N⁶-Formylation of Lysine: A Pathological Secondary Modification of Proteins

by

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Bahar Edrissi

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Abstract

There is increasing recognition that aberrant protein modifications play an important role in the pathophysiology of inflammation and oxidative stress in cells. We recently discovered that N\(^6\)-formylation of lysine is an abundant endogenous modification of histone and chromatin proteins. The high abundance of N\(^6\)-formyllysine in histone proteins and its chemical similarity to the biologically important N\(^6\)-acetyllysine has raised questions about its mechanisms of formation and biological consequences. Using novel ultrasensitive and specific liquid chromatography-coupled tandem mass spectrometry methods (LC-MS/MS) to quantify N\(^6\)-formyllysine lesions in proteins, we aimed to investigate the sources as well as the fate of this abundant endogenous protein modification. We present evidence that endogenous formaldehyde is a major source of N\(^6\)-formyllysine and that this adduct is widespread among proteins in all cellular compartments. We observed in vitro as well as in vivo that formaldehyde exposure leads to a dose-dependent increase in N\(^6\)-formyllysine protein adducts, with the use of isotopically-labeled formaldehyde to dissect endogenous from exogenous formaldehyde as sources of the adduct. Further, other isotope labeling studies revealed that lysine demethylation in histone proteins is not a source of N\(^6\)-formyllysine. With regard to N\(^6\)-formyllysine persistence in cells, our investigation of histone deacetylases revealed that despite chemical similarity of N\(^6\)-formyllysine to N\(^6\)-acetyllysine, the former is refractory to removal by histone deacetylases, which suggests that they will persist throughout the life of individual histone proteins. If not repaired, lysine formylation could accumulate to significant levels. The resemblance of N\(^6\)-formyllysine to N\(^6\)-acetyllysine, together with recent studies that mapped its location on many conserved lysine acetylation and methylation sites along histone proteins, support the idea that this abundant protein modification could interfere with normal regulation of gene expression, potentially leading to an epigenetic mechanism of disruption of cell function.

Thesis Supervisor: Peter C. Dedon
Title: Professor of Toxicology and Biological Engineering
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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>AGEs</td>
<td>advanced glycation end products</td>
</tr>
<tr>
<td>CO$_3^-$</td>
<td>carbonate radical anion</td>
</tr>
<tr>
<td>HATs</td>
<td>histone acetyl transferases</td>
</tr>
<tr>
<td>HDACs</td>
<td>histone deacetylases</td>
</tr>
<tr>
<td>HNE</td>
<td>4-hydroxy-2-nonenal</td>
</tr>
<tr>
<td>HO$^*$</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HOCl</td>
<td>hypochlorous acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography-coupled tandem mass spectrometry</td>
</tr>
<tr>
<td>LSD1</td>
<td>lysine-specific demethylase 1</td>
</tr>
<tr>
<td>MDA</td>
<td>malondialdehyde</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO$_2^*$</td>
<td>nitrogen dioxide radical</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>nitrite</td>
</tr>
<tr>
<td>N$_2$O$_3$</td>
<td>nitrous anhydride</td>
</tr>
<tr>
<td>$^1$O$_2$</td>
<td>singlet oxygen</td>
</tr>
<tr>
<td>O$_2^{•-}$</td>
<td>superoxide anion radical</td>
</tr>
<tr>
<td>O$_3$</td>
<td>ozone</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>ONOOCO₂⁻</td>
<td>nitrosperoxycarbonate</td>
</tr>
<tr>
<td>PITC</td>
<td>phenylisothiocyanate</td>
</tr>
<tr>
<td>PTMs</td>
<td>post-translational modifications of proteins</td>
</tr>
<tr>
<td>QQQ</td>
<td>triple quadrupole mass spectrometer</td>
</tr>
<tr>
<td>SAHA</td>
<td>suberoylanilidehydroxamic acid</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>SRM</td>
<td>selected reaction monitoring</td>
</tr>
<tr>
<td>TCE</td>
<td>trichloroethylene</td>
</tr>
<tr>
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In loving memory of my grandmother,

Mahin Shahabpour
Chapter 1

Introduction
Chapter 1: Introduction

Goals and summary of this thesis

This thesis project is focused on defining the mechanism of formation and biological significance of N^6^-formyllysine, a recently discovered abundant endogenous secondary modification of proteins that resembles the physiologically important N^6^-acetyllysine in histone proteins. This thesis is organized in 5 chapters. Chapter 1 presents a comprehensive review of protein secondary modifications with an emphasis on lysine acetylation, due to the resemblance of N^6^-formyllysine to N^6^-acetyllysine. The chapter subsequently discusses the role of reactive oxidants and electrophiles in generating pathological modifications of proteins and then focuses on N^6^-formyllysine and its potential effects in terms of disrupting the epigenetic roles of histone modifications. Chapter 2 provides detailed development of an ultrasensitive and specific liquid chromatography-coupled tandem mass spectrometry method for quantifying this adduct in proteins. Topics such as distribution of this adduct among different histone classes, mechanisms for N^6^-formyllysine generation in addition to the DNA oxidation pathway we originally reported, as well as its possible pathological consequences in cells are explored in Chapter 3, with the discovery of formaldehyde as a major endogenous source of N^6^-formyllysine. Chapter 4 extends our studies to measurement of N^6^-formyllysine protein adducts in tissues of rats exposed to formaldehyde by inhalation. In Chapter 5, we conclude this thesis by summarizing our results regarding sources as well as fate of N^6^-formyllysine adducts and assess possible biological significance of this secondary protein modification, particularly in terms of interfering with regulatory roles of histones. N^6^-Formylation of lysines in histones may represent an epigenetic mechanism of disruption of cell function leading to cancer and other diseases.
Chapter 1: Introduction

Post-translational modifications of proteins and their regulatory roles

In eukaryotes, the level of complexity of the proteome is several orders of magnitude greater than what is dictated by its genome sequence [1], with each gene effectively encoding several proteins. In addition to alternative RNA splicing that generates different mRNA transcripts from a single gene [2], the post-translational modification of proteins (PTMs) significantly enhances the complexity of the proteome [1,3]. This type of protein modification involves covalent alterations of the polypeptide backbone or side chains, leading to changes in a protein’s structure and/or function [1,3,4]. In most cases, these modifications are reversible and under tight regulation of specific enzymes [5]. Eukaryotes dedicate a relatively high percentage of their genomes to enzymes modulating PTMs. For instance, in humans, greater than 5% of the genes encode such enzymes [3].

Protein modifications modulate protein-protein and protein-nucleic acid interactions and regulate the cellular environment by affecting a multitude of biological functions and processes such as protein activity, localization, turnover, signaling cascades, and cellular metabolism [1,5,6]. It is then of no surprise that dysregulation of PTMs leads to a plethora of disorders and diseases.

From more than 300 PTMs discovered to date [5], the most common are phosphorylation, acetylation, methylation, glycosylation, hydroxylation, palmitoylation, sulfation, and ubiquitination [1,2,5], as briefly discussed:

- **Phosphorylation.** Phosphorylation and dephosphorylation of serine, threonine, or tyrosine residues mediated by protein kinases (PKs) and phosphatases (PPs), respectively, is a key modification with many roles in regulating cellular
physiology and metabolism, mainly through modulating enzymatic activities and intracellular signaling pathways [2,7,8].

- **Methylation.** Another key PTM is methylation of lysine and arginine. Specific residues in histones as well as non-histone proteins such as p53, NF-κB, ERα are subject to methylation in cells [9]. In histones, lysine methylation is dynamically regulated by histone methyltransferases and histone demethylases, with important roles in gene expression and chromatin organization [10]. Protein methylation, in addition to DNA methylation/demethylation, has implications in embryonic and postnatal development [1,11,12].

- **Glycosylation.** Protein glycosylation, or the addition of sugar moiety to proteins, is an enzymatic mediated and reversible PTM [13], as opposed to the random non-enzymatic glycation that mostly renders proteins inactive [14]. Protein glycans formed by glycosylation have important structural and modulatory roles such as recognition elements for glycan binding proteins (GBPs), and therefore regulate a variety of processes such as development and survival [15].

- **Hydroxylation.** Hydroxylation of amino acids such as proline, asparagine, phenylalanine, tryptophan, and tyrosine is mediated by enzymes called hydroxylases [16,17]. Hydroxylation is critical in cellular detoxification reactions [1], as well as in the structural stability of proteins (e.g., hydroxyproline is an essential element of collagen) [18].

- **Palmitoylation.** Covalent attachment of fatty acids to proteins is another type of PTM. S-Palmitoylation of the cysteine thiol is a reversible modification with important physiological roles in G-protein-coupled receptor (GPCR) trafficking
and protein activity, as well as localization (as attachment of the hydrophobic moiety is important for membrane association) and T-cell signaling [19,20].

- **Sulfation.** The post-translational sulfation of tyrosine residues, a common modification of secreted and transmembrane proteins, has essential roles in chemokine signaling, leukocyte adhesion, immune response, extracellular protein–protein interactions, intracellular protein transport, protein activity and degradation [21-23].

- **Ubiquitination.** Ubiquitination is a covalent attachment of ubiquitin (Ub) to lysine residues of proteins in order to tag them for degradation and recycling. Attachment of Ub to proteins is a multi-step process, carried out in order with a set of 3 enzymes: Ub activating enzymes (E1), Ub conjugating enzymes (E2), and Ub ligases (E3) [24]. The Ub tag (mediated by mono-Ub or poly-Ub), is a crucial protein modification for proteasomal degradation, intracellular signaling cascades, protein binding, localization, and activity [25,26].

- **Acetylation.** Lysine acetylation is a key protein modification with inherent importance to this thesis due to the chemical similarity of lysine acetylation to lysine formylation and the critical role of lysine acetylation of histone proteins in regulating gene expression. To address this phenomenon, the next section of thesis introduction addresses histone protein structure and function followed by the biochemistry of histone acetylation.
Histone proteins and their post-translational modification

In eukaryotes, histone proteins are intimately associated with DNA and a plethora of other protein complexes forming chromatin [27,28]. The fundamental repeating unit of eukaryotic chromatin is nucleosome, an octamer of core histones (consisting of two H2A-H2B dimers and a H3-H4 tetramer) around which 147 base pairs of DNA are tightly wrapped in a left-handed super-helix 1.7 times around the core octamer [29-32]. The linker histone H1 binds the nucleosome and locks in place the DNA wrapped around the core histones [33-35]. Additionally, it binds to linker DNA (10-60 base pairs of DNA linking the nucleosomes) and allows for formation of the beads on a string structure and higher order assemblies, such as 30 nm solenoids and 100 nm fibers [32,34,36]. Chromatin is a dynamic structure, capable of unfolding and refolding [37], with two general states of tightly compact heterochromatin and the more open euchromatin that are in general associated with transcriptionally silent and active genomic regions, respectively [38,39].

In addition to serving as structural scaffolds for packaging DNA inside the nucleus, histones have regulatory roles affecting a variety of cellular processes such as chromatin organization and gene expression [33,40,41]. Besides the globular domains, all core histones have long (~20-35 residues) and highly conserved amino-terminal tails that protrude from the histone core domain [42] and compose ~25-30% of the mass of individual histones [43]. An exception is the histone H2A that has an additional long carboxyl terminal segment protruding from the nucleosome [37]. These tail domains may be structured or unstructured, may interact with nucleosomal or linker DNA, and participate in protein-protein interactions [44].
Histone tail segments, rich in basic amino acids, are subject to a variety of post translational modifications including acetylation, methylation, phosphorylation, ubiquitylation, and ADP ribosylation with some locations modified in more than one way [30,31,40,45,46]. For instance, acetylation can occur on lysines, methylation on lysines and arginines, phosphorylation on serines and threonines, and ubiquitylation and sumoylation on lysines. Just to add to the complexity, lysine can be mono-, di-, and tri-methylated, and arginine can be mono- and di-methylated. Most of these enormous secondary modifications identified to date occur on the long amino-terminal regions of histones, although there are some that occur on the main globular region [37]. Singly or in combination, these secondary modifications can act as “control switches” and play diverse regulatory roles in chromatin remodeling, regulation of transcription, DNA repair, and replication [40,47,48]. For instance, in higher eukaryotes, the combination of H3 K14 acetylation, H4 K8 acetylation, and H3 S10 phosphorylation usually signals transcriptional activation, and tri-methylation of H3 K9 and the lack of H3 and H4 acetylation are associated with transcriptional repression [37]. It is thought that through these covalent modifications the tail structure or contacts change, thus modulating histone:DNA interactions as well as creating new sites of dynamic interactions that can attract binding of regulatory proteins and affect the chromatin stability and DNA accessibility within the nucleosomes [40,44,49].

Most physiological histone modifications have been shown to be dynamic, reversible, and under regulation of histone modifying enzymes [30]. An exception is arginine demethylation during which arginine is changed into citrulline [30]. Lysine acetylation in histone proteins is among the best characterized PTMs [50], with inherent
importance to this thesis due to chemical similarity of N$^6$-acetyllysine to N$^6$-formyllysine (Figure 1-1). The following section presents a detailed review of lysine acetylation.

**Lysine acetylation is a key regulatory post-translational modification**

Acetylation of the lysine ε-amino group is an abundant protein modification with key regulatory roles in transcription factors, histone proteins, and other chromatin proteins. Histone acetylation is among the best characterized PTMs [50] in its regulation of chromatin organization, gene expression, and DNA repair [36]. Lysine acetylation occurs at highly conserved sites along the tails of core histones and is under tight control of two sets of enzymes: histone acetyl transferases (HATs) and histone deacetylases (HDACs) (Figure 1-2) [51]. HATs transfer an acetyl group from acetyl coenzyme A to conserved lysine residues while HDACs catalyze the removal of the acetyl moiety [39,52]. In addition to disrupting the DNA/histone electrostatic interactions, acetyl lysine is recognized by the bromodomains of chromatin remodeling proteins, which in turn recruit other downstream factors involved in chromatin remodeling and DNA
Chapter 1: Introduction

Figure 1-2. Lysine acetylation is under tight control of two sets of enzymes: histone acetyl transferases (HATs) and histone deacetylases (HDACs).

transcription [48,53]. Acetylation is almost exclusively associated with transcriptional activation [30]. The chemical similarities of N^6^-formyllysine and N^6^-acetyllysine (Figure 1-1) suggest a disruptive role for the former in signaling by histone acetylation. Indeed, N^6^-formyllysine has been detected at conserved sites of lysine acetylation and methylation in histone proteins [35,54].

The state of acetylation in cells is also important in the pathophysiology of a variety of diseases including neurodegenerative diseases. For instance, it has been shown that β-amyloid hyperacetylation and acetylation of tau proteins are associated with Alzheimer’s disease and dementia, respectively [1]. However, dysregulation of physiological and (in most cases) enzymatically controlled protein modifications are not the only ones affecting cellular physiology and disease. Protein modification by reactive oxidants and electrophiles constitute an important change to protein structure and/or function. The next sections examine some of the sources of these damaging agents and the protein modifications that they produce.
Chapter 1: Introduction

Production of reactive chemical species in cells

A wide range of physiological or pathophysiological processes in cells generate oxidants and electrophiles that directly damage biomolecules or lead to the formation of other reactive intermediates [55,56], thus affecting crucial cellular processes.

