concentrator. Aqueous buffer of 10 mM sodium phosphate, pH 7.2, containing 0.15 M NaCl (except for chymotrypsin which was resuspended in 1 mM HCl) was then added to give a 1 mg/ml enzyme solution.

IV. RESULTS AND DISCUSSION

4.1 Thermal Destruction Processes in Proteins Involving Cystine Residues

One of the most popular alterations in protein molecules by means of site-directed mutagenesis has been the introduction of new disulfide bonds, in particular to enhance enzyme thermostability and stability against heat-induced autolysis (Wetzel, 1987; Creighton, 1988; see literature survey). Recent work in our laboratory has elucidated the mechanisms of irreversible thermoinactivation of two model enzymes, lysozyme (Ahern and Klibanov, 1985) and ribonuclease (Zale and Klibanov, 1986). In both cases, one the causes of irreversible loss of enzymatic activity at high temperatures ($90°$ - $100°$ C) and neutral pH is the destruction of disulfide bonds (see literature survey). Furthermore, cystine residues in proteins undergo significant degradation at alkaline pH even at ambient temperatures (Florence, 1980; Whitaker and Feeney, 1983). These data cast in doubt the wisdom of engineering additional -S-S- bonds in enzymes when intended for use under extremes of pH or temperature.

In the present study we verified the validity and generality of the foregoing conclusion. More than one dozen unrelated proteins with different numbers of disulfide bonds were examined for cystine destruction at extreme temperatures. The reactions destroying cystine residues, at high temperatures ($100°$ C) and neutral pH (from 4 to 8), are characterized and quantitatively examined. Their implications for the thermostability of enzymes are also analyzed.

4.1.1 Kinetics of cystine destruction

The objective of this research was to identify and investigate the processes leading to destruction of cystine residues in proteins at $100°$ C in the pH range where most enzymes function (pH 4 to 8), and to determine the dependence of these processes on the nature of the protein. Bovine pancreatic insulin was selected as the initial subject: this small (6,000 daltons)

protein contains 3 cystine residues, no cysteines, and its structure has been thoroughly characterized (Meienhoffer, 1979). A 50 mM aqueous solution of insulin was incubated at 100° C and pH 8.0 (0.1 M phosphate buffer). Periodically, aliquots were withdrawn, reduced with sodium borohydride, and the free sulfhydryl groups formed analyzed with Ellman's reagent. The open circles in Figure 5 show the data obtained. One can see that the number of potential thiols (which reflects the number of intact cystine residues in insulin) rapidly decreases with time of heating. The experimental points closely obeyed the first order kinetic rule (the correlation coefficient of 0.999); a first order rate constant of 1.04 hr^{-1} was calculated in semilogarithmic coordinates which corresponded to the half-life of 40 min.

The fact that the destruction of cystine residues in insulin at 100° C and pH 8 is a first order process is highly significant. We followed that process to the degree of conversion of about 90%. Since there are 3 cystine residues in insulin, this means that more than 2.5 of them (on average) have been destroyed. The excellent agreement observed between the experimental data and a theoretical curve corresponding to first order kinetics (Figure 5) indicates that the thermal stabilities of different disulfide bonds in insulin are comparable.

We then investigated the time courses of destruction of cystine residues at 100° C and pH 8 in eleven other natural proteins plus two hybrid ones prepared by us: mixed disulfides of lysozyme and trypsinogen with glutathione. Most of these proteins formed precipitates upon heating. Since this aggregation would severely complicate all subsequent measurements, 6 M guanidine hydrochloride was added to the protein solutions to avoid it. This denaturant should disrupt non-covalent interactions leading to aggregation, and yet is not expected to appreciably affect covalent reactions (Klibanov, 1983). The last assertion was experimentally confirmed (also see below) with insulin: when the protein was heated in the presence of 6 M guanidine hydrochloride (closed circles in Figure 5), the time course of destruction of cystine residues did not significantly differ from that obtained in the absence of the denaturant.

Table 2 presents the half-lives (obtained from the first order rate constants) of destruction of -S-S- bonds in different proteins at 100° C (in the presence of 6 M guanidine hydrochloride to
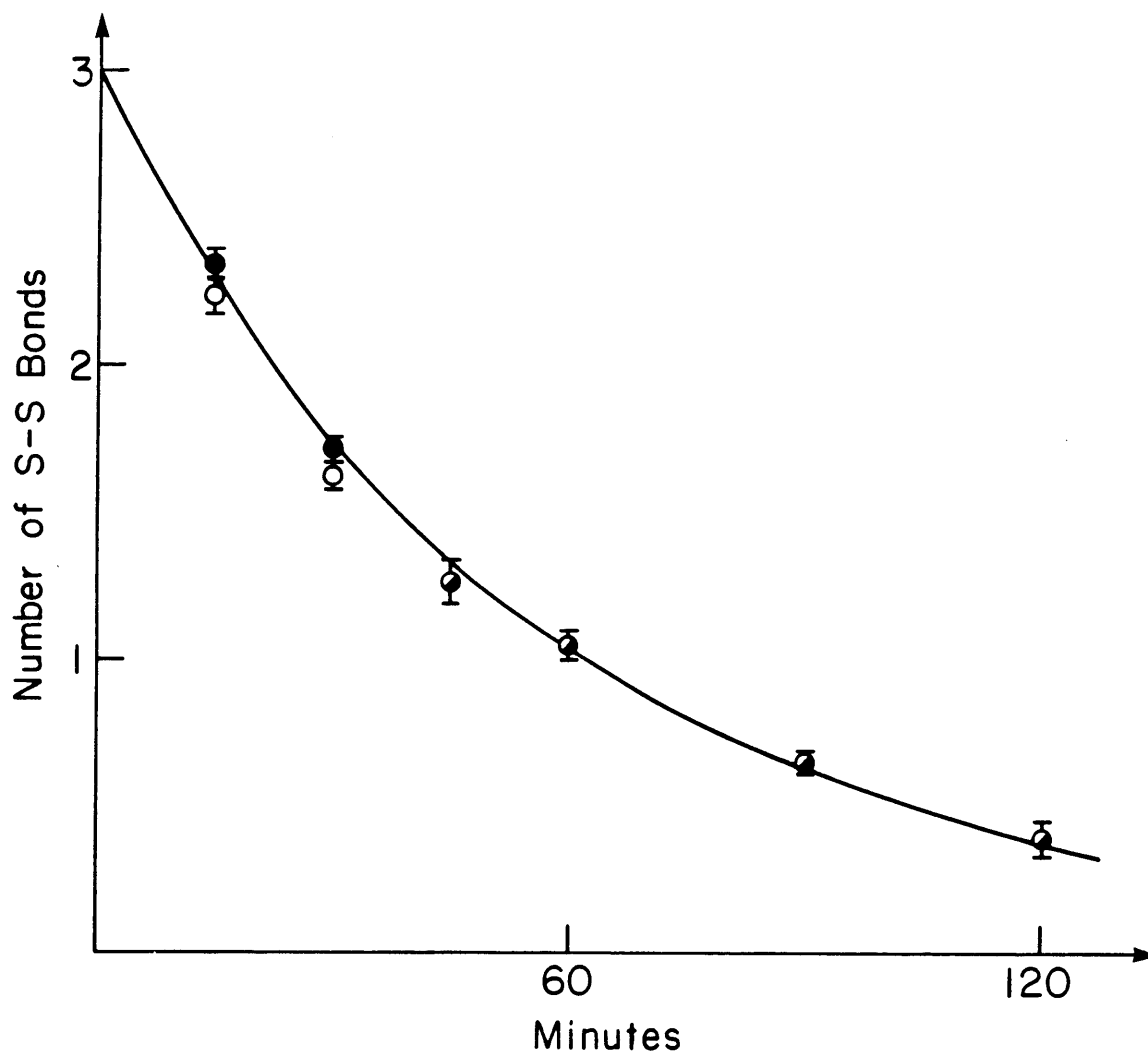
Figure 5 - The time course of the destruction of disulfide bonds in insulin at 100° C and pH 8.0 in the absence (open circles) and in the presence of 6 M guanidine hydrochloride (closed circles). The experimental protocol is described in the Experimental Section; 50 μM initial insulin concentration, 0.1 M phosphate buffer. All experiments were carried out in triplicate, and both the average values and the error bars are given in the Figure 5. The line shown is a theoretical curve corresponding to the first-order model with the rate constant of 1.0 hr $^{-1}$.

prevent protein aggregation) and the correlation coefficients between the experimental results and the corresponding first order kinetics. These data afford a number of important conclusions. First, the correlation coefficients obtained (see Table 2) are very high (greater than 0.99 for 12 out of 14 proteins and greater than 0.97 for all). This suggests that, despite a large diversity in the number of cystine residues in the proteins examined (from 1 to 17), there are no dramatic differences in the inherent thermal stabilities among different disulfides in each protein. Second, the rate constants of destruction of cystine residues in fourteen proteins, that vary in terms of their origin, size, amino acid composition, and sequence, are surprisingly similar: the difference in k values between the most stable and the most labile protein is only about two fold.

The two-fold difference in k values, along with a correlation coefficient below 0.99 for first order kinetics, was mainly due to the hybrid proteins (mixed disulfides between protein and glutathione). Oxidized glutathione, with its γ-glutamyl linkage to cysteine, does not have the same electron withdrawing groups to labilize the α-hydrogen of protein bound cystine; therefore, one does not expect ß-elimination (see next section) to occur as rapidly. Indeed, studies by Schneider and Westley (1969) show that the destruction of cystine in oxidized glutathione (0.5 N NaOH, 37° C) proceeds via ß-elimination, but at a slower rate than the protein-bound cystine of insulin. We experimentally measured cystine destruction in oxidized glutathione at pH 8 and 100° C and found it to be similarly slow with a half-life of 126 minutes (corr. coeff. 0.997).

The differences in thermostabilities of disulfide bonds in various proteins are even lower at pH 6, where the half-life for the most stable one is less than double of that for the most labile protein (the right part of the Table 2). Again, excellent correlation coefficients (all better than 0.995) were obtained indicating a close adherence to first order kinetics. The half life of the cystine residue in oxidized glutathione is 13.5 hrs (corr. coeff. 0.995) at pH 6 and 100° C; therefore, the detectable differences between oxidized glutathione versus protein bound cystine diminishes as the rate of ß-elimination slows down (proportional to the pH value; see next section).

Protein	Number of -S-S- bonds	pH 8		pH 6	
		half-life (hours)	correlation coefficient	half-life (hours)	correlation coefficient
chymotrypsinogen	5	0.83	0.994	9.6	0.997
conalbumin	15	0.63	0.998	9.8	0.998
insulin	3	0.67	0.999	9.3	0.997
lysozyme	4	0.63	0.997	9.2	0.996
lysozyme-glutathione mixed disulfide	8	1.42	0.971	10.0	0.996
ovalbumin	1 (4SH)	1.08	0.994	15.8	0.997
papain	3 (1SH)	0.78	0.994	10.0	0.999
pepsinogen	3	0.78	0.998	9.5	0.998
peroxidase	4	0.93	0.996	13.9	0.997
ribonuclease	4	0.67	0.998	13.5	0.998
serum albumin	17 (1SH)	0.90	0.996	9.0	0.999
transferrin	19	0.68	0.994	13.6	0.998
trypsinogen	6	0.62	0.996	9.4	0.999
trypsinogen-glutathione mixed disulfide	12	1.37	0.973	10.3	0.995

Table 2 - Half-lives of cystine residues, during their destruction by heat-induced ß- elimination, in different proteins at 100° C. The time courses of ß-elimination of disulfide bonds in proteins were followed as described in the Experimental Section. All protein solutions contained 6 M guanidine hydrochloride to prevent aggregation both at pH 8.0 and at pH 6.0 (0.1 M phosphate buffer in both instances). As described in the text, the presence of the denaturant does not appreciably affect the half lives of cystine residues. All proteins (see Experimental Section for their origins) were electrophoretically pure. The numbers in parentheses correspond to free cysteines. All data are taken from Barker et al. (1986). All experimental points were taken in triplicate, and the first order rate constants, subsequently converted to half-lives, were determined by linear regression in semilogarithmic coordinates. A degree of conversion in the heat-induced ß-elimination of disulfide bonds was no less than 80-90%.

One would expect that at 100° C in the presence of 6 M guanidine hydrochloride no appreciable secondary or tertiary structure remains (Tanford, 1968). Therefore, the data in Table 2 suggest that thermal stability of disulfide bonds in proteins is not dramatically affected by the primary structure, i.e., by the cystine residues' neighbors. Two of the fifteen proteins listed in Table 2 do not noticeably aggregate when heated at 100° C even without guanidine hydrochloride. Consequently, we determined the half-lives of thermal destruction of -S-S- bonds at pH 8.0 in ribonuclease and insulin in the absence of the denaturant and found them to be 0.65 and 0.70 hours, respectively. These values are nearly identical to those obtained in the presence of 6 M guanidine hydrochloride (Table 2). Since heating alone does not disrupt all secondary and tertiary structures in proteins, and the remaining ordered structures tend to concentrate around disulfide bonds (Tanford, 1968), the data obtained suggest that thermal stability of cystine residues in proteins is not affected by those remaining elements of ordered structure. Moreover, the coincidence of the rate constants of destruction of -S-S- bonds in the absence and presence of 6 M guanidine hydrochloride confirms that, as assumed before, the denaturant does not affect this covalent reaction.

4.1.2 ß-Elimination of cystine residues in proteins

Inspection of Table 2 reveals that destruction of disulfides in proteins at 100° C is much slower at pH 6 than at pH 8. The process is slower still at pH 4: for example, for ribonuclease and insulin the half-lives of thermal destruction of -S-S- bonds at pH 4 are 5.6 and 6.1 days, respectively. These data indicate that the examined process is catalyzed by hydroxide ions. The most likely candidate for this process is ß-elimination of cystine residues which readily takes place in dilute alkali at room temperature (Whitaker and Feeney, 1983) and hence can be expected to occur at 100° C even at neutral pH (Zale and Klibanov, 1986).

ß-Elimination of cystine residues involves a heterolytic cleavage of the -S-S- bond to form dehydroalanine and thiocysteine residues (Whitaker and Feeney, 1983), as shown

previously in Figure 1 (see literature survey). Dehydroalanine residues can further react with nucleophiles, chiefly ε-amino groups of lysine residues in proteins to form a new cross-link, lysinoalanine (Bohak, 1964). Hence, to verify the ß-elimination mechanism, one has to identify dehydroalanine and lysinoalanine among the products.

Curve c in Figure 6 shows the time course of accumulation of dehydroalanine residues upon incubation of transferrin (one of the proteins from Table 2) at 100° C and pH 8.0. Curve d represents formation of lysinoalanine residues during that heating. For comparison, curves a and b depict the time course of thermal destruction of cystine residues (measured by two independent methods). One can see that both residues, which are hallmarks of ß-elimination, are indeed produced in substantial quantities. Furthermore, the sum of dehydroalanines and lysinoalanines formed (curve e) approaches the number of cystines destroyed: e.g., after 1 hour of heating the former two processes combined account for more than three quarters of the cystines lost. A similar result has been obtained with ribonuclease (Zale and Klibanov, 1986).

The foregoing data strongly indicate that at 100° C and pH 8 disulfides in proteins are destroyed via the ß-elimination reaction. Further evidence for this mechanism was obtained using pepsinogen as a model for cysteine destruction where ß-elimination has been shown to occur at 3-5% the rate of cystine residues under alkaline conditions (Whitaker and Feeney, 1983). Pepsinogen's cystine residues were reduced to cysteine residues with dithiothreitol (DTT). The reduced protein was heated in the presence of 10 mM DTT at 100° C and pH 8, desalted on a Sephadex G-25 column, and then assayed for free thiols. The half-life of cysteine residues was found to be 23.1 hrs (corr. coeff. 0.999) which is 3.4% of value obtained for cystine destruction in pepsinogen under the same conditions.

4.1.3 Formation of Free Thiols

The thiocysteine residues formed as a result of ß-elimination in protein bound cystines are rather unstable at alkaline pH and can undergo decomposition via a complex mechanism to yield

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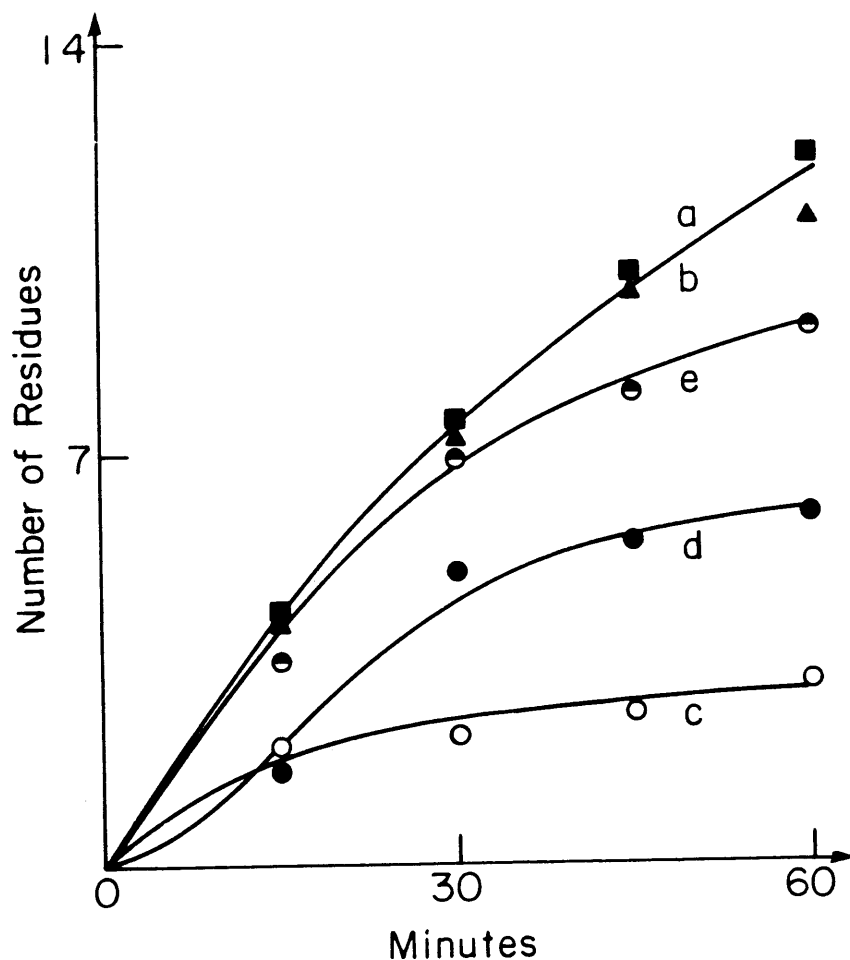
Figure 6 - ß-elimination of cystine residues in transferrin at 100° C and pH 8.0. (a) and (b) - time courses of destruction of cystine residues measured on the basis of disappearance of potential sulfhydryl groups (following reduction) and decrease of carboxymethylated cysteine residues determined by HPLC amino acid analysis (following reduction, carboxymethylation and acid hydrolysis), respectively; (c) - time course of accumulation of dehydroalanine residues; (d) - time course of accumulation of lysinoalanine residues; and (e) - the sum of (c) and (d) (dehydroalanines plus lysinoalanines). Conditions: 40 µM initial transferrin concentration, 0.1 M phosphate buffer containing 6 M guanidine hydrochloride; the experimental protocol is described in the Experimental Section.

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a mixture of hydrosulfide, elemental sulfur, and cysteine residues (Nashef et al., 1977; Whitaker and Feeney, 1983). Curve a in Figure 7 shows the accumulation of free thiols during ß-elimination of cystine residues in lysozyme at 100° C and pH 8.0, while curve b in Figure 7 shows the predicted concentration of thiols based on the kinetics of ß-elimination. A likely reason that less than stoichiometric amounts of thiols are observed after the initial few minutes of ß-elimination (Figure 7) is the destruction or volatilization of persulfide and other sulfhydryl species.

Similar data were obtained with lysozyme at pH 6.0 and for trypsinogen both at pH 8.0 and pH 6.0: the kinetics of thiol generation were identical for the two proteins with free thiol formation more rapid at pH 8 than pH 6 (a steady state level of thiols was reached in 20 minutes at pH 6 vs. 10 minutes as seen in Figure 7), as would be expected from the base-catalyzed mechanism of ß-elimination. Moreover, regardless of protein concentration (50-500 μM lysozyme), the amount of detectable thiols at pH 8, 100° C were 33% of that predicted from the kinetics of ß-elimination at 10 minutes and 5.5% at 20 minutes. In all cases, during the initial time period, each act of ß-elimination resulted in one thiol formed. No color was detected when Ellman's reagent was used to titrate the solution of lysozyme heated in the presence of 10 μM Cu^{2+} (which did not appreciably affect the assay); since the latter catalyzes autooxidation of sulfhydryl groups (Cecil and McPhee, 1959), this result confirms the thiol nature of the products previously formed.

To elucidate the nature of these thiols, a 50 μM lysozyme solution, which had been incubated at 100° C and pH 8.0 for 7.5 minutes, was passed through an Amicon ultrafiltration membrane with a molecular weight cutoff of 10,000 daltons. Titration of sulfhydryls in both high molecular weight and low molecular weight fractions revealed that about 70% of the thiols were protein-bound (presumably, thiocysteines and/or cysteines) and the rest penetrated through the molecular sieve membrane. At least a portion of the later was a hydrosulfide (HS^-): after 7.5 minutes that fraction was about 35%. Very similar values were obtained with a mixed disulfide of lysozyme and glutathione and with ribonuclease (Zale and Klibanov, 1986).
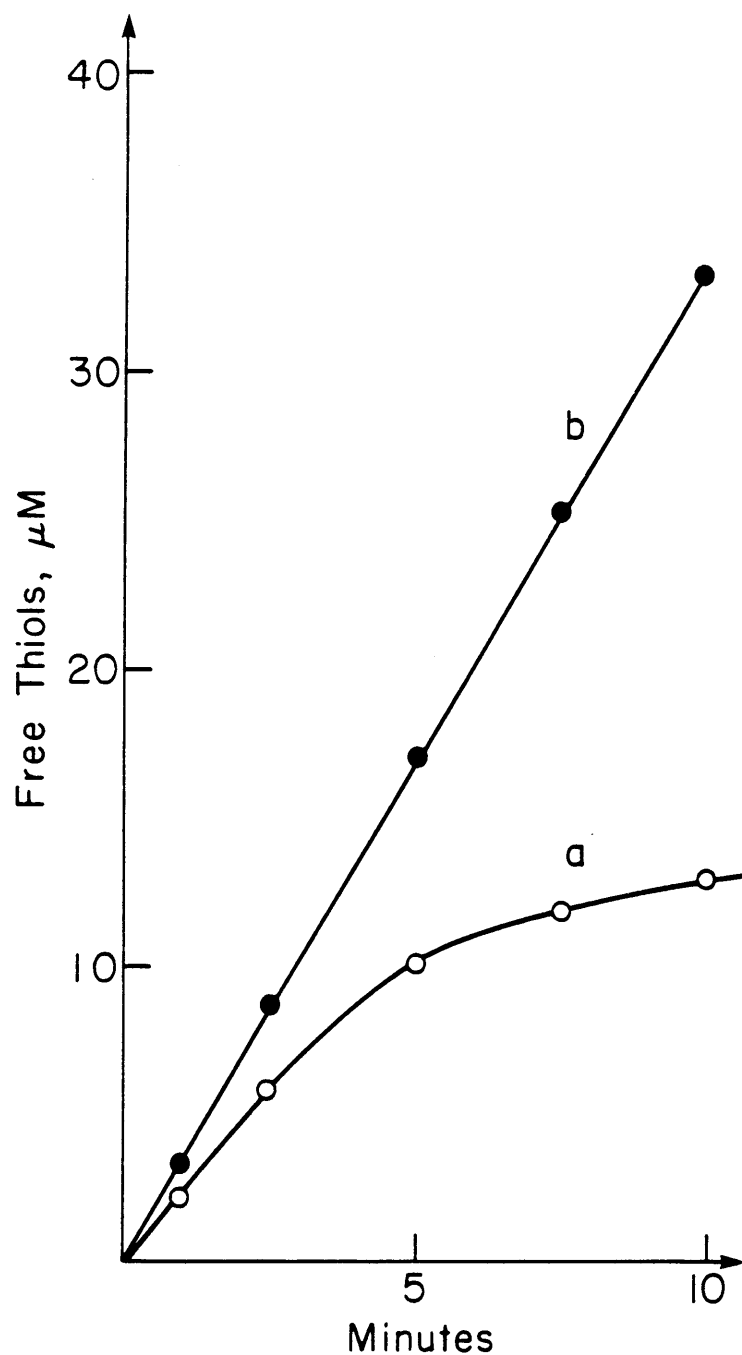
Figure 7 - Formation of free thiols during ß-elimination of cystine residues in lysozyme at 100° C and pH 8.0. (a) - The experimentally determined time course of generation of free SH groups; (b) - the time course predicted assuming that each ß-elimination event results in the production of a sulfhydryl group. Conditions: 50 μM initial lysozyme concentration, 0.1 M phosphate buffer containing 6 M guanidine hydrochloride (to prevent protein aggregation); for experimental procedures, see Experimental Section.

4.1.4 Thiol-catalyzed disulfide interchange

Having established that when proteins are heated at 100° C at neutral pH they undergo ß-elimination which yields free thiols, it was then prudent to ascertain whether these thiols can cause any further damage in protein molecules. Ryle and Sanger (1955) discovered that at neutral and alkaline pH short peptides can undergo disulfide interchange reaction at 35° C which is catalyzed by thiols. The latter carry out nucleophilic attack on the sulfur atom of a disulfide in the same way as in thiol-disulfide interchange (see literature survey). Therefore, we have undertaken a study into the possibility of such a process in proteins at 100° C.

A straightforward way to detect a disulfide interchange in a protein is to subject it to proteolytic digestions, followed by two dimensional paper-chromatographic analysis of the peptides formed and comparison with those for the native protein. Unfortunately, this methodology was not feasible in our case because (i) other processes occur in proteins upon heating (Ahern and Klibanov, 1985; Zale and Klibanov, 1986) that will also change the peptide patterns, and (ii) more than a single scrambled protein can be expected, making the subsequent analysis difficult. Thus, we were forced to develop another approach. Trypsinogen (50 μM) was incubated with 50 μM ^{3}H-labelled oxidized glutathione at 100° C and pH 8.0. Then the unreacted glutathione and other low molecular weight products were separated by gel permeation chromatography, and the radioactivity incorporated into the trypsinogen was measured. It was found that after a 5 minute incubation, 0.31 moles of glutathione were bound to 1 mole of trypsinogen. This binding was indeed via a -S-S- bond because (i) when the complex was rechromatographed, its radioactivity did not change, and (ii) when the complex was rechromatographed following an overnight incubation with 0.1 M dithiothreitol, its radioactivity diminished by more than 10 fold. The observed reaction was catalyzed by thiols, and when the protein and radiolabeled peptide were heated in the presence of a thiol scavenger (100 μM Cu^{+2}), virtually no radioactivity was incorporated into trypsinogen.

Although the above data unequivocally show that thiol-catalyzed disulfide exchange takes place at 100° C and pH 8.0, the methodology employed is hardly amenable to a quantitative investigation. Therefore, an alternative was elaborated. Trypsinogen and lysozyme were reduced and then glutathione moieties were attached to sulfhydryl groups in each protein. As a result, hybrid proteins - mixed disulfides of trypsinogen and lysozyme with glutathione - were prepared. When an act of a disulfide exchange occurs in such a protein, a glutathione moiety is released which can be readily detected.

