Modification of proteins by reactive lipid oxidation products and biochemical effects of lipoxidation

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Abstract

Lipid oxidation results in the formation of many reactive products, such as small aldehydes, substituted alkenals, and cyclopentenone prostaglandins, which are all able to form covalent adducts with nucleophilic residues of proteins. This process is called lipoxidation, and the resulting adducts are called advanced lipoxidation end products (ALEs), by analogy with the formation of advanced glycoxidation end products from oxidized sugars. Modification of proteins by reactive oxidized lipids leads to structural changes such as increased β-sheet conformation, which tends to result in amyloid-like structures and oligomerisation, or unfolding and aggregation. Reaction with catalytic cysteines is often responsible for loss of enzymatic activity in lipoxidized proteins, although inhibition may also occur through conformational changes at more distant sites affecting substrate binding or regulation. On the other hand, a few proteins are activated by lipoxidation-induced oligomerisation or interactions, leading to increased downstream signalling. At the cellular level, it is clear that some proteins are much more susceptible to lipoxidation than others. ALEs affect cell metabolism, protein-protein interactions, protein turnover via the proteasome, and cell viability. Evidence is building that they play roles in both physiological and pathological situations, and inhibiting ALE formation can have beneficial effects.

Introduction

Many types of post-translational modification (PTMs) of proteins exist, and can be broadly divided into those that are enzymatically catalysed and those that occur non-enzymatically, for example through oxidation or other chemical modifications. Although enzymatic PTMs have received more attention based on their physiological and regulatory roles, non-enzymatic modifications also cause changes in protein structure and function, often with important downstream cellular effects. While advanced glycation (or glycoxidation) end products (**Box 1**) are well recognized as the outcomes of proteins attacked by sugars or oxidized sugars and thought to contribute to disease (1-4), the formation of advanced lipoxidation end products (ALEs) is less studied. Lipoxidation refers to the formation of covalent adducts between reactive products of lipid oxidation and macromolecules such as proteins (2, 5), DNA (6, 7) and phospholipids (8, 9). Lipid oxidation can arise in vivo during situations of oxidative stress and redox imbalance, such as inflammation or environmental toxicity (10), and generates many different primary and secondary oxidation products, as reviewed previously (11-13). Lipid oxidation products (LOPs) containing aldehydes and α,β-unsaturated moieties are most reactive and can form covalent adducts. These include full length oxidized fatty acyl chains, such as isoprostanes containing cyclopentenone rings (e.g. 15-Deoxy-Δ12,14-prostaglandin J_2 and isolevuglandins (isoLGs), or products arising from non-enzymatic Hock cleavage of the oxidized fatty acyl chains, for example malondialdehyde and α,β-unsaturated acyl aldehydes such as acrolein, crotonaldehyde, 4-hydroxyhexenal (4-HHE) and 4-hydroxynonenal (4-HNE). These compounds are the most reactive, best known or most studied small reactive aldehydes derived from lipid peroxidation (12, 14), although it should be noted that some compounds, e.g. acrolein and glyoxal, can be formed from both lipid and sugar oxidation.

Recently, there has been a surge in interest in lipoxidation of proteins and its biological effects, owing to evidence that it may play a role in the pathology of diseases such as atherosclerosis, neurodegenerative diseases, autoimmune diseases and type 2 diabetes (8, 14-16). Oxidative damage to proteins, including by reactive LOPs, has also been implicated in ageing, as the level of modification increases greatly in the last third of the life span, although evidence for a causative effect is still lacking (17). Research in the area of lipoxidation is wide-ranging, but this article focuses on the biochemical aspects of protein modification: the changes to protein primary structure and the concomitant effects on tertiary structure and activity from studies in vitro with model proteins or cells, but also considers exemplar cellular effects of lipoxidation and its role in pathology.

The chemistry of lipoxidation

The electrophilic lipid oxidation products (LOPs) can react with nucleophiles on biomolecules, including proteins, nucleic acids and metabolites, via a number of different mechanisms (**Figure 1**). For proteins, LOPs containing ketones and aldehydes can form Schiff base products with lysine or arginine, via the loss of water (**Figure 1, scheme A**), although only the primary amine nucleophiles found on lysine or the protein N-terminus form generally form a relatively stable Schiff base (5, 18). The nucleophilic reaction is favoured when the amine is unprotonated, for instance under basic conditions or where the protein structure results in an increase in the pK_a of the amine (19). While reaction of aldehydes and ketones could occur with other nucleophilic amino acids such as serine or cysteine, the hemi-acetal or hemi-thial products formed are unstable in aqueous solution and are not observed. The formation of the Schiff base itself is freely reversible in aqueous solution, especially under acidic conditions, and so for isolation from a biological sample and subsequent analysis, e.g. by mass spectrometry, they are often reduced in situ to the amine using a mild reductant such as sodium borohydride to stabilize the adduct (20).

