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Bioactivation and Protein Modification Reactions of Unsaturated Aldehydes

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9.1. Processes that Produce Aldehydes

Among the foreign compounds that have been shown to promote protein modification, aldehydes are notable due to their reactivity and prevalence in the environment and production in vivo. Humans are exposed to aldehydes from several exogenous and endogenous sources. Combustion processes from forest fires, open heating and cooking facilities, cigarette smoke, and energy production (coal-powered electrical plants) are prevalent sources of aldehydes. Furthermore, their use as chemicals to preserve organic materials like wood or their use as herbicide, fungicide, and germicide materials increases the likelihood of exogenous aldehyde exposure. Endogenously, the metabolism of biological and exogenous amines, alcohols, and lipids produces aldehydes. Although many of these processes are necessary metabolic pathways for energy production, other processes also produce aldehydes. These include unproductive lipid oxidation, infection, inflammation, chemically induced lipid peroxidation, or foreign compound metabolism involved in the detoxification of drugs or toxic chemicals. Some of these aldehydes are highly reactive, such as the α,β-unsaturated aldehydes, for example, 4-hydroxy-*trans*-2-nonenal (HNE) or acrolein, formed during lipid peroxidation or combustion. As the process

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of lipid peroxidation associated with oxidative stress becomes better understood, we note that some of the products formed may have biological functions as signaling molecules (Ruef et al. 1998; Ji, Kozak and Marnett 2004; Robino et al. 2001). Their properties require continued study to fully appreciate the effects these molecules have in biological systems. Several recent reviews thoroughly explore the possible role of endogenously generated aldehydes in mitogenic or proliferative processes, including those associated with aberrant glucose metabolism associated with diabetes and atherosclerosis (Ruef et al. 1998).

9.2. Metabolism of Aldehydes

Since there are a number of recent reviews on the metabolism of aldehydes, the focus in this article will not be directed toward the disposition of aldehydes by enzymatic oxidation or reduction reactions. However, our intent is to address how aldehydes form adducts with cellular nucleophiles in general, and with proteins in particular. A classic example of the adduction of thiols is the reaction catalyzed by glutathione S-transferases. These enzymes play important roles in metabolizing aldehydes and the reaction catalyzed by them is similar to the reactions that occur when aldehyde–protein adducts are formed spontaneously. Enzymatic pathways for aldehyde disposition have been recently reviewed by Conklin, Prough and Bhatnagar (2006).

In addition to oxidative, reductive, and conjugative metabolic transformations, aldehydes also participate in adduction reactions with several nucleophilic species in cells, including nucleic acids and proteins. Much of the toxicity of aldehydes may be linked with their ability to form adducts with proteins. Like metabolism, adduction of proteins is not static; it is a dynamic process. To examine the intracellular fate of protein–aldehyde adducts, we treated rat aortic smooth muscle (RASM) cells with reagent HNE. After removal of media containing HNE, fresh media was added and the cells were cultured in media without HNE. At appropriate times, the cells were collected, lysed, and protein adducts were quantified by

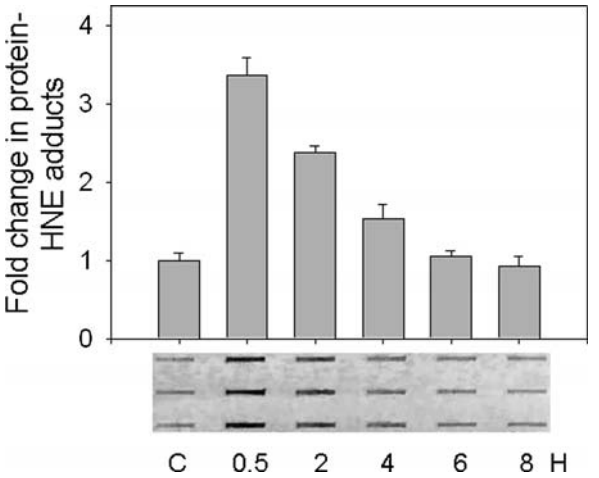


Figure 9.1 Removal of protein–HNE adducts in vascular cells. Anti-protein–HNE immunoblot analysis was performed on lysates from HNE-treated rat aortic smooth muscle (RASM) cells. RASM cells (5×10^5 cells ml^{-1}) were exposed to 50-μM HNE for 30 minutes. The medium was then removed and the cells were either collected or HNE-free growth medium was added to the cells for the indicated times. Cells were then lysed and protein–HNE adduct levels were assessed by slot immunoblotting with anti-protein–HNE antibodies. The figure is representative of at least three experiments. $n = 6$ per group.

immunoslot blotting using anti-protein–HNE antibodies. As shown in Figure 9.1, the level of anti-protein–HNE immune complexes increased more than three- to fourfold after 30 minutes of HNE treatment. The protein–HNE adducts disappeared during the next 8 hours in culture, until the level of protein–HNE complexes returned to the levels seen before treatment. These results clearly show that in exposed cells aldehydes rapidly form protein adducts and further that these adducts are removed from cells with time. The processes that allow adduct formation and the removal of protein–HNE adducts in these vascular cells are processes of importance in understanding aldehyde toxicity. Critical questions arise, such as, what are the reactions that form these adducts, are they reversible, and are they processed in the cell by proteolytic mechanisms? Studies are in progress in our laboratory to address the nature of these reactions and to identify the biochemical processes involved.