Curve a in Figure 8 depicts the time course of the disulfide interchange in the hybrid of lysozyme with glutathione at 100° C and pH 6.0 which was measured as follows: 30 μM solution of the protein was heated in the presence of 6 M guanidine hydrochloride (to prevent aggregation), cooled, passed through the ultrafiltration membrane to separate glutathiones released from the protein, and then the remaining protein was subjected to acid hydrolysis and amino acid analysis. One can see that the number of glutamic acid residues per protein molecule (used as an indicator of the number of glutathiones, see experimental section) steadily decreases. The disulfide interchange process was confirmed by an independent method of measurement (Figure 8, curve b), where the sulfhydryl groups of the released glutathiones were assayed with Ellman's reagent following ultrafiltration and reduction with sodium borohydride. Similar results were obtained for a mixed disulfide of trypsinogen with glutathione (Figure 9). In this case, a radioactive hybrid protein was prepared by using ^{3}H-labelled glutathione. The release of glutathione from the hybrid protein could now be determined via a second method - accumulation of radioactivity in the low molecular fraction over time.

It should be stressed that the process reflected in Figures 8 and 9 (with a half-life of about 10 minutes at pH 6) is almost two orders of magnitude faster than ß-elimination under the same conditions (Table 2), and hence the two processes are entirely different. As shown in Figure 10, the rate of thiol-catalyzed disulfide interchange in the lysozyme hybrid increased when the pH was raised from 6 to 8, was inhibited by thiol scavengers such as Cu^{+2}, and was accelerated by an exogenous sulfhydryl (cysteine). At pH 8.0 and 100° C both hybrid proteins had half-lives
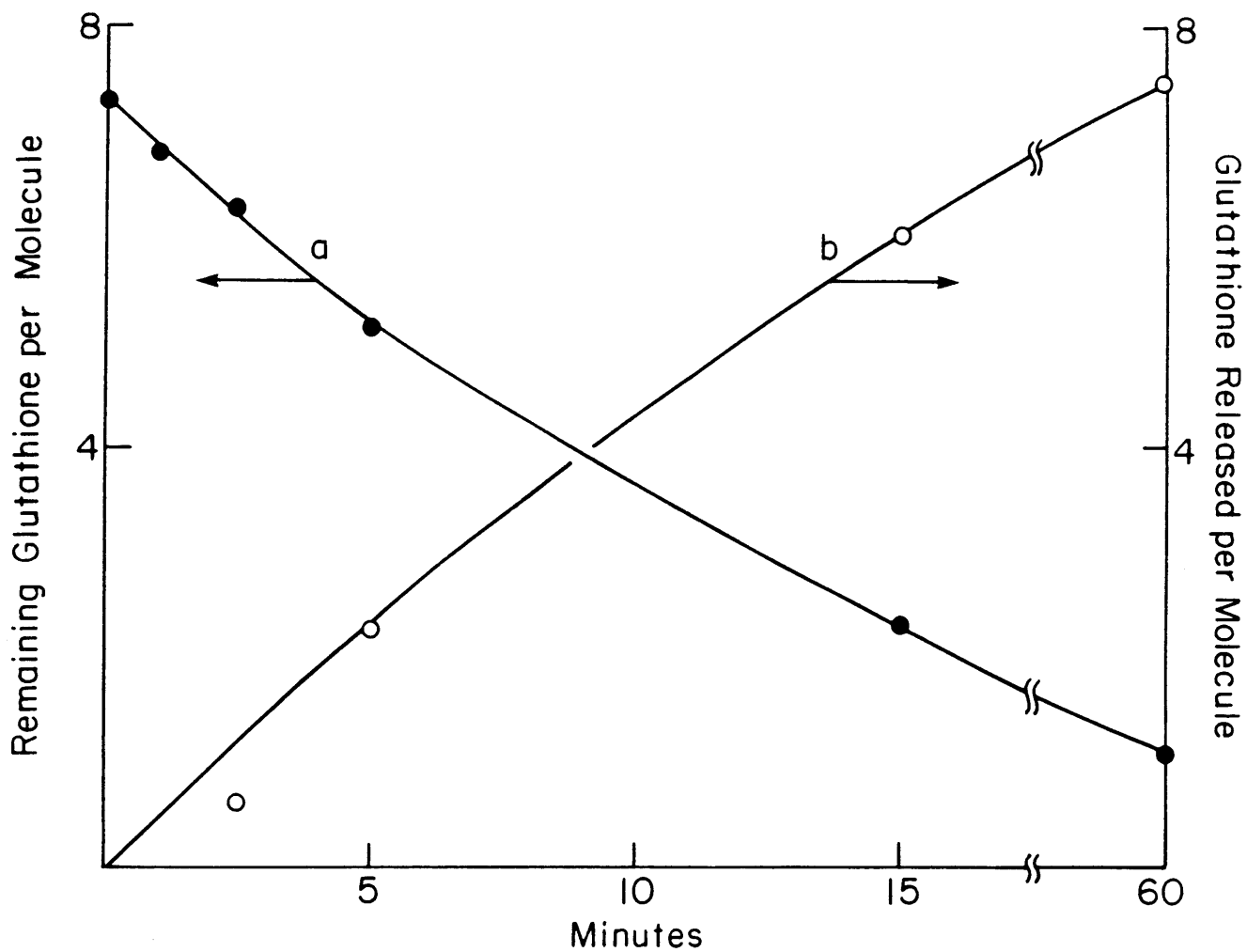
Figure 8 - Disulfide interchange in the lysozyme-glutathione hybrid protein at 100° C and pH 6.0. (a) - Release of glutathione from the mixed disulfide, as measured by amino acid analysis; (b) - release of glutathione as measured by its accumulation in the low molecular weight fraction. Conditions: 30 μM lysozyme-S-S-glutathione, 0.1 M phosphate buffer containing 6 M guanidine hydrochloride (to prevent protein aggregation) and 1 mM EDTA; for experimental procedures, see Experimental Section and text.
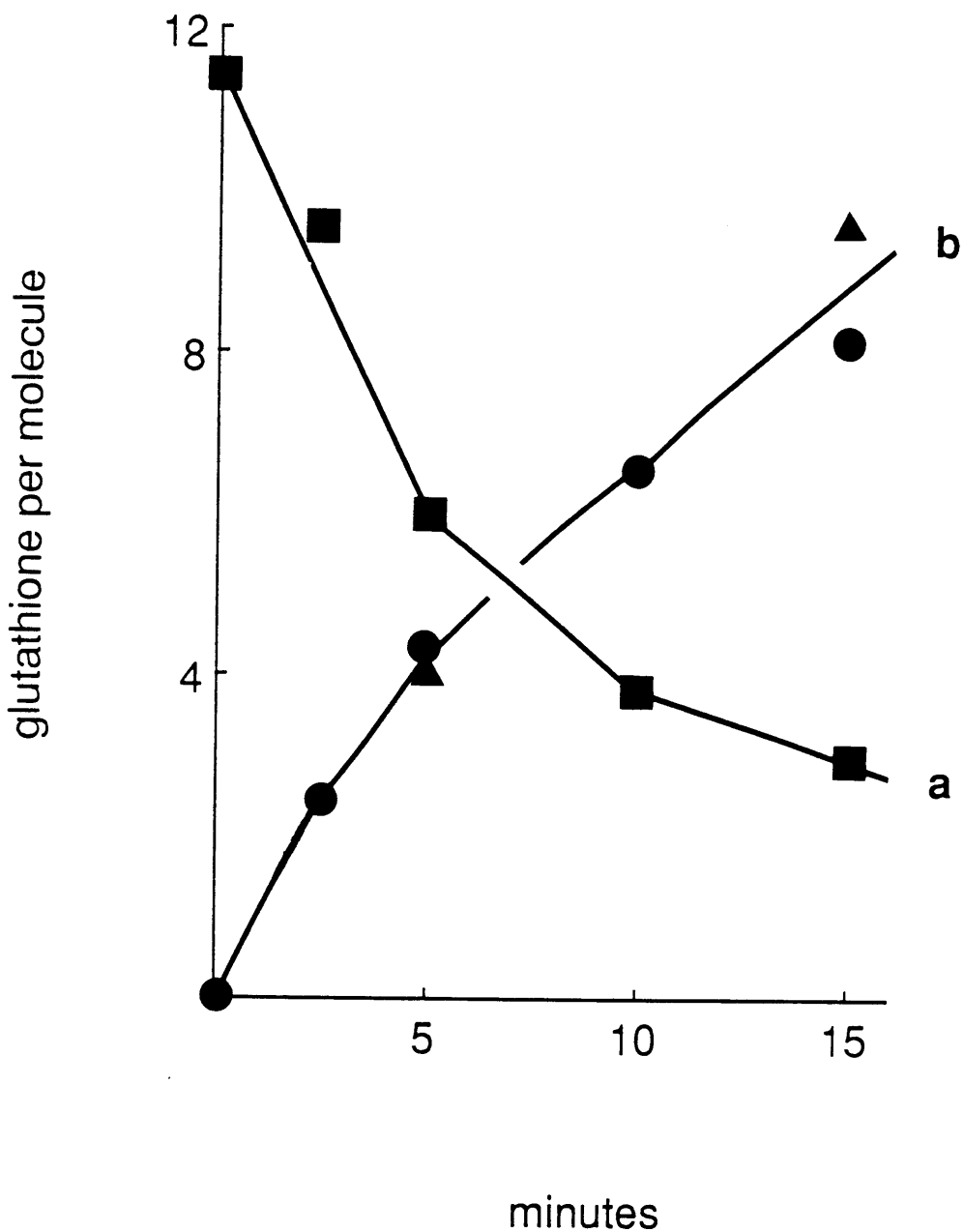
Figure 9 - Disulfide interchange in the trypsinogen-glutathione protein at 100° C and pH 6.0. (a) - Release of glutathione from the mixed disulfide as measured by amino acid analysis; (b) - release of glutathione as measured by appearance of potential sulfhydryl groups (following reduction) (●), or ^{3}H-labelled glutathione (▲) in the low molecular weight fraction. Conditions: 30 μM hybrid protein, 0.1 M phosphate buffer containing 6 M guanidine hydrochloride (to prevent aggregation) and 1 mM EDTA; for experimental procedures, see Experimental Section.
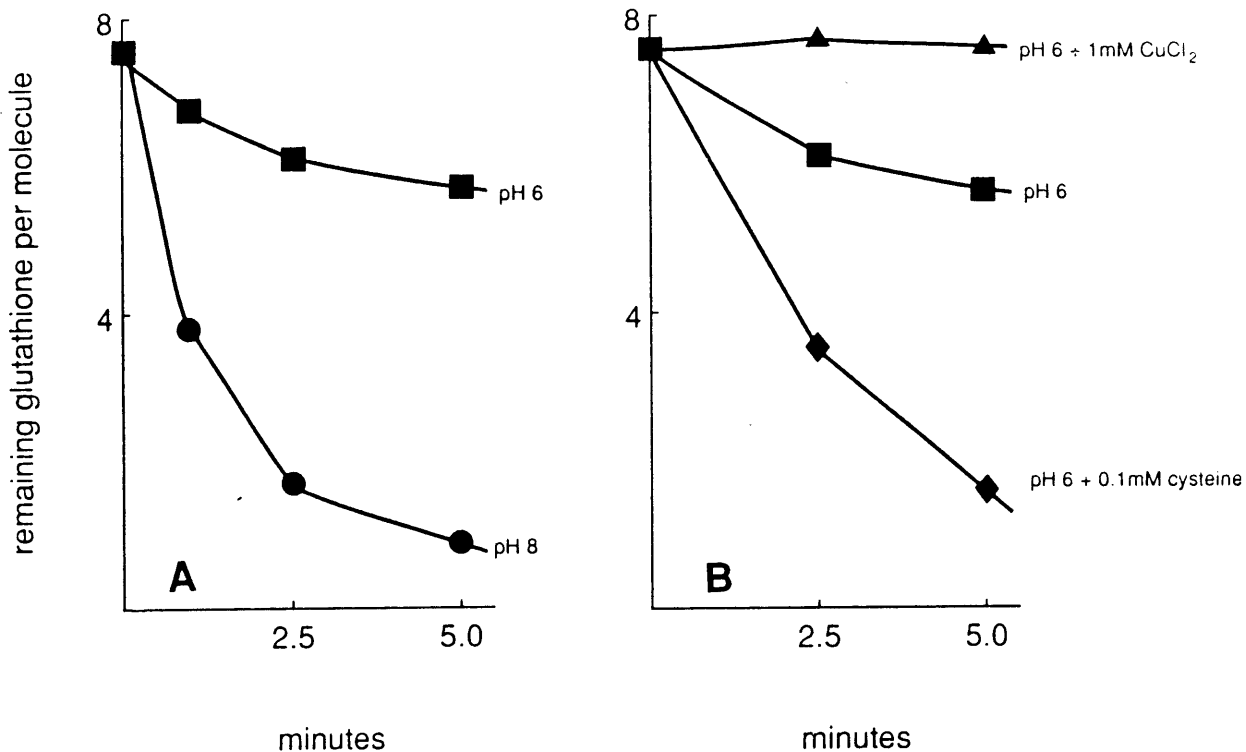
Figure 10 - Effect of additives on disulfide interchange in the lysozyme-glutathione hybrid protein at 100° C. (A) Release of glutathione from mixed disulfide at pH 6 (■) and pH 8 (●) (B) Release of glutathione from mixed disulfide at pH 6 (■), in the presence of 1 mM $CuCl_2$ (▲), or 0.1 mM cysteine (◆). Experimental conditions were the same as Figure 9. Time course of release of glutathione was measured by HPLC amino acid analysis as described in Experimental Section.

for S-S interchange of about 1.6 minutes, again much faster than ß-elimination. This value (when adjusted for temperature) compares favorably with the one determined by Zale and Klibanov (1986) where the rate of disulfide interchange was measured indirectly via thermal inactivation-reactivation experiments. As a final note, the measurements of the ß-elimination process described in the previous section were unaffected by the disulfide interchange as the latter does not change the total number of potential thiols.

Although these kinetic studies of disulfide interchange quantitatively characterize the process, the actual order and mechanism of the reaction(s) are difficult to determine for several reasons: (i) the exact stoichiometry of the thiols produced and destroyed during ß-elimination is not known. (ii) Although ß-elimination is a prerequisite step for disulfide interchange, it is not necessarily the rate-limiting step once it has occurred. In fact, the kinetics of disulfide interchange in the lysozyme hybrid did not appreciably change over a 100 fold range of protein concentrations; thus, although more free thiols were present at higher protein concentrations, the rate of S-S exchange remained constant. (iii) The reaction is clearly not first-order since both ß-elimination, and subsequent reaction(s) involving free thiols, must initially occur before the thiol-catalyzed disulfide exchange can proceed.

During the heating of the hybrid-protein at 100° C, the protein-bound cystine residues are destroyed via ß-elimination at high temperatures and neutral pH. This process generates free thiols which carry out nucleophilic attack on the sulfur atom of a disulfide. The newly formed protein-bound thiol also acts as a nucleophile toward another protein-bound cystine resulting in disulfide interchange. This exchange reaction releases a glutathione molecule which can be measured experimentally allowing us to directly observe the disulfide interchange reaction.

In summary, the data obtained in this work clearly show that at 100° C and neutral pH, cystine residues in proteins undergo destruction via two distinct mechanisms: ß-elimination and disulfide interchange catalyzed by thiols formed during the ß-elimination reaction. The first process is rate-limiting and has a half-life in the range of 1.0 ± 0.4 hrs and 12.4 ± 3.4 hrs at pH 8.0 and 6.0, respectively (and around 6 days of pH 4.0). These half-lives have been found to be

remarkably independent of the nature of the protein and hence demarcate the upper limit of thermal stability of proteins containing cystine residues.

4.2 Mechanism of Thermoinactivation of Immobilized Glucose Isomerase

Recent work in our laboratory has elucidated the mechanisms of irreversible enzyme thermoinactivation for hen egg-white lysozyme (Ahern and Klibanov, 1985), bovine pancreatic ribonuclease (Zale and Klibanov, 1986), yeast triose phosphate isomerase (Ahern et al., 1987), and microbial α-amylases (Tomazic and Klibanov, 1988a). In the present work the conceptual and experimental methodology developed in those studies is applied, for the first time, to an immobilized enzyme, glucose isomerase from Streptomyces olivochromogenes. Specifically, the following questions are addressed:

(1)   Do the same covalent reactions identified in model enzymes limit the thermostability of practical biocatalysts under conditions used in commercial biochemical reactors?

(2)   What mechanism(s) limit the thermostability of immobilized glucose isomerase under extreme conditions (90° C) and near bioreactor temperatures (70° C)?

(3)   What is the upper limit of glucose isomerase thermostability? Is it possible to directly isomerize dextrose to 55% fructose at high temperatures in order to bypass the currently used costly enrichment steps?

4.2.1 Kinetics of thermoinactivation of immobilized glucose isomerase

Glucose isomerase from Streptomyces olivochromogenes appeared to be an attractive and pertinent subject for our investigation because (i) it is currently used commercially (Enzyme Bio-

System's G-ZYME™ G993 Glucose Isomerase); and (ii) its physicochemical (Anthrim et al., 1979) and enzymatic (Suckling, 1985) properties , amino acid sequence (Farber and Petsko, 1987a) and tertiary structure (Farber and Petsko, 1987; Glasfeld et al., 1988) are known (see literature survey), thus facilitating analysis of the experimental data. Therefore, the enzyme was purified to homogeneity, as discussed in experimental section, and then its behavior at elevated temperatures was investigated.

Upon heating at 80 to 90° C and pH 8.0 (the pH optimum of catalytic activity), soluble glucose isomerase (0.1 - 1.0 mg/ml) readily inactivates with concomitant heavy aggregation. Even the presence of xylitol (1 M), a potent competitive inhibitor of glucose isomerases (Suckling, 1985), does not prevent the aggregation process. Molecular examination of aggregated enzymes is extremely difficult (Ahern and Klibanov, 1987). Also, in industry, all glucose isomerases are currently used in the immobilized form (see literature survey). Therefore, in order to eliminate heat-induced aggregation (Klibanov, 1983), we decided to continue our study with the enzyme covalently attached to a solid support. Controlled pore glass (Weetall, 1976) was selected as a model support for immobilization due to its chemical and mechanical robustness. Upon covalent attachment to aminated porous glass beads via glutaraldehyde and sodium borohydride, glucose isomerase retains as much as 80 to 90% of its specific activity, thus making the immobilized enzyme a realistic and meaningful analog of its free predecessor in that diffusional limitations do not occur.

When immobilized glucose isomerase is incubated in an aqueous buffer (pH 8.0) at 80° C, it gradually loses its activity with a half-time of 120 minutes. This process closely adheres to the first order kinetic law (correlation coefficient of 0.98). The thermal inactivation is irreversible, for no appreciable reactivation was observed following even after a prolonged incubation of the immobilized enzyme at ambient temperature. Interestingly, the half-life of free glucose isomerase (0.1-0.5 mg/ml) under the same conditions is 76 minutes. Thus, in contrast to a commonly held belief, immobilization does not necessarily dramatically stabilize enzymes. Furthermore, since the free enzyme aggregates upon heating and the immobilized one does not, and yet the two have

comparable thermostabilities, aggregation is likely to be an accompanying phenomenon, rather than a cause of irreversible thermoinactivation (Tomazic and Klibanov, 1988a).

The investigation of the behavior of immobilized glucose isomerase at different temperatures began by examining the temperature dependence of the initial rates of the isomerization reaction. As shown in Figure 11, two distinct regions of interest can be identified. First, in the temperature range of 65-85° C, we see an Arrhenius-type increase in the reaction rate. We chose 70° C as a representative of the "low" temperature region in order to investigate thermoinactivation near operating conditions. Second, the temperature optimum is reached at 88-90° C followed by rapid inactivation at higher temperatures. We began our mechanistic investigation of irreversible thermoinactivation of immobilized glucose isomerase in this "high" temperature region (90° C).

First, it should be noted that the experiment described in Figure 11 is carried out at low substrate concentrations (0.1 M fructose). However, high fructose corn syrup (HFCS), used industrially, is a concentrated sugar solution (2-3 M). Substrates and ligands usually stabilize enzymes against heat-induced unfolding (inactivation) by shifting the equilibrium between the native and thermounfolded forms of the enzyme toward the former (Klibanov, 1983; Ahern and Klibanov, 1987). Therefore, one would expect that in the presence of high ligand concentrations, such as those in HFCS, glucose isomerase will definitely remain folded in the vicinity of 90° C. Hence, thermoinactivation experiments conducted at this temperature involve the essentially folded, catalytically competent enzyme. This mechanistic element complements our previous work with other enzymes where, in order to observe noticeable thermoinactivation, studies often had to be done at temperatures where enzymes are (reversibly) thermounfolded.

We found that the enzyme was greatly stabilized in the presence of commercial HFCS (Figure 12): at 90° C the half-life of immobilized glucose isomerase is 37 minutes, while it is less than half a minute without the substrate. An even higher thermostability is observed in the presence of the competitive inhibitor xylitol (2 M): half-life of 77 minutes at 90° C (Figure 12). Since the two ligands exert similar effects and it is technically difficult to work with HFCS (high
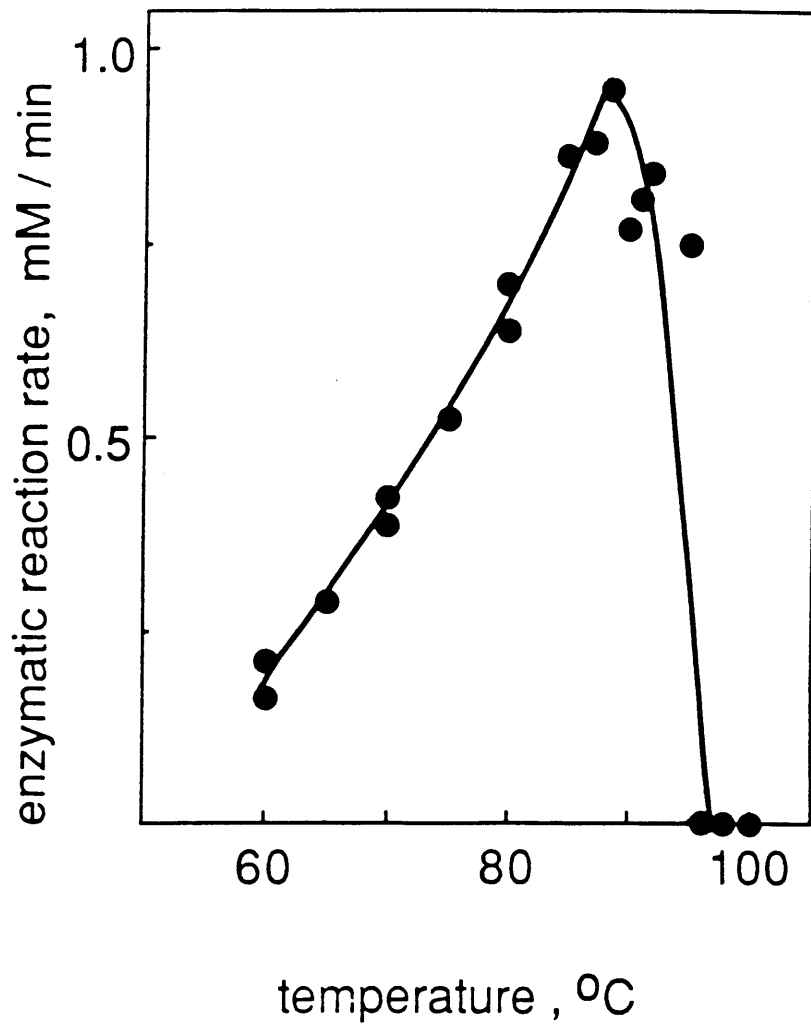
Figure 11 - Temperature dependence of the initial rates of fructose (0.1 M) isomerization catalyzed by immobilized glucose isomerase in 0.1M Tris-HCl buffer (pH 8.0) containing 10 mM $MgSO_4$.
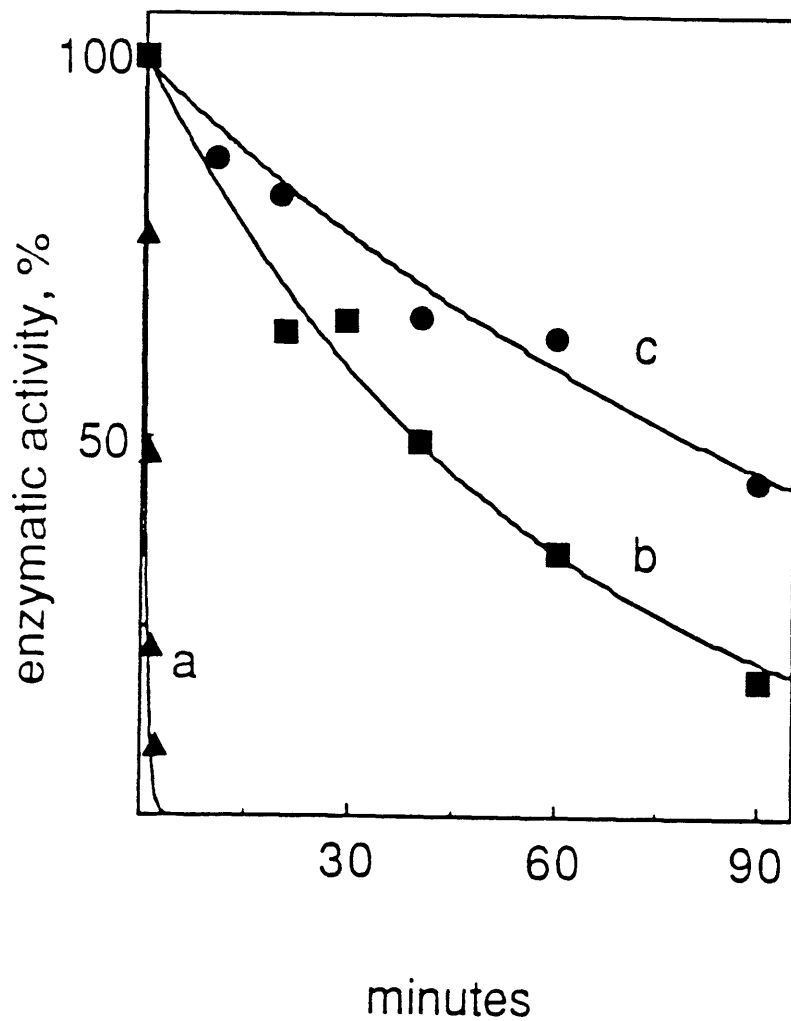
Figure 12 - Time course of irreversible thermoinactivation of immobilized glucose isomerase at 90° C (pH 8.0) in (a) - 0.1 M Tris-HCl containing 10 mM MgSO$_4$; (b) - commercial high fructose corn syrup containing 0.1 M NaHSO$_3$ and 10 mM MgSO$_4$; and (c) - 0.1 M Tris-HCl containing 2 M xylitol and 10 mM MgSO$_4$.

viscosity, a constant pH drop, and a gradual formation of colored by-products), xylitol was employed as a stabilizing ligand in subsequent experiments.

Now that we have established the kinetics, the next step is to determine the molecular mechanism of irreversible thermoinactivation of immobilized glucose isomerase in the presence of a ligand (curve c in Figure 12).

