

Understanding Oxidative Instability of Protein Pharmaceuticals

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Abstract: Mechanism of oxidation of methionine residues in protein pharmaceuticals by hydrogen peroxide was investigated via *ab initio* calculations. Specifically, two reactions, hydrogen transfer of hydrogen peroxide to form water oxide and the oxidation of dimethyl sulfide (DMS) by hydrogen peroxide to form dimethyl sulfoxide, were studied as models of these processes in general. Solvent effects are included both via including explicitly water molecules and via the polarizable continuum model. Specific interactions including hydrogen bonding with 2-3 water molecules can provide enough stabilization for the charge separation at the activation complex. The major reaction coordinates of the reaction are the breaking of the O-O bond of H₂O₂ and the formation of the S-O bond, the transfer of hydrogen to the distal oxygen of hydrogen peroxide occurring after the system has passed the transition state. Reaction barriers of the hydrogen transfer of H₂O₂ are in average of 10 kcal/mol or higher than the oxidation of DMS. Therefore, a two step oxidation mechanism in which the transfer of hydrogen atom occurs first to form water oxide and the transfer of oxygen to substrate occurs as the second step, is unlikely to be correct. Our proposed oxidation mechanism does not suggest pH dependence of oxidation rate within a moderate range around neutral pH (i.e. under conditions in which hydronium and hydroxide ions do not participate directly in the reaction), and it agrees with experimental observations over moderate pH values.

Index Terms—Methionine Oxidation, Hydrogen Peroxide, Protein Pharmaceuticals

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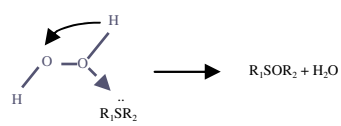
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I. INTRODUCTION

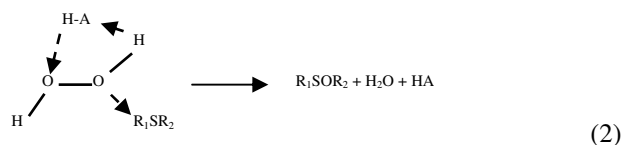
Oxidation is an important chemical process, prevalent throughout chemistry. It leads to significant changes in the properties of organic and biological compounds^{1,2}. Peroxides, including hydrogen peroxide, hydroperoxides, and peroxy acids are efficient oxidants of organic sulfides in aqueous solutions³. For instance, the oxidation of methionine groups by hydroperoxides is a major way by which therapeutic proteins are degraded⁴⁻⁸. Designing a storage formulation that hinders oxidation of these sites and thus provides an acceptable shelf-life is one of the most challenging tasks in the process of drug development. In order to move beyond trial and error, empirical approaches to design a storage formulation, an accurate quantitative, molecular-level understanding of the process would be helpful. Another example is the oxidation of dimethyl sulfide (DMS) in the atmosphere. DMS is emitted steadily by ocean phytoplankton and is the major source of sulfur in the troposphere⁹. Dimethyl sulfoxide (DMSO) is the first product of the aqueous phase oxidation of DMS by hydroperoxides, and has occurrence in both rain¹⁰⁻¹² and snow¹³. Knowledge of the kinetics and mechanism of DMS oxidation should be helpful in understanding the contribution of DMS to acid rain formation and its effects on the climate¹⁴. In summary, a correct understanding of the oxidation mechanism of organic sulfides by peroxides would be of use in organic chemistry, biochemistry and atmospheric chemistry.

Before 1968, the oxygen transfer from hydroperoxides to nucleophilic substrates in solution was generally accepted to be an S_N2 type displacement (eq.(1)) reaction^{3,15,16}:

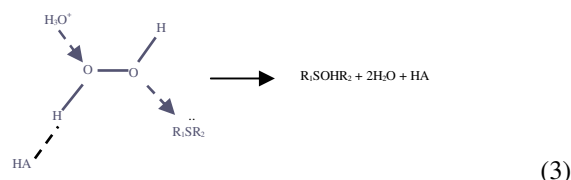


In this mechanism, the transfer of oxygen is associated with a hydrogen shift to the distal oxygen. In 1968, Dankeff et al¹⁶, proposed the mechanism in eq.(2), based on their data

of solvent effects on the oxidation of thioxane by hydrogen peroxide. HA is a general acid, for example, a solvent molecule, and was thought to serve as an intermediate agent which reduced the charge separation of the activation complex via *proton transfer*¹⁶. Specifically, they found that the rates of oxidation in different solvents correlate better to solvent acidity (pK_a) than to solvent polarity (dielectric constant). Moreover, transferring the reaction to an aprotic solvent, lead to an increase in the reaction order of hydrogen from 1 to 2, strong evidence that the second hydrogen peroxide played the role of the generalized acid. The schematic picture of eq.(2) has been generally accepted to be the mechanism of oxidation of organic sulfides via peroxides^{3,6,7,15-19}.