Many LOPs have multiple functional groups that allow the formation of more stable adducts (21). Bifunctional aldehydes such as malondialdehyde and methylglyoxal can form stable adducts with arginine through intramolecular reaction of the second carbonyl group following the initial Schiff base formation (**Figure 1, scheme B**). α,β-Unsaturated carbonyl-containing LOPs, can react via Schiff base formation with the carbonyl group, but more usually react by Michael addition to the β-carbon of a range of different nucleophilic amino acid side chains, most commonly lysine, cysteine and histidine (**Figure 1, scheme C**). Nitroalkenes from nitrated fatty acids also react via Michael addition (22, 23). While occasionally lipoxidation adducts with arginine have been reported, the basicity of arginine means that it is usually protonated and relatively unreactive with α,β-unsaturated carbonyl compounds. For the other amino acids, the propensity of the adduct to form depends on:

i) the nucleophilicity of the attacking atom of the amino acid side chain, which can be affected significantly by the local protein environment, but with thiol generally accepted to be the best nucleophile;
ii) the reactivity of the LOP, with the shorter chain products such as acrolein and crotonaldehyde being more reactive, and electron-withdrawing groups in substituted alkenals increasing reactivity (24);
iii) the stability of the product, which depends on the leaving group ability of the added moiety.

In practice, cysteine adducts are often observed due to good nucleophilicity of the thiol, while histidine adducts are generally observed as the most stable due to the poor leaving group nature of the imidazole at neutral pH. Additional functionality at the 4-position, such as the 4-hydroxy group in HNE, can help to stabilize the product, for example the formation of cyclic acetal in the case of 4-HNE (**Figure 1, scheme D**).

The initial adducts formed by reaction with bifunctional LOPs, such as α,β-unsaturated carbonyls, can react further with an additional molecule of the electrophile, often giving rise to stable cyclic products that appear to be particularly important in aberrant recognition of proteins (25, 26). Examples are the formation of *N*ε-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) from the reaction with two molecules of acrolein or the formation of a dihydropyridyl lysine (DHP-lysine) from reaction with two molecules of malodialdehyde and a molecule of acetaldehyde (**Figure 2, scheme A**). Alternatively, the adducts of one, two or more bifunctional LOPs initially formed can react with another protein nucleophile, resulting in inter or intramolecular crosslinks with a wide range of linear or cyclic structures. Cyclic structures such as pyrroles and furans (**Figure 2, scheme B**) are the most common ones observed from the reaction of 4-substituted α,β-unsaturated carbonyl compounds such as HNE, presumably due to their stability.

Effects on protein structure and function

Lipoxidation clearly alters the structure of the protein, and for larger, longer chain adducts, introduces relatively hydrophobic and bulky species. Thus it can have a variety of different effects at the structural and functional level, which are dependent both on the structure of the target protein and the nature of the specific aldehyde. The common types of effects are summarized in **Figure 3** and some examples are described in more detail below.

Unfolding and Aggregation

With globular proteins, modification by lipid electrophiles at low levels is initially likely to target the reactive surface residues, but small aldehydes, e.g. acrolein, are able to penetrate into binding pockets or folds, causing structural instability that subsequently leads to protein unfolding. Studies carried out with model proteins in vitro suggest that the effects are concentration dependent, and that once a threshold of modification that triggers unfolding and exposure of normally buried residues is reached, the protein becomes more susceptible to further modification. Although this has been demonstrated more extensively for direct oxidative modifications, such as metal-catalysed oxidation or HOCl-induced modification (27), there have been few studies testing lipoxidation. Even for protein