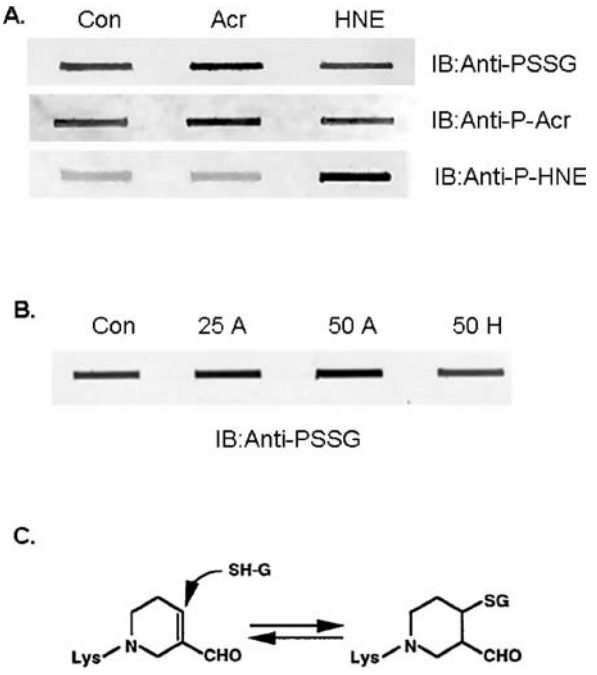


Figure 9.2 Complex protein adducts formed by the environmental pollutant and lipid peroxidation product, acrolein. Immunoblots of glutathiolated proteins and protein-bound acrolein or HNE. (**a**) Monkey kidney fibroblasts cells were exposed to vehicle, acrolein, or HNE for 30 minutes and protein modifications were assessed by immunoblotting with anti-protein glutathione (anti-PSSG), anti-protein acrolein (anti-P-Acr), or anti-protein HNE (anti-P-HNE) antibodies. Only acrolein increased the formation of protein–glutathione adducts. (**b**) Concentration-dependence of glutathiolation with acrolein. Acrolein at 25-μM (25 A) and 50-μM (50 A) concentrations increases protein glutathiolation; HNE (50 μM; 50 H) does not promote protein glutathiolation. (**c**) Potential mechanism for the formation of glutathiolated proteins due to protein–acrolein adducts. Acrolein can form a *bis*-adduct with lysine side chains (not shown) that condensates to form the N^{ε}-(3-formyl-3,4-dehydropiperidino) lysine (FDP-lysine) adduct. This adduct itself possesses α, β-unsaturation that can react with glutathione or can promote protein crosslinking reactions (scheme derived from the results of: Furuhata A. et al. 2002).

A subsequent series of experiments were performed to compare the effects of two different α,β-unsaturated aldehydes, acrolein and HNE, on the formation of protein–aldehyde and protein–glutathione adducts. Monkey kidney fibroblast (COS7) cells were exposed to either vehicle, acrolein or HNE, for 30 minutes and the protein modifications were assessed by immunoblotting with anti-protein glutathione, anti-protein acrolein, or anti-protein HNE antibodies. As shown in Figure 9.2a, row 2, acrolein formed protein adducts distinct from that observed in cells treated with HNE (row 3). Conversely, HNE formed protein adducts that were not recognized by anti-protein–acrolein antibodies. Of most interest, in row 1, acrolein treatment caused formation of protein–glutathione adducts, but HNE did not. This result clearly indicates that although both α,β-unsaturated aldehydes form protein adducts, only acrolein increases the formation of protein–glutathione modifications. Figure 9.2b shows the concentration dependence of acrolein-dependent protein glutathiolation reactions and the inability of HNE to enhance protein thiolation. We postulate that protein–acrolein–glutathione adducts are formed in reactions like that shown in Figure 9.2c. While these approaches allow us to study aspects of the kinetics of adduct formation and removal, they do not provide a method to clearly deduce the chemistry of these processes and explain why acrolein specifically increases the formation of glutathione-modified proteins. Our preliminary studies suggest that using mass spectrometric (MS) approaches to study these protein adducts offers considerable promise in understanding the processes of oxidative stress and the spectrum of products formed during oxidative stress or even normal metabolic processes.

9.3. The Chemistry of Aldehyde–Protein Adducts

Aldehydes owe their chemical reactivity to an activated carbonyl functional group with a proton attached to it. The separation of charge in this carbonyl allows the aldehyde functional group to interact readily with nucleophilic groups, such as amine groups, to form a reversible adduct commonly know as a Schiff base (Figure 9.3). The level of reactivity of aldehydes is determined by the electron deficiency at the carbonyl carbon caused by a variety of electron-withdrawing groups. The carbonyl carbon reacts with primary amine groups to form a hemiaminal intermediate, and after loss of water forms an aldimine Schiff base. As described by Klotz and coworkers studying the effect of aldimine formation with hemoglobin, many of these Schiff base intermediates were shown to be reversible, suggesting that their presence in proteins may be transient in nature (Zaugg, Walder and Klotz 1977).

For α,β-unsaturated aldehydes, electron withdrawal from the double bond by the conjugated carbonyl group decreases electron density at the β-carbon of the double bond, making the β-carbon very reactive with

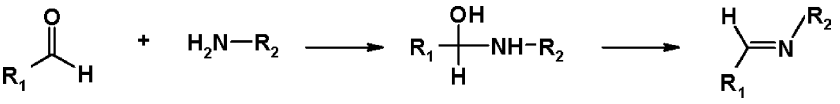


Figure 9.3 Schiff base formation.

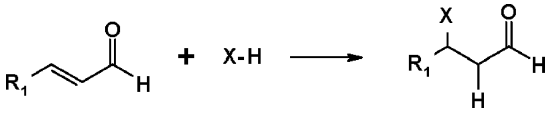


Figure 9.4 Michael addition reaction.

protein nucleophiles. The electrophilic β-carbon can react as a Michael acceptor with nucleophiles, such as the thiol group in cysteine (Cys), amine groups in lysine (Lys) and N-terminal α-amino acids, and the C3 of histidine imidazoles leading to the Michael addition reaction (Figure 9.4). Aldehydes that form Michael adducts are particularly reactive and can subsequently crosslink nucleic acids, proteins, and lipids. For many in this field, these crosslinked aldehyde products are thought to be an indicator of oxidative stress in general and important toxicological endpoints that are a phenotype of aldehyde toxicity.

In α,β-unsaturated aldehydes, the contribution of electrons from the double bond decreases the electron deficiency at the carbon in carbonyl groups, which makes the α,β-unsaturated aldehydes less reactive in Schiff base formation. In Michael addition products of α,β-unsaturated aldehydes, however, the double bond at α, β-carbon becomes saturated. The further reaction of Michael addition products with primary amine groups in proteins through Schiff base formation can result in further crosslinking of proteins. For example, acrolein causes crosslinking of proteins such as RNase (Burcham and Pyke 2006) and links glutathione (GSH) to proteins through putative lysine residues involved in formation of Michael addition products (Figure 9.2c). Thus, α,β-unsaturated aldehydes have multiple reactive functional groups available for protein adduct formation. In addition to the two basic reactions mentioned above, they also participate in more complex reactions to form other adducts.