Starting in 1991, in order to evaluate the plausibility of eq.(2) and other hypothesized reaction mechanisms, ab initio studies on peroxide oxidation have been performed for both the hydrogen transfer reaction of hydrogen peroxide^{20,21} and for the oxidation of amines and sulfides with hydrogen peroxide^{20,22}. The attempt of those studies was to develop a more detailed understanding of the oxidation mechanism. Important questions that were discussed include: what are the roles of the general acid? What are the essential reaction coordinates? What is the order by which the transfer of oxygen and hydrogen proceeds? And what are the predicted activation energies and how do they compare with experimental values? The last question was the most important in that proposed reaction mechanisms were accepted or eliminated depending on how their energy barrier compared to experimental values. For example, the direct 1,2 hydrogen transfer of H_2O_2 in vacuum was found via ab initio computations to have a very high energy barrier (56 kcal/mol)^{20,23}, but typical observed activation barriers for peroxide oxidation of amines and organic sulfides in aqueous solutions are in the range of 10-20 kcal/mol^{3,16,24,25}. On this basis, the direct 1,2 hydrogen transfer of H_2O_2 was assumed not to be the correct mechanism. In general, however, ab initio calculations of the mechanism eq.(2) yielded activation barriers in the range of 28-50 kcal/mol^{18,20-22}, all much higher than the range of experimental data (10~20 kcal/mol)^{3,16,24,25}. Therefore, Bach et al.¹⁸ concluded that general acid alone cannot catalyze the oxidation reaction. On the other hand, when both a protonated solvent and a general acid are present (eq.(3)), the calculated energy barriers lie between 5-15 kcal/mol, within the experiment range¹⁸.



This was, thus, concluded by Bach et al. to be the reaction mechanism^{18,26}. Note that by this mechanism (eq.(3)), the oxidation rate should decrease with increasing pH. However, subsequent to the proposal of this mechanism, it was found that the dependence of rate of oxidation on pH of DMS (pH=2 to 10)¹⁴ and of methionine residues in human parathyroid hormone (pH=2 to 8)²⁷ was negligible in aqueous solution. In general, experimental observations do not indicate a pH dependence of peroxide oxidation between low (pH=2-5) and high (pH=7-10) pH values. An increase in oxidation rate was observed when pH is low (<2), indicating that the mechanism proposed by Bach et al. (eq.(3)) can be valid at low pH values but is unlikely to occur at moderate pH values. Thus, the questions remains: what is the mechanism of oxidation of sulfur sites in organics?

In this study, we address this question by analyzing proposed oxidation mechanisms using high level ab initio simulations with the objective of finding a mechanism that is consistent with all experimental observations in moderate pH ranges. We carefully include both the specific effects of solvent molecules and the effects of the solvent as a polarizable dielectric continuum. It turns out that including water molecules explicitly leads to a new reaction mechanism, which is consistent with all experimental data. We studied both the hydrogen transfer in H_2O_2 and the oxidation of dimethyl sulfide.

II. METHODS OF CALCULATION

Ab initio calculations were performed using the GAUSSIAN 98 package²⁸. B3LYP (Becke's three-parameter functional²⁹) with the 6-31++G(d,p) basis set was employed for geometry optimizations, transition-state searches and frequency calculations throughout this study; a scaling factor of 0.9806³⁰ was used for zero-point-energies (ZPEs) corrections. We have tested the accuracy of the B3LYP functional and the 6-31++G(d,p) basis set against higher levels of theories (MP2, MP4^{31,32} and CCSD, CCSD(T)^{33,34}) and larger basis sets (6-31++G(3df,3dp), cc-pVDZ and aug-cc-pVDZ) for the 1,2 hydrogen transfer reaction of hydrogen peroxide²⁰. The results indicate that the B3LYP/6-31++G(d,p) theory is sufficient to predict accurate geometries and energetics with MP2 post-SCF correction; details can be found in the supporting information. Unless specifically mentioned, the energetics reported in this study is based on MP2//B3LYP/6-31++G(d,p) theory. All transition states reported have only