4.2.2 Deamidation of asparagine and/or glutamine residues and the irreversible thermo-inactivation of immobilized glucose isomerase

In considering the possible cause(s) of irreversible thermoinactivation of immobilized glucose isomerase in the presence of a ligand (pH 8), we can eliminate both cystine destruction and thiol-disulfide interchange since this glucose isomerase contains no disulfide bonds (Farber and Petsko, 1987b). Furthermore, hydrolysis of the polypeptide chain at aspartic acid residues occurs only at acidic pH (see literature survey). We noticed, however, that the half-life of the enzyme at 90° C (77 minutes) was similar to those for bovine pancreatic ribonuclease A (53 minutes) and Bacillus stearothermophilus α-amylase (80 minutes) brought about by deamidation of asparagine and/or glutamine residues. Since deamidation has emerged as a major mechanism of irreversible enzyme thermoinactivation (see literature survey and Appendix B), we tested its applicability to glucose isomerase.

As shown in Figure 2, the deamidation reaction involves intramolecular cyclization of aspartyl (or glutamyl) residues under neutral to basic conditions. Formation of a cyclic imide intermediate requires the main chain amide to act as a nucleophile, attacking the electrophilic carbonyl moiety of the aspartyl residue with ammonia being released as the leaving group. Subsequent hydrolysis of the cyclic imide leads to a mixture of α and ß- aspartyl residues. Glutamine side chains also deamidate but at much slower rates (Robinson and Rudd, 1974). Furthermore, recent work by Clarke (1987) and Kossiakoff (1988) indicates that the tertiary structure is a principal determinant of protein deamidation (for details, see literature survey).

Deamidation results in the evolution of ammonia, and as can be seen in Figure 13, NH_3 is indeed released upon heating of the immobilized enzyme. The ammonia production observed is due to deamidation of asparagine and/or glutamine residues as opposed to destruction of other amino acid residues because (i) the amino acid analysis of completely thermoinactivated glucose isomerase shows no appreciable loss of any amino acids (data not shown), and (ii) exhaustive heating of both immobilized and free glucose isomerase reveals (Figure 14) that NH_3 evolution followed first-order kinetics ($k_{deamidation}$ = 0.066 hr $^{-1}$ with a half-life of 32 hours and a corr. coeff. of 0.99) and levelled off in the range between 20 and 25 mole-equivalents per monomer (or 44 mole-equivalents per dimeric enzyme), i.e., in a reasonable agreement with the total number of 22 amide residues (asparagine (14) and glutamine (8)) in the monomeric subunit of the enzyme (Farber and Petsko, 1987a). It was of interest to see if this first-order release of ammonia was a general process occurring in all proteins when heated at high temperatures. The results are discussed in Appendix B.

Thus, deamidation of asparagine and/or glutamine residues does occur upon incubation of immobilized glucose isomerase at 90° C, pH 8.0, and in the presence of 2 M xylitol, but is it actually the cause of thermoinactivation? As a first step toward answering this question, we employed the following approach. Previous thermoinactivation studies in our laboratory (see literature survey) have dealt with inactivation of several free enzymes brought about by heat-induced deamidation. We combined the data for different enzymes under a variety of conditions and plotted on the same graph the dependence of the remaining enzymatic activity on the number of mole-equivalents of ammonia released. As one can see in Figure 15, a common, general trend emerges. Despite a wide scatter in the data (understandable given the diversity of the assumptions made and the data sources; see Appendix C), one observes that, upon deamidation of asparagine and/or glutamine residues in various enzymes, there is a gradual decline in catalytic activity. Moreover, assuming that the immobilized glucose isomerase follows a similar pattern, the results depicted in Figure 15 afford a prediction of the range of residual enzymatic activity at any degree of ammonia evolution, i.e., extent of deamidation. If one combines this information
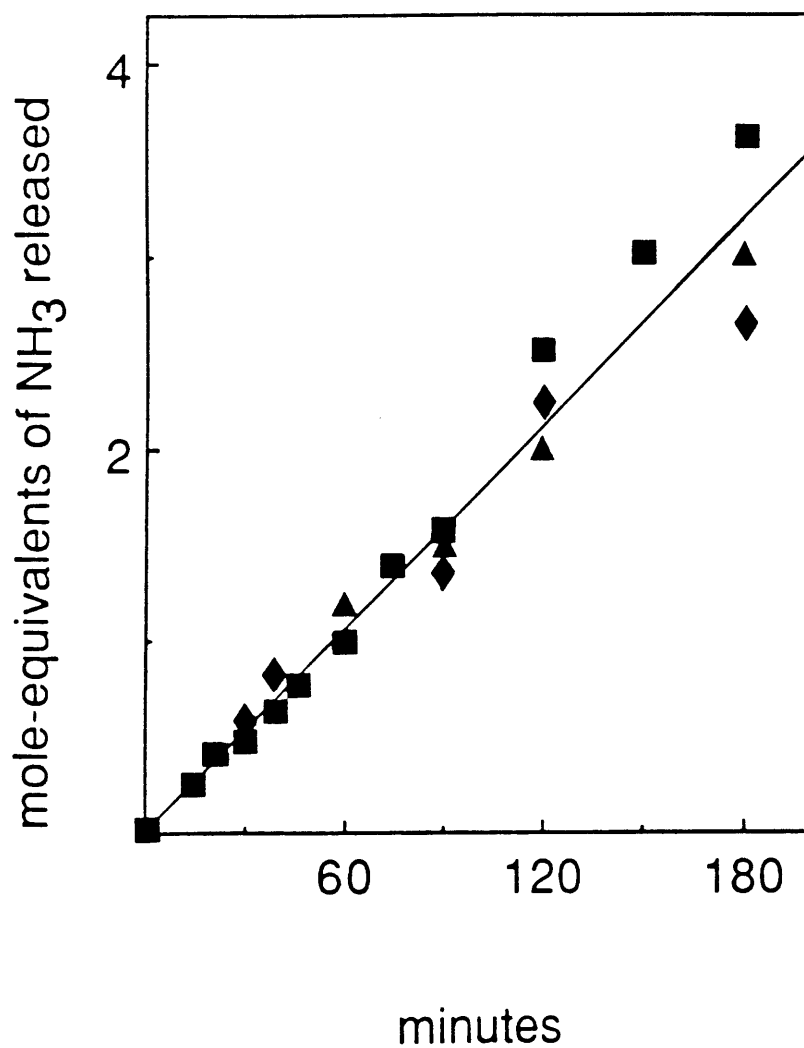
Figure 13 - Time course initial release of ammonia during incubation of immobilized glucose isomerase at 90° C in 0.1 M Tris-HCl buffer (pH 8.0) containing 2 M xylitol and 10 mM MgSO$_4$. Different symbols correspond to different experiments. The number of mole-equivalents of ammonia is expressed per dimeric enzyme.
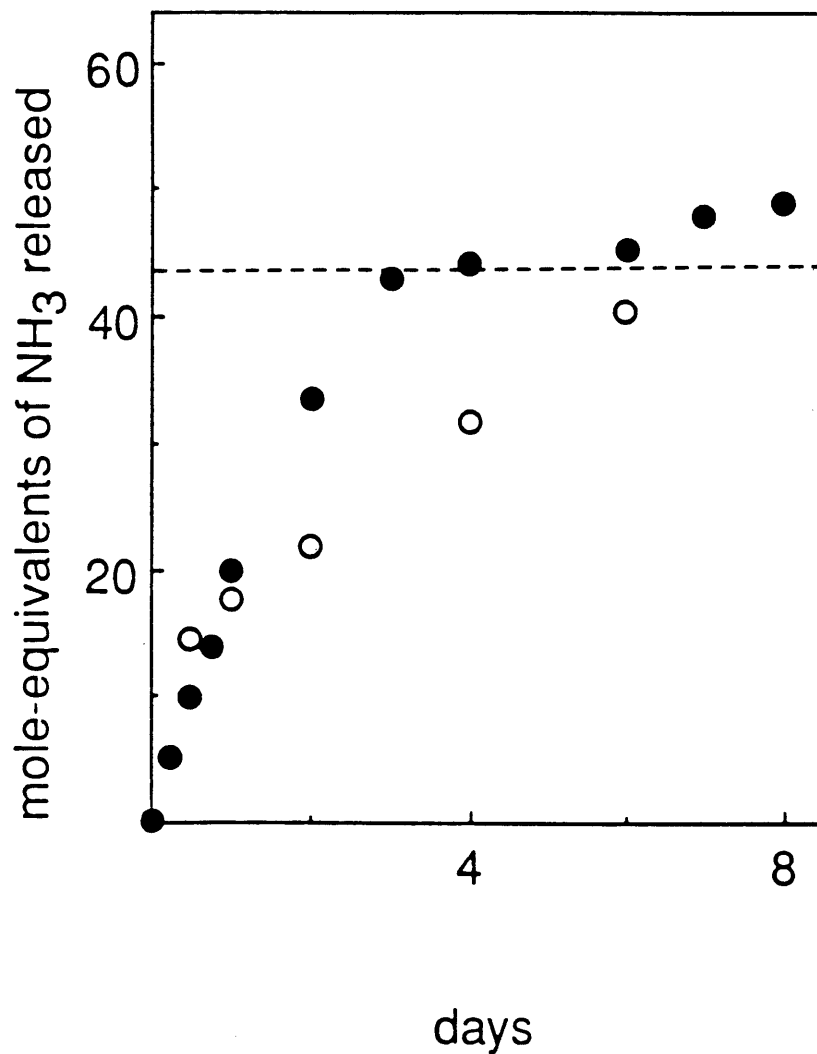
Figure 14 - Time course of ammonia evolution during incubation of glucose isomerase at 90° C in 0.1 M Tris-HCl buffer (pH 8.0) containing 2 M xylitol and 10 mM $MgSO_4$. Long term NH_3 release for the immobilized (open symbols) and free (closed symbols) enzyme. The dashed line represents 44 mole-equivalents of ammonia produced in agreement with the total number of 22 (Asn + Gln) residues per monomer of dimeric glucose isomerase. The number of mole-equivalents of ammonia is expressed per dimeric enzyme.
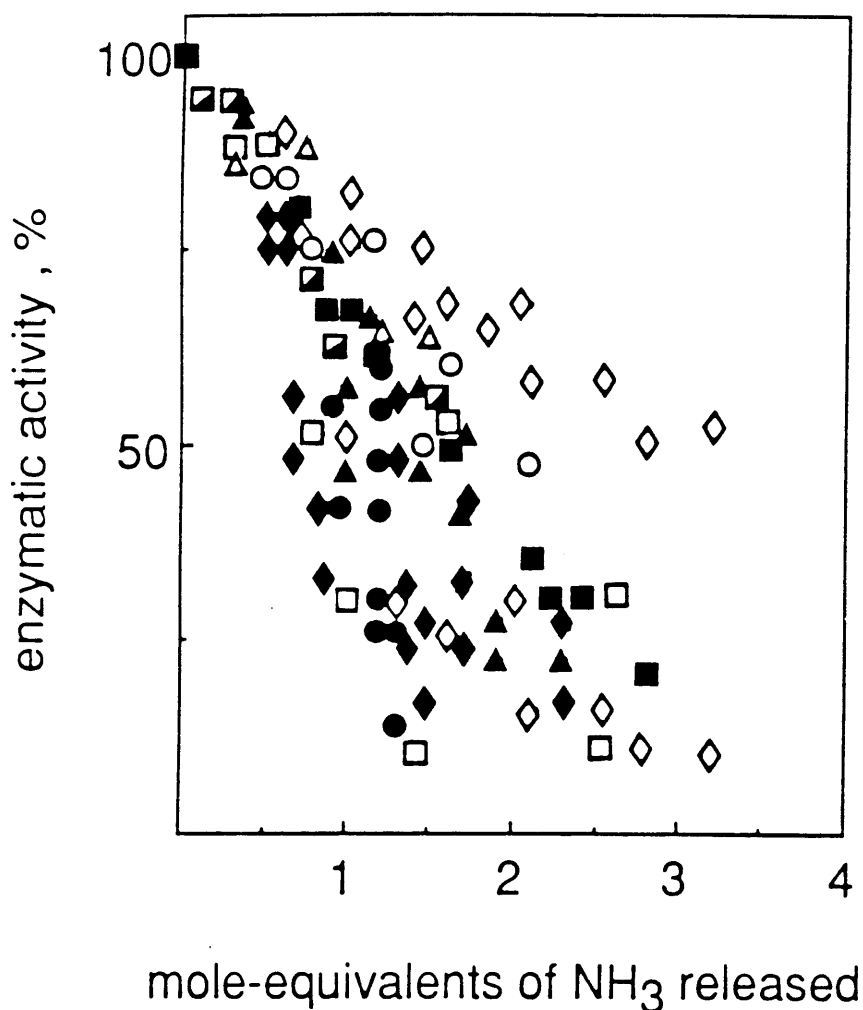
Figure 15 - Modeling of irreversible thermoinactivation of immobilized glucose isomerase due to deamidation of its (Asn + Gln) residues. The literature data on the dependence of the residual activity of various enzymes on the number of mole-equivalents of NH_3 released during irreversible thermoinactivation caused by deamidation of the amide amino acid residues. Enzymes and conditions: (▲) lysozyme, 100° C, pH 4.0; (●) lysozyme, 100° C, pH 6.0; (◆) lysozyme, 100° C, pH 8.0; (◇) triose phosphate isomerase, 100° C, pH 6.0; (■) ribonuclease, 90° C, pH 6.0; (□) B. amyloliquefaciens α-amylase, 90° C, pH 8.0; (△) B. stearothermophilus α-amylase, 90° C, pH 8.0; (○) B. stearothermophilus α-amylase, 90° C, pH 6.5; (◪) B. licheniformis α-amylase, 90° C, pH 6.5. See Literature Survey for references and Appendix C for a more detailed explanation of the modeling.

with the time course of NH_3 release during heating of immobilized glucose isomerase (Figure 13), then one can estimate the expected pace of thermoinactivation of this enzyme due to deamidation. The calculated profile (reflected by error bars in Figure 16) turns out to be in surprisingly good agreement with the experimentally observed time course of irreversible thermoinactivation of the enzyme. Needless to say, this coincidence does not prove that at 90° C (pH 8.0, 2 M xylitol) immobilized glucose isomerase thermoinactivates due to deamidation of its asparagine and/or glutamine residues, but simply demonstrates that it is feasible.

To obtain more definitive proof, we developed a strategy in which the effect of different environmental factors was investigated concomitantly with the time courses of thermoinactivation and heat-induced deamidation. We know that both the hydroxide ion concentration (i.e., the pH value) and water are involved in the protein deamidation reaction (see mechanism, Figure 2). Therefore, we can manipulate these environmental conditions to affect the rate of protein deamidation, and then observe the kinetics of irreversible thermoinactivation. Clearly, if the effects are invariably similar, then a causal relationship must exist between the two; if the effects are uncoupled, then the two phenomena merely take place simultaneously but are not related.

The first variable parameter employed by us was the pH. As one can see from Table 3, and perhaps more clearly in the normalized rates in Table 4, a reduction of pH from 8.0 to 7.0 results in a 6.6-fold stabilization of immobilized glucose isomerase and also decreases the rate constant of its thermal deamidation by 6.8-fold. Conversely, when the pH is raised from 8.0 to 9.0, the half-life of the enzyme drops 15.4-fold, and the rate constant of deamidation also increases 9.2-fold. In the second set of experiments, H_2O is replaced with D_2O as the reaction medium. This substitution leads to a 4.9-fold stabilization of the immobilized enzyme and also lowers the rate constant of heat-induced deamidation by 5.5-fold. The magnitude of both changes is in the range of a typical H-D kinetic isotope effect (Schowen, 1978). In addition, the replacement of H_2O with D_2O as the reaction medium is known to increase the conformational stability of proteins (Lewin, 1974; Hattori et al., 1965). Therefore, the above environmental factors affect thermostability and thermal deamidation of immobilized glucose isomerase in a

Figure 16 - Modeling of irreversible thermoinactivation of immobilized glucose isomerase due to deamidation of its (Asn + Gln) residues. The experimentally obse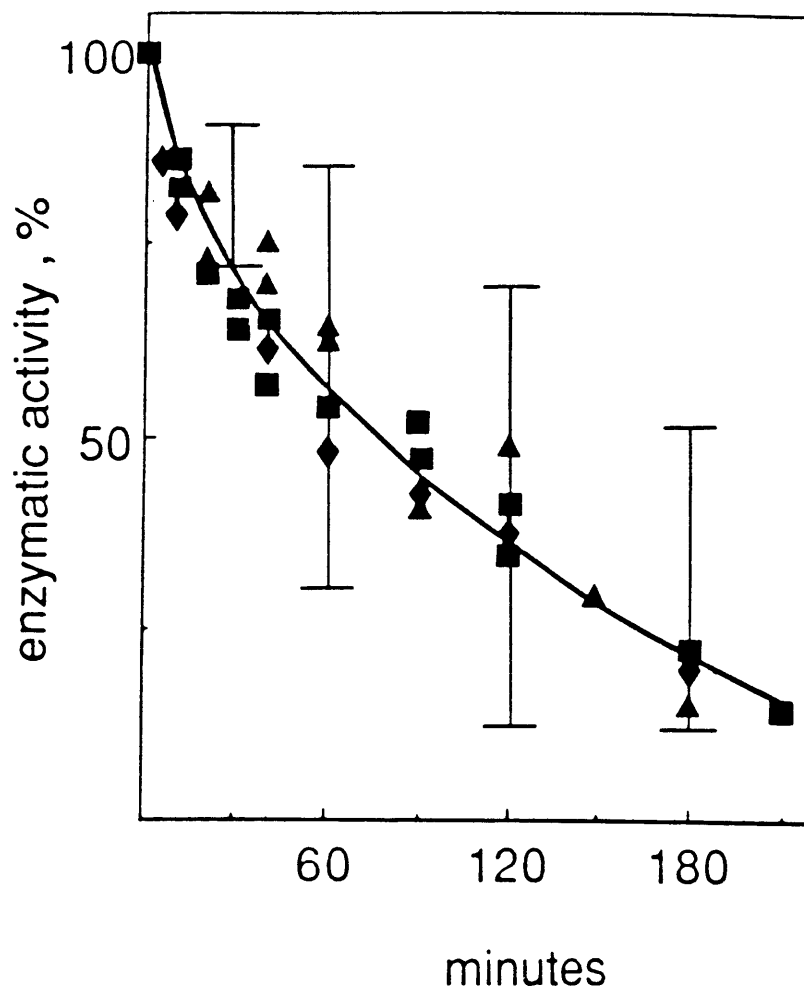rved time course of irreversible thermoinactivation of immobilized glucose isomerase at 90° C in 0.1 M Tris-HCl buffer (pH 8.0) containing 2 M xylitol and 10 mM $MgSO_4$ (solid symbols corresponding to independent experiments) and the time course of irreversible thermoinactivation of the enzyme predicted on the basis of superposition of the data in Figures 15 and 13 (error bars).

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Additives, pH	Half-time of thermoinactivation (min)	First order rate constant of deamidation [a] $\times 10^4$ (min^{-1})	Half-time for release of the initial mole equivalent of NH_3 (min)
2 M xylitol, pH 8.0	77	3.8	30
2 M xylitol, pH 7.0	510	0.56	200
2 M xylitol, pH 9.0	5	35	3
2 M xylitol in D_2O, pD 8.0[b]	380	0.69	160
none, pH 8.0	< 0.5	11	10
2 M xylitol, 1 M KCl, pH 8.0	4	3.8	30
2 M xylitol, 1 mM EDTA, pH 8.0	17	3.8	30

Table 3 - The effect of various experimental conditions on the kinetics of irreversible thermo-inactivation and of heat-induced deamidation of immobilized glucose isomerase at 90° C.

Immobilized glucose isomerase was incubated at 90° C in the solutions indicated in the first column of the Table and periodically assayed for enzymatic activity and for ammonia evolution as described in the experimental section. The following buffers were used (the pH values listed are those at 90° C): at pH 8.0 and 7.0, 0.1 M Tris-HCl containing 10 mM $MgSO_4$; at pH 9.0, 0.1M glycine containing 10 mM $MgSO_4$. [a]The first order rate constants of deamidation were determined on the basis of the evolution of the initial 2-3 mole-equivalents of NH_3 per dimeric enzyme. [b]Adjusted at 25° C, as described in the Experimental Section, to a pD value that results in pD 8.0 at 90° C as determined by our calibration curves.

Additives, pH	Relative half-life of thermoinactivation	Relative rate of deamidation
2 M xylitol, pH 8	1.0	1.0
2 M xylitol, pH 7	6.6	6.8
2 M xylitol, pH 9	0.06	0.10
2 M xylitol in D_2O, pD 8.0	4.9	5.5
No ligand, pH 8	0.006	0.40
2 M xylitol, 1 M KCl, pH 8.0	0.05	1.0
2 M xylitol, EDTA, pH 8.0	0.22	1.0

Table 4 - Effect of various experimental conditions on kinetics of irreversible thermo-inactivation and deamidation of immobilized glucose isomerase at 90°C; values are normalized to the kinetic data for pH 8, 2 M xylitol as shown in the first row of Table 3.

substantial and parallel fashion. This finding confirms that at 90° C and pH 8.0 in the presence of the ligand xylitol (2 M) the immobilized enzyme thermoinactivates due to deamidation of its asparagine and/or glutamine residues.

As a control experiment, a set of environmental conditions was chosen which will not affect protein deamidation, but should alter the thermostability of the enzyme. In general, at every given temperature, irreversible thermoinactivation of an enzyme is caused by a combination of covalent and conformational processes (Ahern and Klibanov, 1987). Sometimes, one of these reactions is much faster than the others and hence is predominant, e.g., deamidation in the case of immobilized glucose isomerase under the conditions listed in the first half of Tables 3 and 4. However, when experimental conditions are altered, relative contributions of various thermoinactivation processes also change, for, generally speaking, they are affected to different extents. For example, inspection of the first line in the second-half of Table 3 reveals that in the absence of a ligand, enzyme thermoinactivation cannot be attributed to deamidation because it is too fast: even if each deamidation led to the complete inactivation of the enzyme, the half-life of the latter would be 10 minutes (third column in Table 3), whereas in fact it is less than 0.5 minutes. Without ligand, immobilized glucose isomerase probably inactivates due to a conformational process, formation of incorrect (scrambled) structures (Klibanov, 1983). The ligand xylitol (and presumably HFCS) is not the only factor that can "switch" irreversible thermoinactivation from one regime to another. For instance, the addition of 1 M KCl (breaks apart ionic interactions) or 1 mM EDTA (binds magnesium ions), as seen in the last two lines in Table 3 and Table 4, drastically reduces the thermostability of the enzyme without affecting the rate of its thermal deamidation. In summary, comparison of the kinetics of inactivation and deamidation of the initial amide residue indicates, as predicted, that deamidation cannot possibly be the cause of irreversible thermoinactivation under those conditions, but rather it is likely brought about by conformational scrambling.

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4.2.3 Oxidation of cysteine residues and the irreversible thermal inactivation of immobilized glucose isomerase

The literature contains many reports about the oxygen sensitivity of glucose isomerases (Anthrim et al., 1979); in fact, in industrial bioreactors this problem is routinely dealt with by pretreating glucose solutions via sparging with nitrogen, adding sodium bisulfite, or evacuating under reduced pressure. Oxygen sensitivity of enzymes usually stems from oxidation of cysteine residues (Friedman, 1973), and we explored this possibility in our particular case.

A solution of glucose isomerase reveals no indication of the presence of free thiol groups when titrated with Ellman's reagent (see experimental section). However, when a strong denaturant, 6 M guanidine hydrochloride, is added to the enzyme solution, 0.9 ± 0.3 free sulfhydryl groups per monomer of glucose isomerase are detected. This finding was confirmed by the reduction and carboxymethylation of the enzyme in a denaturant solution, followed by dialysis, acid hydrolysis, and amino acid analysis: 1.1 mole-equivalents of carboxymethylated cysteine residues per monomer were detected. Hence glucose isomerase from Streptomyces olivochromogenes, in contradiction to a literature report (Suekane et al., 1978), contains one cysteine residue per monomer, and this amino acid residue is buried in the interior of the enzyme.