oxidation, there is disagreement over the extent of effects. For example, it has been calculated that a single oxidation event is able to destabilize susceptible proteins (28), but a study using molecular dynamics simulations of the effect of carbonyl formation by direct oxidative modification on lysine, arginine, or proline residues in the headpiece of villin suggested that high levels of modification were required to destabilize and unfold the protein (29). This was despite the fact that carbonyl formation on a lysine or arginine residue was found to be equivalent to a hydrophobic neutral mutation, which was suggested to increase the intrinsic aggregation propensity of the protein. This discrepancy between reports probably reflects differences in susceptibility of individual proteins, and is also probably the case for lipoxidative modifications. In addition to direct alteration of hydrophobicity by introduction of acyl groups, lipoxidation often alters the charge state of the protein, as adducts are formed on basic residues (lysine, arginine, histidine) leading to loss of positive charge. Michael adduct formation on cysteine may also alter the charge, as low pK_a cysteines exist in the thiolate form (negatively charged) under physiological conditions (28).

Some studies have demonstrated changes in secondary structure following treatment of proteins in vitro with reactive lipid oxidation products, typically involving an increase in the proportion of β-sheet structure compared to α-helix. Modification of the plasma protein fibrinogen by methylgyoxal, which is a product of both sugar and lipid oxidation, was found to alter the structure from approximately 86% α-helix with little β-sheet to 65% β-sheet (30). This change was associated with increased protein aggregation, which fits with the general observation that β-sheet structures provide a better environment for the intermolecular interactions required for oligomerization and fibril formation. A wider range of lipid peroxidation-derived aldehydes (4-HNE, malondialdehyde, glyoxal, and secosterols A and B) were tested on a human antibody and a light chain; they were also found to result in increased β–sheet conformation and propensity for aggregation (31). In this case, further investigation by transmission electron microscopy showed that two different types of conformational change occurred: HNE caused amyloid fibril formation, while the other aldehydes caused an amorphous aggregation. The mechanisms were also aldehyde dependent, as the secosterols induced a classic nucleated polymerization-type aggregation following a substantial lag phase, in contrast to the small aldehydes HNE, MDA and glyoxal, which caused a lag-free seeded-type aggregation. These structural changes may be induced by the stereochemical or electrostatic structural changes mentioned above.

Inhibition of enzymatic activity

As lipoxidation involves reaction with histidine, cysteine, lysine and occasionally arginine residues, which are often key residues in enzymatic catalytic sites, it is not

surprising that treatment with reactive lipid products tends to cause loss of enzymatic activity. Even if not directly involved in catalysis, modification of these residues at or close to the active site can block binding of the substrate or alter the active conformation through the types of hydrophobic effects described above. Consequently, lipoxidation is often perceived as a detrimental modification resulting in loss of function. Inhibition by lipid oxidation derived aldehydes has been observed for several enzymes, usually in vitro but data from cellular treatments and in vivo situations also exist (**Table 1**). Much early work focused on glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), owing to their high abundance and convenient activity assays, and these continue to be well-studied models. For GAPDH it is clear that inhibition by HNE or acrolein is associated with attack on cysteine residues, although at higher treatments histidine and lysine residues can also be modified (32). Similar effects have been observed more recently, and key sites of modification have been identified with the catalytic (C150) and regulatory cysteines (C152) noted as most sensitive and other cysteines (e.g. C156; C282) modified at higher acrolein levels (33, 34). Another enzyme with a catalytic cysteine that has been found to be susceptible to modification by aldehydes both in vitro and in cells is the peptidyl-prolyl isomerase Pin1. HNE forms Michael adducts on Pin1, while its keto analogue 4-oxononenal (ONE), which is more than 100-fold more reactive, led to the formation of a pyrrole-containing cross-link between C113 and K117 in the active site (35). This modification is thought to be irreversible and block the active site; although the enzyme activity was not measured in this study, it is known that oxidative damage to Pin1 occurs in Alzheimer's disease brain and causes inactivation of the enzyme (36). As tau is a substrate of Pin1 and cis-trans isomerisation is important in protein stabilization, this clearly offers a mechanism by which tau neurofibrillary tangles could occur in disease. HNE and ONE are also able to inhibit mitochondrial aldehyde dehydrogenase (ALDH2), an enzyme that detoxifies aldehydes by oxidation and contains three adjacent cysteine residues in its active site, although at lower levels they can act as substrates (37). Larger LOPs also cause enzyme inhibition, for example, the modification of the catalytic C298 of aldose reductase AKR1B1, a related enzyme, by PGA_1 and other cyclopentenone prostaglandins (38).