one imaginary vibrational mode found without imposing constraints during the process of geometry optimization. The polarized continuum model^{35,36}, PCM, was used to describe the long-range electrostatic polarization of the surrounding environment. Water is the only solvent considered ($\epsilon_r=80$), and the united atom topological model³⁷ was used to build up the solute cavity.

III. RESULTS AND DISCUSSIONS

Hydrogen transfer of hydrogen peroxide to form water oxide

Water oxide, H_2OO , is a local energy minimum in the potential energy surface of hydrogen peroxide and is 46~50 kcal/mol higher in energy than H_2O_2 . The reported activation energies of the hydrogen transfer reaction of H_2O_2 in earlier ab initio studies were in the range of 29~50 kcal/mol^{20,21}, depending on the number of water molecules involved. The lower end of reported energy barriers is still much higher than the measured activation barriers (10~20 kcal/mol) for the oxidation of organic sulfides.

Fig.1 shows the reactant and the transition state of the hydrogen transfer reaction of H_2O_2 with three water molecules. Compared to the results of earlier theoretical studies of the hydrogen transfer reaction of H_2O_2 (the lowest value reported: 29.5 kcal/mol²¹), the activation energy of reactant cluster **1** and **TS-1** shown in Fig.1 is lower (25.5 kcal/mol). The major cause of this reduction is the specific inclusion of water molecules within the reactant. Two water molecules are involved in the hydrogen-transfer process in **TS-1**, and this type of 1,6 of hydrogen transfer has been reported²¹. The third water molecule in **TS-1** is not involved in the hydrogen transfer process, but it is hydrogen bonded to H_2O_2 and the presence of the third water molecule has reduced the barrier by ~5 kcal/mol. This type of configuration has not been explored in earlier studies. This result indicates that specific interactions with solvent molecules can have significant stabilization effects on the reaction.

In addition, the effects of long-range electrostatic polarization of solvent ($\epsilon_r=0$ in gas phase and $\epsilon_r=80$ in aqueous solution) have been analyzed by incorporating Tomasi's polarizable continuum model (PCM)^{35,36} in locating the geometries of the reactant clusters and transition states and in total energy calculations. Including polarization in this way does not lead to a significant difference (< 1.5 kcal/mol) in reaction activation energies compared to those in vacuum. This result indicates that solvent molecules stabilize the hydrogen transfer reaction of H_2O_2 via specific interactions rather than dielectric polarization; it is also consistent with the experimental observation that the reaction rate of peroxide oxidation is insensitive to solvent dielectric constants¹⁴. Although the minimum activation energy obtained (25.5 kcal/mol) is lower than that obtained in previous studies^{18,20,21}, it is still too high compared to experimentally determined activation

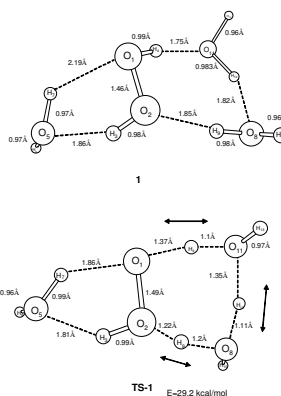


Fig. 1. The hydrogen transfer reaction of hydrogen peroxide with three water molecules..

energies of the oxidation of organic sulfides by hydrogen peroxide (10~20 kcal/mol).