We then investigated the stability of the cysteine residue at high temperatures by adopting the same strategy as previously described for deamidation: (i) does a deteriorative, covalent reaction occur? and (ii) does it play a role in the thermoinactivation process? As one can see in Figure 17 (curve a), upon heating at 90° C (in the presence of 2M xylitol) the number of free SH groups in immobilized glucose isomerase gradually decreases. As expected, this autooxidation of thiols can be prevented by deoxygenating the solution and adding the O_2 scavenger ß-mercaptoethanol (curve b in Figure 17). However, the ß-mercaptoethanol/argon treatment has no appreciable effect on the irreversible thermoinactivation of immobilized glucose isomerase under the same conditions (Figure 18). Therefore, the two processes are uncoupled, and although cysteine oxidation does indeed occur and may inactivate glucose isomerase, it does not
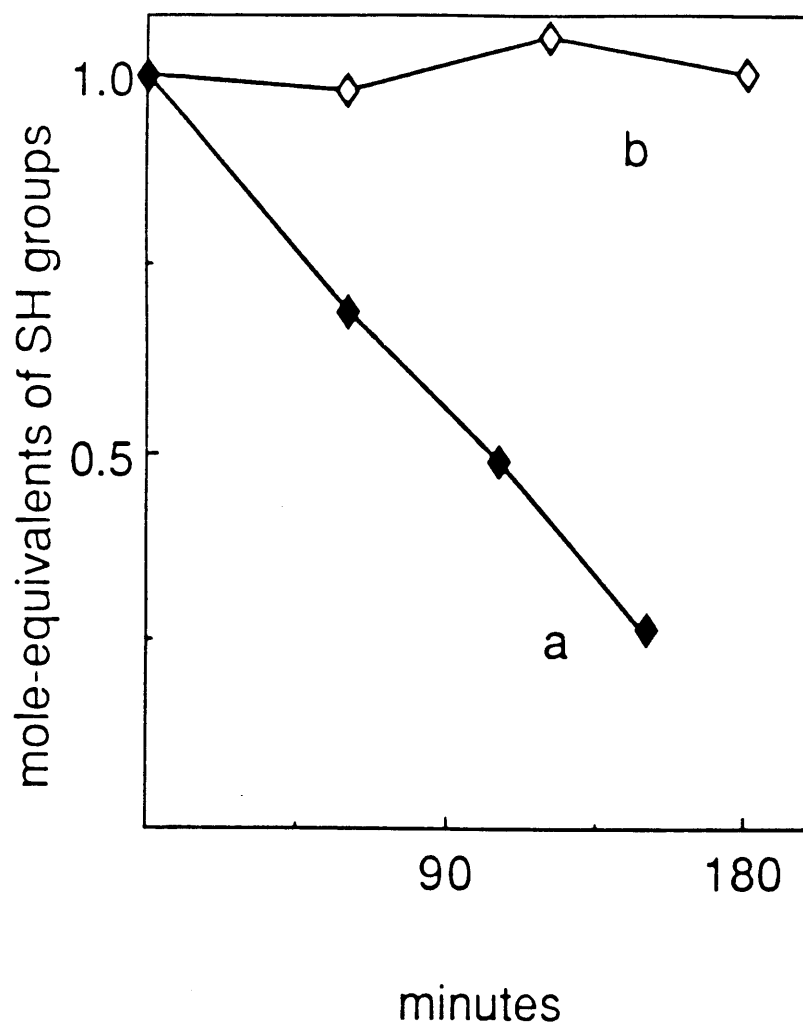
Figure 17 - Time course of air oxidation of immobilized glucose isomerase at 90° C in 0.1 M Tris-HCl buffer (pH 8.0) containing 2 M xylitol and 10 mM MgSO$_4$ in the absence (a, closed symbols) or presence (b, open symbols) of 20 mM ß-mercaptoethanol placed under argon. Different symbols correspond to independent experiments. The amount of free sulfhydryl groups (detected using Ellman's reagent in the presence of 6 M guanidine hydrochloride) is expressed per monomer of the dimeric enzyme.
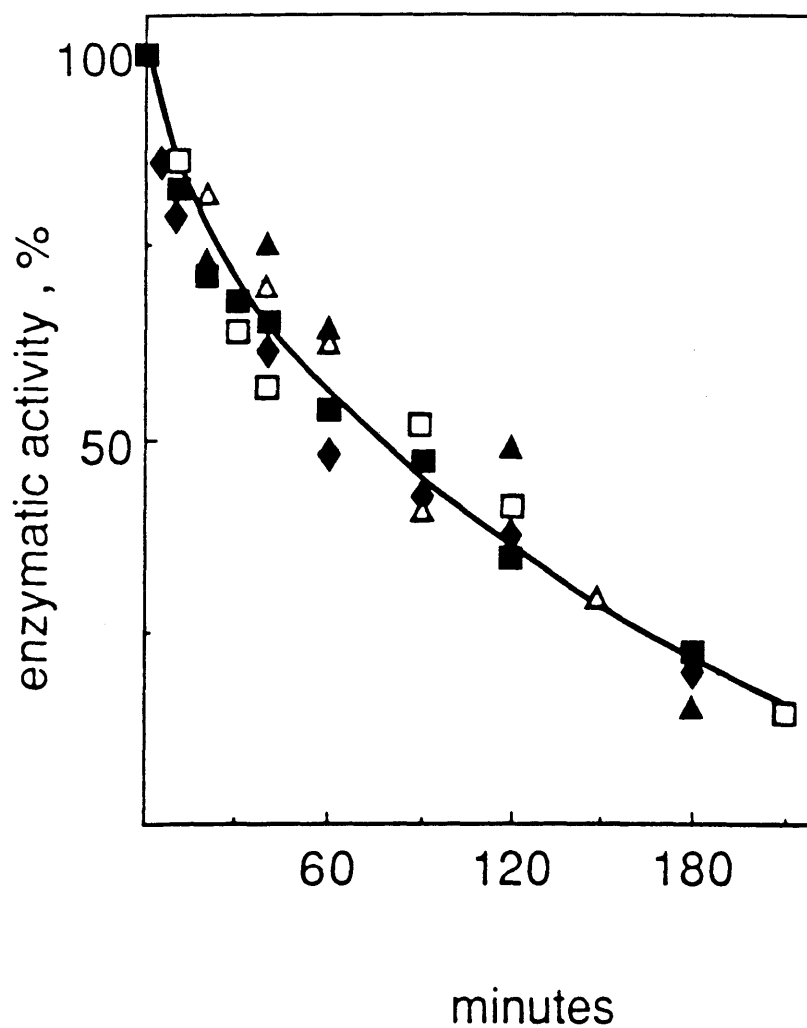
Figure 18 - Time course of irreversible thermoinactivation of immobilized glucose isomerase at 90° C in 0.1 M Tris-HCl buffer (pH 8.0) containing 2 M xylitol and 10 mM $MgSO_4$ in the absence (a, closed symbols) or presence (b, open symbols) of 20 mM ß-mercaptoethanol placed under argon. Different symbols correspond to independent experiments.

significantly contribute to the observed thermoinactivation of the enzyme at 90° C (see Appendix D). This conclusion is further supported by the observation that effects similar to those presented in Table 3 and 4 are obtained in the presence of ß-mercaptoethanol under argon. Under these conditions, oxidation of cysteine residues does not take place (Figure 17), but thermoinactivation due to deamidation still occurs.

4.2.4 Thermoinactivation of immobilized glucose isomerase near bioreactor conditions

It has now been shown that at 90° C oxidation of glucose isomerase's cysteine residues is too slow to substantially contribute to the irreversible thermoinactivation of the enzyme. In order (i) to assess the situation at a temperature closer to the range employed in industry, and (ii) to answer the question whether oxidation of the cysteine residue results in the loss of glucose isomerase activity, we investigated this process at 70° C. As one can see in Figure 19, incubation of immobilized glucose isomerase under air in the presence of 10 mM Cu^{2+} results in complete inactivation of the enzyme after 2 days. Importantly, after a 24 hour incubation, when the residual enzymatic activity is 22%, 76% of free sulfhydryl groups of the enzyme also disappear. When the oxidation of cysteine residues is prevented by carrying out thermo-inactivation under argon and in the presence of 20 mM ß-mercaptoethanol, no appreciable loss of glucose isomerase activity is observed. Hence we can conclude that the two processes are coupled and there is a causal relationship between them. Interestingly, no inactivation (or cysteine oxidation) occurs in the presence of xylitol (with or without 10 mM Cu^{2+}) or in the buffer devoid of copper ions. These data indicate that the cysteine residue is not very reactive, is hidden in the interior of the enzyme molecule, and must be exposed in order to be oxidized which is prevented by the binding of the ligand xylitol.

In order to obtain a better understanding of thermoinactivation in the "low" temperature region (70° C), we examined the temperature dependence of the rate constant of irreversible thermoinactivation of immobilized glucose isomerase in the presence of the ligand xylitol (under

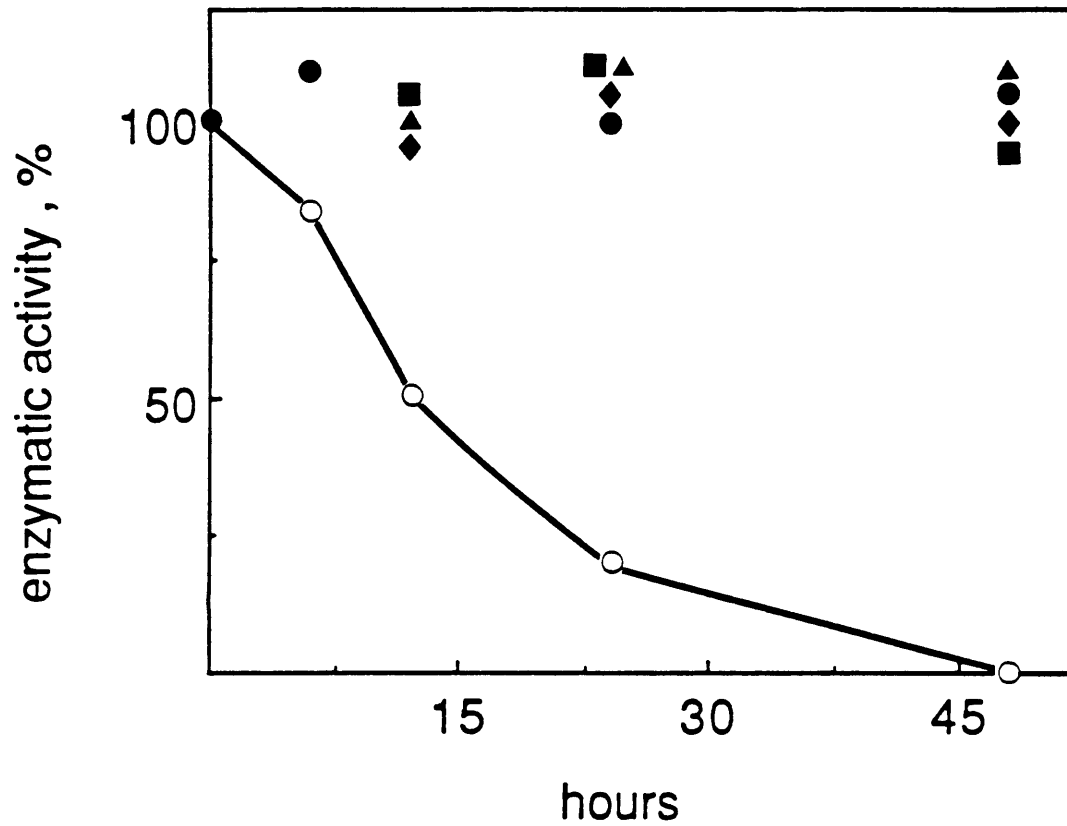**Figure 19** - The time course of irreversible thermoinactivation of immobilized glucose isomerase at 70° C (pH 8.0) in 0.1 M Tris-HCl containing 10 mM $MgSO_4$ under different conditions. (○) - in the presence of 10 μM $CuCl_2$; (●) - in the presence of 20 mM ß-mercaptoethanol under argon; (◆) - in the presence of 2 M xylitol without or with (■) 10 μM $CuCl_2$; and (▲) - no additives.

anaerobic conditions to prevent the possibility of cysteine oxidation). When plotted in Arrhenius coordinates, these data (line a in Figure 20) yield an activation energy of 180 kcal/mol. This huge activation energy is totally uncharacteristic of a covalent reaction. In fact, the activation energy for deamidation of immobilized glucose isomerase under the same conditions is found to be only 44 kcal/mol (line b in Figure 20). The only logical explanation for this apparent contradiction is that deamidation of just a few out of the total of 22 amide (Asn + Gln) residues per enzyme monomer actually leads to inactivation. It is reasonable to assume that those key amide residues are located in the interior of the enzyme molecule (perhaps near the active center) and thus are not accessible to the solvent. Consequently, the enzyme molecule must partially unfold in order for deamidation of those key residues to take place. Hence the temperature dependence of their deamidation follows the same pattern as enzyme unfolding which is expected to have a very high activation energy (Ahern and Klibanov, 1987). This is in contrast to the behavior of the overall deamidation process which presumably mainly reflects ammonia evolution of the amide residues close to the protein surface. Therefore, the two processes uncouple in the "low" temperature region (70° C) and deamidation is no longer the cause of irreversible thermoinactivation.

Similar data on the temperature dependence of both thermoinactivation and deamidation of immobilized glucose isomerase were obtained in buffer alone without the ligand 2 M xylitol (half-life values for loss of enzymatic activity were 2,160 , 120, 30, and 0.4 minutes at 75° C, 80° C, 82° C and 90° C respectively; $k_{deamidation}$ values were 4.6, 22, and 110 (x 10^{-5}) minutes at 70° C, 80° C and 90° C respectively). These data afford similar activation energies compared to the previous results in the presence of 2 M xylitol: 142 kcal/mole for thermoinactivation and 35 kcal/mole for deamidation. Therefore, the enzyme is less thermostable in the absence of ligand (about 10° C for both the thermoinactivation and deamidation kinetic data); thus, the ligand shifts the equilibrium between native and unfolded enzyme towards the latter at elevated temperatures, but the relationship between thermoinactivation and deamidation does not change.
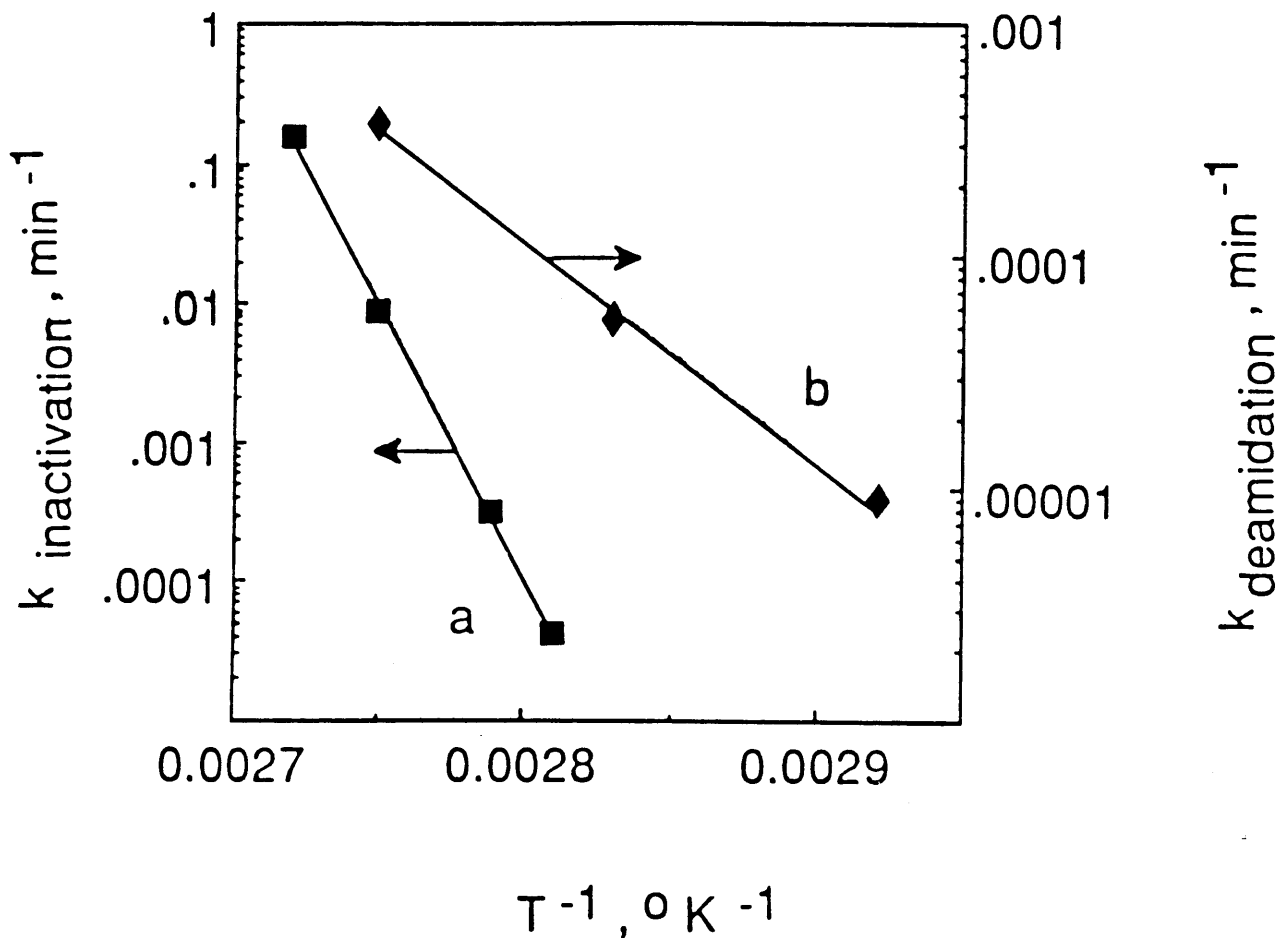
Figure 20 - The Arrhenius dependence for the rate constant of (a) irreversible thermoinactivation and (b) heat-induced deamidation of immobilized glucose isomerase in deoxygenated 0.1 M Tris-HCl buffer (pH 8.0) containing 2 M xylitol, 20 mM ß-mercaptoethanol, and 10 mM MgSO$_4$.

The data presented in Figure 20 afford another important conclusion about "low" temperature (70° C) thermoinactivation of glucose isomerase. Extrapolation of the rate constant of irreversible thermoinactivation of immobilized glucose isomerase to 70° C results in a half-life of the enzyme of 260 years. We obviously could not carry out the experiment for so long but after 3 months at that temperature (under anaerobic conditions, as attempted in industry, in order to prevent the possibility of cysteine oxidation) over 90% of enzyme activity is retained. Moreover, at 65° C, i.e., the temperature of the industrial process (see introduction), the half-life of the enzyme would be an incredible 20,000 years. Thus, assuming that the ratio of stabilizing powers of 2 M xylitol to HFCS is the same at 65° C as it is at 90° C (see Figure 12), one would predict the half-life of immobilized glucose isomerase in a commercial bioreactor to be about 10,000 years which is quite different from the actually observed few weeks. Even if the extrapolation is not totally valid, the difference between thousands of years and several weeks is too enormous to be explained by that. It was mentioned earlier (Figure 12) that HFCS undergoes a pH drop and colored by-product formation at 90° C. Under industrial conditions (65° C in a packed-bed column with short residence times) these reactions must surely occur, only much more slowly. Therefore, one is forced to conclude that the irreversible inactivation of immobilized glucose isomerase in industrial bioreactors is due to oxidation of the enzyme's cysteines (because total exclusion of oxygen is hardly feasible) or deleterious reactions of the enzyme with HFCS or impurities, exacerbated by elevated temperatures.

In summary, the results of this study provide a mechanistic description of irreversible thermoinactivation of immobilized glucose isomerase under biotechnologically relevant conditions. They demonstrate that thermal inactivation of immobilized enzymes can be successfully analyzed using the same experimental methodology as that employed for their free counterparts. The experimentally obtained data show that deamidation of asparagine and glutamine residues demarcates the upper limit of the immobilized enzyme's thermostability. In addition, at temperatures near bioreactor conditions (pH 8, 70° C), the loss of glucose isomerase

activity is brought about by either the oxidation of cysteine residues and/or heat-induced deleterious reactions with HFCS.

4.3 Thermostability of Enzymes in Anhydrous Organic Solvents

The recent discovery that enzymes can function in organic solvents has greatly increased the potential for their use as practical catalyts. Perhaps the most neglected aspect of this emerging technology is the stability of enzymes in nonaqueous media. It has been reported that enzymes not only function, but display markedly increased thermostability in organic solvents (see literature survey). These observations afford the opportunity to quantitatively investigate the cause and mechanism of this greatly increased thermostability.

Five covalent pH-dependent processes leading to the irreversible thermal inactivation of enzymes have been identified (see introduction): destruction of cystine residues, thiol-catalyzed disulfide interchange, oxidation of cysteine residues, deamidation of asparagine and/or glutamine residues, and hydrolysis of peptide bonds at aspartic acid residues. Water is a reactant in all of these degradative reactions (except for cysteine oxidation). In addition, water is the solvent which facilitates protein mobility leading to reversible unfolding, incorrect structure formation and aggregation (see literature survey). Therefore, the substitution of a nonaqueous medium for water should stabilize enzymes against both conformational processes and covalent reactions that cause irreversible thermal inactivation. In fact, this rationale may explain the several literature reports of increased thermostability cf enzymes in organic solvents (see literature survey).

In the present study, we investigate the mechanism of irreversible thermoinactivation of enzymes in anhydrous organic solvents. Bovine pancreatic ribonuclease A (RNase) has been chosen as a model system because its mechanism of irreversible thermoinactivation in aqueous buffer has been thoroughly investigated (Zale and Klibanov, 1986). This data base will allow for direct comparisons between water and nonaqueous systems. Specifically, we aim at obtaining the answers to the following questions:

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(1) What mechanism(s) cause irreversible thermoinactivation of enzymes in neat anhydrous organic solvents?

(2) Are these mechanism(s) covalent or conformational? Are conformational reactions reversible or irreversible? Are the covalent reactions the same or different as compared to aqueous solutions?

(3) What is the role of water and the solvent (hydrophobic vs. hydrophilic) during thermoinactivation in nonaqueous media?

(4) What is the upper limit of enzyme thermostability in anhydrous organic solvents?

By elucidating the mechanism(s) of thermoinactivation of enzymes in anhydrous organic solvents, we can increase our understanding of how and why enzymes inactivate at high temperatures, and perhaps learn more about enzymes that function naturally in hydrophobic environments (see literature survey). Furthermore, we can develop strategies not only to stabilize, but to design (Arnold, 1988) enzymes for nonaqueous environments, thereby increasing their usefulness as practical catalysts.

4.3.1 Kinetics of irreversible thermoinactivation of enzymes in anhydrous organic solvents

The objective of this research is to identify and characterize the processes that cause irreversible thermoinactivation of enzymes in anhydrous organic solvents. Bovine pancreatic ribonuclease A (RNase) was chosen as an initial model enzyme because (i) the structure and conformational dynamics of this small, monomeric protein have been thoroughly characterized (Blackburn and Moore, 1982; Richards and Wyckoff, 1970), (ii) the reversible thermal

denaturation process has been studied extensively (Hermans and Scheraga, 1960; von Hippel and Wong, 1965) and, (iii) the mechanism of irreversible thermoinactivation in aqueous solution (90° C, pH range of 4-8) has been elucidated (Zale and Klibanov, 1986).

When ribonuclease is dissolved in aqueous solution at pH 8 and then heated at 90° C, the enzyme rapidly inactivates with a half-life of 2 minutes (at low protein concentrations to ensure monomolecular processes, Zale and Klibanov (1986)). As an initial experiment, we lyophilized ribonuclease from an aqueous solution at pH 8, placed the enzyme powder in a hydrophobic organic solvent (anhydrous nonane, boiling point of 152° C), and heated the enzyme in sealed vials for various time periods. The samples were then allowed cool to room temperature, the solvent removed by evaporation under reduced pressure, and the protein was resuspended in aqueous buffer (see experimental section). We subsequently analyzed the aqueous solution (containing protein heated for various time periods in anhydrous nonane) for enzymatic activity and soluble protein. As shown in Figure 21, ribonuclease displays markedly increased thermostability compared to aqueous solution; it retains over 95% of its enzymatic activity following heating for six hours at 110° C and after prolonged heat treatment was found to have a half-life of 43 hours. Even upon a 55° C increase in temperature (over that employed in aqueous solution) to 145° C, the half-life of the enzyme in anhydrous nonane is one hour. As the enzyme loses enzymatic activity at 145° C in nonane, there is a concomitant loss of soluble protein as measured by the Lowry protein assay, e.g. there is less than 50% of the initial amount of protein in solution after six hours. This observation is confirmed visually by noting the appearance of macroscopic aggregates.

In order to better quantitate the loss of soluble protein after heating, we subjected the ribonuclease samples obtained to FPLC gel filtration chromatography. As can be seen in Figure 22, the extent of the loss of the soluble, monomeric ribonuclease peak on the gel filtration column closely parallels that of the loss of enzymatic activity. Therefore, the Lowry protein assay measured macroscopic aggregation, while gel filtration chromatography also detected microscopic aggregates (i.e., dimers, trimers, tetramers, etc.) which, when taken together, then
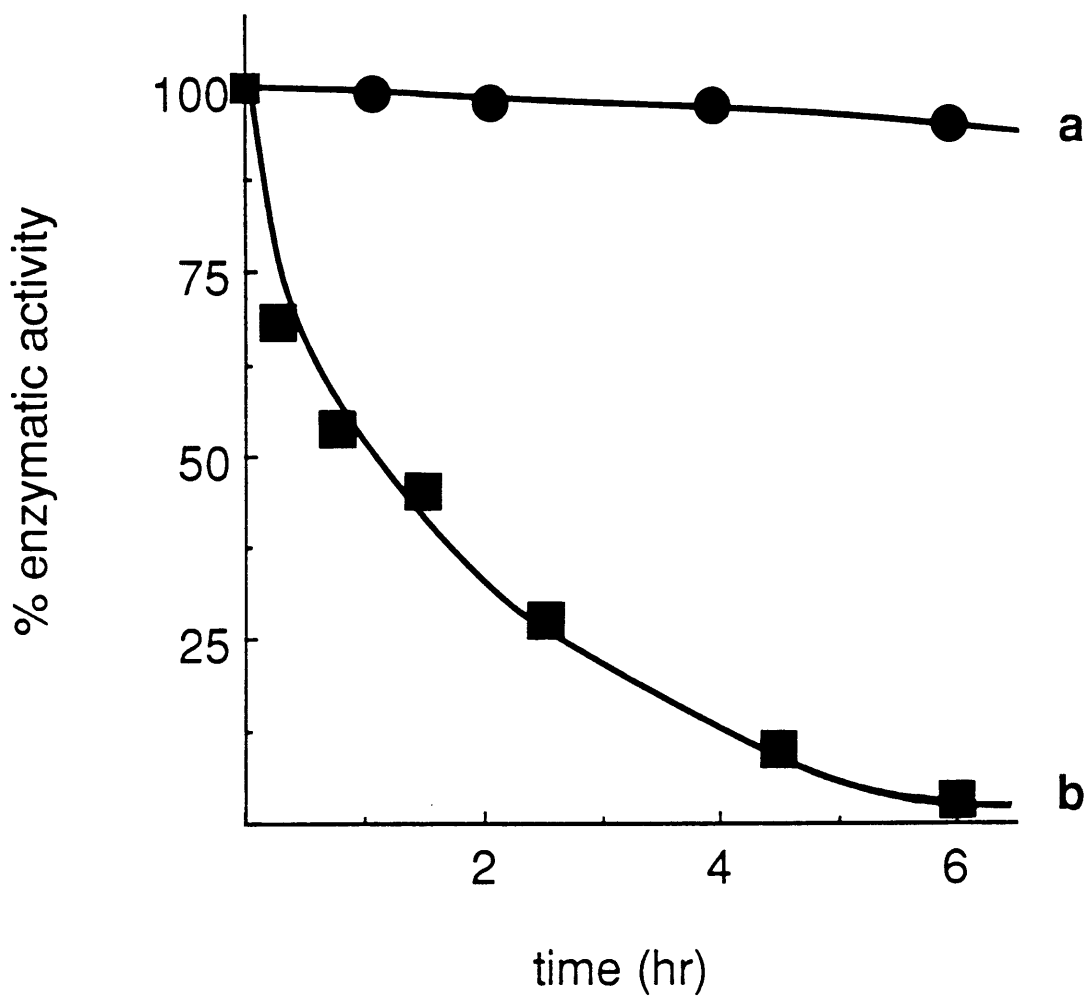
Figure 21- Time course of irreversible thermoinactivation of ribonuclease in anhydrous nonane. (a) at 110° C; and (b) at 145° C. The enzyme was lyophilized from aqueous solution at pH 8.0. The powder was than added to the anhydrous organic solvent (note that enzymes are insoluble in nonane; Singer, 1962), heated, allowed to cool to room temperature, followed by removal of the solvent under reduced pressure until dryness. The enzyme was then resuspended in aqueous buffer (1 mg/ml) and assayed for enzymatic activity (see Experimental Section).
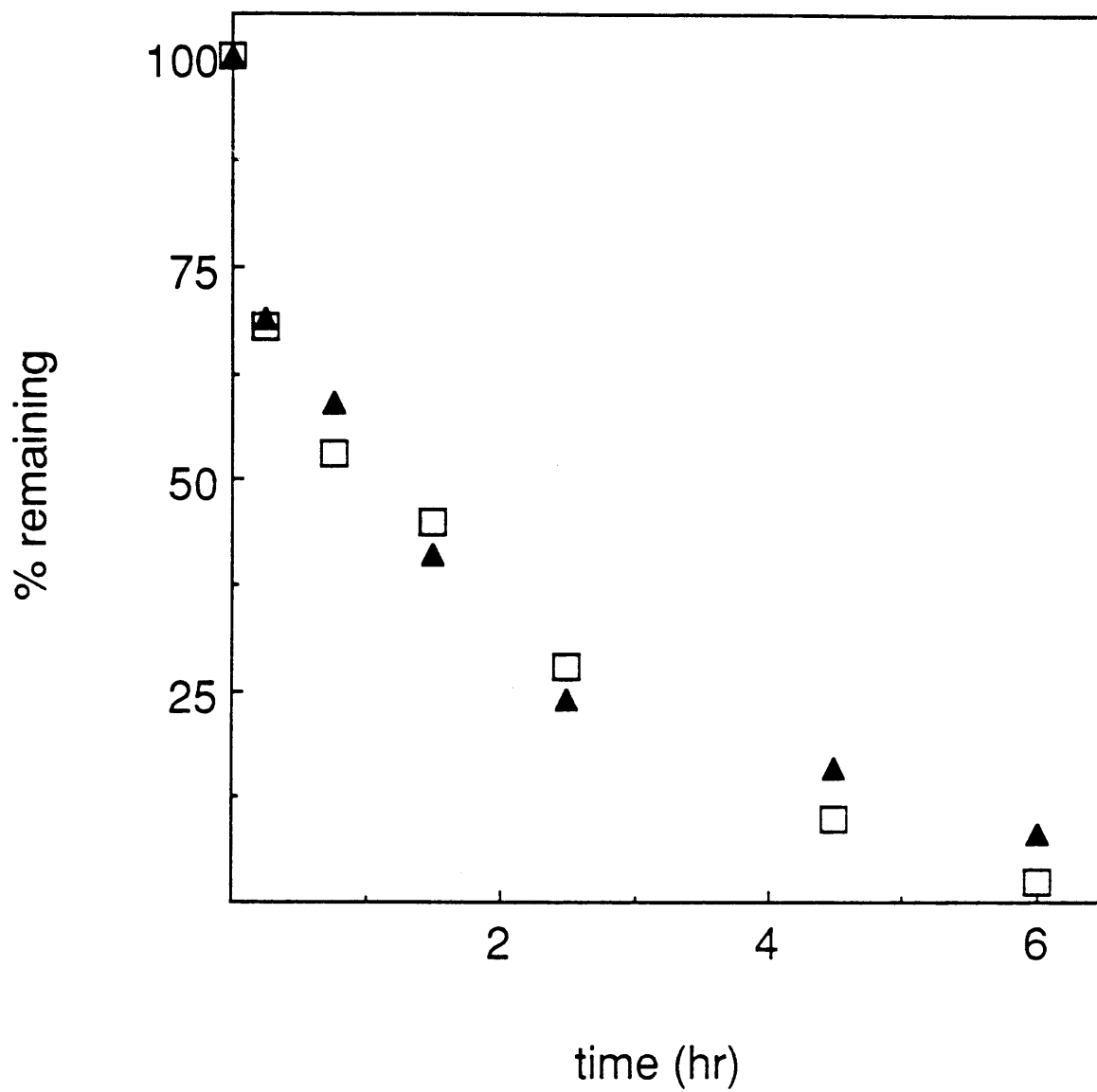
Figure 22 - Irreversible thermoinactivation of ribonuclease in anhydrous nonane at 145° C. (□) fraction of initial enzymatic activity; (▲) fraction of soluble, monomeric protein as determined by FPLC gel filtration chromatography (see Experimental Section). Thermoinactivation protocol was the same as for Figure 21.

account for the loss of all of the enzymatic activity. Thus, ribonuclease undergoes irreversible thermoinactivation at 145° C in anhydrous nonane with a half-life of one hour, and that this process is caused by the loss of soluble, monomeric protein due to microscopic and macroscopic aggregation.