In pyruvate kinase, which does not contain a catalytic cysteine, modification and inhibition in vitro by several aldehydes has also been observed: the 9-carbon LOPs HNE and ONE modified both cysteine and histidine residues, decreased enzymatic activity and increased protein cross-linking (39). Acrolein, malondialdehyde and HHE were found to cause different modification profiles of pyruvate kinase, reflecting their size and reactivity, and all caused inhibition although acrolein had the strongest effect at physiological concentrations (40). While some of the residues modified were close to substrate binding

and regulatory sites, it was concluded that the severe inhibition probably related to destabilization of the globular structure, especially with longer treatment times. Similarly, the zinc-containing enzyme SIRT3 was found to be inhibited by modification at C280, distal from the active site H248, which was not adducted (41). Here it also appears that inhibition involves structural changes in the protein, specifically a shift in the Zn-binding motif leading to alteration in the catalytic cleft that prevents substrate binding.

Increased enzymatic or biological activity

Interestingly, in a small number of cases, LOP adduct formation can activate or alter the function of some proteins. One example is the GTP-binding protein H-Ras. PTMs are known to play a role in its function; it is normally isoprenylated at the C-terminal domain but additionally has two cysteines that can be palmitoylated, C181 and C184. However, cyclopentenone prostaglandins such as 15-deoxy-$\Delta^{12,14}$-Prostaglandin J_2 (15dΔ-PGJ_2) and Δ^{12}-PGJ_2 have been found to bind selectively to the H-Ras hypervariable domain at these residues, cross-linking the cysteines and activating ras signalling (42). In fact, the sites of adduct were dependent of the CyPG structure, as PGA_1 and 8-iso-PGA_1 bound to cysteine 118 in the GTP-binding motif. Activation of H-ras by CyPGs led to increased proliferation and protection from apoptosis, showing the physiological relevance of this mechanism (43). Similar studies have been carried out on GSTP1-1, a cellular defence enzyme that conjugates electrophilic compounds to glutathione for subsequent export, but also regulates stress signalling pathways. 15d-PGJ_2, HNE and other reactive lipid products caused cross-linking of GSTP1-1 via C47 and C101, but while the small aldehyde effects were reversible, the CyPG effects were not (44, 45). 15d-PGJ_2-induced oligomerisation of GSTP1-1 caused sustained c-Jun NH_2-terminal kinase activation and apoptosis. It has also been found that the mammalian intracellular lipid-binding protein epithelial fatty acid-binding protein (E-FABP) can be covalently modified by HNE at C120, which normally forms a disulfide with Cys-127 within the ligand binding cavity. As retinal epithelial cell lines from E-FABP null mice contain higher levels of HNE-modified proteins, it has been suggested that the protein may protect the cell by scavenging reactive oxidized lipids (46).

Differential susceptibility to modification

There is increasing evidence that some cellular proteins are particularly susceptible to modification by reactive electrophilic LOPs in vivo. Exposure of cells to an analogue of HNE that gave the same phenotypic response, but which had an additional azido or alkynyl

reactive group at the distal end of the alkyl chain, enabled the direct enrichment of HNE-modified proteins using highly selective click chemistry, and their subsequent identification by mass spectrometry (47, 48). Proteins involved in stress response (e.g. HSP70 and HSP90) and in redox regulation (GSTP1, peroxiredoxin-1) were identified as being particularly sensitive to modification in vivo. A broader screen using a similar approach identified >400 proteins commonly modified in 2 phenotypically-distinct cell lines using HNE and ONE. These were classified by cellular functional protein systems; protein synthesis and catabolism, mitochondrial electron transport and the cytoskeleton were identified as common targets (49). Interestingly, the cell lines also differed substantially in their intracellular GSH concentrations, and high GSH gave much lower levels of modification. This approach was developed further using an isotopically-tagged, photo-cleavable HNE derivative, to identify the sites of modification of 398 adducts (386 cysteine and 12 histidine) on 335 proteins, and demonstrated that these adducts are rapidly lost in intact cells and the rate of loss is also site-selective (50). Targets of reactive environmental electrophiles (e.g. hydroquinone and the antifungal chlorothalonil) showed a similar selectivity, with modification of enzymes from glycolysis and lipid metabolism, and cytoskeletal proteins predominating (51), while a different sub-set has been found for 2-trans-hexadecenal adducts (52). In an orthogonal approach, thiol groups on proteins were identified using a thiol-reactive click chemistry probe to enrich cysteines unmodified by the added electrophile, which enabled targets of HNE (53) and acrolein (54) to be mapped. These chemical proteomics approaches overcome some of the limitations of previous approaches using biotin hydrazide, which reacts with any aldehyde- or ketone-containing moiety, and antibody approaches where cross-reactivity is a significant issue. A common finding is that the susceptible proteins tend to be modified on hyper-reactive cysteine residues with low pK_as. This is supported by a recent meta-analysis of electrophilic adduct formation, which suggested that lysine and histidine modification was more random than that of cysteines (55).