9.3.1. Adducts Formed by Acrolein

Alkyl groups at the β-carbon decrease the reactivity of α,β-unsaturated aldehydes in Michael addition reactions by increasing electron density and causing steric hindrance at the β-carbon. Therefore acrolein, with no alkyl substitution at β-carbon, is by far the most reactive compound among the α,β-unsaturated aldehydes. Due to its high reactivity, acrolein readily forms covalent adducts with nucleophilic protein residues. As discussed in the Chapter 8, two types of such adducts, N^{ε}-(3-formyl-3,4-

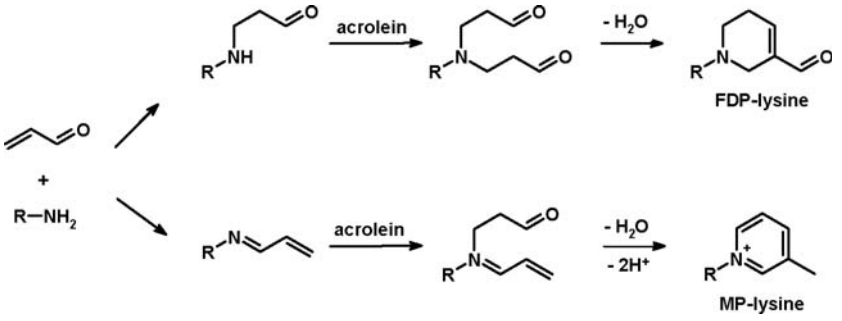


Figure 9.5 Proposed mechanism of MP-lysine and FDP-lysine formation.

dehydropiperidino)lysine (FDP-lysine) and N^{ε}-(3-methylpyridinium)-lysine (MP-lysine) have been identified by Uchida's group (Uchida et al. 1998a, 1998b; Furuhata et al. 2003; Figure 9.5).

FDP-lysine apparently is formed by the addition of two molecules of acrolein to the amino group at lysine by Michael addition, followed by cyclization and loss of water. The intermediates were not detected when N^{α}-acetyllysine was incubated with acrolein (Uchida et al. 1998a). An alternative mechanism might be involved in FDP-lysine formation. The possibility of an alternate mechanism is supported by the observation that a large excess of aldehyde over the amine is not necessary for the formation of FDP-lysine. Similar adducts can also form from other α,β-unsaturated aldehydes (Ichihashi et al. 2001). FDP-lysine is also a α,β-unsaturated aldehyde and it can react with glutathione to form a glutathione conjugate (Furuhata et al. 2002; Figure 9.2C).

Many pyridinium adducts, some that cause crosslinking of proteins, can form from α,β-unsaturated aldehydes through complex reactions (Alaiz and Barragan 1995; Baker et al. 1998; Ichihashi et al. 2001). Unlike many other adducts, the pyridinium adducts are highly stable. They carry a fixed positive charge and may have significant effect on protein function. MP-lysine adduct, a major antigenic adduct generated in acrolein-modified proteins (Furuhata et al. 2003), is one such adduct derived from acrolein. The mechanism of formation proposed by Furuhata et al. is shown in Figure 9.5. However, the mechanism of formation is still not clearly defined. However, existence of such adducts have been confirmed by nuclear magnetic resonance (NMR), and have been shown to be readily formed in acrolein-treated apolipoprotein AI (ApoAI) (Shao et al. 2005).

The possibility of complex adduction reactions between α,β-unsaturated aldehydes and proteins is supported by our data obtained using actin as a model protein. To examine its reactivity with α, β-unsaturated aldehydes, we

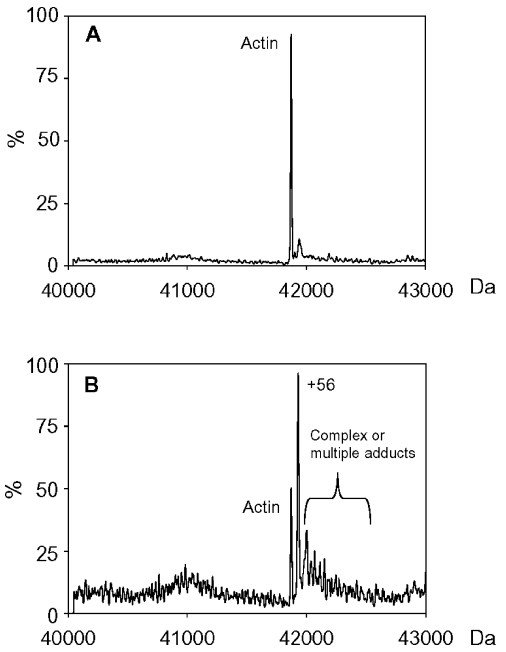


Figure 9.6 Detection of acrolein–actin adducts by electrospray ionization-mass spectrometry (ESI-MS). Deconvoluted spectra of actin: Actin was incubated with 100-mM DTT in 100-mM Tris, pH 7.5, for 1 hour at 37°C. Actin was then subjected to gel filtration on a Sephadex G25 column to remove DTT and to exchange buffer systems. The actin was then either analyzed by positive-mode ESI-MS (**a**) or incubated with 100-μM acrolein for 1 h at 37°C in 100-mM potassium phosphate buffer, pH 7.5. Excess acrolein was then removed by gel filtration and the modification state of actin was assessed by positive-mode ESI-MS (**b**).

first reduced actin with 100-mM dithiothreitol (DTT) in 100-mM Tris, pH 7.5, for 1 hat 37°C. Subsequently, the sample was desalted and DTT was removed by gel filtration. The actin was analyzed by electrospray ionization-mass spectrometry (ESI-MS) after incubation in the absence (Figure 9.6a) or presence (Figure 9.6b) of 100-μM acrolein for 1 hour at 37°C in 100-mM potassium phosphate buffer, pH 7.5. Excess acrolein was then removed by gel filtration and the modification state of actin was assessed by ESI-MS. Figure 9.6a and b show the deconvoluted mass spectra of actin and acrolein-modified actin, respectively. As seen in Figure 9.6b, one can observe a number of chemical species of actin in the spectrum suggesting that this single protein forms multiple protein–acrolein adducts, obviously with a variety of chemical modifications. The presence of intrachain cross-links cannot be determined without further analysis.

9.3.2. Adducts Formed by HNE

HNE has a hydroxyl group at C4. In Michael addition reactions of HNE, the aldehyde group can react with the hydroxyl group to form cyclic semiacetal as shown in Figure 9.7. The dihydrofuran adduct forms upon loss of water from the semiacetal (Liu, Minkler and Sayre 2003). This intermediate has the same mass as a Schiff base adduct, but it cannot be reduced by $NaBH_4$ as expected of a Schiff base. The Michael addition product of HNE has an aldehyde group. It can react with the ε-amino group of lysine side chains of proteins to form a Schiff base and subsequently cause crosslinking in proteins (Uchida and Stadtman 1993). However, Michael addition adducts exist predominantly in semiacetal form which prevents the further reaction of Schiff base formation.