To ensure that this correlation of loss of enzymatic activity and soluble, monomeric protein is not a function of the enzyme assay (either the size of the enzyme substrate or the enzyme aggregate), we reanalyzed samples from the previous experiment with both a large (yeast RNA) and small (cytidine 2':3' cyclic monophosphate) molecular weight substrate. Figure 23 clearly shows that the kinetics of the loss of enzymatic activity are independent of the size of the enzyme substrate. Furthermore, when assaying for enzymatic activity via potentiometry (see experimental section), we can reduce the size of the heat-induced enzyme aggregate by sonication of the enzyme solution until a very fine dispersion is observed. Since no significant changes in the kinetics of the loss of enzymatic activity are seen (Figure 23), the heat-induced aggregates do not trap active enzyme.

After establishing that the mechanism of irreversible thermoinactivation in our model system (ribonuclease in anhydrous nonane at 145° C) is aggregation, we set out to test the generality of this observation by examining other enzymes and solvents. First, we chose two unrelated enzymes whose physicochemical properties are also well established, chicken egg white lysozyme and bovine pancreatic α-chymotrypsin. Using the same protocol as described above for ribonuclease, lysozyme and chymotrypsin (both enzymes were lyophilized from pH 8 aqueous solution) were heated at 145° C in anhydrous nonane for various periods of time. As can be seen in Figure 24A for lysozyme and Figure 24B for chymotrypsin, both enzymes lose enzymatic activity during heating (with a half-life of 90 and 45 minutes, respectively) and this loss correlates with an aggregational event. It is worth noting that lysozyme and chymotrypsin both exhibit markedly increased thermostability compared to aqueous solution where the half-life for lysozyme is 1 minute at 90° C (Ahern and Klibanov, 1985) and 15 minutes at 55° C for chymotrypsin (Martinek et al., 1977). Interestingly, when comparing the half-lives of these

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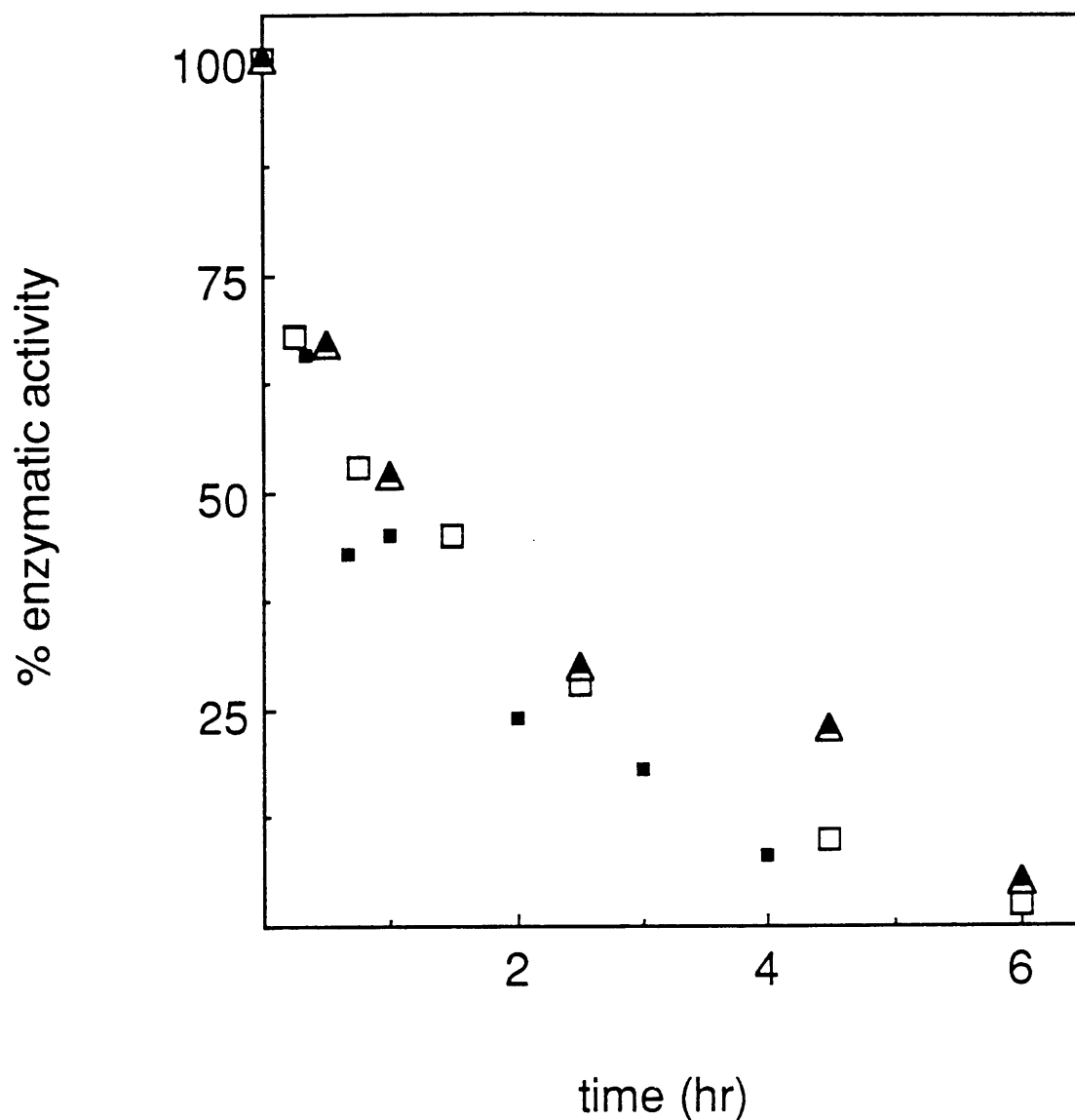
Figure 23 - Irreversible thermoinactivation of ribonuclease in anhydrous nonane at 145° C as a function of enzymatic assay conditions. Determination of enzymatic conditions via (i) potentiometry both with (△) and without (▲) sonication of the aggregate; and (ii) spectrophotometrically with both a large (yeast RNA, □) and small (cytidine 2':3' cyclic monophosphate, ■) molecular weight enzymatic substrate. Thermoinactivation protocol was the same as in Figure 21.
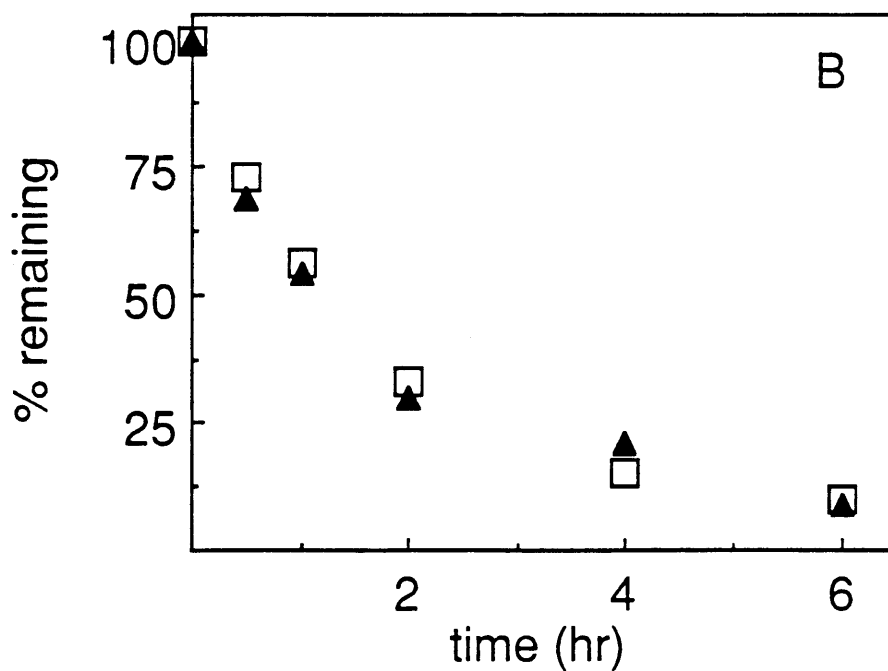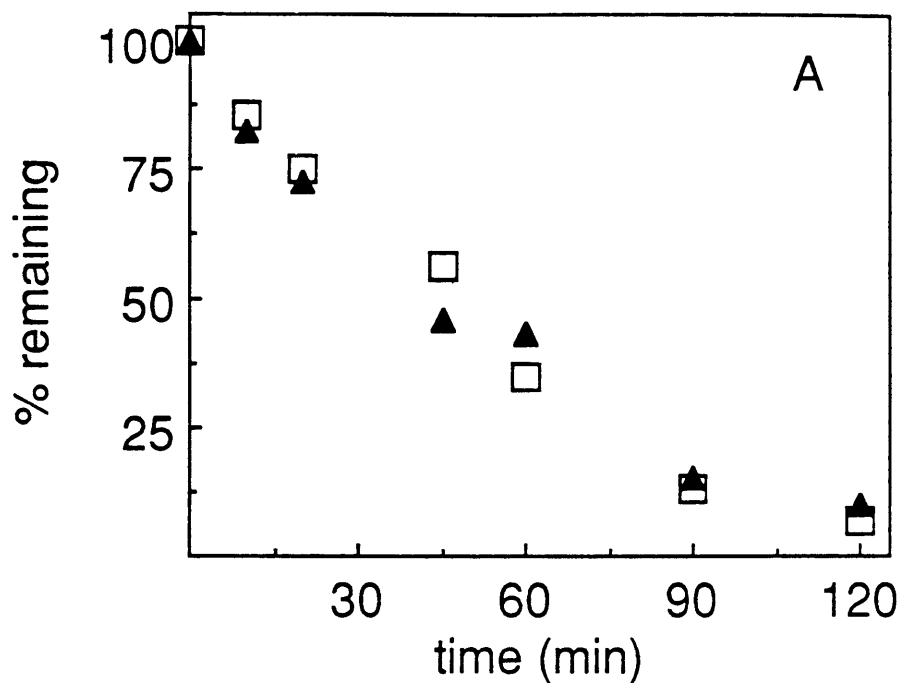
Figure 24 - Time course of irreversible thermoinactivation of chymotrypsin (A) and lysozyme (B) in anhydrous nonane at 145° C. (□) percentage of initial enzymatic activity; and (▲) percentage of soluble, monomeric protein as determined by FPLC gel filtration chromatography. Thermoinactivation protocol is described in Figure 21.

three enzymes (at 145° C in nonane) at equal water contents (13-14% H_2O (g/g); see next section), a range of values is seen from 30 minutes (ribonuclease) to 45 minutes (chymotrypsin) to 90 minutes (lysozyme). Therefore, the nature of the protein molecule itself affects the kinetics of this process to a modest degree, and the mechanism of enzyme irreversible thermoinactivation remains identical.

Several investigators have noted that enzymes are more thermostable in hydrophobic than in hydrophilic solvents, however, the mechanism of this observation has not been pursued (see literature survey). We therefore examined the effect of the solvent's physical properties on enzyme thermostability. For our purposes, organic solvents can be divided into three broad categories: non-polar, moderately polar and aprotic dipolar solvents. Nonane is an example of the first type with a high log P value (5.1), a measure of the hydrophobicity of a solvent (the logarithm of the partition coefficient between octanol and water; Laane et al., 1987), and a low dielectric constant ($\varepsilon = 2.0$), a measure of the polarity of the solvent (Gordon and Ford, 1972). We chose anhydrous 1-butanol (log P = 0.8 and $\varepsilon = 17.1$) and anhydrous N,N-dimethyl formamide (DMF) (log P = -1.0 and $\varepsilon = 36.7$) as representatives of moderately polar and aprotic dipolar organic solvents, respectively.

Ribonuclease was placed into both of these hydrophilic solvents, heated and then analyzed as described previously for nonane. Figure 25A shows the kinetics of the loss of enzymatic activity and soluble, monomeric protein in aqueous solution after heat treatment in 1-butanol while Figure 25B is the same experiment in DMF, both at 110° C. Ribonuclease is significantly less stable in these anhydrous, relatively hydrophilic solvents (half-life of 5 hours in butanol and 2 minutes in DMF at 110° C) when compared to anhydrous nonane (half-life of 43 hours at 110° C), yet ribonuclease is still much more stable in these organic solvents than in aqueous solution (half-life of 2 minutes at 90° C). Our results show that the thermostability of ribonuclease in anhydrous organic solvents increases with the hydrophobicity and apolarity of the organic milieu. Clearly, if these hydrophilic solvents can dissolve water, they can also hydrogen bond with donors and acceptors in the protein molecule. Thus, not only do
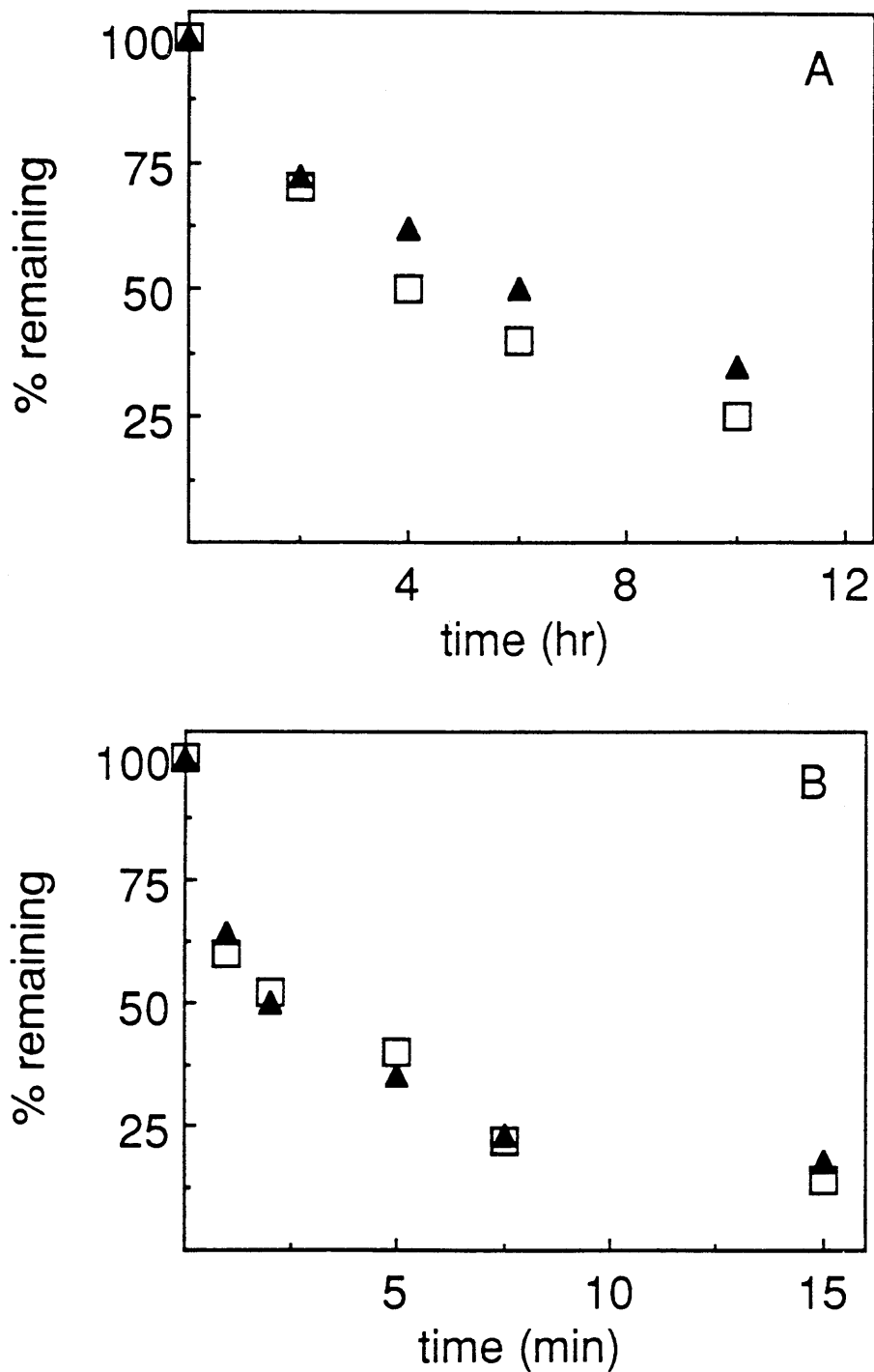
Figure 25 - Time course of irreversible thermoinactivation of ribonuclease at 110° C in anhydrous 1-butanol (A) and anhydrous N,N dimethylformamide (B). (□) fraction of enzymatic activity; and (▲) fraction of soluble, monomeric protein as determined by FPLC gel filtration chromatography. Thermoinactivation protocol is described in Figure 21.

hydrophilic solvents strip water from proteins (see next section), but they can also compete with the native noncovalent interactions in protein molecule, especially at elevated temperatures; an effect which may explain the decreased thermostability of ribonuclease in hydrophilic solvents.

The kinetics of the loss of both enzymatic activity and soluble, monomeric protein are not a simple, first-order exponential processes (typical correlation coefficient of 0.92), but rather a higher order reaction (see Figure 23). This observation is indicative of an aggregational phenomenon because protein coagulation is a multi-molecular event. In summary, ribonuclease undergoes irreversible thermoinactivation in hydrophobic and hydrophilic organic solvents via an aggregational mechanism and the enzyme's thermostability increases with the hydrophobicity of the solvent. Moreover, enzyme thermoinactivation in organic solvents (via aggregation) is not limited to ribonuclease; it is observed with both lysozyme and chymotrypsin. Importantly, for all of the solvents and enzymes tested, enzyme thermostability is greater in organic solvents than aqueous solution. The next step in the investigation is to examine the nature of this aggregational process in greater detail.

4.3.2 Heat-induced protein denaturation in neat organic solvents

The action of heat on proteins can lead to two kinds of conformational changes, denaturation and/or aggregation (Jaenicke, 1967). These two processes are classically related to each other as consecutive reactions with heat denaturation as the first step, followed by heat induced aggregation (Putnam, 1954; Joly, 1965):

$$N \rightleftharpoons pU \longrightarrow A \qquad (3)$$
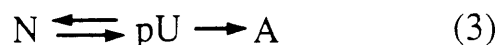
where N is a native protein, pU is a partially thermounfolded protein, and A is an aggregated (inactive) protein. Notice that the denaturation process is typically reversible in aqueous solution

(see literature survey), while aggregation is an irreversible event (even though reactivation of conformational aggregates is potentially possible, see next section).

Differential scanning calorimetry has emerged as a powerful tool to examine the first step in equation (1), protein denaturation, especially in solid samples such as dry enzyme powders or, in our case, enzymes in nonaqueous media. We began our differential scanning calorimetry study by using the same model system as before: ribonuclease (lyophilized from aqueous solution at pH 8) heated in the presence of anhydrous nonane. The spectrum (a) in Figure 26 shows that as the temperature increases from 25 to 90° C, no conformational changes occur in the protein molecule. However, from 92 to 104° C a thermal transition is observed with a peak at 99° C. This peak is called the thermal transition temperature (T_m) and it is caused by the excess heat (enthalpy) required to induce protein denaturation (similar to the excess energy required to melt ice at 0° C which is called the heat (enthalpy) of melting). This T_m value of 99° C for ribonuclease powder in anhydrous nonane is significantly higher than the T_m for this enzyme is aqueous solution (61° C at the optimum pH for conformational stability, von Hippel and Wong, 1965). Spectra (b and c) in Figure 26 are the superposition of two separate control experiments: (i) the anhydrous solvent nonane itself and (ii) ribonuclease was returned to ambient conditions after the first denaturation and then reheated. We can see that the enzyme cannot undergo a second conformational transition after the first thermal transition. Therefore, in contrast to the situation in aqueous solution, the thermal denaturation process for ribonuclease in organic solvents is irreversible, i.e., the enzyme cannot refold into its native conformation in neat organic solvents after returning to ambient temperatures.

Having established that an irreversible denaturation occurs when ribonuclease is heated in anhydrous nonane, the next step was to determine how different parameters affect the system. As discussed in the literature survey, the hydration level of protein powders affects their conformational flexibility, enzymatic activity, and thermostability. In order to determine to what extent water content affects both the thermal denaturation (T_m) and the subsequent irreversible thermoinactivation (aggregation) process of dehydrated enzymes in organic solvents,
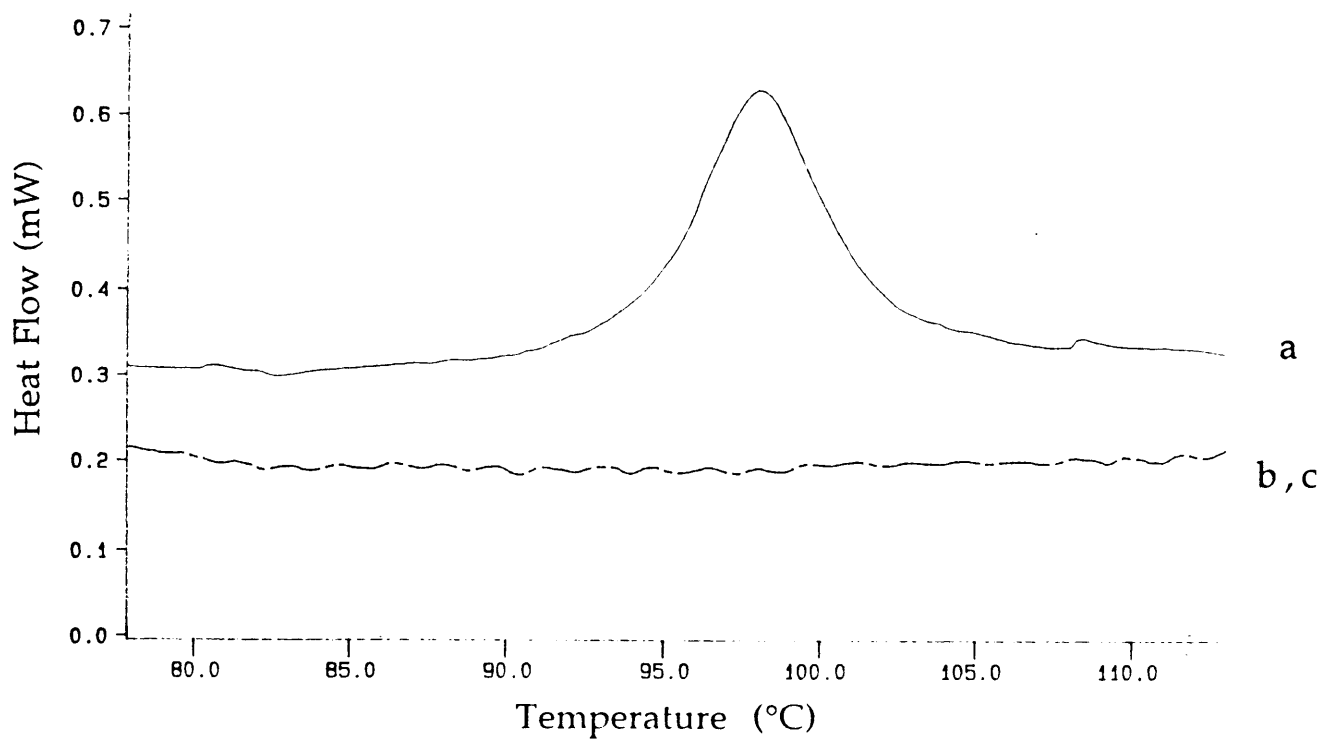
Figure 26 - Differential scanning calorimetry thermogram of (a) ribonuclease (16% H_2O, g/g, lyophilized from aqueous solution at pH 8.0) placed into anhydrous nonane; and (b) anhydrous nonane alone; and (c) ribonuclease rerun for a second time after the first denaturation. For experimental conditions, see Experimental Section.

ribonuclease powders were hydrated to different levels of water content (Leeder and Watt, 1974; see experimental section). These enzyme powders were then examined by both DSC to determine their T_m temperature (thermal denaturation) and by kinetic analysis at 145° C to determine the rate of inactivation by aggregation.

The results are summarized in Table 5. One can see that as the water content of the ribonuclease powder increases from 6% to 20%, the transition temperature decreases from 124° C to 92° C, while the rate of loss of both enzymatic activity and soluble, monomeric protein increases, as shown by the change in half-life from 120 minutes to less than 4 minutes. These results clearly show that (i) the loss of enzymatic activity due to heating at 145° C (column 3) is due to aggregation and (ii) the increasing water content of the enzyme powder has a detrimental effect on the thermostability of the enzyme. We observed that the T_m values of RNase in nonane were exactly the same as the protein powder by itself. In order to better understand the effect of this hydrophobic solvent on the irreversible thermoinactivation process, we measured the kinetics of the loss of enzymatic activity for ribonuclease at 145° C in either nonane, air or argon (Figure 27A). We can see that regardless of the environment, the rates are quite similar (the small differences between the liquid and gas environments are most likely due to differences in thermal conductivity). Moreover, the kinetics of disappearance of the soluble, monomeric protein peak on gel filtration chromatography are also comparable (see Figure 27B). Thus, nonane is essentially an inert solvent where not only are the Tm values of ribonuclease the same in nonane as in air, but the kinetics of the thermal inactivation of the enzyme at 145° C (via an aggregational mechanism) are identical in anhydrous nonane and molecular gases. Interestingly, the removal of oxygen does not affect this process indicating that oxidative reactions do not contribute to the aggregational phenomenon.