One source involves inflammation. As shown in Figure 1-3A, during inflammation, activated macrophages and neutrophils produce a variety of highly reactive oxygen and nitrogen species as part of the host defense system against pathogens. These highly reactive species alter cellular biomolecules through oxidation, nitrosation, nitration, and halogenations among other reactions [57]. The membrane-bound NADPH oxidase of these inflammatory cells catalyzes the transfer of an electron from NADPH to O₂, forming the superoxide anion radical (O₂⁺) [58]. O₂⁺ is not a strong oxidant, but it is a precursor to other reactive species [59]. Most cellular O₂⁺ is converted to hydrogen peroxide (H₂O₂) via superoxide dismutase (SOD) [55,59]. A range of potent oxidants and oxidation/nitration intermediates are produced as H₂O₂ and O₂⁺ react with other molecules (Figure 1-3A). H₂O₂ can be reduced to the highly reactive hydroxyl radical (HO•) by redox reactive metals (e.g., Fe²⁺ and Cu⁺⁺) via Fenton chemistry. Reaction of O₂⁺ with nitric oxide (NO) produced by activated macrophages in high concentration (≤1μM) [60,61] at sites of inflammation, yields peroxynitrite (ONOO⁻). ONOOH (protonated form of ONOO⁻) undergoes rapid homolysis (t₁/₂ ~1 s) to produce HO• and the weak oxidant, nitrogen dioxide radical (NO₂⁺) [55,57]. Reaction of ONOO⁻ with carbon dioxide generates a potent nitrating agent, nitrosoperoxycarbonate (ONOOCO₂⁻), that upon homolysis (t₁/₂ ~ 50 ms) forms carbonate radical anion (CO₃⁻) and NO₂⁺ [57].
Another nitrosating agent, nitrous anhydride (N₂O₃), is derived from oxidation of NO. In activated neutrophils, myeloperoxidase-mediated reaction of H₂O₂ with Cl⁻ yields hypochlorous acid (HOCl), a strong oxidizing and halogenating agent [62].
Myeloperoxidase in these cells also mediates the conversion of nitrite (NO$_2^-$) to NO$_2^*$ [63].

The other sources of reactive oxygen species include mitochondrial respiration (Figure 1-3B) and other O$_2$ metabolism pathways. The use of molecular O$_2$ for the cellular production of energy is responsible for the generation of many reactive oxygen species [65]. A variety of reactive molecules and free radicals are derived from O$_2$ such as O$_2^{•−}$, HO$^•$, H$_2$O$_2$, peroxy radical (ROO$^•$), ozone (O$_3$), and singlet oxygen (¹O$_2$). Reactive species such as O$_2^{•−}$ and H$_2$O$_2$ are produced as a result of electron leakage from the mitochondrial electron transport chain during normal cellular metabolism [66,67]. Oxidoreductase enzymes such as NADPH oxidase, myeloperoxidase, and the cytochrome P450 enzymes also produce reactive species [68].

Reactive oxygen and nitrogen species have the capability of damaging molecules directly. However, damage caused by these reactive species (e.g., lipid peroxidation, carbohydrate oxidation, and direct amino acid oxidation), can yield electrophiles capable of causing further damage to biomolecules [55] (Figure 1-4). For instance, electrophilic species such as reactive aldehydes and epoxides are formed as a result of lipid peroxidation and can react with nucleophilic sites in DNA and proteins to yield adducts [55,69]. Among the products generated, the α,β-unsaturated aldehydes possessing two reactive sites are especially important since they can form cross-links or cyclic adducts with protein or nucleic acid [70-72]. Pathological modifications in proteins can be readily formed through reaction of nucleophilic amino acids lysine, histidine, and cysteine with reactive electrophiles generated from oxidation of polyunsaturated fatty
acids and carbohydrates such as malondialdehyde, 4-hydroxy-2-nonenal (HNE), 4-oxononenal, and glyoxal [73-77].

The next chapter covers in more detail these adventitious protein modifications due to reaction with primary and secondary reactive species. It should be noted that protein oxidative modification can result from other sources such as xenobiotics, cigarette smoke, acetaminophen, γ-irradiation, ultraviolet (UV) light, and ozone [68], among other agents. However, for the purpose of this thesis, we only focus on protein damage due to endogenously derived reactive oxidants and electrophiles.

**Figure 1-4.** Examples of reactive aldehydes generated from damage to biomolecules.
Protein damage due to reactive endogenous oxidants and electrophiles

In addition to physiological secondary modifications that are mostly enzymatically regulated, proteins are subjected to reactions with endogenous oxidants and electrophiles generated by oxidative stress, inflammation, and normal cell metabolic processes [77-80]. Protein targets of reactive species are vast and include structural and membrane proteins, metabolic and detoxification enzymes, or proteins involved in cell signaling and gene expression [55]. Protein modification caused by a single oxidant or electrophile, such as HNE can produce a diverse set of cellular responses with hundreds of genes upregulated and downregulated [58]. Here we review representative examples of the types of damage caused by these reactive species in cells.

Direct oxidation of amino acid side chains is one example of adventitious protein modification. Oxidative modification of proteins is usually specific to type and location of the residue and may occur on polypeptide backbone or the nucleophilic/redox-sensitive side chains. As mentioned previously, modifications of proteins affect their function in a positive or negative way, thus playing important roles in cellular physiology as well as progress of disease. Cysteine, tyrosine, and methionine are among the amino acids mostly modified by endogenous oxidants [55]. Cysteine sulfenic acid is formed via oxidation of the sulfhydryl group of cysteine, helping to absorb the oxidative insult [81]. Cysteine sulfenic acid is unstable and forms disulfide bonds with glutathione or other accessible thiols or undergoes further oxidation to cysteine sulfinic acid and cysteine sulfonic acid [82]. S-nitrosylation of cysteine and 3-nitration of tyrosine residues in proteins is another consequence of oxidative or nitrosative stress [83,84]. In fact, 3-nitrotyrosine is used as a biomarker of protein damage associated with inflammation and
variety of diseases [84]. Methionine oxidation to form methionine sulfoxide, reversed by methionine sulfoxide reductases, is speculated to serve as a defense mechanism against oxidative stress by preventing other residues from oxidative damage [85]. Elevated levels of oxidized aromatic amino acid residues (e.g., 3-nitrotyrosine, 3-chlorotyrosine, and 3,4-dihydroxyphenylalanine) are seen in age-related diseases such as neurodegenerative and cardiovascular diseases [55]. For instance, 3-nitrotyrosine adducts were detected in tyrosine hydroxylase isolated from brain tissues of Parkinson’s disease model mice that exhibited reduced enzymatic activity [86]. Oxidation of proline, arginine, and lysine residues result in protein carbonyls such as glutamate and aminoadipate semialdehydes [73,78]. In general, protein carbonyls can be generated via direct damage to polypeptide backbone, oxidation of amino acids proline, lysine, arginine, threonine, glutamate, aspartic acid [1,55,73,78], or Michael addition of reactive α,β-unsaturated aldehydes to nucleophilic amino acids lysine, histidine, and cysteine [73-77]. Protein carbonylation, an irreversible modification, often leads to loss of protein function [87]. Indeed, total protein carbonyl content is regarded as a biomarker of oxidative stress and inflammation and elevated levels are associated with a variety of human diseases such as cardiovascular and neurodegenerative disorders, and processes such as aging [73,75,78].

Another adventitious protein modification involves reaction of amino acid side chains with physiological sugars and sugar oxidation products. Unlike the enzymatic mediated and site specific glycosylation of proteins, these advanced glycation end products (AGEs) are random events [1]. For instance, the reactive dicarbonyl sugar 3-deoxyglucosone, synthesized via the Maillard reaction and the polyol pathway [88],
results in formation of AGEs such as imidazolone, N⁶-carboxymethyllysine, and pyrraline by reaction with amines [89] (Figure 1-5). AGEs have been shown to cause extracellular matrix dysfunction, to react with cell receptors and change cytokine and hormone levels, and to alter functions of a variety of intracellular proteins [90]. AGEs can also form crosslinks, especially in long lived proteins such as collagen and elastin [91]. The formation and accumulation of AGEs over time has been implicated in the development of diabetes as well as age-related degenerative processes by damaging blood vessels, connective tissue, ocular lens, and nerves [14,90].

Reactive aldehyde induced modifications of proteins typically arise by reaction of the nucleophilic side chains of lysine, cysteine, and histidine with reactive electrophiles such as malondialdehyde (MDA), 4-hydroxynonenal (HNE), acrolein, and glyoxal generated by oxidation of polyunsaturated fatty acids and carbohydrates, among other

**Figure 1-5.** Examples of protein adducts caused by reactive electrophiles.
biomolecules [74,75,77,79,80]. Michael adducts are among the major adducts formed, for example, from the reaction of MDA with lysine residues (Figure 1-5) and 4-hydroxy-2-alkenals with lysine, cysteine, and histidine amino acids [55]. Sequential addition of two acrolein molecules to epsilon-amino group of lysine results in formation of cyclic N^6-3-formyl-3,4-dehydropiperidino-lysine adduct [92] (Figure 1-5). Schiff base adducts are formed from reaction of 4-hydroxy-2-alkenals [93] as well as formaldehyde with lysine residues [94]. Protein adducts generated by products of lipid peroxidation can alter protein function and thus affect the vast range of biological functions they regulate, leading to pathological processes associated with human diseases [77-80,95]. For instance, HNE induces cell toxicity by covalently modifying IkB kinase, thus inhibiting the subsequent transcription of NFkB-dependent genes needed for cell survival [58]. It was also shown that HNE results in loss of microtubule network in neuroblastoma cells by forming Michael adducts with tubulin isoforms [96].

Among adventitious protein adducts (Table 1-1 and Figure 1-5), N^6-formylation of lysine has recently emerged as an abundant protein modification [35,53,54,97]. N^6-Formyllysine is a chemical homolog of the biologically important N^6-acetyllysine and thus may interfere with acetylation signaling in cells. The following section is a more detailed introduction on this pathological protein modification that was originally observed in histones and chromatin proteins.
**Table 1-1.** Examples of oxidative modification of proteins. Adapted from [68].

<table>
<thead>
<tr>
<th>Oxidative Modification</th>
<th>Amino Acids Residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disulfides, glutathiolation</td>
<td>Cys</td>
</tr>
<tr>
<td>S-Nitrosocysteine</td>
<td>Cys</td>
</tr>
<tr>
<td>3-Nitrotyrosine</td>
<td>Tyr</td>
</tr>
<tr>
<td>3-Chlorotyrosine</td>
<td>Tyr</td>
</tr>
<tr>
<td>Methionine sulfoxide</td>
<td>Met</td>
</tr>
<tr>
<td>Cross-links, aggregates</td>
<td>Several</td>
</tr>
<tr>
<td>Lipid peroxidation adducts</td>
<td>Lys, Cys, His</td>
</tr>
<tr>
<td>Carbonyls</td>
<td>All (Lys, Arg, Pro, Thr)</td>
</tr>
<tr>
<td>Amino acid oxidation</td>
<td>Lys, Cys, His</td>
</tr>
</tbody>
</table>

**N⁶-Formyllysine as an adventitious protein modification**

Emerging evidence points to widespread modification of cellular proteins by N⁶-formylation of lysine as a result of adventitious reactions with endogenous electrophiles [35,53,54,97]. These adducts were originally described in histones and other chromatin proteins [35,53,54], although they have since been identified as adducts arising in proteins subjected to nitrosative and oxidative stresses [97].

We have previously shown the generation of N⁶-formyllysine adducts in histone proteins as a result of DNA oxidation [53]. Histones are rich in nucleophilic amino acids, such as lysine and arginine that can react with pathogenic electrophiles generated from oxidized DNA, proteins, and lipids [53]. Our previous study in which DNA was labeled with 5' tritiated thymidine followed by cell treatment with neocarzinostatin (an enediyne
antibiotic that abstracts a 5'-H from 2-deoxyribose) showed that lysines in histones can react with 3'-formylphosphate residue, an electrophile generated from 5'-oxidation of 2-deoxyribose in DNA, to form N6-formyllysine (Figure 1-6) [53].

N6-Formyllysine was reported to be the major adduct formed when proteins were reacted with trichloroethylene (TCE) oxide in vitro [98], and a recent study points to its formation in proteins treated with biological oxidant, peroxynitrite [97], suggesting other
Chapter 1: Introduction

sources for its generation besides DNA oxidation. In 1985, N$^6$-formyllysine was reported to be formed by reaction of l-lysine with formaldehyde [99] and recently, it was shown it could result from silver staining procedures that involve the use of formaldehyde [100]. Considering that formaldehyde reacts with amines to give a carbinolamine intermediate that is only one oxidation state away from a formamide functional group, we hypothesized that endogenous formaldehyde could serve as a major source of N$^6$-formyllysine residues in histone and other proteins.

Formaldehyde is a reactive aldehyde forming adducts with nucleophilic sites in DNA and protein molecules resulting in products such as N$^2$-hydroxymethyl-dG DNA adducts [101] and formaldehyde induced Schiff bases on lysine residues of histones [94], in addition to protein-DNA crosslinks. It has been classified as a known human carcinogen by the International Agency for Research on Cancer in 2005 [102] and by the National Toxicology Program of the National Institute of Environmental Health Sciences in 2011 [103], although it is still considered a probable human carcinogen by the U.S. Environmental Protection Agency [104]. In addition to environmental and occupational sources [101,105,106], formaldehyde arises endogenously from cellular processes such as demethylation of histones and nucleic acids as well as biosynthesis of purines, thymidine, and some amino acids [10,105,107]. Formaldehyde is a relatively abundant metabolite at concentrations ranging from 13 to 97 μM in human plasma [105]. Thus, formaldehyde induced N$^6$-formyllysine generation in proteins could be the major source of this adduct compared to previous sources reported.

The relatively high abundance of N$^6$-formyllysine adducts in histones [35,53] suggests it could interfere with the regulatory roles of histones. The following section
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examines the potential disruptive role of N^6-formyllysine in cellular epigenetics.

The biological implications of N^6-formyllysine modification of histones

N^6-Formyl lysine has been reported to be a major protein adduct in histones occurring on many conserved lysine residues involved in epigenetic regulations [35,53]. Even the DNA oxidation pathway in itself can result in high abundance of this adduct in histones. Indeed, there are thousands of oxidative events in a cell on a daily basis, resulting in hundreds of deoxyribose oxidations in DNA with each site producing different electrophilic products, leading to formation of 10's of N^6-formyllysines per cell per day [53,108]. As discussed later in this thesis, formaldehyde is found to be another major source giving rise to this adduct in histones. Therefore, if not removed, this pathological modification may accumulate to significant levels in cells.