Oxidation of dimethyl sulfide(DMS)

Dimethyl sulfide (CH_3-S-CH_3) is an organic sulfide and can be oxidized with H_2O_2 to dimethyl sulfoxide (DMSO) in aqueous solution¹⁵. DMS represents part of the methionine side chain, and oxidation of the sulfur atom in methionine residues by peroxides is one of the major degradation pathways of therapeutic proteins^{4,6,8,38}. Our starting point is the fact that of the mechanisms previously proposed in the literature, as presented in the introduction and as studied in the previous section, none of them are consistent with experimental data, including both the pH dependence of the rates of oxidation and the estimated activation energies. Aside from the mechanism proposed by Bach et al.¹⁸ (eq.(3)), the activation energies estimated in previous theoretical studies are too high compared to experimental estimations^{3,16,24,25}. The problem with the mechanism of Bech et al. (eq.(3)) is that it implies a marked pH dependence of rates of oxidation of organic sulfides via H_2O_2 , but it was found that the rates of oxidation of DMS is independent of pH over the range of pH=2~10 in aqueous solutions¹⁴. Note that eq.(3) may still be the route at low pH values, since an increase in the rates of oxidation of DMS was observed for pH<2¹⁴. We wish to determine the mechanism of the oxidation process in a moderate pH range (2-10), including understanding the governing factors that bring about this reaction. Not only will this add to our understanding of a class of important chemical reactions, but also, it will enhance our ability to develop ideas to hinder this process, when desired.

Fig.2 shows geometries for the reactant cluster **2** and the transition state **TS-2** for DMS oxidation by H_2O_2 in the presence of a single water molecule. At **TS-2**, the reaction mode is mainly the transfer of O_2 to S_1 and does not exhibit significant hydrogen transfer character. This indicates that the proper reaction coordinate involves the S_1-O_2 distance

and the O₂-O₃ distance, and that our picture is different from the mechanism found earlier for DMS oxidation where the transfer of hydrogen to form water oxide occurs before the transfer of oxygen¹⁸. A reaction path following

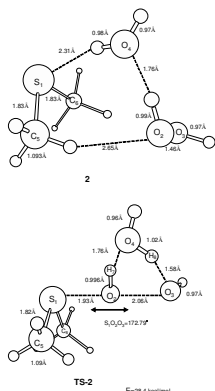


Fig. 2. Oxidation of dimethyl sulfide with a single water molecule.

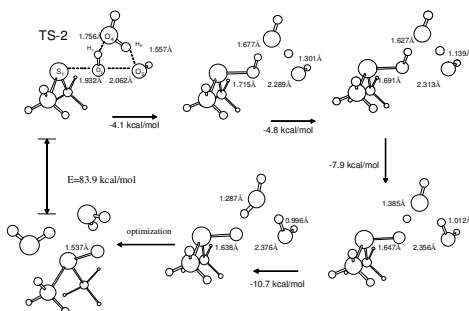


Fig. 3. Reaction path analysis of DMS oxidation with a single water molecule, snapshots are from IRC calculations.

analysis^{39,40} shows that the transfer of hydrogen indeed occurs after the transfer of oxygen. Selected frames and corresponding energies are shown in Fig.3.

The energy difference between the reactant cluster and the transition state in this model is 28.4 kcal/mol at the MP2//B3LYP/6-31++G(d,p) level. Although this value is still too high compared with experimental observations, it is lower than the activation energy of a similar reaction: oxidation of DMS with two H₂O₂ molecules calculated at the MP4//MP2/6-31G(d) level, in which the transfer of hydrogen occurs first to form water oxide. The reported energy barrier of this process was 40.8 kcal/mol¹⁸.

The effects of the electrostatic polarization of the surrounding solvent were also analyzed with the PCM model for DMS oxidation with a single water molecule. The difference in activation energy between the unsolvated phase and the solvated phase is insignificant (0.2 kcal/mol). The insensitivity of activation energy to dielectric polarization of solvent for this reaction is similar to the

hydrogen transfer reaction of H₂O₂ as described earlier, and again, is also consistent with the experimental observation that the reaction rate of peroxide oxidation is insensitive to solvent dielectric constants¹⁴.

Based on the experience of the H₂O₂ hydrogen transfer reaction, we anticipate that the number of water molecules in the theoretical model can dramatically change the reaction energy barrier. Indeed we found that adding the second water molecule has reduced the reaction barrier by ~10 kcal/mol. We also found that the major stabilization effects are specific interactions such as hydrogen bonding with H₂O₂ at the transition state, especially to the more negative leaving oxygen. The more negativity of the leaving oxygen is due to the charge separation of H₂O₂ when the O-O bond is breaking.