We have seen that both the thermal denaturation temperature and kinetics of aggregation at 145° C for ribonuclease at a given water content are the same in the hydrophobic solvent nonane and in dry powders. These findings suggest that the effect of the water content of ribonuclease powder on enzyme thermostability is the same for these two environments. As

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% H_2O (g/g) in enzyme powder [a]	thermal transition temperature, T_m (°C) [b]	half-life ($t_{1/2}$) of enzyme at 145° C (minutes) [c]
6	124	120
11	111	50
13	106	30
16	99	10
20	92	< 4
in water	61	—

Table 5 - The effect of water content of ribonuclease powder on both protein denaturation and irreversible aggregation during heating in anhydrous nonane. (a) The amount of protein-bound water was determined by Karl Fisher titration. (b) The thermal transition temperature was established via differential scanning calorimetry. (c) The kinetics of enzyme thermoinactivation at 145° C was measured by loss of both enzymatic activity and soluble, monomeric protein (see Experimental Section) (d) T_m value of ribonuclease in aqueous solution at optimum pH for conformational stability (von Hippel and Wong; 1965).

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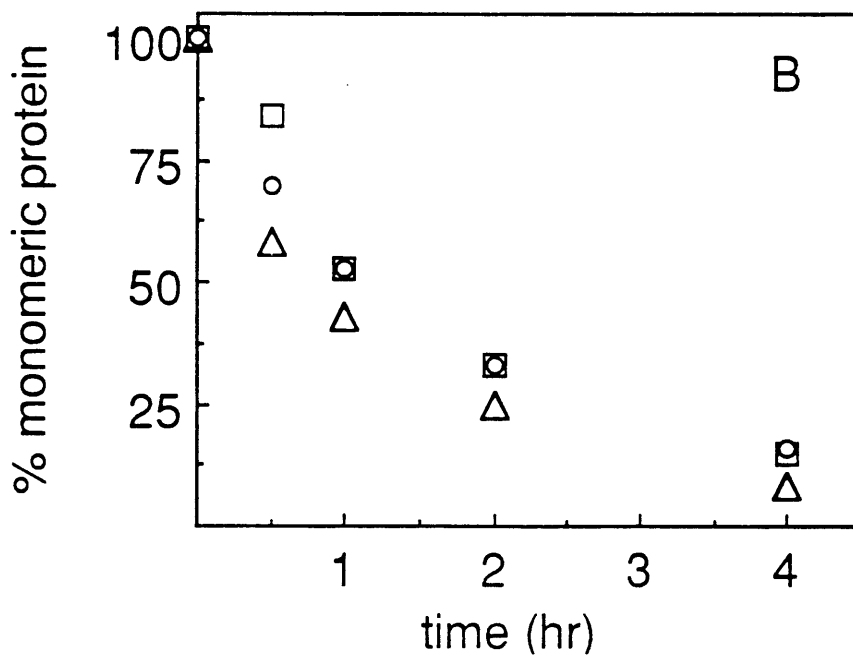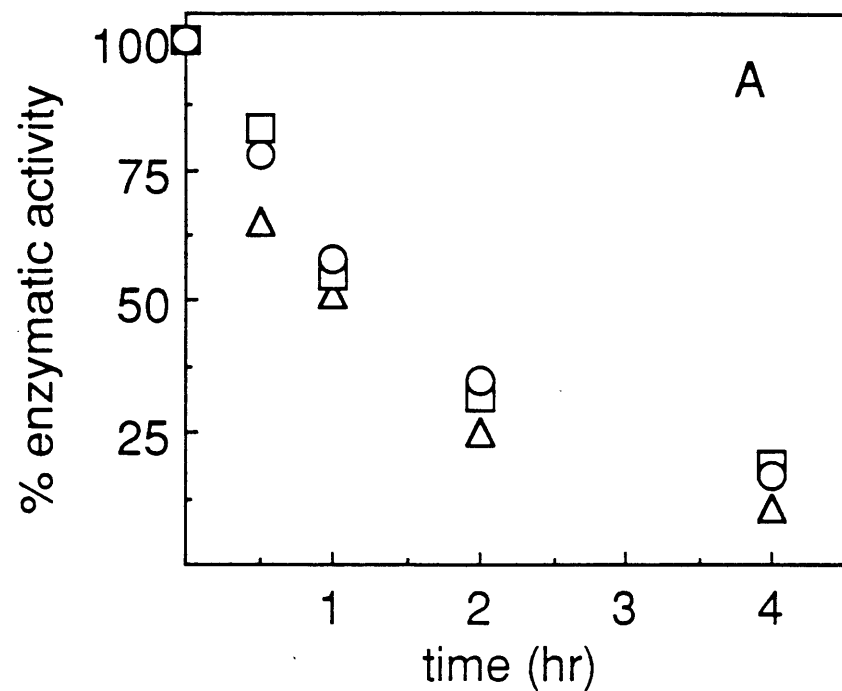
Figure 27 - Irreversible thermoinactivation of ribonuclease at 145° C in different inert environments. (A) Loss of enzymatic activity when heated in anhydrous nonane (△), argon (□), and air (○). (B) Fraction of soluble, monomeric protein as determined by FPLC gel filtration chromatography, when heated in anhydrous nonane (△), argon (□), and air (○). Thermoinactivation protocol is described in Figure 21.

discussed in the literature survey, using a variety of experimental techniques, it has been shown that the hydration of dry enzyme (lysozyme) powders proceeds in three stages (Rupley et al., 1983): From 0 to 7% H_2O g/g (0 to 60 water molecules per protein molecule), water forms clusters around charged surface residues with little protein motion and no enzymatic activity. Between 7 to 25% H_2O g/g (60 to 200 water molecules per protein molecule), water begins to cover most of the protein surface, there is significant protein mobility and concomitant detection of enzymatic activity. Finally, at 38% H_2O g/g (over 300 water molecules per protein molecule) the protein molecule becomes fully hydrated and gains up to 10% of the enzymatic activity of that in aqueous solution. Similarly, it has been shown that as the water content of lysozyme powders increases from 5 to 40 % H_2O g/g, the thermal transition temperature decreases from 130° C to the value in aqueous solution, near 70° C (Fujita and Noda, 1978).

Therefore, we hypothesize that increased protein mobility due to hydration of the enzyme powder (where water acts as a molecular lubricant capable of forming multiple hydrogen bonds) causes the observed decrease in the thermal stability of ribonuclease in nonane. The relationship between the conformational flexibility and thermal stability of an enzyme is based on the classic findings on the molecular mechanisms of thermophilicity, where an increased number of (or strengthened) noncovalent interactions are found in thermostable proteins compared to their mesophilic counterparts (i.e., rigidification; see literature survey). It has also been shown that there is a direct correlation between amide proton exchange rates (flexibility) and the denaturation temperature in proteins related to bovine pancreatic trypsin inhibitor (Wagner and Wuthrich, 1979). Furthermore, molecular modeling studies on the "flexibility indices" of a variety of well defined proteins have been calculated, and shown to increase as the thermostability of the protein decreases (Vihinen, 1987).

The effects of both hydrophobic and hydrophilic organic solvents on the thermal denaturation of ribonuclease was analyzed by means of differential scanning calorimetry. Using ribonuclease powder containing 16% H_2O (g/g), it was found that the transition temperatures (T_m) for the enzyme powder itself, and the enzyme powder in anhydrous hydrophobic solvents

such as nonane, toluene and dibutyl ether were virtually identical (T_m = 99° C, 100° C, 102° C and 101° C, respectively; however, the size of the peaks did decrease in some cases compared to the enzyme powder itself). Moreover, when anhydrous nonane was saturated with water, the T_m of ribonuclease (13% H_2O (g/g), see Table 1) shifted downward from 106° C to 102° C implying that excess water partitions from the hydrophobic organic solvent to the enzyme causing greater mobility (see above). Therefore, we can see that anhydrous hydrophobic solvents do not affect the thermal transition temperature of the enzyme powder in any substantial way.

Conversely, when the same ribonuclease powder was placed into anhydrous, hydrophilic solvents such as 1-butanol or DMF, no thermal transition peaks were observed. Why are thermal transition peaks observed in hydrophobic solvents but not in hydrophilic ones? It has been shown that differences in enzyme activity in hydrophobic versus hydrophilic solvents are due to water partitioning between the solvent and the enzyme (Zaks and Klibanov, 1988 b; Halling, 1989). In hydrophobic solvents water partitions to the enzyme; conversely, in hydrophilic solvents the opposite is the case (in both situations an equilibrium is established between protein-bound and free water). Therefore, in anhydrous, hydrophilic organic solvents, water is stripped from the protein and partitions to the solvent. If water is added to a hydrophilic solvent, the enzyme regains water and enzymatic activity concomitantly (Zaks and Klibanov, 1988 b). Interestingly, when 3% H_2O was added to 1-butanol, a thermal transition for ribonuclease was detected on the DSC with a peak at 83-85° C.

We directly measured the water content of ribonuclease after incubation in both anhydrous butanol and 3% H_2O-butanol (see experimental procedures). The water content of ribonuclease powder (15% H_2O g/g) decreased to 1.3% (g/g) in anhydrous butanol and subsequently increased to 3.1% (g/g) in the water-butanol solution. Thus the solvent does strip water from the enzyme and the enzyme does regain water in 3% H_2O-butanol. These water content values are very similar in magnitude to those reported previously for enzymes in organic solvents (Zaks and Klibanov, 1988 b; Yamane et al., 1988). The penetrating action of the

hydrophilic solvents could have several consequences which may explain the peculiar behavior of the thermal transition of ribonuclease in hydrophilic solvents: (i) creation of a heterogeneous population of enzyme molecules which do not undergo one, unified thermal transition, (ii) the enzyme molecules are not able to unfold to same extent as before, or (iii) perhaps a fewer number of noncovalent bonds can be broken. Either way, upon the addition of water to hydrophilic solvents, the enzyme molecules regain water (and presumably greater flexibility) which may account for the observed thermal transition on the DSC.

Ribonuclease has been shown to be extremely thermostable in anhydrous nonane by differential scanning calorimetry with T_m values far greater than in aqueous solution. Anhydrous hydrophobic solvents do not affect the T_m of ribonuclease when compared to the protein powder itself; in fact, the kinetics of irreversible thermoinactivation of ribonuclease are the same when suspended in nonane or heated as powders under air or argon. An increase in the water content of the ribonuclease powder facilitates both thermal denaturation and the aggregation process in nonane, suggesting that water acts as a molecular lubricant which increases the conformational flexibility of the protein (as is the case for enzyme powders). Finally, hydrophilic solvents are much more interactive than hydrophobic ones; they clearly strip water from ribonuclease and therefore can also hydrogen bond to the protein molecule itself. This effect may be responsible for the decreased thermostability of ribonuclease in hydrophilic organic solvents (see previous section) and the observed effects of hydrophilic solvents on the thermal denaturation of the enzyme.

4.3.3 Characterization of heat-induced aggregates in neat organic solvents

Using the identical model system as before, ribonuclease (lyophilized from aqueous solution at pH 8) in anhydrous nonane at 145° C, we set out to further characterize the nature of the irreversible aggregation reaction (the second step of equation 1) by: (i) determining whether the aggregate can be solubilized and perhaps reactivated, (ii) ascertaining whether the aggregation

process is chemical or physical in nature, and finally, (iii) drawing further comparisons between the processes leading to irreversible thermoinactivation of ribonuclease in organic solvents and aqueous solution.

The protocol for reactivation of thermally-induced conformationally altered monomers and aggregates has been elaborated by Klibanov and Mozhaev (1978) and Rudolph et al. (1979), respectively: inactive enzyme is solubilized in a high concentration of strong denaturants containing a reducing agent. The enzyme is then reoxidized in the absence of denaturant with a redox buffer and analyzed for activity. As can be seen in Figure 28, ribonuclease (6% H_2O g/g) heated in anhydrous nonane for various times could not be reactivated by this protocol. In fact, despite different denaturants (6M guanidine hydrochloride or 9M urea), reducing agents (ß-mercaptoethanol or dithiothreitol) and pH values (8.5 or 2.0), enzymatic activity could not be recovered, even though the heat-treated enzyme had been solubilized by these treatments.

In order to better understand why the solubilized enzyme aggregate could not be reactivated, the heat-treated protein was analyzed first by SDS-polyacrylamide gel electrophoresis and second for degradative, covalent reactions involving protein bound amino acid residues. This strategy is based on the investigation of the irreversible thermoinactivation of ribonuclease in aqueous solution where several pH-dependent reactions which contribute to the thermal inactivation of ribonuclease were identified: polypeptide hydrolysis at aspartic acid residues, deamidation of asparagine and/or glutamine residues, and cystine destruction and thiol-catalyzed disulfide interchange (Zale and Klibanov, 1986).

Ribonuclease was heated in anhydrous nonane at 145° C for 4 hours (6% H_2O g/g; see Figure 28), allowed to cool to room temperature, and the solvent was subsequently removed. The aggregate was dissolved in a buffered 9 M urea solution followed by the addition of a reducing agent and 1% SDS. The sample was then subjected to SDS-polyacrylamide gel electrophoresis (see experimental section). As can be seen in the top half of Table 7, when not exposed to heat treatment in organic solvent, the enzyme migrates as a single band as expected. However, heat-treated enzyme shows high molecular weight bands at the dimer, trimer, and

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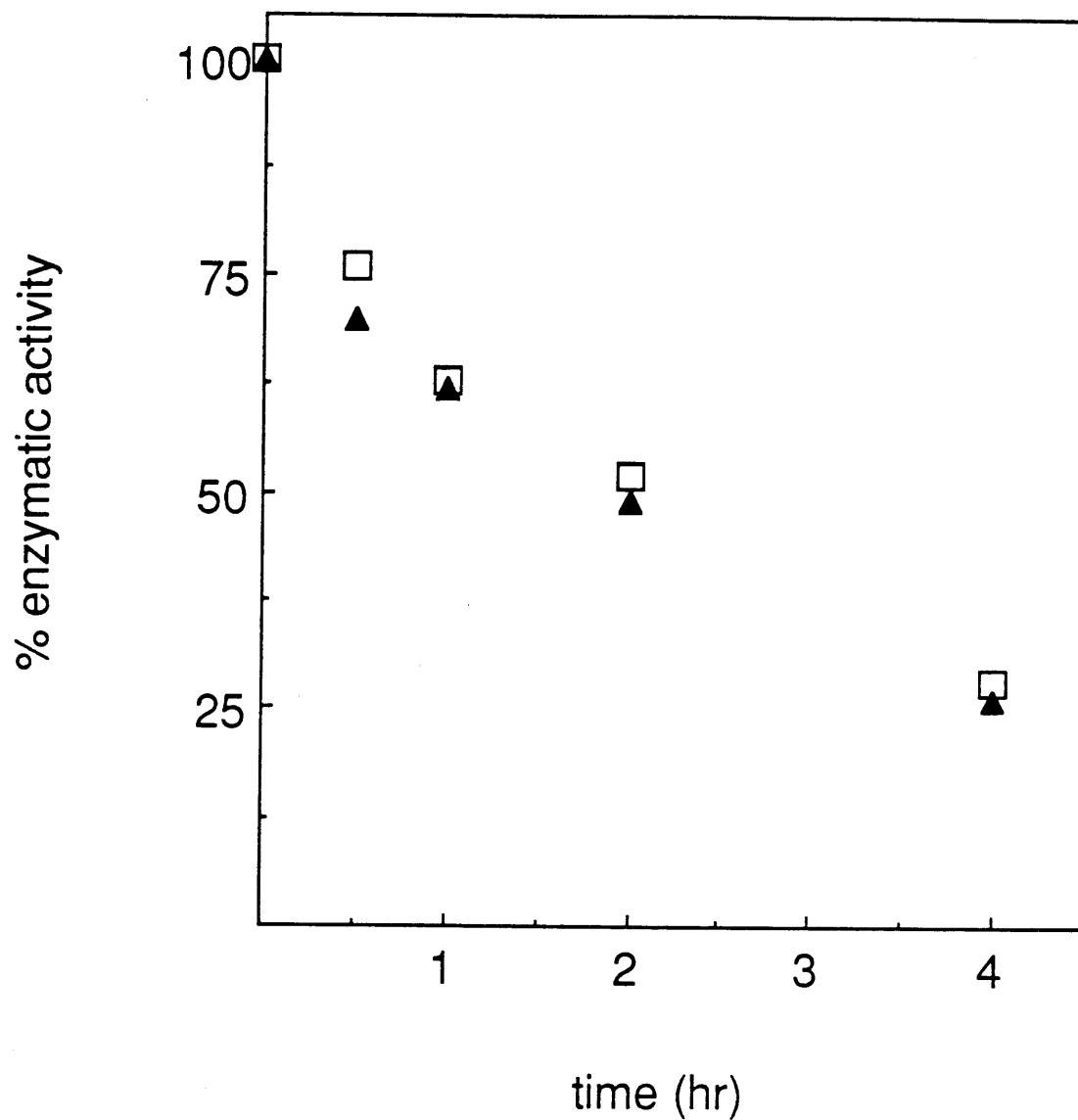
Figure 28 - Attempted reactivation of ribonuclease after irreversible thermoinactivation of the enzyme at 145° C in anhydrous nonane. (□) Loss of enzymatic activity when heated in anhydrous nonane; and (▲) Attempted reactivation of the heat-induced aggregate via solubilization in 6 M guanidine hydrochloride solution containing 0.1 M Tris, 2 mM EDTA and 70 mM dithiothreitol at pH 8.5, followed by desalting and then reoxidation of the enzyme in a redox buffer as described in the Experimental Section.

higher molecular weight positions. It is now clear why the reactivation experiment failed. Despite denaturants, reducing agents, and detergents (SDS), the enzyme molecules remained associated thereby implying that intermolecular, covalent crosslinks may exist.

The same native and heat-treated enzyme (as described above) were also dissolved in 6 N hydrochloric acid, followed by acid hydrolysis and amino acid analysis (see experimental procedures). Within experimental error, we found that no amino acids in ribonuclease were destroyed after heating for 4 hours at 145° C in anhydrous nonane (see Table 6). This result confirms the conformational nature of the thermoinactivation process (except possibly for deleterious reactions not detected by amino acid analysis; see below). Interestingly, one of the degradative, covalent reactions known to cause irreversible thermoinactivation of ribonuclease in aqueous solution, ß-elimination of cystine residues (Zale and Klibanov, 1986), is not occurring since no changes were observed in the total number of cysteine residues before and after heat treatment (Table 6).

Furthermore, no peptide hydrolysis could be detected since low molecular weight bands were not detected on SDS-urea polyacrylamide gel electrophoresis (MW range of 2-20 kd) These lower molecular weight breakdown fragments of ribonuclease are clearly visible after heat treatment in aqueous solution (pH 4). Interestingly, in contrast to the pH dependent aqueous solution thermoinactivation, ribonuclease does not show a pH-dependence toward thermoinactivation in organic solvents at 145° C (the effect of the pH of the aqueous solution from which ribonuclease is lyophilized, pH 4, 6 and 8), although the enzyme is perhaps slightly stabilized at lower pH values as shown in Figure 29 (all enzyme preparations had 8% H_2O g/g).

The next step was to analyze the heat treated RNase for ammonia evolution which, in aqueous solution, is a product of a deamidation reaction. Figure 30 shows that upon heating at 145° C in anhydrous nonane, heat-treated ribonuclease (6% H_2O g/g) does indeed release ammonia at an initial rate of 0.2 mole-equivalents per hour. However, we hypothesized that these high molecular weight bands seen on SDS-PAGE may be related to the observed ammonia evolution by a transamidation-type reaction. It was suggested by Bjarnason and Carpenter

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amino acid	residues [a]	control [b]	no heat [c]	heat-treated [d]
Asn, Asp	10, 5	16.1 ± 0.8	14.1 ± 2.0	14.9 ± 2.0
Gln, Glu	7, 5	12.9 ± 1.2	11.0 ± 1.8	11.8 ± 1.8
Ser	15	14.6 ± 1.0	15.1 ± 0.1	15.8 ± 0.3
His	4	4.6 ± 0.3	4.7 ± 0.2	5.0 ± 0.3
Thr, Gly	10, 3	13.1 ± 0.3	13.9 ± 0.5	14.3 ± 0.3
Ala	4	12.6 ± 1.4	13.5 ± 1.5	13.3 ± 1.0
Arg	4	4.1 ± 0.2	4.3 ± 0.2	4.6 ± 0.4
Tyr	6	5.8 ± 0.4	5.7 ± 0.5	6.1 ± 1.0
Val	9	8.9 ± 0.3	8.8 ± 0.2	9.1 ± 0.2
Met	4	3.4 ± 0.3	3.5 ± 0.4	3.5 ± 0.2
Ile	3	2.6 ± 0.2	2.6 ± 0.2	2.6 ± 0.2
Leu	2	2.2 ± 0.3	2.1 ± 0.1	2.2 ± 0.1
Phe	3	3.0 ± 0.1	2.9 ± 0.1	3.0 ± 0.1
Lys	10	8.9 ± 1.0	9.0 ± 0.3	8.4 ± 0.7
Cys [e]	8	8.2 ± 0.9	8.7 ± 0.8	9.0 ± 0.9

Table 6- Amino acid analysis of ribonuclease. (a) Amino acid composition as reported in the literature (Smyth et al., 1963); ribonuclease does not contain a Trp residue and proline cannot be detected by this method (however, this amino acid is unaffected by 145° C, 27 hours in a powder of bovine serum albumin (Bjarnason and Carpenter, 1970). (b) Ribonuclease lyophilized from aqueous solution at pH 8.0. (c) same as previous sample except placed in anhydrous nonane followed by evaporation of the solvent under reduced pressure. (d) same as previous sample except enzyme was heated in anhydrous nonane for 4 hours. (e) Determined spectrophotometrically prior to acid hydrolysis with Ellman's reagent (see Experimental Section).
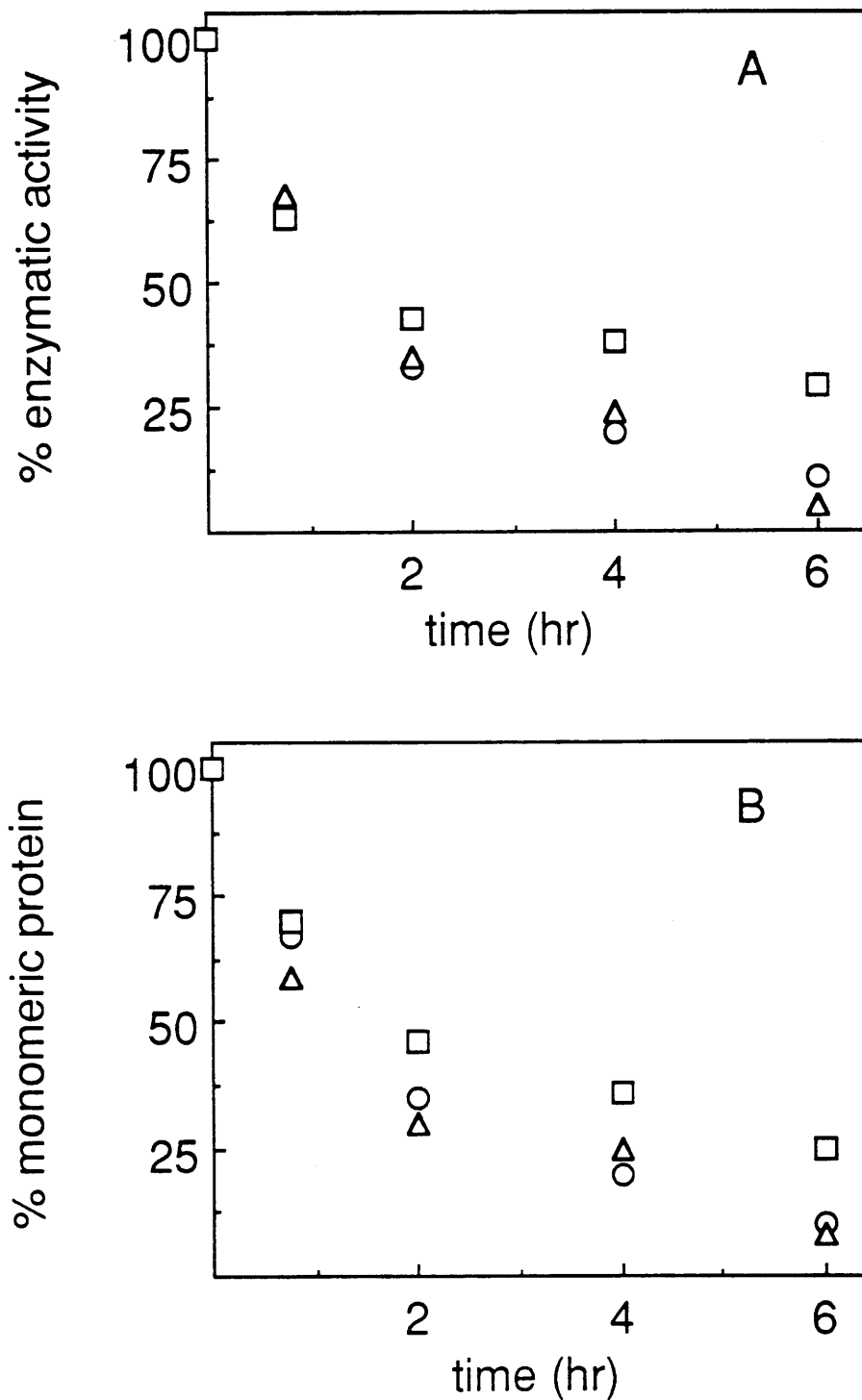
Figure 29 - Irreversible thermoinactivation of ribonuclease at 145° C after lyophilization from aqueous solution at different pH values. (A) Loss of enzymatic activity at pH 8 ($\bigcirc$), pH 6 ($\triangle$), and pH 4 ($\square$). (B) Loss of soluble, monomeric protein as determined by FPLC gel filtration chromatography at pH 8 ($\bigcirc$), pH 6 ($\triangle$), and pH 4 ($\square$). Thermoinactivation protocol is described in Figure 21.