The high rate of occurrence of N^6-formyllysine in histones raises the possibility of epigenetic interferences through affecting the regulatory roles of these proteins. In chromatin proteins, N^6-formyllysine has the potential to interfere with important physiological modifications that perform signaling functions. Indeed, N^6-formyllysine has been detected at conserved sites of lysine acetylation and methylation in histones [35,54]. In addition, the chemical similarities of N^6-formyllysine and N^6-acetyllysine suggest a disruptive role for the former in signaling by histone acetylation. Thus, this thesis project is focused on defining the mechanism of formation and biological significance of N^6-formyllysine. To that end, we developed an ultrasensitive and specific liquid chromatography-coupled tandem mass spectrometry method for quantifying this adduct in proteins (Chapter 2), and used it to answer questions about its mechanisms of
formation and pathological consequences in cells (Chapter 3). Chapter 4 extends our
studies to measurement of N\textsuperscript{6}-formyllysine protein adducts in tissues of rats exposed to
formaldehyde by inhalation, as we discovered formaldehyde to be a major endogenous
source of N\textsuperscript{6}-formyllysine (Chapter 3). We conclude this thesis in Chapter 5 by
summarizing our results regarding sources as well as fate of N\textsuperscript{6}-formyllysine adducts and
assess possible biological significance of this secondary protein modification. The
presence of N\textsuperscript{6}-formylation of lysines in histones may have important biological
consequences leading to pathology and progress of variety of diseases.
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References


Chapter 1: Introduction


Chapter 2

Development of a liquid chromatography-coupled tandem mass spectrometry method for quantification of N⁶-formyllysine adducts in proteins
Chapter 2: Development of an LC-MS/MS method for quantification of N\textsuperscript{6}-formyllysine adducts in protein

**Abstract**

The initial step in investigating the mechanism of formation of N\textsuperscript{6}-formyllysine and assessing its possible pathological consequences in cells involved developing an ultrasensitive and specific liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) method for quantifying this adduct in proteins. The quantification method we developed previously (Jiang et al. PNAS 104: 60-5, 2007) proved to be relatively insensitive and biased, so a new method was developed, which involved proteolytic hydrolysis of proteins to individual amino acids, normal phase chromatographic resolution of the amino acids followed by tandem electrospray mass spectrometry to quantify them. N\textsuperscript{6}-Formyllysine is reported as a percentage of total lysines in order to normalize it across samples with different amounts and types of proteins. The use of *Streptomyces griseus* protease greatly diminished the background levels of lysine and formyllysine observed previously with proteinase K. Further, the direct analysis of amino acids, as opposed to phenylisothiocyanate (PITC) derivatization, improved both the sensitivity and specificity of the assay. In addition to lysine and N\textsuperscript{6}-formyllysine species, N\textsuperscript{6}-acetyl, mono-, di-, and tri-methyllysine modifications are monitored, with limit of detection of 1-50 fmol for all species. Measurement of N\textsuperscript{6}-formyllysine in histones of TK6 cells indicates that this adduct is an abundant endogenous protein modification (~1 per 10\textsuperscript{4} lysines). In addition, the dilution rate of the absolute quantity of N\textsuperscript{6}-formyllysine in histone proteins of TK6 cells (grown in media containing isotopically labeled lysine) reveals that N\textsuperscript{6}-formyllysine is a rather stable modification of histones, with a half-life closely matching TK6 cell doubling rate.
Chapter 2: Development of an LC-MS/MS method for quantification of N°-formyllysine adducts in protein

Introduction

The first step in defining the biological relevance of a new molecule is to quantify its presence in cells and tissues. With this in mind, a new method was developed to quantify the N°-formyllysine protein adducts. The method workflow shown in Figure 2-1 involves hydrolysis of proteins to generate individual amino acids that are then analyzed using liquid chromatography-coupled tandem mass spectrometry. Each step in the method is critical to the precision and accuracy of the analytical data.

Proteolytic digestion using proteinase K has been utilized previously for generating individual amino acids containing N°-formyllysine moiety [1,2] in order to avoid removal of the formyl group by traditional strong acid-mediated protein hydrolysis. However, we found that proteinase K undergoes extensive autolysis and, when used in small quantities to avoid self digestion, it was not an efficient protease. The next step of chromatographic resolution of amino acids was previously accomplished with PITC derivatization and reversed phase HPLC. However, this approach decreased the sensitivity of the method and added an extra step. The final step of tandem mass spectrometric quantification of the amino acids is relatively straightforward.

This chapter covers the new enzyme discovered as well as other advancements to our previous method to make it more reliable and sensitive and presents an example of its immediate applications (i.e., determining the rate of dilution of this adduct in cells). We extend our quantitative analysis of proteins to include other lysine modifications such as acetylation and methylation besides formylation. We report the development of a highly robust and sensitive LC-MS/MS method for measuring all N°-formyl, -acetyl, and -methyllysine modifications, with limits of detection of 1-50 fmol for all species. In
addition to providing insights into N^6-formyllysine content of proteins, there are numerous applications of this method for characterizing the chemical biology of this protein modification as presented in the subsequent chapters.

**Materials and Methods**

**Materials.** Lysine internal standard, 4,4,5,5,-[^2]H]-Lysine, was purchased from Cambridge Isotope Laboratories (Andover, MA). N^6-Formyllysine internal standard, 4,4,5,5,-[^2]H]-N^6-formyllysine, was synthesized from 4,4,5,5,-[^2]H]-lysine according to Jiang *et al.* [1]. N^6-Acetyllysine internal standard, 3,3,4,4,5,5,6,6,-[^2]H]-N^6-acetyllysine
was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). Lysine, \( N^6 \)-formyllsine, \( N^6 \)-acetyllysine, *Streptomyces griseus* protease, and protease inhibitor cocktail (for use with mammalian cell and tissue extracts) were obtained from Sigma-Aldrich (St. Louis, MO). \( N^6 \)-Mono-methyllysine, \( N^6 \)-di-methyllysine, and \( N^6 \)-tri-methyllysine were purchased from Bachem Bioscience Inc. (King of Prussia, PA). Nonidet P-40 was from Roche Diagnostic Corporation (Indianapolis, IN). Human lymphoblastoid TK6 cell line was a generous gift of Wogan Lab at Massachusetts Institute of Technology.

**TK6 cell culture and labeling.** TK6 cells were cultured in RPMI 1640 medium (Cellgro, Manassas, VA) supplemented with 10% heat-inactivated horse serum (Atlanta Biologicals, Lawrenceville, GA), 10,000 U penicillin/ml and 10,000 \( \mu \)g streptomycin/ml (Lonza, Walkersville, MD), and 2mM L-glutamine (Lonza, Walkersville, MD) at 37° C in a 5% CO\(_2\) atmosphere. For labeling proteins with endogenous isotopically labeled \( N^6 \)-formyllsine, TK6 cells were grown in a customized RPMI-1640 medium, with everything identical to traditional RPMI 1640 medium (supplemented with horse serum, antibiotics, and L-glutamine), except for the presence of deuterated lysine (3,3,4,4,5,5,6-[\( ^2 \text{H} \)]-Lysine) instead of non-labeled lysine. After one week of growth (roughly 9 doubling times) in the customized medium, cells were washed and resuspended in non-labeled RPMI medium. Histones were extracted from \( \sim \)10 million cells at 0h, 18h, 36h, and 54h, and the quantity of isotopically labeled formyllsine, as a percentage of total labeled and unlabeled lysines, was measured at each of those time points.
Histone extraction from TK6 cells and bovine liver tissue. Extraction of histones was performed according to Boyne et al. [3], with modifications. Cells (~10^7 per sample) were pelleted by centrifugation at 1000 x g for 5 min at 4 °C and the pellet was washed once with PBS. Cell pellets were then lysed by resuspension in ice-cold lysis buffer (15 mM Tris-HCl, pH 7.5, 15 mM NaCl, 60 mM KCl, 1 mM CaCl₂, 5 mM MgCl₂, 250 mM sucrose, 1 mM dithiothreitol, 10 mM sodium butyrate) containing a 100:1 v:v dilution of protease inhibitor cocktail in the presence of 0.03% Nonidet P-40 and incubation on ice for 5 min with occasional gentle mixing. Nuclei were pelleted by centrifugation at 600 x g for 5 min at 4 °C, and the pellet was washed twice with ice-cold lysis buffer without Nonidet P-40. Histones were extracted by addition of ice-cold 0.4N H₂SO₄ and incubation overnight on ice. The solution was centrifuged at 3000 x g for 5 min and proteins in supernatant were precipitated by addition of 20% v/v trichloroacetic acid and overnight incubation at 4 °C. Samples were then centrifuged at 14000 x g for 10 min at 4°C, washed once with ice-cold acetone containing 0.1% HCl, and once with ice-cold acetone. The extracts were air-dried and stored at -20 °C until use. For extracting histones from bovine liver tissue, 20 mg of tissue was cut into small pieces and washed with PBS. Chromatin bound fraction was obtained using the Subcellular Protein Fractionation Kit from Thermo Scientific (Waltham, MA) and a Kontes all-glass Dounce homogenizer (10 strokes with a type B pestle). Proteins in chromatin bound fraction were precipitated by addition of 20% v/v trichloroacetic acid and overnight incubation at 4 °C. Samples were then centrifuged at 14000 x g for 10 min at 4 °C, washed once with ice-cold acetone containing 0.1% HCl, and once with ice-cold acetone. The extracts were air-dried and stored at -20 °C until use.
**Purification of individual histones.** HPLC purification of total histones was performed according to Boyne et al. [3], with modifications. Total histones (≤ 50 μg) were dissolved in 0.1% trifluoroacetic acid (TFA) in water and fractionated by HPLC on an Agilent 1100 series instrument (Agilent Technologies, Santa Clara, CA), using a Source 5RPC C₁₈ reversed-phase column (4.6 x 150 mm, 5 μm particles; GE Healthcare Life Sciences). The mobile phase flow rate was 1 mL/min and the solvent system was 0.1% TFA in water (A) and 0.094% TFA in acetonitrile (B) with the elution starting at 0% B, linearly increasing to 28% B over 28 min, reaching 37% B at 70 min, 38% B at 100 min, 60% B at 150 min, and finally 100% B at 151 min, before the column was re-equilibrated to 0% B for 10 min. Protein elution was monitored by UV absorbance at 214 nm and histones in each fraction were tentatively identified by resolution on a 13% SDS-PAGE gel with Coomassie Blue staining (see Figure 3-S1).

**Enzymatic hydrolysis of proteins.** Histones extracted from TK6 cells and other protein samples were dissolved in 50 μL of 100 mM ammonium bicarbonate buffer (pH 8.5), 4,4,5,5-[²H]-lysine (2 nmol), 4,4,5,5-[²H]-N⁶-formyllysine (1 pmol), and 3,3,4,4,5,5,6,6-[²H]-N⁶-acetyl lysine (10 pmol) were added as internal standards, and the proteins hydrolyzed by addition of *Streptomyces griseus* protease (freshly prepared solution each time) with incubation at 37 °C for ≥ 16 h. *S. griseus* was used at a ratio of 1 μg of enzyme per 10 μg of protein. Samples were then dried under vacuum and resuspended in 50 μL of water before mass spectrometry analysis.

**Quantification of amino acids.** N⁶-Formyllysine and other amino acids were quantified
as a percentage of the total quantity of lysine, by liquid chromatography-coupled mass spectrometry (LC-MS/MS). HPLC was performed with an Agilent 1100 series instrument. Adducts of interest (Figure 2-2) in the resuspended protein hydrolysates were separated using an aqueous normal phase Cogent diamond hydride column (2.1 x 150 mm, 4 μm) from MicroSolv Technology Corporation (Eatontown, NJ). The mobile phase flow rate was 400 μL/min, and the column temperature was maintained at 20 °C. The solvent system was 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B), with the elution starting at 100% B, the gradient linearly decreased to 25% B over 30

<table>
<thead>
<tr>
<th>N°-Formyllysine</th>
<th>Lysine</th>
<th>N°-Acetyl Lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure" /></td>
<td><img src="image2.png" alt="Structure" /></td>
<td><img src="image3.png" alt="Structure" /></td>
</tr>
<tr>
<td>MW: 174.20 Da</td>
<td>MW: 146.19 Da</td>
<td>MW: 188.22 Da</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N°-Mono-Methyl Lysine</th>
<th>N°-Di-Methyl Lysine</th>
<th>N°-Tri-Methyl Lysine</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image4.png" alt="Structure" /></td>
<td><img src="image5.png" alt="Structure" /></td>
<td><img src="image6.png" alt="Structure" /></td>
</tr>
<tr>
<td>MW: 160.21 Da</td>
<td>MW: 174.24 Da</td>
<td>MW: 189.28 Da</td>
</tr>
</tbody>
</table>

**Figure 2-2.** The structure and MW of lysine species monitored.
Chapter 2: Development of an LC-MS/MS method for quantification of N⁶-formyllysine adducts in protein

min, stayed at 25% B for 3 additional min before the column was re-equilibrated at 100% B for 7 min. In order to separate the co-eluting M+1 citrulline signal from M+2 ion of N⁶-formyllysine, an extended chromatography run time was used, with the elution starting at 100% B, the gradient linearly decreased to 75% B over 75 min, further decreased to 25% B over the next 3 min, reached 15% by 83 min before the column was re-equilibrated at 100% B for 7 min. The species of interest were then analyzed using an Agilent 6410 triple quadrupole mass spectrometer (MS/MS) equipped with an electrospray ionization (ESI) source operated in positive ion mode. The operating parameters were as follows: ESI capillary voltage, 4000 V; gas temperature, 350 °C; drying gas flow, 12 L/min; and nebulizer pressure, 30 psi. Selected reaction monitoring (SRM) transitions are summarized in Table 2-1. Note that in addition to chromatographic separation and presence of internal standards, the unique product ions of m/z 112 and m/z 126 for formyl and acetyl lysines, respectively, distinguish them from their isobaric compounds di- and tri-methyl lysines (Table 2-1 and Figure 2-3). The fragmentor voltage and collision energy were optimized in order to maximize the signal of each product ion monitored (Figure 2-4) and are summarized in Table 2-1. 4,4,5,5-[²H]-lysine, 4,4,5,5-[²H]-N⁶-formyllysine, and 3,3,4,4,5,5,6,6-[²H]-N⁶-acetyl lysine were used as internal standards and external calibration curves for methyl species were prepared using deuterated acetyl lysine (Figure 2-5).
Chapter 2: Development of an LC-MS/MS method for quantification of $N^\alpha$-formyllysine adducts in protein

Table 2-1. Summary of mass spectrometry parameters for each species.