When a Schiff base is formed before Michael addition, HNE can also form a 2-pentylpyrrole adduct as shown in Figure 9.8 (Sayre et al. 1993). The Schiff base can rearrange to form a γ-ketone, followed by cyclization to form an unstable semiaminal and loss of water to form 2-pentylpyrrole. Since Schiff base formation is not a favorable reaction for HNE, pyrrole adduct formation appears to be a minor pathway when compared with the Michael addition reaction. However, unlike other HNE adducts mentioned above, its formation is irreversible and it also removes positive charges carried by the parent lysine residues. Such adducts of HNE have been detected in human plasma and atherosclerotic plaques (Salomon et al.

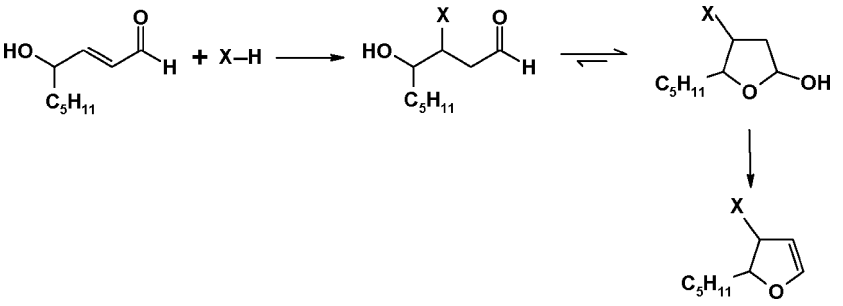


Figure 9.7 Cyclic semiacetal and dihydrofuran adducts from Michael addition adducts of HNE.

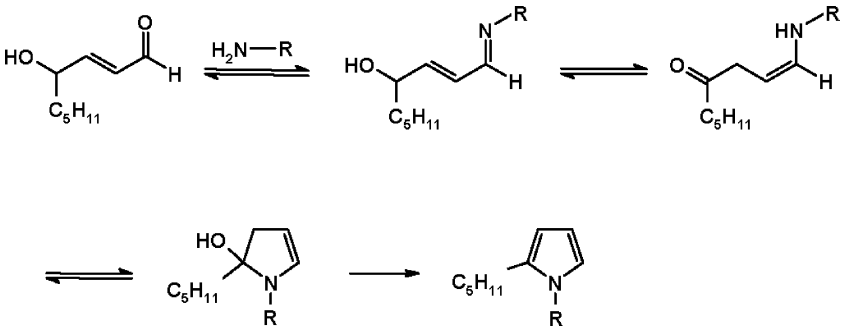


Figure 9.8 Formation of 2-pentylpyrrole adduct from HNE.

2000) and might be more relevant markers of permanent protein damage caused by HNE (Sayre et al. 1996).

9.3.3. Adducts Formed by 4-oxo-trans-2-nonenal

4-oxo-2-nonenal (ONE) has been identified as a common lipid peroxidation product, in addition to HNE (Lee and Blair 2000, Spiteller et al. 2001), and has much higher reactivity than HNE. Both α- and β-carbon can react with histidine (His) and Cys in Michael Addition reactions. The β-carbon is the more reactive position (Zhang et al. 2003). The reaction is irreversible and about 6–31 times faster than HNE (Lin et al. 2005.) However, ONE is less reactive than HNE toward the ϵ-amino group of lysine side chains through Michael addition. Instead it goes through a fast, but reversible reaction to form a Schiff base. Schiff base formation at lysine was found to be the fastest reaction of ONE with model compounds. Although this reaction might be transient and the products difficult to detect due to the reversibility of the reaction, the possible deleterious biological effects from this reaction should be considered (Lin et al. 2005).

The Michael Addition products of 4-oxononenals can further react through Schiff base formation, since they have two carbonyl groups. Under nonoxidative conditions, they can react with amine groups through Paal–Knorr condensation (Amarnath et al. 1995) to form pyrrole adducts as shown in Figure 9.9 (Xu and Sayre 1999, Zhang et al. 2003). Such

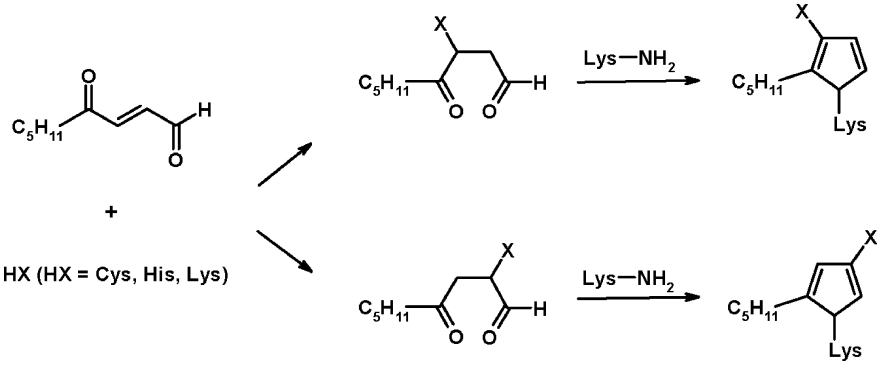


Figure 9.9 Formation of 2-pentylpyrrole adducts from ONE.

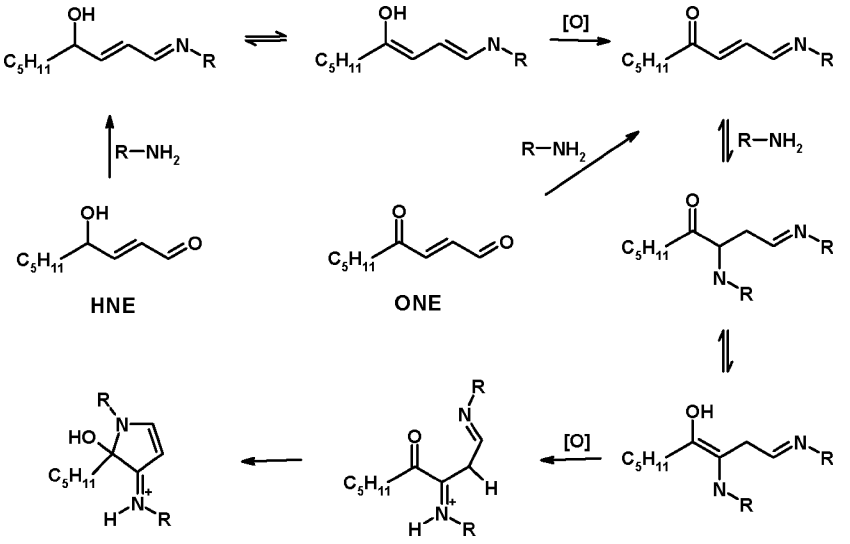


Figure 9.10 Formation of dihydropyrrole adducts from HNE and ONE.

intrachain crosslinking by ONE was found between histidine and lysine at a HAK motif of histone H4 (Oe et al. 2003b).