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(1970) that ammonia evolution from heat-treated bovine serum albumin may be due to a transamidation reaction:

$$ASN-CH_2-C\overset{O}{\underset{NH_2}{\|}} \quad\longrightarrow\quad ASN-CH_2-C\overset{O}{\underset{LYS-(CH_2)_4}{\|}}NH \qquad (4)$$

$$LYS-(CH_2)_4-NH_2 \qquad NH_3$$
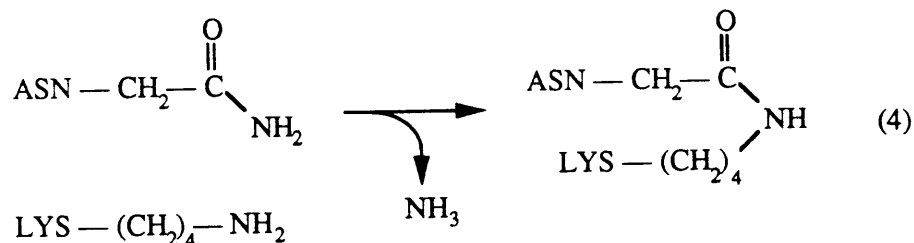
Note that this reaction is also undetectable by acid hydrolysis followed by amino acid analysis, since hydrolysis regenerates a lysine and aspartic acid residue. In order to test this possibility, we chemically modified the lysine residues of ribonuclease using acetic anhydride. We then examined the effect of chemical derivatization of the lysine residues of ribonuclease on both ammonia evolution and protein aggregation as seen on SDS-PAGE during heating in anhydrous nonane. This comparison will determine if ammonia evolution via a transamidation reaction is at least partially responsible for the observed intermolecular crosslinks.

Both unmodified and acetylated ribonuclease (water content 13% H_2O (g/g); see Table 5) were heated for various times at 145° C in anhydrous nonane, and these enzymes were found to lose enzymatic activity and soluble, monomeric protein (aggregate) comcomitantly, and at the same rate (half-life of 30 minutes). However, after four hours the unmodified ribonuclease had released 1.0 equivalents of NH_3 while an equal amount of acetylated enzyme had evolved only 0.42 equivalents NH_3. Thus, the derivatization of 70% of the lysine residues had reduced ammonia evolution by almost 2.5 fold while not affecting the kinetics of thermoinactivation. Clearly, some of the evolved ammonia in this experiment is due to a transamidation reaction, but this reaction is secondary in the inactivation mechanism.

We then solubilized the heat-treated unmodified and acetylated ribonuclease (parallel samples from the ammonia experiment) in a denaturant solution in both the presence and absence of a reducing agent. The addition of a reducing agent is expected to break apart any intermolecular S-S bonds (if present) and therefore reduce the size of the aggregates. These

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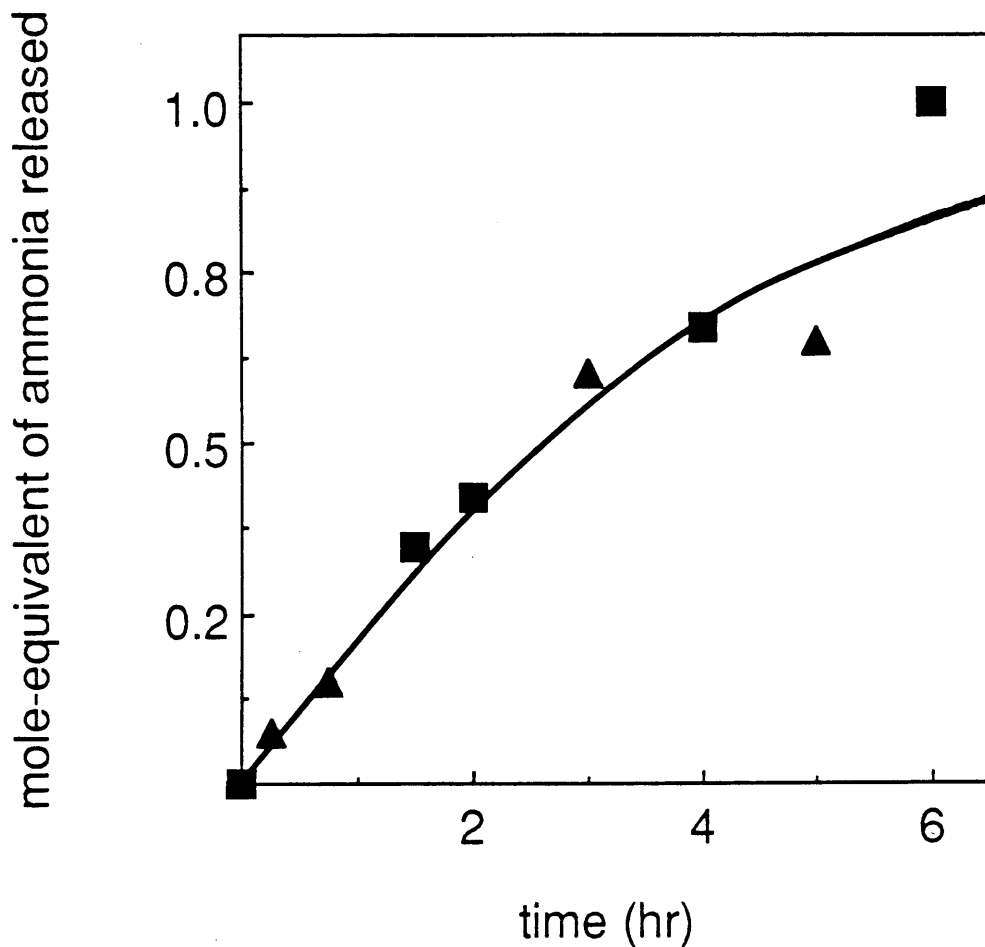
Figure 30 - Kinetics of ammonia evolution from ribonuclease (lyophilized from aqueous solution at pH 8.0) in anhydrous nonane at 145° C. Different symbols represent independent experiments. Number of equivalents of ammonia released were determined by heating the enzyme in a sealed vial, cooling to room temperature, followed by extracting ammonia into an aqueous solution and measuring its concentration enzymatically as described in the Experimental Section.

samples were then subjected to SDS-polyacrylamide gel electrophoresis (after the addition of 1%

SDS), stained with coomassie blue, and then quantitatively analyzed by gel densitometry.

The results of this experiment are summarized in Table 7. The first row of Table 7 clearly shows that the unmodified ribonuclease (unheated) migrated as a single native band in both the absence and presence of reductant. However after heat treatment, the unmodified enzyme shows a higher percentage of the native band upon the addition of a reducing agent (45% vs 60% plus a noticeable reduction in the amount of higher molecular weight species), thereby implying the presence of thiol-crosslinked aggregates. The same basic effect is seen for the acetylated enzyme (65% vs 90% native band for unreduced and reduced, respectively). This observation could be confirmed visually since the heat-treated unmodified ribonuclease remained as a visible aggregate in the denaturant solution until 70 mM dithiothreitol was added.

As seen in Table 7, the heat-treated acetylated protein shows a higher percentage of native band than the heated, unmodified enzyme either under nonreducing (45% vs 65%) or reducing (60% vs 90%) conditions. We can see that chemical modification of the lysine residues of ribonuclease does indeed reduce the amount of intermolecular crosslinking, and in combination with the ammonia evolution data, these findings show a transamidation reaction must be occurring. Therefore, 90% of the native band on SDS-PAGE (Table 7) can be restored by the combination of acetylation (which blocks transamidation) and reducing agent (which breaks apart any disulfide interchange crosslinks). Hence, the aggregates of ribonuclease, caused by heat treatment in anhydrous nonane at 145° C, are partially chemically-crosslinked and at least some of the observed ammonia evolution is due to a transamidation reaction.

At this point, we reattempted our reactivation experiment with acetylated (instead of of the unmodified enzyme as described earlier) ribonuclease because the heat-treated acetylated enzyme no longer contained the covalent crosslinks causing microscopic aggregation as seen on SDS-PAGE. The unmodified and acetylated enzyme were both heated for 4 hours at 145 °C in anhydrous nonane (until only 10% of the initial enzymatic activity remained) and then reactivated as described previously. Surprisingly, the acetylated protein did not regain 90% of its enzymatic

ribonuclease samples [a]	% native band on SDS-PAGE [b]	% dimer, trimer, higher MW bands on SDS-PAGE [b]
Unmodified enzyme		
no heat		
unreduced	98	2
with reducing agent	100	0
heated at 145° C, 4 hours		
unreduced	45	12, 6, 37
with reducing agent	60	24, 3, 13
Acetylated enzyme [c]		
no heat		
unreduced	95	3, 2
with reducing agent	100	0
heated at 145° C, 4 hours		
unreduced	65	20, 10, 5
with reducing agent	90	8, 2, 0

Table 7 - Gel densitometry analysis of SDS polyacrylamide gel electrophoresis of ribonuclease before and after heat treatment in anhydrous nonane at 145° C. (a) Thermoinactivation protocol was to place enzyme in organic solvent (either with or without a subsequent four hour heat treatment) followed by evaporation of solvent under reduced pressure. (b) Enzyme samples were then solubilized in 0.1M Tris-HCl, pH 8.5, containing 9 M urea and 0.03% EDTA with or without 70 mM dithiothreitol, had 1% SDS added, and were subjected to SDS polyacrylamide gel electrophoresis. The protein bands were stained with coomassie blue, and then analyzed by gel densitometry (see Experimental Section). (c) Acetylated ribonuclease had 70% of lysine groups blocked with retention of 60-70% of enzymatic activity (see Experimental Section).

activity as expected, but rather only 32% (the unmodified enzyme did not reactivate at all, as had been seen before).

In order to understand why the heat-treated acetylated enzyme failed to fully reactivate (32% activity instead of 90%), we prepared heat-treated samples of both unmodified and acetylated ribonuclease (as described above), dissolved them in a buffered 9 M urea solution containing a reducing agent and then subjected them to analysis by means of FPLC gel filtration chromatography equilibrated in the same denaturant solution (protein samples were carboxymethylated to prevent any potential reoxidation of thiol groups). We found that, after heat treatment, the unmodified protein had only 10% of its native peak in direct correspondence to its remaining enzymatic activity, while the acetylated protein showed 35% of the native peak (also corresponding to its remaining 32% of initial enzymatic activity). Thus, the denaturant solution alone does not break up the physically associated heat-induced aggregates of the acetylated enzyme (only 32% of the aggregate) to the same extent as the treatment during SDS-PAGE analysis which allows for recovery of 90% of the native protein within the SDS containing polyacrylamide gel (addition of 1% SDS and 5 minutes at 100° C). Unfortunately, we could not use this SDS-treatment during the reactivation experiment because the removal of SDS is extremely difficult since the detergent irreversibly binds to proteins (Helenius and Simons, 1975).

In summary, three unrelated enzymes (ribonuclease, chymotrypsin, and lysozyme) were shown to display markedly increased thermostability in anhydrous organic solvents compared to aqueous solution. The mechanism of irreversible thermoinactivation in nonaqueous media for these three enzymes was determined to be heat-induced protein aggregation. The products of the subsequent heat-induced aggregation were also characterized as being both chemically crosslinked, in part due to transamidation and intermolecular disulfide interchange reactions, and physically associated protein oligomers. Bovine pancreatic ribonuclease A was chosen as a model enzyme to establish the influence of water content and solvent type (hydrophobic or hydrophilic) on thermal denaturation (see next section).

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V. SUMMARY AND CONCLUDING REMARKS

The motivation for this study was to quantitatively establish the upper limit of protein thermostability by analyzing both conformational and covalent thermoinactivation processes in a variety of enzymes and proteins under different environmental conditions. Previous work in our laboratory had identified five pH dependent, covalent reactions that caused irreversible thermoinactivation of enzymes at high temperatures: cystine destruction, thiol-catalyzed disulfide exchange, cysteine oxidation, deamidation of asparagine and glutamine residues, and peptide bond hydrolysis at aspartic acid residues (see introduction). Because of the general nature of these reactions, they afforded the opportunity to define the upper limit of protein thermostability in aqueous solution. The introductory section of the thesis raised four questions which, once answered, would not only establish the upper limit of protein thermostability, but also suggest strategies to stabilize enzymes for use as practical catalysts at elevated temperatures.

The first objective was to determine the general nature of these degradative, covalent reactions by examining them in a variety of proteins at high temperatures. Two of these processes, cystine destruction and disulfide interchange, were quantitatively characterized in over a dozen proteins. All proteins studied undergo heat-induced ß-elimination of cystine residues in the pH range from 4 to 8 at 100° C. The time course of this process closely follows first order kinetics indicating that the stability of a disulfide bond is not significantly affected by its position in the polypeptide chain. Moreover, the first order rate constants of ß-elimination of -S-S- bonds at 100° C are remarkably similar for different proteins: 0.8 ± 0.3 hr^{-1} and 0.06 ± 0.02 hr^{-1} at pH 8 and 6, respectively. Thus, this process is relatively independent of both the primary structure and elements of higher structure remaining in proteins at 100° C. The ß-elimination of disulfide bridges produces free thiols which were found to be both protein-bound and low molecular weight species including hydrosulfide. These byproducts cause yet another deleterious reaction in proteins: heat-induced disulfide interchange. This reshuffling of -S-S- bonds, which is much faster than ß-elimination was also quantitatively characterized (half-life of

10 minutes at pH 6 and 2 minutes at pH 8) using mixed disulfides of lysozyme and trypsinogen with glutathione.

Similar experiments were carried out on ten different proteins at pH 4, 100° C, in order to determine the rate constants of overall deamidation of proteins (Appendix B). Along with our findings on the thermal stability of immobilized glucose isomerase at pH 8, and data from other enzymes previously examined in our laboratory, it was established that protein deamidation at 90-100° C is a pH dependent process occurring in over a dozen different proteins (half-lives of 8 ± 3 hours at pH 8, 41 ± 9 hours at pH 6, and 132 ± 50 hours at pH 4; see Appendix B). The remaining deteriorative, covalent reactions (cysteine oxidation and peptide bond hydrolysis) have also been characterized either through work described herein on the mechanism of thermoinactivation of immobilized glucose isomerase or by analysis of recently published reports on peptide and protein behavior at elevated temperatures (see literature survey).

The general nature of these heat-induced, degradative covalent reactions in proteins is summarized in Table 8. These data on the overall rates of protein thermoinactivation processes show that for each reaction, labile amino acid residues are more or less equally reactive regardless of the nature of the protein. Although a few particularly labile sequences have been identified (deamidation of Asn-Gly and peptide hydrolysis at Asp-Pro; see literature survey), none of the neighboring sequences next to these labile amino acids are observably more stable towards the degradative reactions observed at 90-100° C. The thermoinactivation of enzymes due to these "weak links" is a general phenomenon observed in over half a dozen enzymes (see literature survey). Since cysteine, asparagine, glutamine, and aspartic acid residues are common to proteins regardless of structure or function, these data allow for the prediction of the maximum expected thermal stability of any protein at high temperatures in the pH range of 4-8. We can see from Table 8 that, at the optimum pH, these covalent reactions limit the integrity of the protein molecule to several hours at temperatures of 90-100° C, and therefore, demarcate the upper limit of protein thermostability in aqueous solution.

Covalent reactions shown to cause irreversible thermoinactivation of enzymes	Time for 50% modification (hours)
ß-elimination of cystine residues in twelve different proteins at 100° C (Volkin and Klibanov, 1987)	
pH 4	144
pH 6	9 - 16
pH 8	0.5 - 1.4
Thiol-catalyzed disulfide interchange at 100° C in mixed disulfides of lysozyme and trypsinogen with glutathione (Volkin and Klibanov, 1987)	
pH 6	0.17 (10 min)
pH 8	0.04 (2 min)
Cysteine oxidation at pH 8 in α-amylase (Tomazic and Klibanov, 1988a) and immobilized glucose isomerase (Volkin and Klibanov, 1989a)	
70° C	14
90° C	0.5 - 1.5
Deamidation of asparagine and glutamine residues in proteins (Appendix B) and peptides (Geiger and Clarke, 1987)	
ten different proteins (100° C, pH 4)	132
five different proteins (90° C, pH 6 - 6.5)	41
six different proteins (90° C, pH 8)	8
Asn-Gly hexapeptide (100° C, pH 7.4)	0.15 (9 min)
Asn-Pro; Asn-Leu hexapeptide (100° C, pH 7.4)	5 - 8
Hydrolysis of peptide bonds at aspartic acid residues in proteins (Zale and Klibanov, 1986) and peptides (Marcus, 1985)	
Asp_{121}- Ala_{122} in ribonuclease (90° C, pH 4)	26
Asp-Pro dipeptide (100° C, pH 2)	0.18 (11 min)
Asp-Phe; Asp-Ser dipeptide (100° C, pH 2)	1.8 - 2.2

Thermophilic microorganisms need catalytically active enzymes at high temperatures and Nature accomplishes this goal by preventing reversible denaturation via the incorporation of additional or strengthened noncovalent interactions (see literature survey). It was of interest to inquire if Nature knows of the rules characterized herein concerning the mechanism of the irreversible thermoinactivation of enzymes, i.e., the slow loss of catalytic activity over prolonged periods of time. Analysis of protein sequences and bulk compositions of thermophilic versus mesophilic microorganisms (Barker et al., 1986) shows no such correlation except perhaps in the case of cystine destruction. As concluded by Hochachka and Somero (1984), "...comparisons of proteins from different species, including those of thermophilic bacteria living near the boiling point of water, have not found evidence for widespread utilization of incorporation of additional disulfide links for structural stabilization." However, this deficiency of S-S bonds may be attributable to the absence of an organelle system in prokaryotes (endoplasmic reticulum) or, as discussed in the thesis itself, the heat-sensitive nature of this chemical linkage.

The mechanism of irreversible thermoinactivation of enzymes is of concern to those interested in stabilizing enzymes as practical catalysts at elevated temperatures for prolonged periods of time. Nature probably does not concern itself with the irreversible thermoinactivation of enzymes because it may be easier to simply accelerate the rate of proteolytic degradation and protein biosynthesis (Zale and Klibanov, 1986). Nevertheless, it is quite possible that in tightly folded thermophilic proteins, the thermolabile amino acid residues located in the protein interior will be less accessible to water (required for these degradative reactions). Therefore, thermophilic proteins may be more thermoresistant than their mesophilic counterparts to both reversible and irreversible thermoinactivation when heated at temperatures at or below their melting temperature (Zale and Klibanov, 1986; Tomazic and Klibanov, 1988b).

The remaining objectives of this research project were dedicated to the topic of stabilizing enzymes as practical catalysts at both elevated and extreme temperatures. First, the irreversible thermoinactivation of immobilized glucose isomerase from Streptomyces olivochromogenes was mechanistically investigated at the pH-optimum of enzymatic activity (pH

8.0) in order to ascertain to what extent these deteriorative, covalent reactions limit the thermostability of this practical biocatalyst under both extreme temperature (90° C) and near bioreactor conditions (70° C). The enzyme-catalyzed isomerization of glucose into fructose is carried out in industrial bioreactors at 60-65° C where the half-life of the immobilized glucose isomerase is on the order of several weeks. Most industrially employed glucose isomerases exhibit temperature optima in the range of 80-90° C (in the presence of substrate), but even at these temperatures, insufficient operational stability precludes their use (see literature review).

Ligands (high fructose corn syrup and the competitive inhibitor xylitol) greatly stabilize the immobilized enzyme at high temperatures. At 90° C in the presence of 2 M xylitol, irreversible inactivation of immobilized glucose isomerase is almost exclusively caused by deamidation of its asparagine and/or glutamine residues. This covalent reaction demarcates the upper limit of thermal stability for the enzyme. Additional experiments show that at the temperature close to that of industrial bioreactors (70° C), the time-dependent decay of glucose isomerase activity is brought about by different processes: oxidation of cysteine residues and/or by heat-induced deleterious reactions with high fructose corn syrup or its impurities. Thus the cause of irreversible thermoinactivation of this enzyme depends on the temperature regime.

The next objective of this research project was to establish the mechanism of enzyme thermoinactivation at extreme temperatures (110-145° C) in nonaqueous environments. The recent discovery that enzymes can function in organic solvents has dramatically expanded the range of reactions possible via biocatalysis. Perhaps the most neglected aspect of this emerging technology is the stability of proteins in nonaqueous media (see literature survey). The results of this study provided a mechanistic description of the irreversible thermoinactivation of three unrelated enzymes (ribonuclease, chymotrypsin, and lysozyme) in anhydrous organic solvents. They demonstrated that enzymes display greatly increased thermostability in anhydrous organic solvents compared to aqueous solution. The loss of enzymatic activity due to heating in hydrophobic and hydrophilic organic solvents at 110-145° C correlates with the loss of the soluble, monomeric protein as caused by heat-induced aggregation. The heat-induced aggregates

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of ribonuclease were also characterized as being both physically associated and chemically crosslinked in nature, in part due to transamidation and intermolecular disulfide interchange.

Using bovine pancreatic ribonuclease A as a model protein, it was established that enzymes are more thermostable in hydrophobic solvents (shown to be essentially inert) than in hydrophilic ones (shown to strip water from the enzyme). Using differential scanning calorimetry, we were able to quantitatively analyze the conformational thermostability of enzymes in neat hydrophobic organic solvents. It was determined that the thermostability of RNase in nonane decreases as the water content of the enzyme powder increases. It has been shown that water acts as a molecular lubricant thereby increasing conformational flexibility of enzymes in the dry state (Rupley et al., 1983). Our findings suggest that RNase is conformationally rigid in hydrophobic organic solvents due to lack of water, and therefore, similar to the increased rigidity of thermophilic enzymes (see literature survey), more thermostable in organic solvents than in aqueous solution.

By understanding the causes and mechanisms of enzyme thermoinactivation, we can develop strategies to stabilize enzymes at elevated temperatures. A summary of these general strategies is shown in Table 9 which include both conventional chemical and physical methods and newer technologies such as protein engineering and the screening of thermophilic microorganisms. The experimentally obtained data from this project on the mechanisms of irreversible thermal inactivation of enzymes offer specific solutions to the particular cases examined. For example, since it is likely that the cleavage or reshuffling of -S-S- bonds will inactivate an enzyme, one should conclude that it is unwise to genetically engineer new cystine residues in enzymes that are to work at high temperatures for prolonged periods of time. In other words, although additional disulfide bonds stabilize proteins against reversible thermal unfolding (by reducing the entropy of the unfolded state, see Anfinsen and Scheraga, 1975), they may not be as useful against irreversible thermoinactivation and, in fact, their introduction results in new "weak links" in the enzyme molecule.

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Effectors	Comments
Intrinsic stability	
1. Mesophilic vs. thermophilic enzymes	Rigidification of enzyme conformation
2. Site-directed mutagenesis	Replacement of labile amino acid residues
Additives	
1. Specific	Shift $N \longleftrightarrow U$ toward the native form
2. Nonspecific	Neutral salts and polyhydric compounds
3. Competitors	Outcompete enzyme for inactivating agent or remove catalyst of deteriorative reaction
Immobilization	
1. Partitioning and diffusional effects	Change microenvironment around enzyme
2. Multipoint attachment of enzyme	Rigidification of enzyme conformation or circumvent protein aggregation
Chemical modification	
1. Cross-linking reagents	Rigidification of enzyme conformation
2. Alter ionic state	Add, neutralize, or change charged residues
3. Introduce steric hindrances	Inhibit interactions with other macromolecules

Table 9 - Examples of approaches to minimize irreversible thermoinactivation of enzymes as described by Volkin and Klibanov (1989b).

The studies on immobilized glucose isomerase showed that deamidation of asparagine and glutamine residues demarcates the upper limit of the immobilized enzyme's thermostability. Therefore, it will be very difficult, if not impossible, to carry out isomerization reactions at 100°-105° C to bypass the chromatographic enrichment of HFCS to 55% fructose. The conclusions of this work also have important implications for those wishing to produce more thermostable glucose isomerases for use in current industrial bioreactors. Random mutagenesis or screening of new microorganisms may yield enhanced stabilities by rigidifying the native form of the enzyme, but only by chance. Site-directed mutagenesis can be helpful if used to replace the enzyme's cysteine residue with a non-oxidizable, isosteric amino acid. Another rational approach to more heat-resistant glucose isomerases involves elucidation of the reaction of the enzyme with HFCS and/or impurities (pH drop, reaction with impurities, or Maillard reaction (Gottschalk, 1972; Whitaker, 1983), followed by designing a strategy for avoiding that reaction.

It has been shown that when an enzyme is heated at or below its melting temperature, the thermal stability of the protein molecule is a function of its conformational stability, i.e., resistance against reversible thermal denaturation (see literature survey). In fact, this relationship was seen during our studies with immobilized glucose isomerase. This enzyme was shown to have a thermal denaturation temperature of 88° C (in 0.1 M fructose), and when heated at 90° C (in the presence of 2 M xylitol), to inactivate by means of deamidation of its asparagine and/or glutamine residues. However, when heated well below its thermal unfolding temperature, 70° C, deamidation no longer caused thermoinactivation presumably because the labile (or affecting catalytic activity) interior amide residues were no longer exposed to solvent. Water is the solvent which facilitates protein mobility leading to reversible unfolding, incorrect structure formation and aggregation (see literature survey). Therefore, the substitution of a nonaqueous medium for water should stabilize enzymes against both conformational processes and covalent reactions that cause irreversible thermal inactivation. The experimentally obtained data suggest that enzymes are indeed far more thermostable in anhydrous organic solvents not only due to their conformational rigidity in the dehydrated state, but also because of their resistance to most of the

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the covalent reactions known to cause irreversible thermoinactivation of enzymes in aqueous solution.

In aqueous solution, the driving force in the transition between the unfolded and folded form of a protein molecule is an entropic effect where interactions between the solvent and hydrophobic regions of a protein molecule are minimized by the formation of tertiary structure (Creighton, 1983). The question then arises as to why enzymes remain in their catalytically active conformation and do not unfold in anhydrous organic solvents, where such interactions will be drastically reduced? Zaks and Klibanov (1988 a) suggested that enzymes would indeed prefer to unfold in organic solvents, but are prevented from doing so by the intramolecular noncovalent forces of the native conformation which, in the absence of the "molecular lubricant" water, kinetically trap the enzyme placed into organic solvents. Moreover, protein-protein interactions were shown not to be the reason for the stability of proteins in organic milieu, since immobilized chymotrypsin had the same activity as the free enzyme in organic solvents.

Our thermostability findings support this "kinetically trapped" hypothesis of enzyme stability in organic solvents. Similar to the thermal denaturation of enzyme powders, we observed a significantly higher thermal transition temperature for RNase in (hydrophobic) organic solvents than in aqueous solution as measured by differential scanning calorimetry. However, at a certain temperature there was enough heat added to overcome both the kinetic barriers of the dehydrated enzyme and the intramolecular noncovalent forces that hold together the enzyme molecule. The enzyme molecule then denatured, and this transition was shown to be irreversible, i.e., the molecule did not refold into its native conformation after the first heat-induced unfolding in organic solvents.

VI. SUGGESTIONS FOR FUTURE RESEARCH

Future work in the area of enzyme thermostability will require increasingly more sophisticated techniques and equipment in order to better understand the relationship between reversible protein denaturation and the subsequent irreversible thermoinactivation events. The application of two methodologies, namely protein engineering and biophysical spectroscopic characterization, to the study of irreversible thermal inactivation of enzymes will be proposed based on the results from this research project.

First, we have established the general nature of the covalent reactions that cause irreversible thermoinactivation of enzymes at high temperatures. Specific residues have now been identified and can be replaced by protein engineering techniques in order to increase the thermal stability of practical biocatalysts when used at elevated temperatures. For example, site-directed mutagenesis may be used to replace the labile cysteine residue in glucose isomerase with an isosteric amino acid such as serine or alanine. This strategy eliminates the current protein engineering protocol, dubbed by Knowles (1987) as "gee-whiz" mutagenesis, where an amino acid residue is replaced and the consequences are subsequently determined. Similarly, other "hot-spots" such as Asn-Gly and Asp-Pro may be changed in order to stabilize proteins or peptides at moderate temperatures or for short times at high temperatures. Ironically, despite our interest in enzyme stabilization, the results from this work indicate that the long term stabilization of enzymes at high temperatures (for example, glucose isomerase at 90° C to isomerize dextrose directly to 55% fructose) is not possible since replacement of all asparagine and glutamine residues is likely to cause severe loss of activity or solubility in the mutant protein.