Cellular outcomes of lipoxidation

The cellular outcome of protein lipoxidation depends on the protein being modified, the nature of the modification, and the extent of the modification. The latter is likely a critical factor that may switch the response from protective to deleterious, which can be illustrated by considering the effect of protein unfolding. Slight modification by longer chain adducts increases the hydrophobicity of the protein, both directly and through mild unfolding exposing hydrophobic residues. Exposure of hydrophobic regions leads to enhanced

degradation by the 20S proteasome, the system mainly responsible for degradation of oxidatively modified proteins. In contrast, extensive modification involving covalent cross-linking of proteins causes aggregation, and these stabilized aggregates usually inhibit proteasomal activity, resulting in cellular accumulation of non-degradable products as reviewed by (15, 56, 57). Some amyloid-type aggregates cause similar effects, and this outcome is thought to contribute to neuronal death in Alzheimer's disease and retinal damage in ageing (58). Moreover, protein modification and unfolding can also trigger the unfolded protein response (UPR) (59, 60). On the other hand, proteasomal inhibition following modification by reactive lipids and cross-linking proteins by photodynamic therapy has been suggested as an approach to tumour cytotoxicity (61).

Electrophilic modification of proteins may also alter gene expression, either by direct or indirect effects. KEAP1-Nrf-2 is a prominent example: adduction of KEAP-1 cysteine residues inhibits the E3 ligase activity, causing accumulation of the leucine-zipper transcription factor Nrf-2 in the nucleus, where it binds to the antioxidant response element (ARE, also known as EpRE or the electrophile response element) in target genes encoding phase II detoxifying enzymes, antioxidant and anti-inflammatory proteins (62). KEAP-1 contains many cysteines that can be cross-linked by a variety of electrophilic compounds, including α,β-unsaturated aldehydes and nitro-lipids, as reviewed previously (23, 63). This is a protective response, as is the heat shock response (HSR) regulated by chaperone heat shock proteins (HSPs) and heat shock transcription factors (HSFs). HSP70 and HSP90 can be modified by HNE and nitro-fatty acids (48), allowing detachment of HSF1 and its binding to target genes, which leads to transcription a variety of molecular chaperones, regulatory and repair enzymes (64, 65). A moonlighting transcription factor function is also known for acrolein-conjugated GAPDH, which can translocate to nucleus and induce apoptosis (66). In other cases, epigenetic mechanisms may be involved: methylglyoxal causes specific disruption of histone H2B acetylation, affecting chromatin structure and altering gene transcription, especially in cells lacking glyoxylase-1 (67).

As mentioned above, cytoskeletal proteins, in particular actin, lamins, glial fibrillary acidic protein (GFAP) and vimentin, have been reported as common targets of lipoxidation by electrophilic oxidized lipids (68). These modifications cause significant rearrangements of the cytoskeleton, such as strong filament bundling, retraction from the cell periphery and juxtanuclear condensation of intermediate filaments such as GFAP (69) or vimentin (70). Nitro-phospholipids have similar effects (71). For vimentin, the response depends on C382 and a close interplay with protein phosphorylation and zinc binding has been demonstrated (72). The functional relevance of these effects still not well understood; it has been suggested that they could be protective, by isolating abnormally folded proteins for

lysosomal clearance, or detrimental via mitotic abnormalities or increased immunogenicity. Indeed, increased immunogenicity has been reported for several lipoxidized proteins, such as IgG modified by acetaldehyde (73), and methylglyoxal adducts of histone H2A, which form amorphous aggregates (26).