Under oxidative conditions, both HNE and ONE can react with two Lys residues through crosslinking reaction and oxidation to form a 2-alkyl-2-hydroxy-1,2-dihydropyrrol-3-one iminium adducts (Xu and Sayre 1998) as shown in Figure 9.10. This adduct is characterized by the emission of fluorescence at 430 nm when excited with light at 360 nm. The reaction for ONE is preceded by Schiff base formation at the aldehyde group, followed by Michael addition of another lysine, tautomerization, oxidation, and cyclization. Since Schiff base formation from HNE was very slow (not detected in kinetic studies with spectroscopy), HNE preferentially forms Michael addition products with the ε-amino group of lysine side chains (Lin et al. 2005). The decreased electron density at the carbonyl carbon enhances Schiff base formation. Formation of this adduct from HNE requires two steps and 4-electron oxidation instead of one step and 2-electron oxidation from ONE. The formation of this adduct is much slower for HNE than ONE (Xu and Sayre 1998).

Rapid crosslinking of proteins can occur with either HNE or ONE and lysine is normally involved in this crosslinking (Xu et al. 1999). Both nonoxidative and oxidative reactions can generate crosslinking adducts in proteins. However, the formation of pyrrole adducts from nonoxidative reactions are rare and the formation of the substituted 1,2-dihydropyrrol-3-one iminium adduct from oxidative reactions is enhanced particularly in the case of ONE (Zhang et al. 2003).

9.4. Reactivity of Nucleophilic Residues in Protein

The common nucleophilic residues in proteins include Cys, His, Lys, as well as the amino group at N-terminus. All these nucleophiles can participate in Michael addition, while only Lys and amino group at N-terminus

can participate in Schiff base formation. Multiple reactions can occur with amino groups to form more complex products, such as FDP-lysine and MP-lysine. The nucleophilicity of the residues is determined by their polarizability and ionic state. The nucleophilicity of the sulfhydryl anion is much higher than amines. Therefore, Cys is much more reactive than His, Lys, and the N-terminal amino group. In order to be good nucleophiles, amines must exist in the free-base form. Amines with higher pKa will have lower percentage of amine free base at a given pH, which will decrease their reactivity in nucleophilic reactions. The average pKa values of these residues in protein can be estimated with model compounds that resemble amino acids chains (model pKa values). Some of these are listed in Table 9.1 (Nielsen and Vriend 2001).

The pKa value of the N-terminal amino group is much lower than that of Lys, and reactivity at the N-terminus can be much higher than Lys. A clear example is glycosylation of hemoglobin by glucose in which Schiff base formation is the first step of glycosylation. Higher levels of glycosylation occurs at N-terminus than at the ε-amino group of Lys. When hexanal was incubated with oxidized insulin β-chain, Schiff base formation was found predominantly at N-terminal Phe instead of Lys (Fenaille, Guy and Tabet 2003). Reactivity of HNE and ONE was evaluated with N-acetylated peptides containing one or more nucleophilic residues. Peptides were incubated with excess amount of HNE or ONE. Formation of adducts was monitored with spectrophotometry and confirmed by MS. The reactivity of residues was found to have the order of: Cys >> His > Lys (> arginine (Arg) for ONE) and the reactivity of ONE toward Cys was more than 100-fold higher than HNE (Doorn and Petersen 2002).

The guanidinium group in Arg is a strong base. Arg is mostly protonated at physiological pH. Delocalization of positive charge among the amino groups significantly decreases the reactivity of Arg in nucleophilic reactions. Only a very reactive aldehyde will react with Arg to form adducts. Formation of an imidazole adduct of ONE was reported by Blair's group (Oe et al. 2003a). The proposed mechanism of formation is shown in Figure 9.11. The amino group of guanidine reacts with aldehyde groups to form a carbinolamine intermediate. Then the secondary amine of guanidine reacts with β-carbon of ONE through an intramolecular Michael Addition to form a cyclic carbinolamine intermediate, and loss of water from the intermediate forms the imidazole adduct. Formation of HNE adducts with Arg has also been reported (Isom et al. 2004), and 2-pentylpyrrole was proposed to be the product. However, this has not been confirmed by NMR.

Table 9.1 Model pKa values of selected residues.

Residue type	Model pKa value
Arg	13.0
Lys	10.4
Cys	8.7
N-terminus	8.0

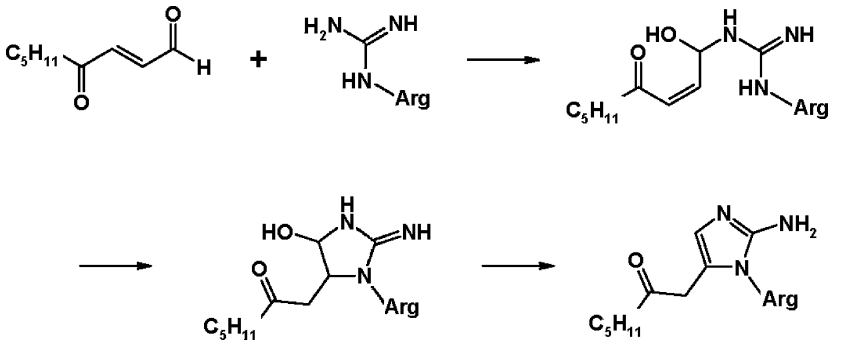


Figure 9.11 Formation of arginine adducts from ONE.

9.5. Effect of Neighboring Nucleophilic Amino Acid Residues

Reactivity of nucleophilic residues can be affected by other amino acid residues located nearby. The presence of an Arg on a Cys-containing peptide increased the reactivity of Cys toward HNE and ONE by a factor of 5–6 (Doorn and Petersen 2002). Formation of MP-lysine in peptide Ac-GEYAHKY is much higher than the peptides that His is replaced with Arg or Glu (Shao et al. 2005). When acrolein was incubated with apolipoprotein A-I, MP-lysine was formed preferentially at peptide sequences containing multiple lysine residues and only one of the Lys formed adduct with acrolein. Interestingly, FDP-lysine was not detected (Shao et al. 2005).