For the three-water model, the reactant **3** and **TS-3** are shown in Fig.4. Since the distal oxygen is more

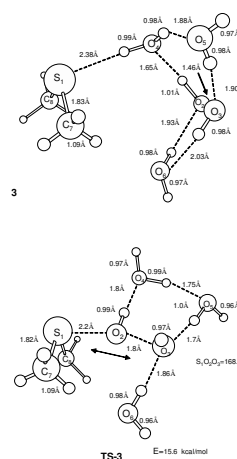


Fig. 4. Oxidation of dimethyl sulfide with three water molecules.

negative than the transferring oxygen at the transition state, it is expected that the third water molecule will have significant stabilization effect. This is indeed the case. The energy barrier is 15.7 kcal/mol, the third water molecule has reduced the activation energy by ~5 kcal/mol. These results indicate that forming a hydrogen bond with the more negative distal oxygen at the transition state has more stabilization effect than doing this with the less negative O₂ atom. When compared with the activation energy (13.5 kcal/mol) for thioxane oxidation by hydrogen peroxide in aqueous solution, the above result is also quite close.

For each model of DMS oxidation, the activation barrier is about 10 kcal/mol or more lower than that of the corresponding hydrogen transfer reaction of hydrogen peroxide to water oxide. Therefore, it is unlikely that oxidation occurs in two steps in which the hydrogen transfers first to form water oxide. Instead, oxidation occurs via stretching of the O-O bond of H₂O₂ and reduction in the S-O distance. Solvent molecules stabilize the charge separation at the transition state via specific interactions, including hydrogen bonding, and may also be

involved in the transfer of hydrogen after the system has passed the transition state. Hydrogen bonding to the more negative distal oxygen at the transition state has a significant stabilization effect as shown in the two-water and three-water models. Water molecules stabilize the charge separation at the activation complex via specific interactions including hydrogen bonding. This mechanism is also in agreement with the experimentally observed pH-independent rates over moderate pH ranges, since an additional proton is not involved in this model. Our results imply that unprotonated water molecules can provide enough stabilization for hydrogen peroxide oxidation of organic sulfides. At extremely low pH conditions, of course, high concentrations of protonated solvent molecules may result in different oxidation pathways^{14,18}.

IV. CONCLUSION

The following conclusions can be drawn from the above theoretical analysis:

1. For moderate pH values, perhaps between at 2 and 12, the reaction coordinate leading to oxidation of dimethyl sulfide by H₂O₂ is the separation (breaking) of the O-O bond together with the formation of the S-O bond. Thus, under these conditions, water molecules stabilize the transition complex via *specific interactions including formation of hydrogen bonds with H₂O₂* but not *proton transfer* as previously assumed in the literature^{3,6,7,15-19}.
2. Hydrogen transfer does occur during oxidation of DMS, but it is not the determining factor of the activation barrier. Hydrogen transfer can occur via multiple different pathways depending on the local solvent configuration.
3. During DMS oxidation, an uneven charge distribution between the oxygen atoms of H₂O₂ is developed. The distal oxygen is more negative than the transferring oxygen. Water molecules stabilize the charge separation by local polarization and via formation of hydrogen bonds with H₂O₂.
4. For hydrogen transfer reactions involving H₂O₂ and for DMS oxidation with a single water molecule, including solvent via a polarizable continuum model results in small effects on activation energies (<1 kcal/mol in most cases). This is also consistent with the experimental observation that the rates of oxidation of organic sulfides are not a strong function of solvent dielectric constant¹⁶.
5. The activation energy of DMS with 3 water molecules is 14.2 kcal/mol, within the experimental range of barriers of oxidation of organic sulfides^{3,16,24,25}.
6. The activation energies of the hydrogen transfer reaction of hydrogen peroxide are an average of 10

kcal/mol or more higher than those for the oxidation of DMS by H₂O₂. This, combined with conclusions 1-5, indicates that a two-step mechanism in which the hydrogen transfer of H₂O₂ occurs first to form water oxide followed by second the transfer of oxygen to the nucleophile from water oxide is not the mechanism by which organic sulfides are oxidized by hydrogen peroxide.

7. Our proposed oxidation mechanism does not suggest a pH dependence of oxidation rates within a moderate range around neutral pH (i.e. under conditions in which hydronium and hydroxide ions do not participate directly in the reaction). In this respect, it agrees with experimental observations over moderate pH values^{14,27}. It also predicts activation energies corresponding to measured activation energies without including a protonated solvent molecule.

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