Modification of extracellular proteins can also affect cells via binding and activation of cell surface receptors; for example, RAGE is a known receptor for AGEs that can lead to activation of NFκB and inflammatory signalling (74). Recent work has demonstrated that ALEs on albumin that involve neutralization of a positive charge surrounded by acidic residues are also binders of RAGE (75), unveiling a novel mechanism for influencing immune function. AGEs and ALEs are essentially damage-associated molecular patterns (DAMPs); AGEs can also interact with other pattern recognition receptors, such as Toll-like receptors (76), and ALEs are considered to be oxidation-specific epitopes with effects on innate immunity (77).

Conclusions and perspectives

In this short overview, some examples of cellular proteins known to be modified by reactive electrophilic lipids and their effects have been described, but the full list is long and steadily expanding. Several reviews address this, and consider their occurrence and importance in a variety of diseases (8, 13, 15, 78). Consequently, there is steadily growing interest in the therapeutic potential of interventions targeting the formations of both AGEs and ALEs. “AGE-breakers” were reported more than 20 years ago and reported to be beneficial in diabetes (79) and arterial disease (80). More recently, carnosine-based scavengers of ALEs have been investigated (81) and found to improve inflammatory conditions associated with obesity (82). As well as biochemical and biological in mammalian cells, it is important to bear in mind that plant proteins are also susceptible to modification, and plants have analogous response systems that are of considerable interest in the development of stress resistance (83). Research is also ongoing in the food industry, where lipoxidation of plant- or meat-derived proteins is considered to contribute to food deterioration (84-86). In view of the importance across several disciplines, advanced methods to analyse lipoxidized proteins and determine the nature and location of the modification are important, as has been described elsewhere (5, 12). In particular, it is important to appreciate that methods other than liquid chromatography tandem mass spectrometry may not discriminate between carbonyls formed by direct oxidation and lipoxidation adducts (**Box 2**). Characterization of specific adducts is important, as it may offer more potential for diagnosis of different inflammatory diseases, although this is likely to remain a challenging area for some years.

Summary Points

- Reactive oxidized lipid products form a wide variety of covalent structural adducts with proteins, a process called lipoxidation.
- Proteins containing hyper-reactive cysteine residues are most susceptible to modification, although adducts on lysine and histidine residues also occur.
- Lipoxidation can cause inactivation of enzymes, altered macromolecular interactions, unfolding and enhanced proteasomal degradation, but also upregulate cellular defence responses.
- Under extreme conditions, lipoxidized proteins form aggregates that disrupt proteasomal function and cytoskeletal structure, causing cytotoxicity and contributing to pathology.

Abbreviations list

AGE, advanced glycation / glycoxidation end product; ALE, advanced lipoxidation end product; CyPG, cyclopentenone-containing prostaglandin; DHP-lysine, dihydropyridyl lysine; 15dΔ-PGJ_2, 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2; E-FABP, epithelial fatty acid-binding protein; FDP-lysine, *N*ε-(3-formyl-3,4-dehydropiperidino)lysine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GSH, glutathione; GSTP1-1, glutathione-S-transferase P1-1; HHE, 4-hydroxyhexenal; HNE, 4-hydroxynonenal; HSP; heat shock protein; isoLG, isolevuglandin; KEAP-1, Kelch-like ECH-associated protein 1; LOP, lipid oxidation product; MDA, malondialdehyde; NFκB, nuclear factor κB; Nrf2, Nuclear factor erythroid 2-related factor 2; ONE, 4-oxononenal; PGA_1, prostaglandin A_1; PTM, post-translational modification; UPR, unfolded protein response.

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Figure Legends

Figure 1. Formation of lipid peroxidation product adducts with protein residues. (A) shows the formation of the Schiff base between an aldehyde (nonanal in this case), and stabilization of the adduct by reduction with sodium borohydride to form the secondary amine. (B) shows the intramolecular reaction of the bis-carbonyl containing compounds methylglyoxal and malondialdehyde with arginine to give stable imidazole and pyrimidine products respectively. (C) shows the mechanism of Michael addition to 2-nonenal as a model αβ-unsaturated aldehyde, where X can be lysine, cysteine, histidine or arginine, and stabilization of the adduct by reduction with sodium borohydride to form the alcohol. (D) shows the additional stabilization of the Michael adduct of HNE by the formation of the cyclic acetal. Red denotes Schiff base formation, blue denotes Michael addition.