<table>
<thead>
<tr>
<th>Species</th>
<th>SRM Transition ($m/z$)</th>
<th>Internal Standard Transition ($m/z$)</th>
<th>Qualifier Ion Transition ($m/z$)</th>
<th>Fragmentor Voltage (V)</th>
<th>Collision Energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>147 $\rightarrow$ 130</td>
<td>151 $\rightarrow$ 134</td>
<td>NA</td>
<td>100</td>
<td>8</td>
</tr>
<tr>
<td>$N^\alpha$-Formyllysine</td>
<td>175 $\rightarrow$ 112</td>
<td>179 $\rightarrow$ 116</td>
<td>NA</td>
<td>105</td>
<td>10</td>
</tr>
<tr>
<td>$N^\alpha$-Acetyllysine</td>
<td>189 $\rightarrow$ 126</td>
<td>197 $\rightarrow$ 134</td>
<td>NA</td>
<td>105</td>
<td>10</td>
</tr>
<tr>
<td>$N^\alpha$-Mono-methyllysine</td>
<td>161 $\rightarrow$ 84</td>
<td>NA</td>
<td>161 $\rightarrow$ 130</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>$N^\alpha$-Di-methyllysine</td>
<td>175 $\rightarrow$ 84</td>
<td>NA</td>
<td>175 $\rightarrow$ 130</td>
<td>100</td>
<td>14</td>
</tr>
<tr>
<td>$N^\alpha$-Tri-methyllysine</td>
<td>189 $\rightarrow$ 84</td>
<td>NA</td>
<td>189 $\rightarrow$ 130</td>
<td>100</td>
<td>14</td>
</tr>
</tbody>
</table>

Figure 2-3. Proposed structures for product ions monitored.
Chapter 2: Development of an LC-MS/MS method for quantification of N⁶-formyllysine adducts in protein

Figure 2-4. Examples of product ions for each lysine species monitored, after optimization. In all cases, the highest count was used as the product ion for MRM transitions in triple quadruple mass spectrometer, as described in Materials and Methods. An exception was lysine, for which the 130 m/z ion was selected due to lysine’s high abundance compared to other species monitored.
Figure 2-5. Examples of calibration curves for the isotope dilution LC-MS/MS analysis of lysine species, as described in Materials and Methods. Abbreviations: FK, N\(^6\)-formyllysine; DFK, deuterium-labeled N\(^6\)-formyllysine; AK, N\(^6\)-acetyllysine; DAK, deuterium-labeled N\(^6\)-acetyllysine; K, lysine; DK, deuterium-labeled lysine; MK, N\(^6\)-mono-methyllysine; M\(_2\)K, N\(^6\)-di-methyllysine; M\(_3\)K, N\(^6\)-tri-methyllysine.
Results

An improved method to quantify N⁶-formyllysine in proteins. Our previous method for quantifying N⁶-formyllysine in proteins involved proteinase K-mediated hydrolysis of proteins, derivatization of the resulting amino acids with PITC, HPLC pre-purification of amino acid derivatives, and final LC-MS/MS analysis of the derivatized amino acids [1]. This method proved to be relatively insensitive and biased as a result of using proteinase K, which produced only partial hydrolysis of some proteins when used in small quantities to minimize background autolysis (Figure 2-6). To overcome these problems, we used Streptomyces griseus protease at a ratio of 1 µg enzyme per 10 µg proteins, which resulted in efficient and complete digestion of all proteins as judged by the total amount

![Graph showing comparison of released lysine in protein digestion using Streptomyces griseus protease versus proteinase K. Proteinase K produced only partial hydrolysis of TK6 histone proteins when used in small quantities to minimize background autolysis.](image)

Figure 2-6. Comparison of released lysine in protein digestion using Streptomyces griseus protease versus proteinase K. Proteinase K produced only partial hydrolysis of TK6 histone proteins when used in small quantities to minimize background autolysis.
of lysine released per µg of purified histone proteins (Figure 2-6). In addition, the method was optimized to eliminate the HPLC pre-purification step and the need for PITC derivatization to achieve chromatographic resolution of amino acids. This was achieved using aqueous normal phase HPLC with a diamond-hydride column to resolve individual amino acids. This chromatographic system resolved N6-acetyllysine, mono-, di-, and tri-N6-methyllysines, as well as N6-formyllysine and lysine, as shown in Figure 2-7. With isotopically labeled internal standards added prior to protease digestion, identification and quantification of amino acids were accomplished by isotope-dilution LC-MS/MS in positive ion mode, using multiple reaction monitoring (MRM) transitions. The limits of detection were found to be 1 fmol for N6-formyl- and N6-acetyllysine, 10 fmol for lysine, and 50 fmol for each of N6-mono-, di-, and tri-methyl lysine. Data for the various lysine modifications are expressed here as proportions of the total number of lysines in the sample.

**Measurement of N6-formyllysine adducts in total histone proteins.** The first application of the new method involved quantification of the various modified lysines in a mixture of total histone proteins extracted from TK6 lymphoblastoid cells (Table 2-2). Data show that N6-formylation of lysine occurs at a low frequency (e.g., 1 per 10⁴ lysines) in total histones compared to higher frequencies of acetylation and methylation (40 – 230 per 10⁴ lysines). Further, an analysis of total histone proteins, either extracted from TK6 cells or from commercial sources, show a similar background level of N6-formyllysine in all samples (Table 2-3). The next chapter covers the distribution of various lysine adducts in different classes of histones separated by HPLC.
Chapter 2: Development of an LC-MS/MS method for quantification of N\(^6\)-formyllysine adducts in protein

Table 2-2. Quantification of lysine modifications in a sample of total histone proteins.

<table>
<thead>
<tr>
<th></th>
<th>Formyl(^1)</th>
<th>Acetyl</th>
<th>Methyl</th>
<th>Dimethyl</th>
<th>Trimethyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK6 Cells</td>
<td>1.0 ± 0.1(^2)</td>
<td>40 ± 10</td>
<td>230 ± 50</td>
<td>130 ± 40</td>
<td>25 ± 5</td>
</tr>
</tbody>
</table>

\(^1\)Column titles denote different N\(^6\)-modifications of lysine.

\(^2\)Data are expressed as modifications per 10\(^4\) total lysines and represent mean ± SD for 3 biological replicates.

Figure 2-7. An example of different lysine species detected in purified histone H4 from TK6 cells. Lysine adducts were monitored by tandem mass spectrometry, as described in Materials and Methods.
Chapter 2: Development of an LC-MS/MS method for quantification of N^6-formyllysine adducts in protein

### Table 2-3. Quantification of N^6-Formyllysine in total histones.

<table>
<thead>
<tr>
<th>Histone source</th>
<th>N^6-Formyllysine (per 10^5 Lys)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK6 cells</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Calf thymus</td>
<td>2 ± 0.2</td>
</tr>
<tr>
<td>Bovine liver</td>
<td>2 ± 0.6</td>
</tr>
</tbody>
</table>

**Resolving co-elution problem of a contaminant with M+2 ion of N^6-formyllysine.** In addition to monitoring N^6-formyllysine ion (m/z transition of 175 → 112), some applications of the quantification method, as will be discussed in Chapters 3 and 4, involve monitoring the heavy isotope of N^6-formyllysine (m/z transition of 177 → 114). However, we encountered the presence of 177 m/z ion in control samples (without the heavy isotope) that was well beyond the background abundance of M+2 ion of N^6-formyllysine (~0.7%), suggesting the co-elution of a contaminant peak with the analyte. We have determined the contaminant to be the M+1 ion of citrulline (Figures 2-8 and 2-9) and thus substantially extended the chromatography to separate the two.
Figure 2-8. Citrulline co-elutes with M+1 ion of N⁶-formyllysine. The co-eluting peaks of equal amounts of citrulline and N⁶-formyllysine standards injected (A), are separated by substantially extending the chromatography (B). C is the comparison of the product ion of citrulline standard with the product ion of the co-eluting peak in a total histone sample from bovine liver.
Chapter 2: Development of an LC-MS/MS method for quantification of N⁶-formyllysine adducts in protein

Figure 2-9. An example of co-elution of a contaminant peak with M+2 ion of N⁶-formyllysine in total histone sample from bovine liver. In applications that require monitoring the heavy isotope of N⁶-formyllysine (m/z transition of 177 → 114), the co-eluting peaks (A) are separated by substantially extending the chromatography (B).

Measuring the rate of dilution of N⁶-formyllysine. The next application of the method involved determining the rate of loss of N⁶-formyllysine in TK6 cells containing isotopically-labeled lysine in proteins. The chemical similarity of N⁶-formyllysine and N⁶-acetyllysine (Figure 1-1) suggested that the former might be subject to removal by histone deacetylases [4] or other protein repair or degradation processes. The dilution experiment was based on labeling TK6 cell proteins with [²H₈]-lysine by growing the cells in customized RPMI medium containing 3,3,4,4,5,5,6,6-[²H]-lysine for one week prior to resuspending them in non-labeled medium. Due to the average doubling time of 18 h for TK6 cells, histones were extracted at 0 h, 18 h, 36 h, and 54 h, corresponding to 0-3 replication cycles. The quantity of [²H₈]-N⁶-formyllysine, as a percentage of total labeled and unlabeled lysines, was then measured at each time point (Figure 2-10A).
comparison of labeled and unlabeled lysine signals revealed > 90% labeling of all lysines and modified lysines, including N⁶-formyllysine. Upon removal of the [²H₈]-lysine, the rate of loss of [²H₈]-N⁶-formyllysine in histones was detected by mass spectrometry as ions that are 8 mass units heavier than the unlabeled counterparts. The results show a dilution rate of 15 ± 0.5h for [²H₈]-N⁶-formyllysine, which closely matches the cell doubling time of 16 ± 0.9h for this experiment (calculated based on [²H₈]-lysine dilution curve as shown in Figure 2-10B).

**Figure 2-10.** Dilution rate of N⁶-formyllysine in TK6 cells. The rate of dilution of [²H₈]-N⁶-formyllysine (A) closely matches the exact cell doubling time calculated based on [²H₈]-lysine rate of dilution (B).
Chapter 2: Development of an LC-MS/MS method for quantification of $N^\delta$-formyllysine adducts in protein

Discussion

We set out to develop a highly sensitive and specific LC-MS/MS method to accurately quantify $N^\delta$-formyllysine lesions in proteins extracted from cells and tissues, as a first step towards defining the biological relevance of lysine $N^\delta$-formylation. The quantification method we developed previously [1] proved to be relatively insensitive and biased, and thus major improvements were required. We were able to implement significant improvement to increase the efficiency as well as sensitivity and specificity of our previous $N^\delta$-formyllysine quantification method.

First, we discovered and optimized a new proteolytic enzyme, *Streptomyces griseus* protease, that proved to be highly efficient at digesting proteins to individual amino acids compared to proteinase K previously used (Figure 2-6). Using proteinase K at concentrations low enough to prevent the autolysis of enzyme resulted in only 10% digestion compared to complete digestion performed by comparable amount of *S. griseus* enzyme. In addition, we extended our quantitative analysis of proteins to include other lysine modifications such as acetylation and methylation besides formylation, and utilization of the new highly efficient enzyme allowed for detection of all lysine species (Table 2-2).

The HPLC step was further optimized to make the method more robust. The pre-purification step was eliminated and the need for PITC derivatization for chromatographic resolution of amino acids with reversed phase HPLC, was obviated by switching to normal phase HPLC. We were able to achieve baseline separation of analytes of interest in the resuspended protein hydrolysates (Figure 2-7). The elimination
Chapter 2: Development of an LC-MS/MS method for quantification of N\(^6\)-formyllysine adducts in protein

of the derivatization step removed an extra step and further enhanced the sensitivity of the method.

The identification and quantification of amino acids were accomplished by isotope-dilution LC-MS/MS in positive ion mode, using MRM transitions. The discovery of the unique product ions of \(m/z\) 112 and \(m/z\) 126 for formyl and acetyl lysines, respectively, distinguished them from their isobaric compounds di- and tri-methyl lysines (Table 2-1 and Figures 2-3 and 2-4) while lowering the limits of detection by more than 10 fold (10 fmol to 1 fmol). The optimization of fragmentor voltage and collision energies (Table 2-1 and Figure 2-4) further decreased the limits of detection for all species. The new sensitive and specific method allows for robust quantification of all N\(^6\)-methyl-, -acetyl- and -formyl-lysine modifications, with limits of detection of 1 fmol for N\(^6\)-formyl- and N\(^6\)-acetyllysine, 10 fmol for lysine, and 50 fmol for each of N\(^6\)-mono-, di-, and tri-methyl lysine.

In addition to providing insights into N\(^6\)-formyllysine content of proteins, there are numerous applications of our quantification method for characterizing the chemical biology of this protein modification, as presented in the subsequent chapters. Here, we provided two examples of its immediate application. The first example was quantification of the background levels of lysine adducts in a sample of total histones extracted from TK6 cells. As shown in Table 2-2, N\(^6\)-formyllysine adducts occur at a low frequency (e.g., 1 per \(10^4\) lysines) in total histones compared to higher frequencies of acetylation and methylation (40 – 230 per \(10^4\) lysines). The next chapter covers the distribution of N\(^6\)-formyllysine adducts in different classes of histones separated by HPLC. Our quantification data is consistent with published studies of relative quantities
of histone modifications using immunologic and radiolabeling techniques [5-8]. Moreover, using our sensitive methods, we report ~1 lysine formylation per $10^4$ lysines in total histones extracted from TK6 cells compared to 4-10 modification per $10^4$ previously reported [1].

The other application of the quantification method presented here was to determine the rate of dilution of [$^2$H$_8$]-N$^6$-formyllysine in TK6 cells. Data showed that the dilution rate of 15 h for [$^2$H$_8$]-N$^6$-formyllysine closely matches the cell doubling time of 16 h (Figure 2-10), suggesting that N$^6$-formyllysine is either a stable modification of histones or is formed and removed at the same frequency, resulting in no overall change in its steady state levels. The observation that cell replication did not result in an apparent repair or removal of N$^6$-formyllysine adducts in histone proteins is consistent with a previous study of conservation of histone carcinogen adducts during replication [9].

Our robust and sensitive analytical method could readily be extended for quantification of other amino acid modifications in proteins, with the advantage of using a highly efficient yet mild proteolytic digestion as opposed to the traditionally used strong hydrochloric acid for protein hydrolysis that could remove the adduct of interest. The absolute quantification of a specific protein modification, in addition to mapping studies, could provide insights on various biological pathways in cells. For instance, mapping studies of N$^6$-formyllysine sites reveal that this adduct occurs on conserved lysines involved in epigenetic regulation [5], and through absolute quantification, we showed that this modification is rather stable or is formed and removed at the same frequency, resulting in no overall change in its steady state levels. Furthermore, this
method is used in subsequent chapters to quantify various lysine modifications in individual classes of histones. Histone modification-based signaling involves the location and number of specific modification targets within a histone protein as well as the frequency of modification of a target among all copies of a particular histone protein. Our quantification data provide some insight into this issue. Further, N^6-formyllysine quantification is used to prove possible mechanisms for its formation and shed light on its fate.
References


Chapter 3

Quantitative analysis of histone modifications:
Formaldehyde is a source of pathological 
$N^6$-formyllysine that is refractory to histone deacetylases
Abstract

Aberrant protein modifications play an important role in the pathophysiology of many human diseases, in terms of both dysfunction of physiological modifications and the formation of pathological modifications by reaction of proteins with endogenous electrophiles. Recent studies have identified a chemical homolog of lysine acetylation, N^6^-formyllysine, as an abundant modification of histone and chromatin proteins, one possible source of which is the reaction of lysine with 3'-formylphosphate residues from DNA oxidation. Using a new liquid chromatography-coupled to tandem mass spectrometry method to quantify all N^6^-methyl-, -acetyl- and -formyl-lysine modifications, we now report that endogenous formaldehyde is a major source of N^6^-formyllysine and that this adduct is widespread among cellular proteins in all compartments. N^6^-Formyllysine was evenly distributed among different classes of histone proteins from human TK6 cells at 1-4 modifications per 10^4 lysines, which contrasted strongly with lysine acetylation and mono-, di- and tri-methylation levels of 1.5-380, 5-870, 0-1400 and 0-390 per 10^4 lysines, respectively. While isotope labeling studies revealed that lysine demethylation is not a source of N^6^-formyllysine in histones, formaldehyde exposure was observed to cause a dose-dependent increase in N^6^-formyllysine, with use of [^{13}C,^{2}H_2]-formaldehyde revealing unchanged levels of adducts derived from endogenous sources. Inhibitors of class I and class II histone deacetylases did not affect the levels of N^6^-formyllysine in TK6 cells and the class III histone deacetylase, SIRT1, had minimal activity (<10%) with a peptide substrate containing the formyl adduct. These data suggest that N^6^-formyllysine is refractory to removal by histone deacetylases, which supports the idea that this abundant protein modification could interfere with normal regulation of gene expression if it arises at conserved sites of physiological protein secondary modification.
Chapter 3: Sources and fate of N⁶-formyllysine adducts in proteins

Introduction

In addition to physiological secondary modifications, proteins are subjected to reactions with endogenous electrophiles generated by oxidative stress, inflammation, and normal cell metabolic processes [1-5]. These adventitious or pathological modifications typically arise by reaction of the nucleophilic side chains of lysine, histidine, and cysteine with reactive electrophiles such as malondialdehyde, 4-hydroxynonenal (HNE), and glyoxal generated by oxidation of polyunsaturated fatty acids and carbohydrates, among other biomolecules [2-4,6,7]. The resulting adducts, which can alter protein function and lead to protein degradation, are associated with a variety of pathological processes and human diseases [1-5,8]. Among these pathological adducts, N⁶-formylation of lysine has recently emerged as an abundant protein modification [5,9-11]. While originally described in chromatin proteins [9-11], it has since been identified as an adduct arising in proteins subjected to nitrosative and oxidative stresses [5]. In chromatin proteins, N⁶-formyllysine has the potential to interfere with the functions of other post-translational modifications that perform signaling functions [12-15], such as acetylation, methylation, phosphorylation, ubiquitylation, and ADP ribosylation, with some locations modified in more than one way (e.g., refs. [16-18]). The chemical similarities of N⁶-formyllysine and N⁶-acetyllysine suggest a disruptive role for the former in signaling by histone acetylation. Indeed, N⁶-formyllysine has been detected at conserved sites of lysine acetylation and methylation in histones [10,11].