It is well known that the pKa and reactivity of amino acid side chains of proteins is greatly influenced by their microenvironment. Effect of environment on reactivity of residues with HNE was studied recently by Liebler's group (Szapacs et al. 2006). After incubation with HNE, human serum albumin (HSA) samples were reduced with $NaBH_4$, digested with trypsin, and analyzed by liquid chromatography (LC)/MS/MS. Ten Michael addition products at His and Lys were identified. The reactivity of different residues varied considerably. The kinetic study indicated the order of reactivity was $H_{242} > H_{510} > H_{67} > H_{367} > H_{247} \sim K_{233}$, which correlates well with the calculated pKa values of these residues (reactivity increases with decrease in pKa). The only exception was H_{367}, which has the second lowest pKa. Crystal structure of HSA indicates that it sits in a cleft and steric hindrance may limit its reactivity with HNE. The H_{242} residue sits in the hydrophobic binding cavity and has an unusually low pKa of 0.81; hence, its reactivity with HNE is much higher than that of any other residue. Finally, it should be noted that these reactivity considerations are for proteins in an essentially aqueous environment, so that membrane-incorporated protein reactivities would be expected to differ.

9.6. Reversibility of Aldehyde–Protein Adduction Reactions

Some reactions between aldehydes and amino acid residues in proteins are reversible. There is an optimal pH for Schiff base formation, depending on the reactants. Significant deviation from the optimal pH for Schiff base

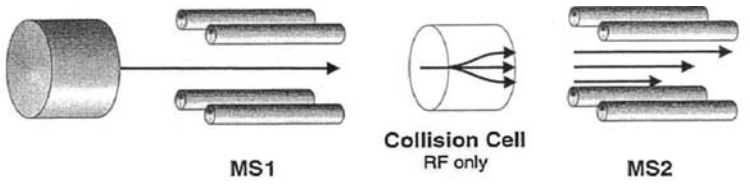
formation decreases the rates of formation and stability of the products. Schiff base formation of HNE and ONE with model compounds have been studied (Lin et al. 2005). Excess amounts of nucleophiles were used to react with HNE or ONE and the reactions were monitored by spectrophotometry. Michael addition of HNE with amines and Schiff base formation of ONE with amines were found to be reversible. The reversibility of adduct formation can complicate the detection of these adducts. For example, the thiol group in Cys is generally the most reactive nucleophile in proteins and acrolein is the most reactive α,β-unsaturated aldehyde. Acrolein adduct formation at Cys has not been reported to date and reversibility of reaction is the likely explanation because the thiol anion is an excellent leaving group. However, the effect caused by formation of reversible adducts in proteins should not be ignored. Adduct formation and its reversal are pH-dependent. This phenomenon may vary in different cellular microenvironments, may change the reactivity of proteins, and may even favor formation of species that serve as mediators or modulators of various biological responses.

9.7. Detection and Characterization of Aldehyde–Protein Adducts

Immunological assays have been widely used to monitor aldehyde–protein adduct formation. These have excellent sensitivity and specificity and are very useful in localizing adducts in tissues or cellular organelles. However, this technique cannot be used to establish final specific location of adducts in proteins or to elucidate structures of new adducts. For this, MS has emerged as a technique of choice. The basic MS techniques used in protein analysis have been reviewed recently (Domon and Aebersold 2006).

In qualitative studies, two types of data (MS and MS/MS) are usually acquired. In MS mode, MS spectra are dominated by peaks from intact peptides/proteins when soft-ionization techniques such as matrix-assisted laser desorption/ionization (MALDI) and ESI are used to ionize samples. Mixtures of peptides/proteins can be analyzed with this technique and intact molecular weight and charge state are the main information obtained from this technique. Sometimes limited fragmentation can occur during analysis. While this might provide structural information about the adduct, it might also lead to false identification or incorrect assignments. For example, in-source collision-induced dehydration of the Michael adduct of HNE could be incorrectly identified as a Schiff base adduct (Bolgar and Gaskell 1996).

In tandem MS/MS analyses, ions from samples of interest can be selected by the first mass analyzer (separated from other ions to avoid interference), the selected ion can be fragmented in a collision cell, and the fragment ions can be separated with a second mass analyzer and recorded to obtain MS/MS spectra (Figure 9.12). In other instruments (e.g., ion trap and Fourier transform (FT)-MS), ion selection, fragmentation, and separation often occurs within the trap, allowing the process to be repeated several times to generate higher-order tandem mass spectra. From MS/MS spectra of peptide/protein, sequences and specific location of modifications can be



Linked Scan Type	MS1	CID	MS2	Result
Product ion scan	Static (on precursor ion of interest)	Collision	Scan	Discovers all product ions of a given precursor ion
Precursor ion scan	Scan	Collision	Static (on product ion of interest)	Discovers all precursor of a given product ion
Constant neutral loss scan	Scan	Collision	Scan (offset from MS1 by constant neutral loss mass)	Discovers all decompositions that lose same mass

Figure 9.12 Schematic depiction of use of tandem mass spectrometry experiments. The instrument configuration for MS/MS spectrometer (triple quadrupole) is shown in the *top panel*. The methods utilizing MS/MS mass spectrometry are shown in the *lower panel*: **(a)** product ion scanning; **(b)** precursor ion scanning; and **(c)** constant neutral loss scanning.

obtained. For unknown adducts from model compounds, MS/MS spectra can also provide useful information for structure elucidation.

9.7.1. Top-Down Approaches to Identify Protein Modifications Using MS

Two approaches, top-down and bottom-up, have been used to identify type and location of adducts or post-translational modifications in protein. In a top-down approach, the intact protein is fragmented by mass spectrometers. To obtain good efficiency in fragmentation, special fragmentation techniques are used. Application of top-down approach has historically been limited to small- or medium-sized proteins. With development of new techniques, such as high-resolution Fourier transformation ion cyclotron resonance MS, larger proteins can be analyzed by this approach (Han et al. 2006). Fragmentation of proteins will generate many fragments that could exist in a multiplicity of charge states. These factors make the MS/MS spectra from top-down experiment exceedingly complex. Capability of determination of charge states of peaks with large number of charges is essential for determining mass of fragments in these experiments. Therefore, mass spectrometers with very high sensitivity and mass resolution are required for these experiments. The advantage of this approach is that it has potential to determine the entire sequence of a protein (and adducts at any residue). It may also be useful for detecting unstable adducts in proteins.