Figure 2. Rearrangements and cross-linking by LOP adducts. (A) shows the reaction of lysine with two molecules of acrolein, a model αβ-unsaturated aldehyde, to form the formyldihydropyridyl lysine (FDP lysine), or two molecules of malondialdehyde and a molecule of acetaldehyde to give the dihydropyridinyl adduct (DHP-lysine). (B) shows the formation of a pyrrole crosslink formed from 4-HNE, an example of the many crosslinking reactions of LOPs.

Figure 3. Overview of the effects of lipoxidation on proteins and enzymes. The main types of effects are shown, including activation, inactivation, secondary structural changes

from α-helix to β-sheet motifs leading to oligomerization, and unfolding leading to random aggregation. Key cellular outcomes are written in *italics*.

Boxes

Box 1. AGEs and ALEs

- Advanced Glycation End products (AGEs): the formation of non-enzymatic covalent adducts of proteins with sugars. *Precursors: e.g. glucose, fructose and galactose*. [Sometimes also used to refer to glycoxidation end products.]
- Advanced Glycoxidation End products (also called AGEs): the adducts formed from the reaction of oxidized sugars with proteins. *Precursors: e.g. glyoxal and methylglyoxal, glycolaldehyde, dehydroascorbate*.
- Advanced Lipoxidation End products (ALEs): covalent adducts formed by the analogous reaction of lipid oxidation products with proteins, DNA or phospholipids. *Precursors: e.g. glyoxal and methylglyoxal, common with AGEs, and also lipid-specific products e.g. MDA, acrolein, HNE, 15dΔ-PGJ$_2$.*

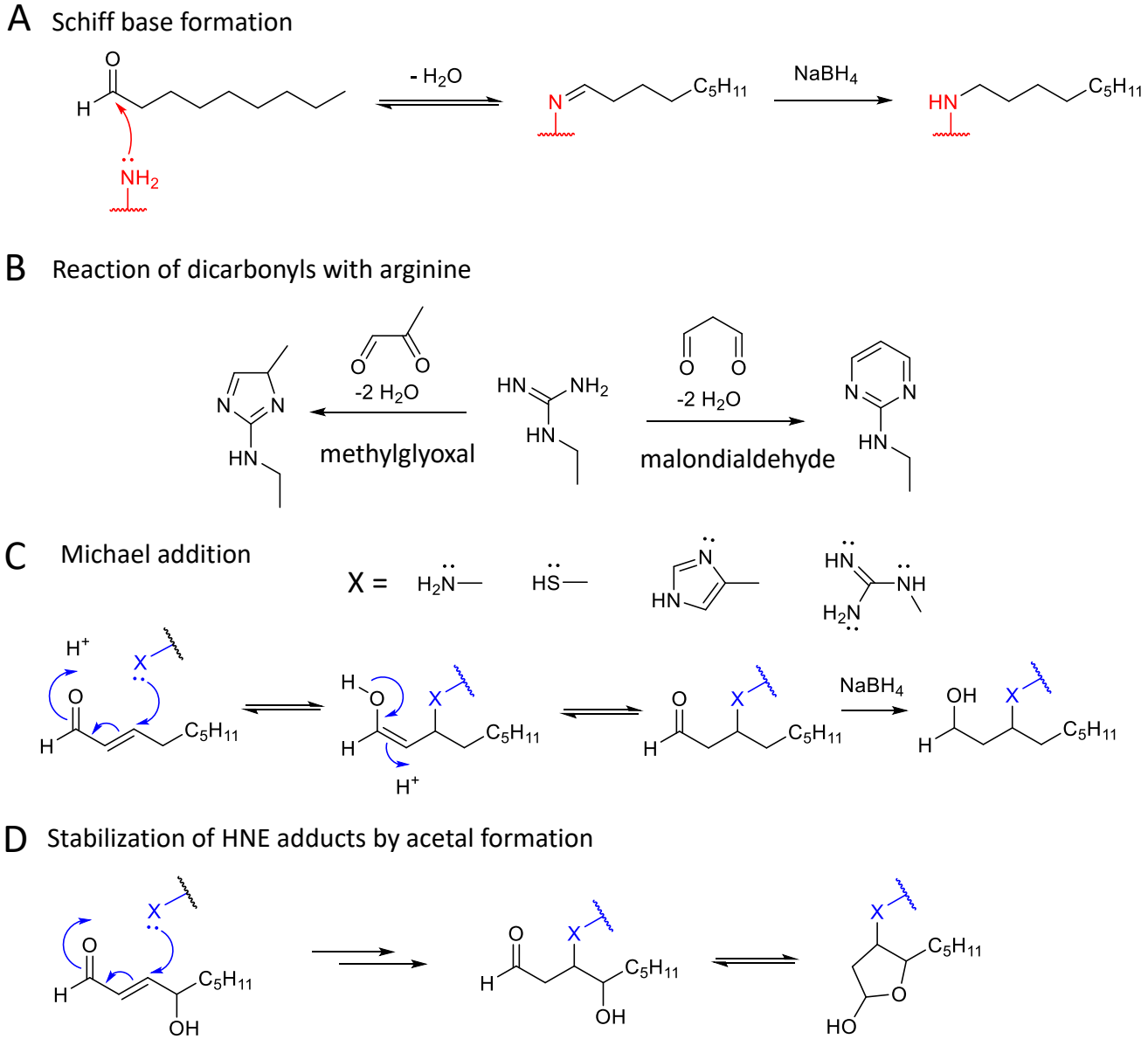
Box 2. "Carbonylation" vs carbonyl formation vs lipoxidation