While N⁶-formyllysine adducts are now well recognized as abundant protein modifications in cells, the source of these pathological adducts remains unclear. We recently showed that some portion of N⁶-formyllysine arises in chromatin proteins by
reaction of lysine side chains with the 3'-formylphosphate residue derived from 5'-oxidation of 2-deoxyribose in DNA in cells (Figure 3-1) [9]. However, the observation of this adduct in proteins treated with the biological oxidant, peroxynitrite, suggests other sources for the formyl species [5]. Considering that formaldehyde reacts with amines to give a carbinolamine (N6-hydroxymethyllysine) intermediate that is only one oxidation state away from a formamide functional group (Figure 3-1), we hypothesized that endogenous formaldehyde could serve as a source of N6-formyllysine residues in histone and other proteins. In addition to environmental and occupational sources [19-21], formaldehyde arises from cellular processes such as oxidative demethylation of nucleic acid and histone proteins, as well as biosynthesis of purines, thymidine, and some amino acids [20,22,23], making it a relatively abundant metabolite at concentrations ranging from 13 to 97 μM in human plasma [20]. To test this hypothesis and to clarify the cellular locations and quantities of N6-formyllysine relative to other major histone modifications, we developed a novel liquid chromatography-coupled to electrospray tandem mass spectrometry (LC-MS/MS) method to quantify all N6-methyl-, -acetyl-, and -formyl-lysine modifications. Application of this method reveals that endogenous formaldehyde is a major source of N6-formyllysine, that this adduct is widespread among proteins in all cellular compartments, and that, in chromatin proteins, it is refractory to removal by histone deacetylases.
Figure 3-1. Sources of N^6-formyllysine. The adduct can be generated in chromatin proteins from reaction of lysine with 3'-formylphosphate residue derived from 5'-oxidation of 2-deoxyribose in DNA or from reaction of lysine with endogenous or exogenous formaldehyde. Formaldehyde reacts with amines to give a carbinolamine intermediate (N^6-(hydroxymethyl)-lysine) that is in equilibrium with a Schiff base and that is one oxidation state away from the formamide functional group of N^6-formyllysine.

Materials and Methods

Materials. Unlabeled and [^{13}C,^{2}H_2]-labeled formaldehyde were purchased as 37% and 20% aqueous solutions from Amresco (Solon, OH) and Isotec (Miamisburg, OH), respectively. 4,4,5,5-[^{2}H]-Lysine was purchased from Cambridge Isotope Laboratories
(Andover, MA). 4,4,5,5-[²H]-N⁶-Formyllysine was synthesized from 4,4,5,5-[²H]-lysine according to Jiang et al. [9]. 3,3,4,4,5,5,6,6-[²H]-N⁶-acetyllysine were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). L-Methionine-([¹³C₂,²H₃]-methyl) was obtained from Isotec (Miamisburg, OH). Lysine, N⁶-formyllysine, N⁶-acetyllysine, bovine serum albumin, human recombinant HMG-1, human IgG, Streptomyces griseus protease, and protease inhibitor cocktail (for use with mammalian cell and tissue extracts) were obtained from Sigma-Aldrich (St. Louis, MO). N⁶-Mono-methyllysine, N⁶-di-methyllysine, and N⁶-tri-methyllysine were purchased from Bachem Bioscience Inc. (King of Prussia, PA). Nonidet P-40 was from Roche Diagnostic Corporation (Indianapolis, IN). Suberoylanilidehydroxamic acid (SAHA) and SIRT1 (human recombinant) enzyme were purchased from Cayman chemical (Ann Arbor, MI). Peptide substrates for SIRT1 (GGAKRHR and its lysine-acetylated and -formylated forms) were synthesized at Massachusetts Institute of Technology Biopolymers Laboratory. The human lymphoblastoid TK6 cell line was a generous gift of Prof. Gerald Wogan (Massachusetts Institute of Technology).

**TK6 cell culture, exposure, and labeling.** TK6 cells were cultured in RPMI 1640 medium (Cellgro, Manassas, VA) supplemented with 10% heat-inactivated horse serum (Atlanta Biologicals, Lawrenceville, GA), 10,000 U penicillin/ml and 10,000 μg streptomycin/ml (Lonza, Walkersville, MD), and 2 mM L-glutamine (Lonza, Walkersville, MD) at 37 °C in a 5% CO₂ atmosphere. For formaldehyde exposure studies, TK6 cells were pelleted, washed, and resuspended in RPMI 1640 medium without any supplements, prior to addition of formaldehyde to the medium. Following
addition of formaldehyde, cells were incubated at 37 °C for 2 h with occasional mixing prior to extracting chromatin proteins. Histones were extracted from ~10^7 cells after exposure and the quantity of formyllysine, as a percentage of total lysine, was measured as described below. For lysine demethylation studies, TK6 cells were grown in a customized RPMI-1640 medium identical to the traditional medium (e.g., supplemented with horse serum, antibiotics, and L-glutamine), except for the presence of labeled methionine (L-methionine-([13C,2H₃]-methyl)) instead of non-labeled methionine. Histones (from ~10^7 cells) were extracted every 2 d for 20 d in order to investigate the formation of N⁶-[13C₂,2H]-formyllysine. For histone deacetylase studies, TK6 cells were incubated with the histone deacetylase inhibitor, SAHA, for 20 h at 37 °C in a 5% CO₂ atmosphere prior to histone extraction. SAHA was dissolved in a 50:50 solution of DMSO:PBS prior to addition to cell media. Control cells (~10^7) were treated with the DMSO:PBS vehicle.

**Histone extraction from TK6 cells and subcellular protein fractionation from tissue.**

For detailed procedure regarding histone extraction, refer to *Materials and Methods* section of Chapter 2. For collecting membrane, cytosolic, and nuclear fractions from tissues, 20 mg of bovine liver tissue was cut into small pieces and washed with PBS, and proteins were fractionated using the Subcellular Protein Fractionation Kit from Thermo Scientific (Waltham, MA) and a Kontes all-glass Dounce homogenizer (10 strokes with a type B pestle). Proteins in subcellular extracts were precipitated by addition of 20% v/v trichloroacetic acid and overnight incubation at 4 °C. Samples were then centrifuged at 14000 x g for 10 min at 4 °C, washed once with ice-cold acetone containing 0.1% HCl,
and once with ice-cold acetone. The extracts were air-dried and stored at -20 °C until use.

**Purification of individual histones.** HPLC purification of total histones was performed according to Boyne *et al.* [24] with modifications. For detailed procedure of histone purification into individual amino acids, refer to *Materials and Methods* section of Chapter 2.

**Enzymatic hydrolysis of proteins.** Proteins were hydrolyzed to individual amino acids using *S. griseus* protease (freshly prepared solution each time) with incubation at 37 °C for ≥ 16 h. For detailed procedure regarding protein digestion into individual amino acids, refer to *Materials and Methods* section of Chapter 2.

**Quantification of amino acids.** N⁶-Formyllysine and other amino acids of interest were quantified as a percentage of the total quantity of lysine, by liquid chromatography-coupled mass spectrometry (LC-MS/MS). For detailed procedure regarding LC-MS/MS based quantification of individual amino acids, refer to *Materials and Methods* section of Chapter 2.

**SIRT1 peptide experiment.** SIRT1 peptide substrates (GGAKRHR, GGAK_{acetyl}RHR, and GGAK_{formyl}RHR) were HPLC purified on an Agilent 1100 series instrument using Vydac 218TP52 C18 reverse-phase column (2.1 x 250 mm, 5 μm) from Grace Vydac (Hesperia, CA). The mobile phase flow rate was 200 μL/min, and the column
temperature was maintained at 30 °C. The solvent system was 0.05% trifluoroacetic acid in water (A) and 0.05% trifluoroacetic acid in 80% acetonitrile (B), with the isocratic elution of 5% B for the first 5 min, then a linear increase to 42% B over 25 min, reaching 100% B at 31 min followed by column re-equilibration at 5% B for 10 min. Each purified SIRT1 peptide substrate (100 pmol) was incubated overnight (12 h) at 25 °C with 1 μg SIRT1, in 50 mM Tris-HCl (pH 8) buffer containing 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, and 6 mM NAD⁺. The removal of acetyl and formyl groups from SIRT1 peptide substrates was determined using liquid chromatography-coupled mass spectrometry. HPLC was performed on an Agilent 1100 series instrument using Agilent Exclipse XDB C18 reverse-phase column (4.6 x 150 mm, 5 μm). The mobile phase flow rate was 200 μL/min, and the column temperature was maintained at 40 °C. The solvent system was 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B), with the elution starting at 20% B, the gradient linearly increased to 50% B over 5 min, reached 100% B at 6 min, and kept at 100% B for 9 minutes before the column was re-equilibrated at 20% B for 10 min. The species of interest were then analyzed using the Agilent 6410 MS/MS system equipped with an electrospray ionization (ESI) source operated in positive ion mode. The operating parameters were as follows: ESI capillary voltage, 3500 V; gas temperature, 345 °C; drying gas flow, 8 L/min; and nebulizer pressure, 30 psi. Multiple reaction monitoring (MRM) transitions were as follow: GGAKRHR peptide, m/z 781.1 → 625.2; GGAK₉(formyl)RHR peptide, m/z 809.4 → 516.3; and GGAK₉(acetylated)RHR peptide, m/z 823.4 → 530.4. The fragmentor voltage and collision energy were 200 V and 40 V for GGAKRHR peptide, respectively; 100 V and 46 V for GGAK₉(formyl)RHR peptide; and 100 V and 52 V for GGAK₉(acetylated)RHR peptide.
Chapter 3: Sources and fate of N\textsuperscript{6}-formyllysine adducts in proteins

Results

Quantification of N\textsuperscript{6}-lysine modifications in proteins. In order to quantify N\textsuperscript{6}-formyllysine adducts, proteins are first subjected to proteolytic digestion to generate individual amino acids that are then analyzed using our improved LC-MS/MS based method, as outlined in detail in Chapter 2. To validate the new analytical method for lysine modifications, we compared the frequency of N\textsuperscript{6}-formyllysine among different classes of histone proteins extracted from TK6 cells and resolved by reversed-phase HPLC. As shown in Figure 3-S1A, all of the major histone classes were separated, with further resolution of variant forms of histones H1 and H3 (SDS-PAGE verification in Figure 3-S1B), which is consistent with previous observations in cultured human cells [10]. N\textsuperscript{6}-Formyllysine was detected in all histone classes at a frequency of 1-4 modifications per 10\textsuperscript{4} lysines. This 3- to 4-fold variation among histone classes stands in contrast to the 10- to 100-fold variation in the frequency of other modifications (Table 3-1). The data in Table 3-1 represent the first absolute quantification of the various lysine acetyl and methyl modifications in histone proteins, and are consistent with published studies of relative quantities of histone modifications using immunologic and radiolabeling techniques [10,25-27]. Histone modification-based signaling involves the location and number of specific modification targets within a histone protein, as well as the frequency of modification of a target among all copies of a particular histone protein. Our data provide some insight into this issue. For example, we observed low-level acetylation and methylation in histone H1, which is consistent with studies using radiolabeled acetate [25], while this low level of modification maps to specific sites in the globular domain and N-terminal tail of histone H1 [10]. This low-level of acetylation
and methylation in histone H1 stands in contrast to the high level of acetylation of H2, H3 and H4 (Table 3-1), which is again supported by studies using radiolabeled acetate [25].

<table>
<thead>
<tr>
<th></th>
<th>Formyl $^2$</th>
<th>Acetyl</th>
<th>Methyl</th>
<th>Dimethyl</th>
<th>Trimethyl</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1 (1)</td>
<td>1.5 ± 0.3$^3$</td>
<td>1.6 ± 0.2</td>
<td>8.0 ± 2.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>H1 (2)</td>
<td>1.2 ± 0.2</td>
<td>1.5 ± 0.3</td>
<td>7.0 ± 2.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>H2A</td>
<td>2.7 ± 0.2</td>
<td>26 ± 20</td>
<td>20 ± 8</td>
<td>60 ± 30</td>
<td>ND</td>
</tr>
<tr>
<td>H2B</td>
<td>1.7 ± 0.7</td>
<td>66 ± 20</td>
<td>5.0 ± 7.0</td>
<td>ND</td>
<td>85 ± 30</td>
</tr>
<tr>
<td>H3(1)</td>
<td>3.9 ± 0.5</td>
<td>280 ± 130</td>
<td>680 ± 80</td>
<td>1100 ± 220</td>
<td>220 ± 60</td>
</tr>
<tr>
<td>H3(2)</td>
<td>3.6 ± 1.3</td>
<td>380 ± 100</td>
<td>870 ± 30</td>
<td>1400 ± 270</td>
<td>390 ± 140</td>
</tr>
<tr>
<td>H4</td>
<td>2.6 ± 0.4</td>
<td>73 ± 60</td>
<td>260 ± 80</td>
<td>740 ± 40</td>
<td>3.0 ± 6.0</td>
</tr>
</tbody>
</table>

$^1$Classes of histone proteins resolved by reversed-phase HPLC, with putative isoforms denoted in parentheses.

$^2$Column titles denote different N$^6$-modifications of lysine.

$^3$Data are expressed as modifications per 10$^4$ total lysines and represent mean ± SD for 3 biological replicates.
The new analytical method was next applied to quantify N^6-formyllysine in non-histone proteins. We had previously observed N^6-formyllysine mainly in histone proteins [9], perhaps as a result of biased proteolysis or subsequent steps in the technique. However, using the new method, we are now able to detect N^6-formyllysine modifications in a variety of different proteins, as shown in Table 3-2. Further, an analysis of proteins in nuclear, cytosolic, and membrane compartments in bovine liver revealed the presence of N^6-formyllysine in all three locations (Table 3-2). These observations are consistent with a source for N^6-formyllysine other than the 3'-formylphosphate residues of DNA oxidation previously identified for histone proteins [9].