9.7.2. Bottom-Up Approaches to Identify Protein Modification Using MS

Currently, most protein characterization studies are done with bottom-up approaches. In a bottom-up approach, either purified proteins or protein

mixtures are first subjected to limited hydrolysis, usually using a purified and well-characterized endoprotease and then the peptides from partially digested proteins are analyzed by MS. In most cases, not all peptides are detected. Coverage might be increased by using a bank of proteases with different hydrolysis site specificities to digest proteins and/or by using different MS techniques to detect the peptides. However, coverage of the entire sequence is rare and adducts may not be detected if modification occurs on peptides undetected by the spectrometer. The most often used protease is trypsin. It cleaves protein at C-termini of Lys and Arg. Lys is one of the residues that reacts with aldehydes. Formation of adducts at Lys may prevent cleavage by trypsin. For example, trypsin does not cleave at Lys residues that form MP-lysine adducts (Shao et al. 2005). In these cases, other proteases such chymotrypsin, pepsin, or glutamic-C can be used to digest proteins.

9.7.3. Analysis of Unstable Aldehyde–Protein Adducts

Some protein adducts of aldehydes are not stable. These may be lost during digestion or during sample preparation before MS analysis. In sample preparation for MS analysis, low-pH solvents are often used. Schiff base formation is a reversible reaction and it can be fully reversible at low pH (Esterbauer, Schaur and Zollner 1991). To prevent loss of Schiff base adducts, protein samples can be reduced with $NaBH_4$ before MS analysis or digestion. The Michael addition product of HNE mainly exists as a semiacetal, and it can also be reduced with $NaBH_4$ to form 1,4-dihydroxy derivatives. Sometimes $Na(CN)BH_3$ is used to reduce Schiff bases to imines. It is a weaker reducing reagent compared with $NaBH_4$ and does not reduce carbonyl groups (Fenaille, Guy and Tabet 2003). In some cases, the use of sodium borodeuteride $NaBD_4$ is useful for simultaneous reduction and mass addition.

9.7.4. Techniques for Aldehyde–Protein Adduction Detection by MS

The type and location of adduct can be identified by a shift in the mass of peptides relative to the molecular weight of peptides from unmodified proteins. When multiple reactive residues exist in the same peptide, molecular weight alone is not enough to determine which residue forms the adduct. In these cases, specific sites of modification can be identified by MS/MS analysis. Some adducts introduce the same mass shifts in peptide spectra, and these may be distinguished by their pattern of fragmentation in MS/MS experiment or by their difference in chemical properties, such as reducibility by $NaBH_4$ or $Na(CN)BH_3$ or reactivity with 2,4-dinitrophenylhydrazine (DNPH).

The MS/MS data can be processed with special software, such as P-MOD (Hansen et al. 2005) and SALSA (Hansen et al. 2001), to reveal adducts at unknown locations. Other MS methods have also been used to selectively detect adducted peptides from peptide mixtures. A method to detect HNE adduct by MALDI-TOF was developed by Guy's group (Fenaille, Tabet and Guy 2004). In this method, the Michael addition adducts of HNE are derivatized with DNPH and analyzed by

MALDI-TOF with DNPH as matrix. When DNPH is used as the matrix, the hydrazone derivatives from Michael addition adducts of HNE have much better desorption/ionization efficiency while signals from unmodified peptides are suppressed.

Some additional MS/MS techniques can also be used to identify unknown location of modifications in proteins. Precursor ion scans and constant neutral loss scans are two of such techniques, both of them use the characteristic fragmentation of adducts to identify peptides with those adducts. In precursor ion scans (Figure 9.12), the instrument is set to identify which precursor ion (intact peptide with adduct) produces a specific product ion in MS/MS mode, which is a characteristic ion from an adduct. The Michael addition adducts of HNE at His, for example, produces an immonium ion of modified His at m/z 266. Precursor ion scans can be set to identify all peptides with a Michael addition adduct of HNE at His (precursors) that produce m/z 266 fragment in MS/MS, and MS/MS spectra from these precursors can be acquired to confirm the sequences and location of modification of the peptides (Bolgar and Gaskell 1996). In precursor ion scans, the selectivity of precursor ion identification is determined by the exclusiveness of formation of the fragment ion. All Michael addition adducts of HNE can produce a fragment ion at m/z 139 (dehydrated HNE). However, this fragment is not exclusively generated from HNE adducts. Using m/z 139 as fragment ion in precursor scan can result in identification of some precursor ions that do not have HNE adducts (Bolgar and Gaskell 1996). In constant neutral loss scans (Figure 9.12), intact peptides that can generate a fragment with known fixed difference in mass from the intact peptide are identified. This technique is useful for unstable adducts that can be easily lost in fragmentation. The Michael addition adduct of HNE can easily lose HNE (M-156) in fragmentation. It is possible to identify all Michael addition adducts of HNE by identifying peptides that have M-156 fragment in a constant neutral loss scan. However, there is difficulty in predetermining the appropriate fragmentation energy for different peptides and their different charge states (Bolgar and Gaskell 1996).

The sensitivity of precursor ion scanning and constant neutral loss scanning is relatively low. An alternative precursor detection method with a hybrid quadrupole-time of flight (Q-TOF) instrument was developed recently (Bateman et al. 2002). In this method, MS scans at alternating low and high collision energies are acquired. The spectra are compared to ascertain whether there are fragment ions in high-collision-energy spectrum that match with ions in low-collision-energy spectrum with a fixed mass difference. Once such precursor ions are identified, MS/MS spectra from these precursor ions are immediately acquired in a data-dependent acquisition mode. With this technique, better sensitivity and selectivity can be obtained because the Q-TOF instrument has excellent mass resolution and can identify constant neutral loss with higher mass accuracy.

9.7.5. Quantification of Protein and Adduct by MS

Mass spectrometry can also be used to measure protein adduct levels with high sensitivity and selectivity. Quantification is usually done by analyzing

the digested samples by LC/MS. Adduct levels can be calculated from the peak areas of ion chromatogram specific to the peptide with adduct. To increase the specificity of adduct quantification, a technique called multiple ion monitoring can be used. In this technique, ions of adduct-containing peptides are selected and fragmented. The fragment ions specific to the peptides can be selectively detected. Adduct levels can be calculated from the peak areas of the ion chromatograms, and peak area ratios from different fragments can be used to confirm that the detected levels are indeed of the targeted adduct. Signals from different peptides vary significantly; therefore, an internal standard must be used in quantification. The best internal standard should have similar chemical and physical properties as the compounds to be quantified and stable isotope-labeled compounds are the best internal standards in MS quantification. For quantification of absolute amount, authentic compound to be quantified and internal standard are required.