Protein engineering techniques (both random and site-directed mutagenesis) have been successfully used to increase the stability of enzymes against reversible thermal denaturation in aqueous solution (see literature survey). It would be of interest to attempt to increase the conformational stability of enzymes in organic solvents during exposure to elevated temperatures. Site-directed mutagenesis may prove helpful to understand which noncovalent interactions within the protein molecule affect the stability of enzymes in organic solvents. For

example, modification of surface residues may affect the amount of bound water while changes in the interior of the molecule could increase hydrophobic and electrostatic interactions as well as hydrogen bonding.

Results from this work, along with data from the other enzymes examined in our laboratory, indicate that at temperatures above the thermal transition temperature of a protein, all asparagine and glutamine residues are more or less equally labile (Appendix B). When proteins are exposed to elevated temperatures which are at or below the T_m of the protein, there is a correlation between reversible thermal denaturation (conformational rigidity) and irreversible thermoinactivation processes, i.e., the tertiary structure of a protein affects the reactions which cause irreversible thermoinactivation of enzymes at moderate temperatures. In fact, recent work has shown that deamidation of asparagine residues in enzymes under physiological conditions is controlled by the tertiary structure of the protein molecule (see literature survey). It would be of interest to determine the role of the tertiary structure as a determinant of the covalent reactions occurring during the irreversible thermoinactivation of enzymes in aqueous solution at moderate temperatures.

For example, the effect of the protein tertiary structure on the destruction of S-S bonds could be examined. Differential scanning calorimetry was used to measure the thermal denaturation temperatures of RNase in this study. In addition, there are many optical spectroscopic techniques which can monitor heat-induced changes in the conformation of a protein (both tertiary and secondary structure) such as circular dichroism, fluorescence, UV absorbance, and infrared and Raman spectroscopy. The last technique is particularly valuable since it not only can measure protein thermal denaturation, but it can specifically monitor disulfide bonds. Therefore, the effect of the changes in the secondary and tertiary structure of a protein due to thermal denaturation could be correlated to both the temperature dependence of the -S-S- bond signal and the kinetics of cystine destruction via the ß-elimination reaction.

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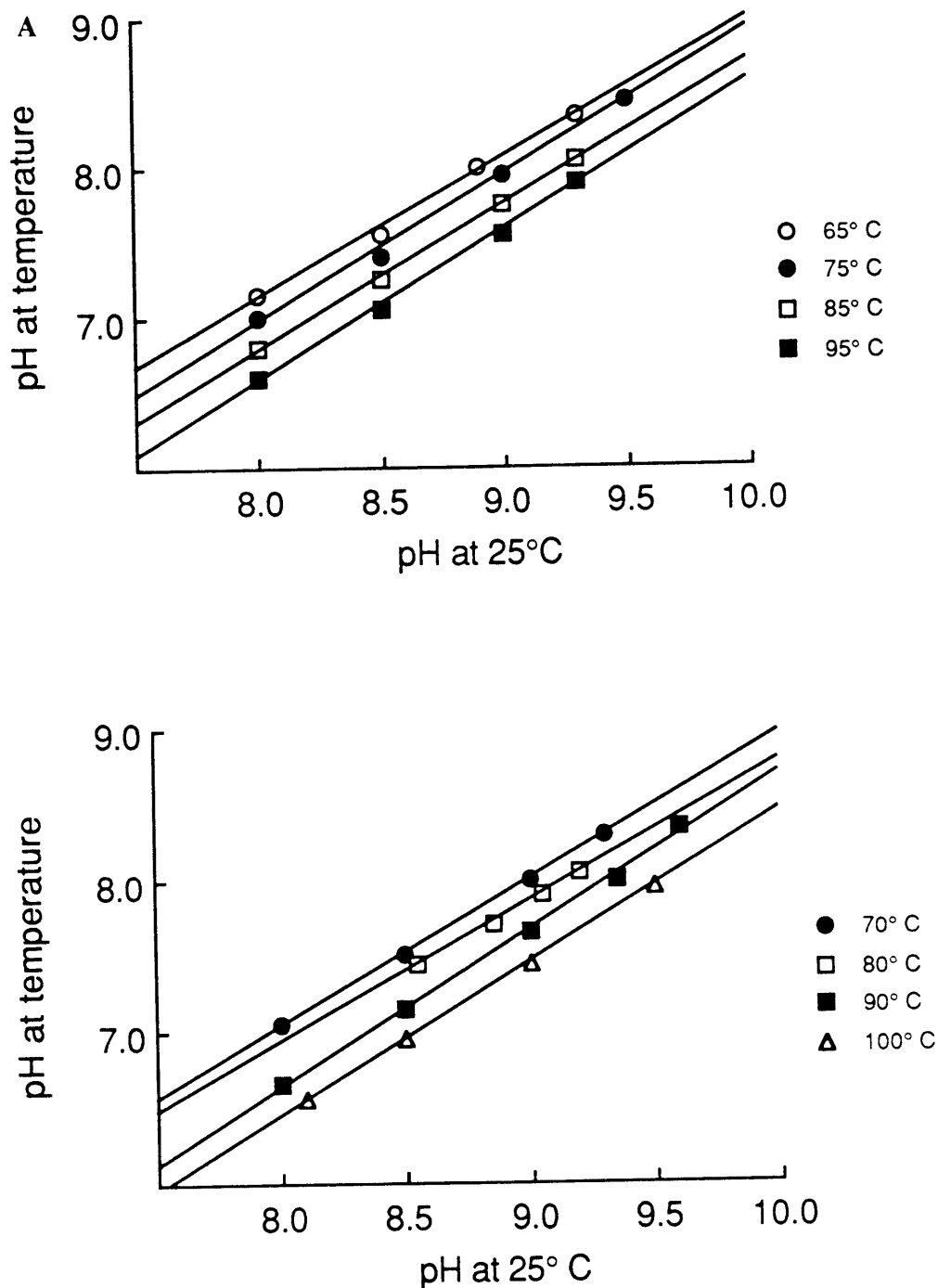
Figure A- The temperature dependence of the pH of 0.1 M Tris-HCl buffer containing 10 mM MgSO₄ (either with or without 2 M xylitol). Buffers were prepared at room temperature, and then equilibrated at various temperatures in a thermostatically controlled glycerol bath. The pH value was directly recorded at the elevated temperature, and were in reasonable agreement with published temperature correlations for Tris-HCl buffer itself (Perrin and Dempsey, 1974).

Appendix B - Rate constants of overall deamidation of proteins at 90-100° C, pH 4-8

The deamidation of asparagine and glutamine residues has emerged as one of the major mechanisms of irreversible thermoinactivation of enzymes at high temperatures. This degradative reaction has been shown to cause, solely or in part, the thermoinactivation of lysozyme at 100° C, ribonuclease at 90° C, triose phosphate isomerase at 100° C, several microbial α-amylases at 90° C, and immobilized glucose isomerase at 90° C (see literature survey). Because of the generality of this deleterious reaction, these amino acid residues are "weak links" in the protein molecule and demarcate the upper limit of protein thermostability.

The deamidation reaction involves the intramolecular cyclization of asparagine (and glutamine) residues under neutral to basic conditions. Formation of the cyclic imide intermediate requires the main chain amide to act as a nucleophile attacking the electrophilic carbonyl moiety of the asparagine residue with the concomitant release of ammonia. This reaction is pH dependent with enhanced rates at higher pH values. The subsequent hydrolysis of the cyclic imide leads to a mixture of α and ß-aspartic acid residues.(see literature survey).

The question naturally arises, are there any sequence dependent environments during the deamidation of either Asn or Gln residues at elevated temperatures? It has been recently shown that the first order rate constant of deamidation of three Asn containing hexapeptides show a marked sequence dependence at 100° C (pH 7.4) where a labile sequence of Asn-Gly deamidates 30-50 times faster than two bulkier (Pro and Leu) peptide sequences (Geiger and Clarke, 1987). This work did not examine peptides containing either Gln residues or other Asn sequences. The earliest research effort aimed at the deamidation of many different sequences of Asn and Gln was carried out by Robinson and coworkers who synthesized pentapeptides of sequence (Gly X_{xx} Asn X_{xx} Gly) or (Gly X_{xx} Gln X_{xx} Gly) and then measured the rate of amide loss under physiological conditions (Robinson and Rudd, 1974). Their rate data showed that (i) both asparagine and glutamine residues deamidate 30 to 2,000 times faster than a simple aliphatic amide; (ii) asparagine deamidates about ten times faster than glutamine (on average) with half-

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lives of 6-507 and 96-3400 days, respectively; and (iii) the rate of deamidation is sequence dependent (Wright and Robinson, 1982). In general, polar, charged or lower steric bulk residues neighboring the asparagine or glutamine residue cause a rate acceleration (there are exceptions to this trend). However, the authors state (Robinson and Rudd, 1974)," ... It is evident from these experiments that deamidation is strongly temperature dependent and that this dependence is a function of the peptide sequence." Thus, this appendix addresses the question of what is the sequence dependence of protein deamidation at elevated temperatures?

Instead of synthesizing the many possible combinations of peptides containing Asn and Gln residues to examine of sequence dependencies, we decided to simply incubate pure proteins (over a dozen) containing numerous Asn and Gln residues, and then measure the kinetics of overall deamidation at high temperatures. If deviations from first order kinetics are observed, then there must be a range of sequence-dependent deamidation sites as has been reported for pentapeptides of Asn and Gln at 37° C (Robinson and Rudd, 1974).

By examining the time course of the deamidation reaction in the pH range of 4-8 in pure proteins at extreme temperatures (90°-100° C), we are looking at a temperature regime above the thermal denaturation temperature (T_m) of most proteins (Bull and Breese, 1973 a,b). Therefore, we do not anticipate any significant tertiary structure effects on the deamidation reaction at these temperatures. In contrast, under physiological conditions, recent studies have confirmed that a protein's tertiary structure is a principal determinant to protein deamidation (using neutron diffraction techniques time-aged, deamidated protein crystals of trypsin were examined (Kossiakoff, 1988), kinetic studies were carried out on the deamidation of a particularly labile Asn-Gly sequence in ribonuclease (Creighton, 1989), as well as a protein sequence data base analysis by Clarke (1987)). By determining the variation in the rate constant of deamidation amongst different proteins at elevated temperatures, we can directly determine the effect of remaining higher ordered structure on protein deamidation at elevated temperatures.

As an initial experiment, the kinetics of the deamidation of insulin, a small, well-characterized protein containing three asparagine and three glutamine residues, were determined.

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Insulin was dissolved in sodium acetate buffer (0.1 M) at pH 4, placed into glass ampoules, sealed, and then heated for various times at 100° C. The protein solution was then cooled, opened, and the concentration of dissolved ammonia was determined by means of a chemical assay described by Forman (1964). As shown in the accompanying Figure B, ammonia is indeed released upon the heating of the protein solution, and this evolution of NH_3 closely follows first order kinetics (corr. coeff. 0.99) with a corresponding half-life for the overall deamidation process of 70 hours. As this reaction proceeds to completion, it levels off at the expected amount (six mole equivalents) which is the total number of asparagine and glutamine residues in the insulin molecule.

We then repeated this experiment at pH 4 and 100° C with nine other proteins, and the results are summarized in the top half of Table B. Regardless of whether the protein remained soluble or aggregated during 300 hours of heat treatment, the release of ammonia followed first order kinetics (corr. coeff. of 0.99 in most cases and 0.97 or better for all proteins examined) with a range of half-lives between 70-183 hours. After the exponential release of ammonia, a very slow, linear increase in the detectable amounts of ammonia was observed with some of the proteins after 300 hours of heating at 100° C, pH 4. In order to understand why this phenomenon was occurring, we subjected all ten heat-treated (for 300 hours) proteins to amino acid analysis (see experimental section), and it was found that no other amino acids were destroyed with the exception of small amounts of histidine (10-20%); this amino acid, along with arginine, have been shown to be labile and susceptible to ammonia release during the extended heat treatment of food proteins (Ledward, 1979). Therefore, the rapid, exponential release of ammonia must be due to the deamidation of asparagine and glutamine residues, while the slow, linear increase during exhaustive heating is due to the destruction of other amino acids.

The time course of overall deamidation in proteins follows first order kinetics, which implies that at extreme temperatures protein-bound asparagine and glutamine residues are more or less equally labile. This result is contrary to the behavior of Asn and Gln containing penta-peptides under physiological conditions where asparagine deamidates faster than glutamine

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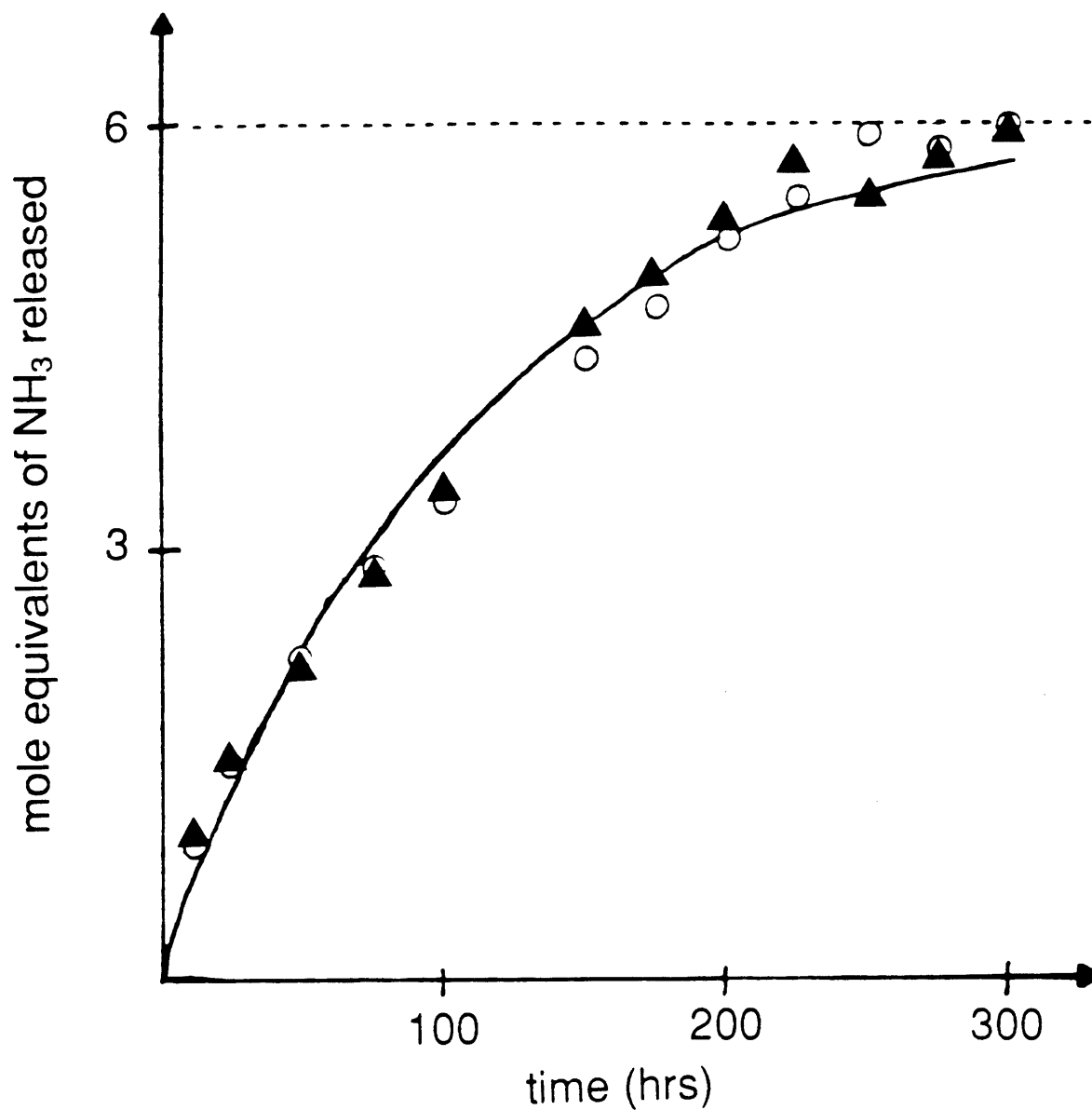
Figure B- The time course of the release of ammonia from insulin at 100° C and pH 4. The experimental protocol is described in Appendix B: 50 μM insulin in 0.1 M acetate buffer. The dashed line represents the total number of Asn + Gln residues per insulin molecule. The solid line is a theoretical curve corresponding to the first order kinetic model with a rate constant of 0.0099 hr^{-1}. Different symbols represent separate experiments.

enzyme	Deamidation half-life (hrs) at pH 4 and 100° C	visible aggregate during heat treatment?
ribonuclease	148	no
lysozyme	97	no
trypsinogen	183	yes
chymotrypsinogen	90	yes
transferrin	113	no
ovalbumin	100	yes
papain	83	yes
soy bean trypsin inhibitor	100	yes
bovine serum albumin	75	yes
insulin	70	no

enzyme	Deamidation half-life (hrs) at pH 6-6.5	Deamidation half-life (hrs) at pH 8
ribonuclease at 90° C (Zale and Klibanov, 1986)	38	5
lysozyme at 100° C (Ahern and Klibanov, 1985)	25	6
three different microbial α-amylases at 90° C (Tomazic and Klibanov, 1988a)	32 - 43	7.7 - 8.2
glucose isomerase at 90° C	—	10.5
glucose isomerase with 2 M xylitol at 90° C (Volkin and Klibanov, 1989a)	—	33

Table B- The kinetics of the release of ammonia from proteins at 90-100° C at pH 4, 6 and 8. All half-life values are calculated from first order kinetic rate constants fitted to the experimental data as explained in Figure B.

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residues (Robinson and Rudd, 1974). Apparently, any structural or conformational restraints on the deamidation of glutamine versus asparagine residues (see literature survey) are overcome at such high temperatures.

These data on the rates of overall deamidation in a variety of proteins also show that there is no significant fraction of amide residues in proteins that are observably more stable towards the deamidation process at 90-100° C, and thus it establishes the upper limit of the thermal stability of amide residues in proteins at high temperatures. The Asn-Gly sequence has been shown to be labile (Geiger and Clarke, 1987; the only known deamidation "hot-spot" due to its conformational flexibility), but it occurs no more frequently in proteins than would be expected from the bulk amino acid composition (Clarke, 1987). Therefore, this hot-spot was not detected in our system because these proteins contain, on average, over 20 amide side chain residues, and therefore, one labile sequence (if present at all) would not be distinguishable from the numerous other sequences. Nevertheless, these findings show that the vast majority of asparagine and glutamine residues are, on average, equally labile with no pronounced sequence dependence at 90-100° C.

The next step in this investigation was to examine the pH dependence of the heat-induced deamidation. As shown in the bottom half of Table B, the rate of overall deamidation in proteins (in the case of α-amylase calculated from the initial release of ammonia) increased with higher pH values, as would be expected from the cyclic intermediate mechanism (see literature survey). For example, the half-life of the overall deamidation of lysozyme at 100° C decreases from 97 to 25 to 6 hours as the pH changes from 4 to 6 to 8, respectively. Similar trends are seen with the initial release of ammonia from immobilized glucose isomerase at pH values of 7, 8, and 9 (see Table 3). The overall time course of ammonia evolution from glucose isomerase at pH 8 and 90° C (Figure 14) closely follows first order kinetics (corr. coeff. 0.99) as had been observed previously for proteins at pH 4.

Despite the wide variety of proteins examined, the half lives of overall deamidation in proteins are quite similar with variations of only 2-3 fold within each pH regime. Since

temperatures such as 90-100° C are above the thermal denaturation temperature of most proteins, there are no significant amounts of remaining tertiary structure to limit protein deamidation as is observed under physiological conditions. It has been shown that immobilized glucose isomerase thermally denatures at 88° C at pH 8 (see Figure 11). As can be seen on the last two lines of the bottom half of Table B, the deamidation of glucose isomerase proceeds at a rate similar to completely thermounfolded enzymes such as lysozyme and ribonuclease. However, when the ligand xylitol (2 M) is added, it stabilizes the enzyme against thermal denaturation at 90° C by shifting the equilibrium between folded and unfolded enzyme toward the former (see Figure 12). Interestingly, the ligand simultaneously inhibits (by three fold) the rate of the deamidation in glucose isomerase at 90° C, implying that the remaining elements of the enzyme's tertiary structure have only a modest effect on the deamidation process. This modest effect is most likely due to the increased amount of conformational flexibility due to thermal motion at 90° C, which overcomes any remaining structural restraints on the deamidation process.

In summary, we have established that protein deamidation at 90-100° C is a general process occurring in over a dozen different proteins. The observed time course of this reaction follows first order kinetics implying that the vast majority of asparagine and glutamine residues are more or less equally labile (no sequence dependence) at such extreme temperatures. In addition, this reaction is pH dependent, and as expected from the mechanism of protein deamidation, the process is faster at higher pH values. Furthermore, despite the wide diversity amongst the proteins examined, the rate constants of overall deamidation in proteins are very similar with half-lives of 8 ± 3 hours at pH 8, 41 ± 9 hours at pH 6, and 132 ± 50 hours at pH 4. Thus, the remaining elements of higher ordered structure do not significantly affect the deamidation of proteins when heated above their thermal denaturation temperature.

APPENDIX C - Calculation of irreversible thermoinactivation of enzymes due to deamidation: strategy, assumptions, and technical problems

Sections (i) through (iv) describe how to calculate irreversible thermoinactivation of enzymes due to deamidation including all assumptions made for any particular enzyme. Part (v) is a discussion of why this protocol could not be applied to glucose isomerase. Finally, part (vi) briefly explains why the data in Figure 15 contains so much scatter.

(i)   <u>Determination of Distribution of Deamidated Species</u> - After thermoinactivation of an enzyme solution, the distribution of mono, di, tri, etc. deamidated species is determined via isoelectric focusing. Since deamidation results in the transformation of a neutral species (Asn) to a charged one (Asp), separation by means of charge differences is then possible. The quantitative determination of the relative amounts of each species is done by gel densitometry. For triose phosphate isomerase, the distribution of deamidated species was calculated via decrease in native band intensity instead of the increase in secondary bands due to differences in the binding of the coomassie blue stain.

(2)   <u>Specific Activity of Each Deamidated Species</u> - Proteins from the stained bands in the isoelectric focusing gel are then extracted from the gel; the protein is refolded and assayed for enzymatic activity. This procedure works well for small, monomeric proteins, but is difficult with oligomeric ones which cannot quantitatively refold from the denatured state. Therefore, for α-amylases, average values of decrease in specific enzymatic activity per successive deamidation must be used as shown in Table C:

	Specific Activity (% of Native) Number of amide residues hydrolyzed			
	0	1	2	3
Ribonuclease	100	65	38	19
Lysozyme	100	52	----21-----	
Cytochrome C	100	60	20	
Triose phosphate isomerase	100	66		

Table C- Specific activity of deamidated proteins as described by Ahern et al., 1987.

(iii) Normalization by Molecular Weight - The literature data was normalized to account for differences in molecular weights between the small protein molecules listed above and α-amylases. For example, when 1 out of 17 amide residues deamidates in ribonuclease, a 35% of the specific activity is lost. Therefore it was assumed that for B. stearothermophilus α-amylase to undergo a comparable loss of specific activity, 2.4 out of 40 amide residues had to deamidate.

(iv) Inactivation Due to Deamidation - The time course of thermoinactivaton due to deamidation is calculated by multiplying the specific activity (part ii) by the percent distribution of each species (part i) followed by summation of these activities at each time point:

$$\text{Activity at time (t)} = \sum^{\text{total species}} (\text{specific activity}) \times (\% \text{ distribution})$$

(v) Glucose Isomerase - The strategy outlined above (used to determine inactivation due to deamidation) could not be applied to this enzyme because (1) isoelectric focusing with an immobilized enzyme is impossible, and (2) soluble glucose isomerase is an acidic protein

(pI of 4.0), thus the initial few deamidations do not change the net charge of the protein sufficiently to allow for separation of deamidated species by means of isoelectric focusing, and (3) this dimeric enzyme does not quantitatively refold from the denatured state.

(vi) Scatter in Modelling Data (Figure 15) - Since it is not clear which of the assumptions listed above are more realistic, in the interests of fairness and objectivity, Figure 15 incorporates the data resulting from all of them (sections i-iii). We were not interested in minimizing the scatter in order to beautify the figure. The only objective was to obtain a "ball park" estimate.

Appendix D- Cysteine Oxidation and Amide Deamidation at 90° C vs. 70° C

The data presented in this work showed that both deamidation of a key asparagine and/or glutamine residue and oxidation of a cysteine residue cause thermal inactivation of glucose isomerase. However, the relative contribution of each degradative reaction to the thermal inactivation of glucose isomerase changes at different temperatures. For example, let us first examine the irreversible thermoinactivation of glucose isomerase at 90° C. The rate of loss of enzymatic activity can be expressed with the following rate equation:

$$v_{inact.} = v_{deamidation} + v_{cysteine\ oxidation} \quad (1)$$

According to the first-order rate equation (a parallel reaction system; Smith, 1981), we can rewrite equation (1) as:

$$-\frac{dE}{dt} = k_d E + k_{co} E$$

where E is the molar concentration of enzyme, and k_d and k_{co} are the rate constants of deamidation and cysteine oxidation, respectively. We can now rearrange the equation as follows:

$$-\frac{dE}{dt} = (k_d + k_{co})\ E$$

$$\frac{dE}{E} = -\ (k_d + k_{co})\ dt$$

then integrate both sides of the equation,

$$\int \frac{dE}{E} = -\ (k_d + k_{co}) \int dt$$

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$$\ln E = - (k_d + k_{co}) t + C$$

and solve for the integration constant and rearrange,

$$\text{at } t = 0, \ E = E_0 \ ; \text{ therefore, } C = \ln E_0$$

$$\ln \frac{E}{E_0} = - (k_d + k_{co}) t$$

by exponentiating each side of the equation, we can develop an expression which describes the remaining concentration of active enzyme at any time t:

$$E = E_0 \exp - (k_d + k_{co}) t \quad (2)$$

Figures 13 and 17 show that the half-life for deamidation and cysteine oxidation is 30 minutes ($k_d = 0.023$ min $^{-1}$) and 90 minutes ($k_{co} = 0.0077$ min $^{-1}$), respectively. Since the ratio of $k_d/k_{co} = 3$, we should see, according to equation 2, a 1.25 fold (or 25%) stabilization of glucose isomerase when heated at 90° C under reducing conditions. However, no apparent stabilization was observed experimentally (see Figure 18). Although individual experiments could detect a slight stabilization (1.10-1.15 fold), this minor effect can no longer be seen when these individual experiments are graphed together as shown in Figure 18. This "masking" is due to the high activation energy of the thermal inactivation process (Figure 20), where differences as small as 1° C between individual experiments can affect the experimentally observed rate of thermal inactivation by two fold. In summary, our experimental techniques were not sensitive enough to detect this anticipated stabilization at 90° C. Conversely, during the irreversible thermoinactivation of glucose isomerase at 70° C, the enzyme molecule is more folded (Figure

11) thereby shielding the key amide residue from the solvent and preventing its deamidation (see text); therefore, under these conditions, cysteine oxidation dominates the inactivation process as shown in Figure 19. Thus the cause of irreversible thermoinactivation of immobilized glucose isomerase depends on the temperature regime.