<table>
<thead>
<tr>
<th>Identity of Protein</th>
<th>N^6-Formyllysine per 10^4 Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>5 ± 0.5</td>
</tr>
<tr>
<td>IgG</td>
<td>2 ± 0.4</td>
</tr>
<tr>
<td>Collagen</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>HMG-1</td>
<td>&lt; 0.2 ± 0.04</td>
</tr>
<tr>
<td>Bovine liver nuclear proteins</td>
<td>2 ± 0.6</td>
</tr>
<tr>
<td>Bovine liver membrane proteins</td>
<td>4 ± 1.0</td>
</tr>
<tr>
<td>Bovine liver cytosolic proteins</td>
<td>4 ± 0.6</td>
</tr>
</tbody>
</table>
Formaldehyde as a source of N⁶-formyllysine. One alternative to 3'-formylphosphate residues as a source of N⁶-formyllysine is oxidation of the carbinolamine (N⁶-hydroxymethyllysine) intermediate in the reaction of formaldehyde with side chain amine of lysine (Figure 3-1). To test this hypothesis, we performed a series of experiments, starting with an *in vitro* reaction of L-lysine with different concentrations of formaldehyde and quantification of N⁶-formyllysine. As shown in Figure 3-2A, there was a concentration-dependent formation of N⁶-formyllysine in reactions with formaldehyde, presumably as a result of oxidation of the carbinolamine adduct by the background of reactive oxygen species generated by trace metals and dissolved oxygen in the solution. This dose-response relationship for N⁶-formyllysine formation was also observed in histone proteins extracted from TK6 cells exposed to formaldehyde for 2 h at 37 °C (Figure 3-2B), with 10 mM formaldehyde producing roughly the same frequency.

![Figure 3-2.](image)

**Figure 3-2.** Formaldehyde is a source of N⁶-formyllysine. Formation of N⁶-formyllysine in (A) *in vitro* reactions of 1 mM L-lysine with formaldehyde for 2 h at 37 °C, and in (B) TK6 cells exposed to formaldehyde, as described in *Materials and Methods*. Data represent mean ± SD for N=3, with asterisks denoting statistically significant differences by Students t-test (p < 0.05).
of N⁶-formyllysine in both in vitro and cellular studies (i.e., 6 per 10⁴ lysine versus 3 per 10⁴ lysine, respectively).

The relatively high endogenous levels of N⁶-formyllysine in histone and other proteins (Tables 3-1 and 3-2) raised the question of the contribution of exogenous formaldehyde exposures to the total load of N⁶-formyllysine in the cells. To address this issue, we exposed TK6 cells to [¹³C₂H₂]-labeled formaldehyde, which led to the formation of N⁶-[¹³C₂H₂]-formyllysine that is 2 mass units heavier than the endogenous adducts (Figure 3-3A). Following extraction of the histone proteins from formaldehyde-treated TK6 cells (2 h, 37 °C), both endogenous and exogenous N⁶-formyllysine were quantified by monitoring the transitions m/z 175 → 112 and m/z 177 → 114, respectively (Figure 3-3A), with a third transition (m/z 179 → 116) for the 4,4,5,5-[²H]-N⁶-formyllysine internal standard. As shown in Figure 3-3B, levels of endogenous (unlabeled) N⁶-formyllysine remained constant at all concentrations of [¹³C₂H₂]-formaldehyde, while N⁶-[¹³C₂H₂]-formyllysine increased as a function of the concentration of labeled formaldehyde.

**Lysine demethylation as a source of N⁶-formyllysine.** The enzymatic demethylation of N⁶-methyllysine modifications represents another possible source of N⁶-formyllysine in histone proteins, given both the carbinolamine intermediate known to form during the process of lysine demethylation and the ultimate release of the methyl group as formaldehyde [23]. Adventitious oxidation of the carbinolamine intermediate or secondary reaction of the released formaldehyde could result in the formation of N⁶-formyllysine locally. To test these hypotheses, TK6 cells were grown in customized
RPIMI medium containing L-methionine with a $^{13}$C,$^2$H$_3$-methyl group for 20 days to label all methyl groups in N$^6$-methyllysine species, and histone proteins were extracted for analysis every 2 days. If N$^6$-formyllysine is a product of disrupted lysine

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**Figure 3-3.** Addition of $^{13}$C,$^2$H$_2$-formaldehyde to TK6 cells distinguishes exogenous from endogenous sources of N$^6$-formyllysine. (A) LC-MS/MS analysis showing signals for the three isotopomeric N$^6$-formyllysine species, as described in Materials and Methods. (B) Plot of N$^6$-formyllysine levels as a function of exposure to $^{13}$C,$^2$H$_2$-formaldehyde. Data represent mean ± SD for N=3.
demethylation in histones and is formed via oxidation of the carbinolamine intermediate known to form during the process of lysine demethylation [23], or by reaction of lysine with the formaldehyde released at the last step of successful lysine demethylation [23], then one would expect to see an increase of 2 mass units corresponding to formation of N6-[13C,2H]-formyllysine (m/z 177 → 114 transition). In order to increase the signal-to-noise ratio for N6-[13C,2H]-formyllysine, N6-formyllysine was HPLC-pre-purified in all samples before LC-MS/MS analysis. Figure 3-4 depicts an example of the analysis using the day 6 sample. As shown in Figure 3-4, N6-mono-methyllysine and N6-di-methyllysine are predominately labeled (>90%) with heavy isotope methyl groups (i.e., [13C,2H3]-methyl). In contrast to methyllysines, the level of N6-[13C,2H]-formyllysine did not increase beyond the natural isotope abundance level of ~0.7% for the [M+2] ion of N6-formyllysine (Figure 3-4C and Figure 3-S2). Note that the HPLC gradient was changed here to fully resolve a contaminant signal from the TK6 cells (identified as the [M+1] ion of citrulline) that otherwise co-eluted with N6-formyllysine and produced an m/z value similar to the [M+2] isotopomer of N6-formyllysine.
Figure 3-4. Analysis of lysine demethylation as a source of N^6-formyllysine. Methyl groups in N^6-methyllysine species in TK6 cells were labeled using L-methionine-([^{13}C,^{2}H_3]-methyl) and N^6-formyllysine and N^6-methyllysine species were quantified by LC-MS/MS as described in Materials and Methods. Panels A and B: N^6-mono-methyllysine and N^6-di-methyllysine are predominately labeled (>90%) with heavy isotope methyl groups (mass increase of 4 m/z and 8 m/z, respectively), with <10% of the modifications containing unlabeled methyl groups. Panel C: the level of N^6-[^{13}C,^{2}H]-formyllysine (m/z 177 → 114 transition) in histones did not show an increase beyond the natural isotope abundance level of ~0.7% for [M+2] ion of N^6-formyllysine.
Persistence of N$^6$-formyllysine in cells. The chemical similarity of N$^6$-formyllysine and N$^6$-acetyllysine suggested that the former might be subject to removal by lysine deacetylases that recognize and remove N$^6$-acetyllysine from histone and other proteins [16,28-31]. Lysine deacetylases fall into several classes, including classes I and II that share a common hydrolytic mechanism and are all inhibited by suberoylanilidehydroxamic acid (SAHA), and the class III enzymes (i.e., sirtuins) that are NAD$^+$-dependent deacetylases [32,33]. In order to assess the activity of lysine deacetylases with N$^6$-formyllysine substrates, TK6 cells were treated with SAHA and the levels of N$^6$-acetyllysine and N$^6$-formyllysine were quantified. The results shown in Figure 3-5A reveal that, while SAHA caused a 3-fold increase in the level of N$^6$-acetyllysine (4 to 14 per $10^3$ lysines), lysine formylation was not affected. To assess sirtuin activity against N$^6$-formyllysine, we performed in vitro reactions of SIRT1 with a consensus peptide (GGAKRHR) containing N$^6$-formyllysine or N$^6$-acetyllysine, and the quantities of the modified and unmodified peptides were analyzed by LC-MS/MS. As shown in Figure 3-5B, SIRT1 removed the acetyl modification completely to generate the unmodified peptide, while only $\sim$10% (± 4%) of N$^6$-formyllysine was removed.
Chapter 3: Sources and fate of $N^6$-formyllysine adducts in proteins

Figure 3-5. Effect of lysine deacetylases on $N^6$-formyllysine. (A) TK6 cells were treated with the class I and class II histone deacetylase inhibitor, SAHA, as described in Materials and Methods. Data represent mean ± SD for N=3, with asterisks denoting statistically significant differences by Students t-test (p < 0.05). (B) Treatment of a peptide substrate containing $N^6$-formyllysine with the class III histone deacetylase, SIRT1.
Discussion

N\textsuperscript{6}-Formyllysine was first reported in 1985 in reactions of lysine with formaldehyde \textit{in vitro} \cite{34} and, more recently, it was shown to form during \textit{in vitro} silver-staining procedures that involve the use of formaldehyde \cite{35}. The recent discovery of N\textsuperscript{6}-formyllysine as a relatively abundant endogenous posttranslational modification of histones and other nuclear proteins in cells \cite{9-11} has raised questions about its mechanism of formation and its potential for interfering with the regulatory function of lysine N\textsuperscript{6}-acetylation. With respect to formation, we previously presented evidence that N\textsuperscript{6}-formyllysine in histones could arise from reactions with 3'-formylphosphate residues derived from DNA oxidation \cite{9}. However, formaldehyde emerged as an alternative source given the high reactivity of formaldehyde toward primary amines, such as the side chain of lysine, and the potential for endogenous oxidation to convert a formaldehyde-derived carbinolamine to a stable formamide (Figure 3-1). We have now demonstrated \textit{in vitro} and in cells that formaldehyde exposure leads to formation of N\textsuperscript{6}-formyllysine residues in proteins. The fact that this modification arises in proteins other than chromatin proteins and in cellular compartments other than the nucleus (Table 3-2) suggests that 3'-formylphosphate residues in oxidized DNA do not account for all N\textsuperscript{6}-formyllysine adducts. This is consistent with recent studies in which N\textsuperscript{6}-formyllysine was detected in a protein oxidized with peroxynitrite \textit{in vitro} \cite{5}. The absence of detectable N\textsuperscript{6}-formyllysine arising from demethylation of N\textsuperscript{6}-methyllysine species (Figure 3-4C and Figure 3-S2) suggests that interruption of histone demethylation reactions to form the carbinolamine precursor of N\textsuperscript{6}-formyllysine occurs at low frequency, or that the formaldehyde produced by complete lysine demethylation \cite{23}
does not occur at concentrations high enough to drive formylation of lysine or cause substantial changes in N^6-formyllysine levels detected by our current analytical method. Furthermore, there is the possibility that the formaldehyde released during lysine demethylation may be scavenged before it could react with lysines in histones. A recent study reports that lysine-specific demethylase 1 (LSD1) is a folate binding protein [36], which led the authors to hypothesize that the biological function of folate is to trap the formaldehyde that is generated during lysine demethylation [36]. In addition, the observation that the formaldehyde equivalent derived from histone demethylation might not account for a significant portion of formyllysine residues is not surprising in light of the abundance of formaldehyde from other cellular processes [20,22,23]. This is clear from the high steady-state concentrations of formaldehyde in human plasma (13-97 μM) [20].

The relative abundance of N^6-formyllysine in histone and other proteins (Tables 3-1 and 3-2) [9-11] and the persistence of these adducts in histone proteins provides insights into both their source and their potential effects on cell function. The N^6-formyllysine residues are relatively evenly distributed among different classes of histone proteins (Table 3-1), while the other functional modifications show very biased distributions over a large frequency range, which is consistent with the known function and conserved locations for lysine methylation and acetylation [16-18]. This random distribution of formyllysine adducts in histone proteins suggests that they are adventitious and not physiological. The fact that N^6-formyllysine levels are similar in histone and non-nuclear proteins and in all cell compartments also suggests that the sources of this protein modification are equally balanced in the various compartments and proteins, or
that there is a single dominant source that distributes uniformly throughout the cell. With regard to their persistence in cells, the \( N^6 \)-formyllysine adducts appear to be refractory to removal by histone deacetylase enzymes, which suggests that they will persist throughout the life of individual histone proteins. Figure 3-6 summarizes our findings presented here on \( N^6 \)-formyllysine adducts.

The isotope labeling studies revealed that lysine demethylation is not a source for \( N^6 \)-formyllysine in histones. Furthermore, our data suggest that \( N^6 \)-formyllysine is refractory to removal by histone deacetylases, which is consistent with the persistence of this pathological adduct throughout the life of individual histone proteins. That the \( N^6 \)-formyllysine adducts have been observed at many of the known conserved functional locations for lysine acetylation and methylation in histones [10,11] suggests that \( N^6 \)-formyllysine could interfere with signaling processes associated with physiological histone modifications [16,28]. The association of \( N^6 \)-formyllysine with a variety of different cell and organismal processes, including metabolism, and the oxidative and nitrosative stresses of inflammation [5,9], suggest that this adduct may play a role in many pathophysiological processes in humans.
Figure 3-6. Summary of findings on N⁶-formyllysine in histones. N⁶-formyllysine can arise from reaction of lysine with the 3'-formyl phosphate residue derived from 5'-oxidation of 2-deoxyribose in DNA or from reaction of lysine with formaldehyde. The isotope labeling studies revealed that lysine demethylation is not a source for N⁶-formyllysine in histones. Furthermore, our data suggest that N⁶-formyllysine is refractory to removal by histone deacetylases, which is consistent with the persistence of this pathological adduct throughout the life of individual histone proteins.
References


Supplementary Figures
Figure 3-S1. Reversed-phase HPLC fractionation of histone proteins. (A) HPLC elution profile for histones extracted from TK6 cells, as described in Materials and Methods section of Chapter 2. (B) SDS/PAGE analysis of the HPLC-fractionated proteins shown in panel A. Lanes 1 and 2 are molecular weight markers, while lanes 3 and 4 refer to total histones from TK6 cells and calf thymus, respectively.
Figure 3-S2. Lysine demethylation is not a source of N⁶-formyllysine in histones. By culturing TK6 cells in customized RPMI medium containing L-Methionine-([¹³C,²H₃]-methyl) for 20 days, it was shown that in contrast to predominant heavy isotope labeling of mono-methyllysines (>90%), even as early as day 2, the level of N⁶-[¹³C,²H]-formyllysine did not show an increase beyond the natural isotope abundance level (~0.7% for [M+2] ion of N⁶-formyllysine), for any day.
Chapter 4

Formaldehyde-induced formation of $N^6$-formyllysine protein adducts in rats
Abstract