The Michael addition adduct of α,β-unsaturated aldehydes with Cys residues in protein has a thioether bond. It can be reduced under strong reducing conditions (Uchida and Stadtman 1992). Methods based on this reduction have been established to detect protein adduct of HNE and 1,4-dihydroxy-*trans*-2-nonene (DHN), the reduced adduct of HNE, in plasma or whole-blood proteins as shown in Figure 9.13 (Veronneau, Comte and Des Rosiers 2002; Asselin et al. 2006). The HNE adduct was first reduced to the DHN adduct with $NaBD_4$, which introduced a deuterium to the DHN from HNE adduct to distinguish the DHN adduct. The proteins were then precipitated and reduced with Raney Ni at 55°C for 20 hours to release DHN. The released DHN was then extracted with ethyl acetate, derivatized with dimethyl-*tert*-butylsilyltrifluoroacetamide (MtBSTFA) to TBDMS, and analyzed by gas chromatography (GC)/MS with D_{11}-DHN as internal standard. This method provides very high sensitivity. It measures the total level of HNE adduct without identifying specific location of adduct in protein and has been used to measure HNE adduct as biomarker of HNE exposure (Asselin et al. 2006).

Some techniques have been developed to measure relative changes, instead of absolute amounts of protein. In these cases, the relative quantification can be accomplished by adding different tags to samples from different treatments. Authentic compounds are not needed and best results are obtained when tags with different isotope compositions are used.

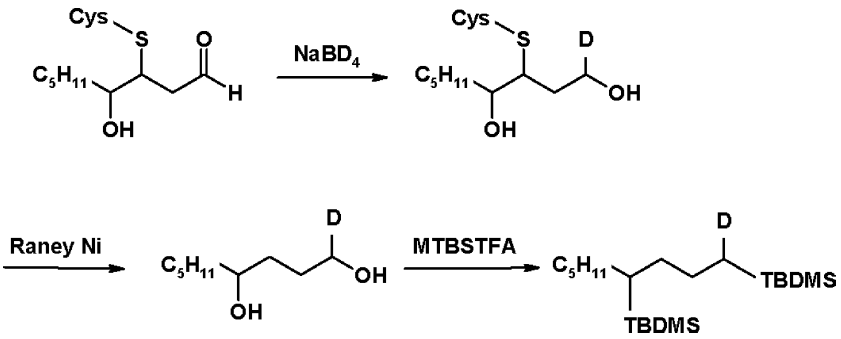


Figure 9.13 Derivatization of HNE adducts at cysteine residues.

Relative quantification of proteins is most often done after digestion of samples with protease. Chemical tags, such as phenylisocyanate (PIC), can be added to proteins before or after digestion. Labeling proteins with tags before digestion will limit the labeling to some of the peptides from proteins. With specially designed tags that include a moiety that is separable using chemical affinity techniques, the labeled peptides can be separated from unlabeled peptides. This simplifies subsequent detection and increases sensitivity by enriching the sample in labeled peptides. This approach has limited application in protein adduct quantification because the adduct can form on peptides without such targeted residues and adducts may form on the same targeted residues. These limitations can be avoided by adding tags to either N- or C-terminus of peptides after digestion. In most cases, all peptides can be labeled with tags (except, in some cases, peptides at N- or C-terminus of proteins). Therefore, relative changes of all proteins at any locations can be monitored, if the peptides are detected by MS analysis. Another advantage of this approach is multiple peptides from same protein can be detected. The agreement of relative changes from different peptides will make the detection of relative change of protein more reliable.

This approach has been used to study the kinetics of HNE adduct formation in HSA (Szapacs et al. 2006). Human serum albumin was incubated with HNE and samples at different time points were collected. The samples were reduced with $NaBH_4$ to stabilize HNE adducts and digested with trypsin. The N-termini of treated samples were labeled with $^{12}C_6$-PIC, referred as light tag, and mixed at 1:1 ratio (based on HSA amount) with a reference sample which was obtained from 24-hours incubation with HNE and prepared the same way except that it was labeled with $^{13}C_6$-PIC (heavy tag). The samples were then analyzed by LC/MS/MS and ion chromatograms of characteristic fragments of sample and reference peptides were extracted. The amount ratios (sample/reference) were obtained from the average of light/heavy ratios of three fragments from same peptide and rate constants of formation of these HNE adducts were calculated from the amount ratios. An alternative approach for labeling involves use of $H_2{}^{18}O$ for labeling the carboxy terminus (reviewed by Miyagi and Rao, 2007.)

9.8. Conclusions and Summary

The purpose of this chapter has been to describe the dynamic process of aldehyde–protein adduct formation and their characterization. Understanding how reactive aldehydes are formed during lipid peroxidation and oxidative stress and how much of these products are formed has challenged research in this area of biomedical sciences for decades. A major reason for the slow progress in understanding these processes has been the lack of clear chemical mechanisms as to how these events occur. Since aldehydes are increasingly shown to be important toxicological agents, new methods to study the chemistry and biochemistry of adduct formation by aldehydes with cellular nucleophiles are required. Mass spectrometry has increasingly become an enabling methodology in toxicology and medicine. The information reviewed in the preceding sections will focus researchers to implement these MS methods, in combination with other biochemical approaches, to

study the formation and turnover of protein adducts with aldehydes and their role in aldehyde toxicity in cells and in vivo.

The application of these MS methods in several recent studies on the reactivity of aldehydes provides encouragement to future studies of how endogenous and exogenous aldehydes evoke their toxicity or how they alter cell signaling. For example, Szapacs et al. (2006) defined in chemical terms the reaction of HNE with HSA and provided a new direction toward establishing defined biomarkers of lipid peroxidation-dependent processes easily obtained from human blood. This approaches hold promise to provide useful biomarkers in the future. In a recent study, Petersen and coworkers (Sampey et al. 2007) have convincingly shown that HNE form covalent adducts with extracellular signal-regulated kinase (Erk-1/2) at residue histidine 178, a process that is linked to inhibition of function of this important kinase. Clearly, these methods provide promise as to our ability to understand the chemical basis of oxidative stress and lipid peroxidation, as well as approaches to develop important biomarkers of human diseases caused by oxidative stress and lipid peroxidation, such as inflammation, atherosclerosis, and diabetes.

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