The emergence of lysine N⁶-formylation as an abundant endogenous modification of proteins has raised questions about its mechanism of formation. In addition to DNA oxidation pathway previously reported (Jiang et al., PNAS 104: 60-5, 2007), we demonstrated in vitro and in cells that formaldehyde exposure leads to formation of N⁶-formyllysine residues in histone proteins (Chapter 3). Here, we extend our studies to quantify N⁶-formyllysine protein adducts in rats exposed to isotope-labeled formaldehyde by inhalation in order to calculate the contribution of exogenous versus endogenous sources of formaldehyde to the total load of N⁶-formyllysine in cells. Moreover, our studies complement previous studies of formaldehyde-induced N²-hydroxymethyl-dG DNA adducts in exposed rats (Lu et al., Chem. Res. Toxicol. 24: 159-61, 2011), where we aim to correlate the protein and DNA adducts given the carcinogenic potency of formaldehyde. Using our ultrasensitive and specific liquid chromatography-coupled tandem mass spectrometry method, we quantified endogenous and exogenous N⁶-formyllysine protein adducts in total and fractionated proteins (cytoplasmic, membrane, and nuclear) extracted from nasal epithelium, lung, and liver tissues of rats exposed to single-dose, nose-only inhalation of 0.7, 2, 6, and 10 ppm [¹³C²H₂]-formaldehyde for 6 h. Similar to the DNA study, exogenous N⁶-formyllysine adducts were only detectable in nasal epithelium and not in distant tissues, with a dose-dependent formation in total as well as in fractionated proteins. At 10 ppm exposure, nearly one-third of the total load of N⁶-formyllysine was derived from exogenous formaldehyde (0.9 ± 0.1 exogenous versus 2 ± 0.1 endogenous N⁶-formyllysine adducts per 10⁴ lysines in total proteins). Moreover, there was a general decrease in exogenous adducts observed in nuclear proteins compared to proteins obtained from other compartments, consistent with expected decrease of inhaled formaldehyde concentration as it reacts with other biomolecules on its way to nucleus. In histone proteins, the exogenous to endogenous adduct ratios for N⁶-formyllysine are observed to occur about one third of ratios observed for DNA adduct, although they follow a similar pattern of increase in response to formaldehyde dosage (e.g., an increase in formaldehyde dosage from 6 ppm to 10 ppm, resulted in ~3-fold increase in exogenous versus endogenous ratios for both protein and DNA adducts). Our formaldehyde studies shed light on yet another potential pathway associated with formaldehyde toxicity and carcinogenicity, that is through epigenetic disruption of histone proteins in cells by permanently formylating conserved sites of important physiological modifications.
**Introduction**

Aberrant protein modifications due to oxidants and electrophiles generated during physiological or pathophysiological processes can lead to altered cellular processes and progression of disease[1-5]. N\(^6\)-Formylation of lysines has recently emerged as an abundant adventitious protein modification [6-9], raising questions about its mechanism of formation and biological consequences in cells. In histone proteins, the chemical similarities of N\(^6\)-formyllysine to N\(^6\)-acetyllysine suggest a disruptive role for the former in signaling by histone acetylation, and the occurrence at many conserved lysine acetylation and methylation sites along histone proteins [7] suggests possible interference with the regulatory roles of post-translational modifications of these proteins. Studies to date point to formaldehyde exposure as a major source of this adduct (Chapter 3) [10,11] in addition to oxidative and nitrosative stresses of inflammation [6,9].

Formaldehyde exposure from environmental and occupational sources[12-14] as well as endogenous cellular processes [13,15,16] make it an abundant electrophile in the human body, as indicated by its concentration range of 13 to 97 μM in plasma [13,17]. Formaldehyde, classified as a known human carcinogen according to IARC [18,19], is reported to cause squamous cell carcinoma in rats [20,21] with sufficient epidemiological evidence for causing nasopharyngeal cancer but limited evidence for leukemia in humans [13,18,19,22].

As a reactive aldehyde, formaldehyde readily forms adducts with nucleophilic sites in DNA and proteins such as N\(^2\)-hydroxymethyl-dG DNA adducts [12] and Schiff bases on the side chain amine of lysines in proteins [23], in addition to extensive DNA-protein and DNA-DNA crosslinks [24]. Our recent studies revealed N\(^6\)-formyllysine
formation in histone proteins of TK6 cells from the reaction of formaldehyde with the ε-amino group of lysine (Chapter 3). Our data showed a dose-dependent formation of N⁶-formyllysine residues as a function of formaldehyde concentration, with use of [\(^{13}\text{C},^{2}\text{H}_2\)]-formaldehyde revealing unchanged levels of N⁶-formyllysinespecies due to endogenous sources.

Here, we extend our cell studies to an in vivo assessment of formaldehyde-induced lysine N⁶-formylation in rats exposed by inhalation, with the use of [\(^{13}\text{C},^{2}\text{H}_2\)]-formaldehyde to differentiate endogenous from exogenous adducts. Using our ultrasensitive and specific liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) method, we quantified the extent to which exposure to [\(^{13}\text{C},^{2}\text{H}_2\)]-formaldehyde by inhalation induces N⁶-[\(^{13}\text{C},^{2}\text{H}\)]-formyllysine. Endogenous N⁶-[\(^{12}\text{C},^{1}\text{H}\)]-formyllysine and exposure-induced N⁶-[\(^{13}\text{C},^{2}\text{H}\)]-formyllysine adducts were quantified in total and fractionated (cytoplasmic, membrane, and nuclear) proteins extracted from nasal epithelium, lung, and liver tissues of rats exposed to single-dose, nose-only inhalation of 0.7, 2, 6, and 10 ppm [\(^{13}\text{C}_2\text{H}_2\)]-formaldehyde for 6 h. These studies complement previous studies of [\(^{13}\text{C},^{2}\text{H}_2\)]-formaldehyde-induced N²-hydroxymethyl-dG DNA adducts in rats from the same formaldehyde exposure studies [12], which allows us to correlate the protein and DNA adducts arising with the same formaldehyde dose.

Application of our sensitive analytical methods revealed that exposure to [\(^{13}\text{C},^{2}\text{H}_2\)]-formaldehyde vapor leads to the formation of exogenous N⁶-[\(^{13}\text{C},^{2}\text{H}\)]-formyllysine in proteins of nasal epithelium, and that the exogenous adducts are formed in proteins extracted from all cellular compartments in a dose-dependent manner, with endogenous adducts dominating at all exposure conditions. Similar to the DNA study,
the exogenous N\textsuperscript{6}-formyllysine adducts were only detected in nasal epithelium and not in distant tissues, with exogenous to endogenous adduct ratios in total proteins closely matching increases seen in DNA adducts.

**Materials and Methods**

**Materials.** Rat tissues were provided by Prof. James Swenberg’s Lab at University of North Carolina at Chapel Hill [12]. Subcellular Protein Fractionation Kit was purchased from Thermo Scientific (Waltham, MA). Lysine internal standard, 4,4,5,5,5-[\textsuperscript{2}H]-Lysine, was purchased from Cambridge Isotope Laboratories (Andover, MA). N\textsuperscript{6}-Formyllysine internal standard, 4,4,5,5-[\textsuperscript{2}H]-N\textsuperscript{6}-formyllysine, was synthesized from 4,4,5,5-[\textsuperscript{2}H]-lysine according to Jiang *et al.*[6]. *Streptomyces griseus* protease was purchased from Sigma-Aldrich (St. Louis, MO).

**Rat exposure to formaldehyde vapor.** Rat exposure to [\textsuperscript{13}C\textsubscript{2}H\textsubscript{2}]-formaldehyde was performed at the Lovelace Respiratory Research Institute, Albuquerque, NM, by our collaborator, Prof. James Swenberg, according to appropriate and approved protocols for the use of vertebrate animals in experiments [12]. Fischer rats (6 w old, male) were exposed to formaldehyde vapor by single-dose, nose-only inhalation of [\textsuperscript{13}C\textsubscript{2}H\textsubscript{2}]-formaldehyde for 6 h to produce final target exposure concentrations of 0.7, 2, 6, and 10 ppm. Rats were euthanized using an intraperitoneal barbiturate injection. For nasal mucosa collection, the skull was split with a slight bias to one side (to preserve septal mucosa) and maxilloturbinates collected, wrapped in aluminum foil, immediately snap
frozen in liquid nitrogen and held at -80 °C pending analyses. Other tissue samples were processed in a similar fashion.

**Subcellular protein fractionation from tissue.** For extracting proteins from tissues, ~10 mg of nasal epithelium, lung, or liver tissue was cut into small pieces and washed with PBS. Tissues were homogenized using a Kontes all-glass Dounce homogenizer, with 10 strokes of a type B pestle, and proteins were separated into cytoplasmic, membrane, soluble nuclear, and chromatin bound fractions using the Subcellular Protein Fractionation Kit from Thermo Scientific. Total protein was obtained by centrifuging tissue lysate, prior to fractionation, for 14000 x g for 15 min at 4 °C and collecting the supernatant. Protein precipitation from each fraction was accomplished by adding 20% v/v trichloroacetic acid and incubating overnight at 4 °C. Samples were centrifuged at 14000 x g for 10 min at 4 °C, washed once with ice-cold acetone containing 0.1% HCl, and once with ice-cold acetone before being air-dried and stored at -20 °C until use. N⁶-Formyllysine adducts were found to be stable under these conditions.

**Enzymatic hydrolysis of proteins.** Extracted proteins from tissues were dissolved in 50 μL of 100 mM ammonium bicarbonate buffer (pH 8.5), 4,4,5,5-[²H]-lysine (2 nmol), and 4,4,5,5-[²H]-N⁶-formyllysine (1 pmol) were added as internal standards, and the proteins were hydrolyzed by addition of 2 μl of freshly prepared *S. griseus* protease solution (1μg/μl) with incubation at 37°C for 16 h. Samples were dried under vacuum and resuspended in 50 μL of water prior to mass spectrometry analysis.
Quantification of amino acids. N^6-Formyllysine amino acids in the resuspended protein hydrolysates were quantified as a percentage of lysine content by liquid chromatography-coupled mass spectrometry (LC-MS/MS). Analytes in total, cytoplasmic, and membrane protein hydrolysates were analyzed without a pre-purification step. Nuclear protein hydrolysates were first HPLC pre-purified to enhance the signal for the M+2 ion of N^6-formyllysine. HPLC pre-purification was done using an Agilent 1100 series system, with an aqueous normal-phase Cogent diamond hydride column (2.1 x 150mm, 4μm) from MicroSolv Technology Corporation (Eatontown, NJ), with mobile phase flow rate of 400 μL/min and column temperature of 20 °C. The solvent system was 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B), with the elution starting at 100% B, the gradient linearly decreased to 25% B over 30 min and held at 25% B for 3 additional min before the column was re-equilibrated with 100% B for 7 min. For N^6-formyllysine quantification, an extended chromatography was used, in order to separate the M+1 ion of citrulline from M+2 ion of N^6-formyllysine (Figure 2-8). The same solvent system of 0.1% acetic acid in water (A) and 0.1% acetic acid in acetonitrile (B) was used, with the elution starting at 100% B, the gradient linearly decreased to 75% B over 75 min, reaching 25% B over the next 3 min and 15% B by 83 min. The column was then re-equilibrated with 100% B for 7 min. N^6-Formyllysine and lysine were then analyzed using an Agilent 6410 triple quadrupole mass spectrometer (MS/MS) equipped with an electrospray ionization (ESI) source operated in positive ion mode. The operating parameters were as follows: ESI capillary voltage, 4000 V; gas temperature, 350 °C; drying gas flow, 12 L/min; and nebulizer pressure, 30 psi, with selected reaction monitoring (SRM) transitions of m/z 175→112 and for N^6-formyllysine (with internal
standard transition of \( m/z \ 179 \rightarrow 116 \) and \( m/z \ 147 \rightarrow 130 \) for lysine (with internal standard transition of \( m/z \ 151 \rightarrow 134 \)). The fragmentor voltage and collision energies were 105 V and 10 V for \( N^6 \)-formyllysine and 100 V and 8 V for lysine, respectively.

**Results**

**Quantification of background endogenous \( N^6 \)-formyllysine in tissues.** In addition to monitoring the formaldehyde-induced (exogenous) \( N^6 \)-formyllysine modifications, it is important to quantify the background (endogenous) levels of this protein adduct to parse the contribution of exogenous formaldehyde sources to the total amount of lysine formylation in cells. To this end, total proteins as well as fractionated proteins from cytosolic, membrane, and nuclear compartments were extracted from nasal epithelium, lung, and liver of exposed rats, and endogenous adducts were quantified using our sensitive and specific LC-MS/MS method with the limits of detection of 1 fmol for \( N^6 \)-formyllysine and 10 fmol for lysine. Table 4-1 summarizes the background levels of \( N^6 \)-formyllysine in all proteins analyzed. There are similar levels of endogenous adducts among different types of tissue analyzed with a range of 2-4 \( N^6 \)-formyllysine residues per \( 10^4 \) lysines. Each tissue shows similar levels in control rats and rats exposed to the highest dose of 10 ppm \( [^{13}C,^2H_2] \)-formaldehyde, which indicates that exposure to formaldehyde did not affect the endogenous adduct levels.
Chapter 4: Formaldehyde-induced formation of N⁶-formyllysine protein adducts in rats

Table 4-1. Quantification of endogenous N⁶-formyllysine protein adducts in total and fractionated proteins.¹

<table>
<thead>
<tr>
<th>Protein source</th>
<th>Nasal Epithelium</th>
<th>Lung</th>
<th>Liver</th>
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</thead>
<tbody>
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<td></td>
<td>0 ppm 9.7 ppm</td>
<td>0 ppm 9.7 ppm</td>
<td>0 ppm 9.7 ppm</td>
</tr>
<tr>
<td>Total protein</td>
<td>2 ± 0.1²</td>
<td>4 ± 0.2</td>
<td>3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2 ± 0.0</td>
<td>3 ± 0.4</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>2 ± 0.4</td>
<td>4 ± 0.6</td>
<td>4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>2 ± 0.4</td>
<td>4 ± 0.6</td>
<td>4 ± 0.1</td>
</tr>
<tr>
<td>Membrane</td>
<td>3 ± 0.8</td>
<td>3 ± 0.5</td>
<td>3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>2 ± 0.4</td>
<td>3 ± 0.4</td>
<td>3 ± 0.2</td>
</tr>
<tr>
<td>Soluble nuclear</td>
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<td>4 ± 1.0</td>
<td>4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>2 ± 1.0</td>
<td>4 ± 0.3</td>
<td>4 ± 0.7</td>
</tr>
<tr>
<td>Chromatin bound</td>
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<td>3 ± 1.0</td>
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</tr>
<tr>
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<td>3 ± 0.3</td>
</tr>
</tbody>
</table>

¹Column titles denote endogenous N⁶-formyllysine modifications in each tissue analyzed (for control versus 9.7 ppm [¹³C,²H₂]-formaldehyde exposed rats).

²Data are expressed as adducts per 10⁶ total lysines and represent mean ± SD for 3 rats.

Formaldehyde-induced formation of exogenous N⁶-formyllysine adducts. The formation of N⁶-formyllysine from inhaled [¹³C,²H₂]-formaldehyde was monitored using the m/z 177→114 transition for N⁶-[¹³C,²H]-formyllysine that is 2 mass units heavier than the endogenous adduct, as noted in Chapter 3, with the 4,4,5,5-[²H]-N⁶-formyllysine internal standard monitored at m/z 179→116 (Figure 4-1). Exogenously-derived N⁶-formyllysine adducts were only detected in proteins extracted from nasal epithelium and not in distant tissues of lung and liver. In all samples analyzed from lung and liver tissues, the exogenous adducts did not show an increase beyond the natural isotope abundance level of ~0.7% for [M+2] ion of N⁶-formyllysine. In addition to total proteins, an analysis of proteins in cytosolic, membrane, and nuclear compartments revealed the dose-dependent formation of exogenous N⁶-formyllysine species in all locations (Table 4-2 and Figure 4-2). Data show that, at all exposures, the endogenous adducts dominate.
Chapter 4: Formaldehyde-induced formation of N$^6$-formyllysine protein adducts in rats

**Figure 4-1.** Inhalation of $[^{13}\text{C}_2\text{H}_2]$-formaldehyde distinguishes exogenous from endogenous sources of N$^6$-formyllysine in rats. LC-MS/MS analysis showing signals for the three isotopomeric N$^6$-formyllysine species, in cytoplasmic proteins extracted from nasal epithelium, as described in Materials and Methods.

There is a clear dose-response relationship for lysine N$^6$-formylation across the range of inhaled formaldehyde doses (0.7, 2, 6, 10 ppm) (Figure 4-2), with exogenous adducts in total proteins rising from < 3% of endogenous adducts to > 40% for a 14-fold increase in formaldehyde dosage (0.7 to 10 ppm). At all exposure levels, the ratio of exogenous to endogenous N$^6$-formyllysine adducts in all proteins, except nuclear proteins, closely correlate with ratios of exogenous and endogenous N$^2$-hydroxymethyl-dG DNA adducts determined by Swenberg and coworkers [12]. For instance, N$^6$-formyllysine ratios of 0.15 and 0.4 in total protein closely match the DNA adduct ratios of 0.2 and 0.6, for 6 and 10 ppm exposures, respectively.
Table 4-2. Quantification of endogenous and exogenous N⁶-formyllysine protein adducts in nasal epithelium from rats exposed to [¹³C₂H₂]-formaldehyde.

<table>
<thead>
<tr>
<th>Dose</th>
<th>0.7 ppm</th>
<th>2 ppm</th>
<th>5.8 ppm</th>
<th>9.7 ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adduct type</td>
<td>Endo</td>
<td>Exog</td>
<td>Endo</td>
<td>Exog</td>
</tr>
<tr>
<td>Total Protein</td>
<td>2± 0.1²</td>
<td>0.06± 0.04</td>
<td>2± 0.2±</td>
<td>0.2± 0.02</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td>2± 0.3</td>
<td>0.05± 0.04</td>
<td>3± 0.2±</td>
<td>0.2± 0.07</td>
</tr>
<tr>
<td>Membrane</td>
<td>2± 0.3</td>
<td>0.06± 0.02</td>
<td>2± 0.2±</td>
<td>0.8± 0.03</td>
</tr>
<tr>
<td>Soluble nuclear</td>
<td>2± 0.3</td>
<td>0.05± 0.05</td>
<td>2± 0.2±</td>
<td>1.0± 1.0</td>
</tr>
<tr>
<td>Chromatin bound</td>
<td>2± 0.4</td>
<td>0.02± 0.02</td>
<td>2± 0.03±</td>
<td>0.8± 0.01</td>
</tr>
</tbody>
</table>

¹Column titles denote endogenous or exogenous N⁶-formyllysine modifications for each [¹³C₂H₂]-formaldehyde exposure dose.

²Data are expressed as modifications per 10⁴ total lysines and represent mean ± SD for 3 rats.

As shown in Table 4-2 and Figure 4-2, there is a general decrease in adduct levels in nuclear proteins compared to proteins from other cell compartments. For example, a 10 ppm formaldehyde exposure produced 0.2 exogenous N⁶-formyllysine adducts per 10⁴ lysines in chromatin proteins compared to 0.7 and 0.8 residues in cytoplasmic and membrane fractions, respectively (p < 0.05).
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Discussion

The results of these in vivo studies point to several important features of $N^6$-formyllysine formation and formaldehyde toxicity. While originally described as an adventitious protein modification of histone and other nuclear proteins [6-8], $N^6$-formylation of lysine has been shown to occur globally in proteins from different cellular compartments as well as plasma proteins (Chapter 3). These observations, together with the in vitro formaldehyde studies of Chapter 3 and the relatively high levels of formaldehyde exposure from environmental [12-14] and endogenous [13,15,16] sources, point to formaldehyde as a major source of $N^6$-formyllysine adducts in cells, as compared to DNA.

Figure 4-2. $[^{13}C,^{2}H_2]$-Formaldehyde causes a dose-response increase in exogenous $N^6$-formyllysine adducts. Ratios of exogenous versus endogenous $N^6$-formyllysine protein adducts in the nasal epithelium of rats exposed to $[^{13}C,^{2}H_2]$-formaldehyde for 6h, as described in Materials and Methods. Data represent mean ± SD for N=3.
oxidation pathway previously reported. Here, our *in vivo* assessment of N⁶-formyllysine formation in rats exposed to formaldehyde by inhalation is consistent with our previous conclusion of formaldehyde as a source of lysine N⁶-formylation. Interestingly, the background levels of N⁶-formyllysine species due to endogenous sources were unaffected even at the highest formaldehyde exposure dosage, which suggests that the formaldehyde doses used for exposure do not alter the cellular production of formaldehyde.

The observation that background N⁶-formyllysine levels are similar in proteins from all cellular compartments suggests that the sources of this protein modification are equally balanced in the various compartments, consistent with cellular abundance of formaldehyde due to environmental and endogenous sources [12-16]. The dose-dependent formation of exogenous N⁶-[¹³C₂H]-formyllysine adducts in all compartments, with decreased levels in nucleus, is consistent with exogenous stable isotope-labeled formaldehyde being the source, as inhaled formaldehyde concentration is expected to decrease on the way to nucleus by reacting with other biomolecules.

To further correlate protein and DNA adducts given the carcinogenic potency of formaldehyde, exogenous/endogenous ratios of N⁶-formyllysine residues in histone proteins are plotted against the published values of N²-hydroxymethyl-dG DNA adducts [12], as shown in Figure 4-3. Data reveal that DNA adducts dominate histone adducts for the same concentration of formaldehyde. For instance, for the case of 10 ppm exposure, the exogenous to endogenous N²-hydroxymethyl-dG adduct ratio is more than 3-fold of that for N⁶-formyllysine (i.e., ~0.6 versus less than 0.2). However, they both follow the same pattern of increase as a response to formaldehyde exposure. For example, going from 2 ppm to 10 ppm, and from 6 ppm to 10 ppm formaldehyde exposure doses result in
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Figure 4-3. Comparison of exposure-response plots for ratios of exogenous to endogenous adducts in DNA (obtained from [12]) and histone proteins in the nasal epithelium of rats exposed to [¹³C,²H₂]-formaldehyde. Data for N⁶-formyllysine adducts represent mean ± SD for 3 rats.

The analysis of N⁶-formyllysine adducts sheds light on the mechanisms of formaldehyde toxicity. The protein adduct data complement previous studies of formaldehyde-induced DNA adducts in rats[12], with our results showing strong correlations between protein and DNA adduct formation during formaldehyde exposure. Our results show that, similar to N²-hydroxymethyl-dG DNA adducts, the exogenously-derived N⁶-formyllysine residues were only detected in nasal epithelium and not in distant tissues of lung and liver, with a dose-dependent formation of exogenous adducts.

same 20-fold and 3-fold ratio increases, respectively, in both DNA and protein adducts (Figure 4-3).
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in total proteins as well as proteins from specific cellular compartments (Figure 4-2 and Table 4-2). With the exception of nuclear proteins, the levels of $N^6$-formyllysine formation in proteins closely correlates with $N^2$-hydroxymethyl-dG adduct formation in DNA. However, as shown in Figure 4-3, the proportion of DNA adducts derived from exogenous formaldehyde was significantly higher than that for the histone adducts for the same concentration of formaldehyde, even though theoretically the same number of exogenous formaldehyde molecules should reach DNA and histones. This difference in ratios could be due to several factors such as DNA guanine content compared to histone lysine content, faster kinetics of $N^2$-hydroxymethyl-dG formation versus $N^6$-formyllysine formation, as well as possible greater solvent exposure of guanine $N^2$-positions compared to lysine amino side chains in histone proteins. Nonetheless, both $N^2$-hydroxymethyl-dG and $N^6$-formyllysine adducts follow the same pattern of increase as a response to formaldehyde dosage (Figure 4-3).

There have been extensive studies to date on possible mechanisms for formaldehyde toxicity and carcinogenicity. For instance, some studies found a non-linear dose-dependent formation of DNA damage products in rats and non-human primates exposed to formaldehyde inhalation [12,17,25,26] with other studies showing long term formaldehyde exposure of higher than 6 ppm dosage resulting in a substantial increases in squamous cell carcinomas in rats [20,21,27]. On the path to understanding formaldehyde's biological consequences, our results shed light on yet another pathway, that is through formation of $N^6$-formyllysine adducts in histones. $N^6$-Formyllysine adducts have been mapped on many conserved lysine acetylation and methylation sites along histones [7,8]. This observation along with chemical similarity of lysine $N^6$-
formylation to lysine $N^6$-acetylation, as well as our results showing $N^6$-formyllysine is refractory to removal by histone deacetylases (Chapter 3) suggests that $N^6$-formyllysine could interfere with the epigenetic roles associated with physiological histone modifications [28,29]. In other words, our studies suggest that prolonged exposure to formaldehyde from environmental and occupational sources can drive substantial and permanent formylation of conserved sites of important physiological modifications in histone proteins, thus interfering with their regulatory roles and further contributing to toxicity and carcinogenicity associated with formaldehyde exposure.
References


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Chapter 5

Concluding remarks
The recent discovery of N\textsuperscript{6}-formyllysine as a relatively abundant endogenous secondary modification of histones and other nuclear proteins [1-3] has raised questions about its mechanism of formation and its potential for interfering with the regulatory roles of these proteins, particularly in terms of disrupting histone acetylation signaling [4,5] due to chemical similarity of N\textsuperscript{6}-formyllysine to N\textsuperscript{6}-acetyllysine (Figure 1-1). With respect to formation, we considered formaldehyde as a source because of its high reactivity toward primary amines, such as the side chain of lysine, and the potential for endogenous oxidation converting the formaldehyde-derived carbinolamine to a formamide bond (Figure 3-1). Using our ultrasensitive and specific liquid chromatography-coupled tandem mass spectrometry method to quantify N\textsuperscript{6}-formyllysine lesions in proteins, we presented evidence for formaldehyde being a major source of this pathological protein modification in addition to products of oxidative DNA damage previously reported [1]. More specifically, the work presented in this thesis demonstrated \textit{in vitro} as well as \textit{in vivo} (Chapters 3 and 4) that formaldehyde exposure leads to a dose-dependent lysine N\textsuperscript{6}-formylation in all cellular proteins, with the use of \([^{13}\text{C},^{2}\text{H}_2]\)-formaldehyde to differentiate endogenous from exogenous adducts. Indeed, our study of formaldehyde exposure in TK6 cells showed that the exogenous formaldehyde contributed to half of the total load of formyllysine in histone proteins at 10 mM concentration (Chapter 3), while in rats exposed to 10 ppm formaldehyde by inhalation, nearly one-third of the total load of N\textsuperscript{6}-formyllysine was derived from exogenous sources in total proteins extracted from nasal epithelium (Chapter 4).

The formaldehyde studies of Chapters 3 and 4 and the high incident of formaldehyde exposure due to environmental [6-8] and endogenous [7,9,10] sources
together with our discovery of global lysine formylation occurring in plasma proteins and proteins from all cellular compartments (Tables 3-2 and 4-1), point to formaldehyde as the major source of $N^6$-formyllysine adducts as compared to DNA oxidation pathway [1]. The fact that $N^6$-formyllysine levels are similar in histone and non-nuclear proteins and in all cell compartments also suggests that the sources of this protein modification are equally balanced in the various compartments and proteins, or that there is a single dominant source that distributes uniformly throughout the cell. Though it is not yet possible to distinguish $N^6$-formyllysine adducts arising from endogenous formaldehyde versus other sources, the fact that human serum concentration of formaldehyde occur in the range of 10 to 100 $\mu$M [7,11,12] is consistent with formylation of lysine in vivo.

In terms of other sources of this adduct, we considered disrupted histone demethylation that would leave the carbinolamine precursor of $N^6$-formyllysine for subsequent oxidation and the formaldehyde produced by complete lysine demethylation [10]. However, we presented evidence in Chapter 3 that lysine demethylation process does not serve as a source of lysine $N^6$-formylation. The absence of detectable $N^6$-formyllysine arising from $N^6$-methyllysine species (Figure 3-4C) suggests that disrupted histone demethylation occurs at low frequency, or that the formaldehyde produced at the last step of lysine demethylation does not occur at concentrations high enough to drive formylation of lysine or cause substantial changes in $N^6$-formyllysine levels detected by our current analytical methods. In addition, there is the possibility that the formaldehyde released during lysine demethylation may be scavenged before it could react with lysines in histone proteins. Indeed, a recent study reports lysine-specific demethylase 1 (LSD1) as a folate binding protein and suggests folate serves as a trap for the formaldehyde
generated during lysine demethylation [13].

With regard to N\textsuperscript{6}-formyllysine persistence in cells, there is still no evidence supporting the enzymatic removal of N\textsuperscript{6}-formyllysine. Our investigation of histone deacetylases (Chapter 3) revealed that despite chemical similarity of N\textsuperscript{6}-formyllysine to N\textsuperscript{6}-acetyllysine, the former is not removed by histone deacetylases, suggesting that they will persist throughout the life of individual histone proteins. Further, the [\textsuperscript{2}H\textsubscript{8}]-N\textsuperscript{6}-formyllysine dilution studies (Chapter 2) indicate that at one extreme N\textsuperscript{6}-formyllysine persists in cells for the life of histone proteins and at the other extreme, its steady state levels doesn't change as it is formed and removed at the same frequency. Although the studies presented in this thesis point to the stability of lysine N\textsuperscript{6}-formylation in proteins, we cannot rule out the possibility of an enzyme that would remove this modification from selected conserved lysine sites in histone proteins, resulting in small change in the quantity of formyllysine that is simply not detectable by our current analytical method.

With respect to pathological consequences of N\textsuperscript{6}-formyllysine in cells, the abundance of lysine N\textsuperscript{6}-formylation in histone proteins points to its potential for epigenetic alterations in gene expression. The observation that N\textsuperscript{6}-formyllysine adducts are randomly and relatively evenly distributed among different classes of histone proteins, as opposed to the very biased distributions of other functional modifications (Table 3-1), suggests that they are adventitious and not physiological. The high abundance of lysine N\textsuperscript{6}-formylation in histone proteins (Table 2-3) as well as its occurrence on many conserved functional locations for lysine acetylation and methylation [2,3] suggest a disruptive role for N\textsuperscript{6}-formyllysine with regards to signaling processes associated with physiological histone modifications [4,5]. In other words, if N\textsuperscript{6}-
formyllysine is not removed, it could accumulate to significant levels in histone proteins and interfere with their epigenetic regulatory processes through sabotaging the lysine acetylation signaling or blocking the conserved lysines sites and preventing them from undergoing important physiological modifications needed for proper functions of cells. This potential pathological consequence of N⁶-formyllysine in cells is consistent with toxicity and carcinogenicity associated with formaldehyde. Indeed, on the path to understanding formaldehyde's biological consequences, our results shed light on yet another pathway, that is through formation of N⁶-formyllysine adducts in histone proteins. This potential epigenetic mechanism of disruption of cell function together with the association of N⁶-formyllysine with oxidative and nitrosative stresses of inflammation [1,14], suggest that this adduct may play a role in many pathophysiological processes in humans.
References