The term "carbonylation" is often used scientific articles on the reactions of lipid-derived aldehydes with proteins. However, in chemistry carbonylation refers to the addition of carbon monoxide (CO) to organic or inorganic compounds, in a process that yields products containing a carbonyl group (-CO). The term has subsequently been adopted to mean the formation of carbonyl groups on proteins by other mechanisms, such as oxidative deamination, oxidative decarboxylation, hydroperoxide degradation, or Michael addition of carbonyl-containing molecules. None of these reactions involve addition of CO to the protein, and therefore chemically are not carbonylation. We recommend "protein carbonyl formation" as a more correct term. Furthermore, it is important to note that protein carbonyls can be formed by direct oxidation of several amino acids, and lipoxidation does not always result in adducts containing a free carbonyl, especially those formed by Schiff's reactions. Therefore methods involving carbonyl-reactive probes do not confirm the occurrence of lipoxidation unless further analysis using mass spectrometry is carried out. Advanced LC-MSMS techniques are required to determine the molecular location of the adduct.

Table 1. Examples of protein targets of lipoxidation, the reactive carbonyl species they have been reported to be modified by, and the biochemical effect.

	Target	Modifier / modification	Effect	Ref
Unfolding, aggregation, structural changes	Villin [a]	Oxidation modification of lys, arg or pro	High levels required to cause destabilization.	29
	Fibrinogen	Methylglyoxal	Alteration of secondary structure, 86% α-helix to 65% β-sheet	30
	Human antibody LC	4-HNE, malodialdehyde, glyoxal, secosterols A and B	Increase in β-sheet leading to aggregation. HNE caused fibril formation, other aldehydes amorphous aggregation	31
	Cytoskeletal proteins (actin, lamins, etc)	Electrophilic LOPs	Cytoskeletal rearrangement	68
	Histone H2A	Methylglyoxal	Aggregation	26
Inhibition	GAPDH	HNE and acrolein	Attack on active site and regulatory cys leading to inhibition. High levels cause other modifications	35
	Pin-1	HNE and ONE	HNE - Michael adducts, ONE - pyrrole crosslinks	36
	ALDH2	HNE and ONE	Cys modification	37
	Aldose reductase AKR1B1	PGA_1 and other cyclopentenone prostaglandins	Cys modification at higher concentrations	38
	Pyruvate kinase	Acrolein, HNE and ONE	Cys and his modification, loss of activity, crosslinking	39, 40
	SIRT3		Cys modification leading to structural changes	41
Enzyme or signaling activation	H-Ras	Cyclopentenone prostaglandins	Cysteine modification. Activate Ras signalling	42
	GSTP1-1	HNE, CyPG, other aldehydes	Crosslinking and activation, CyPG effects irreversible	44, 45
	E-FABP	HNE	Modification of inhibitory cysteine	46
	KEAP-1	Electrophiles	Modification of cys, activation of Nrf2	62
	HSP70 and HSP90	HNE, nitro-FA	Detatchment and activation of HSF1	48, 64, 65
	GAPDH	Acrolein	Moonlighting as TF leading to apoptosis	66
	RAGE	AGEs and ALEs	Activation of NFκB signaling	74

[a] Involved molecular dynamics simulations. All other studies involved practical analysis.



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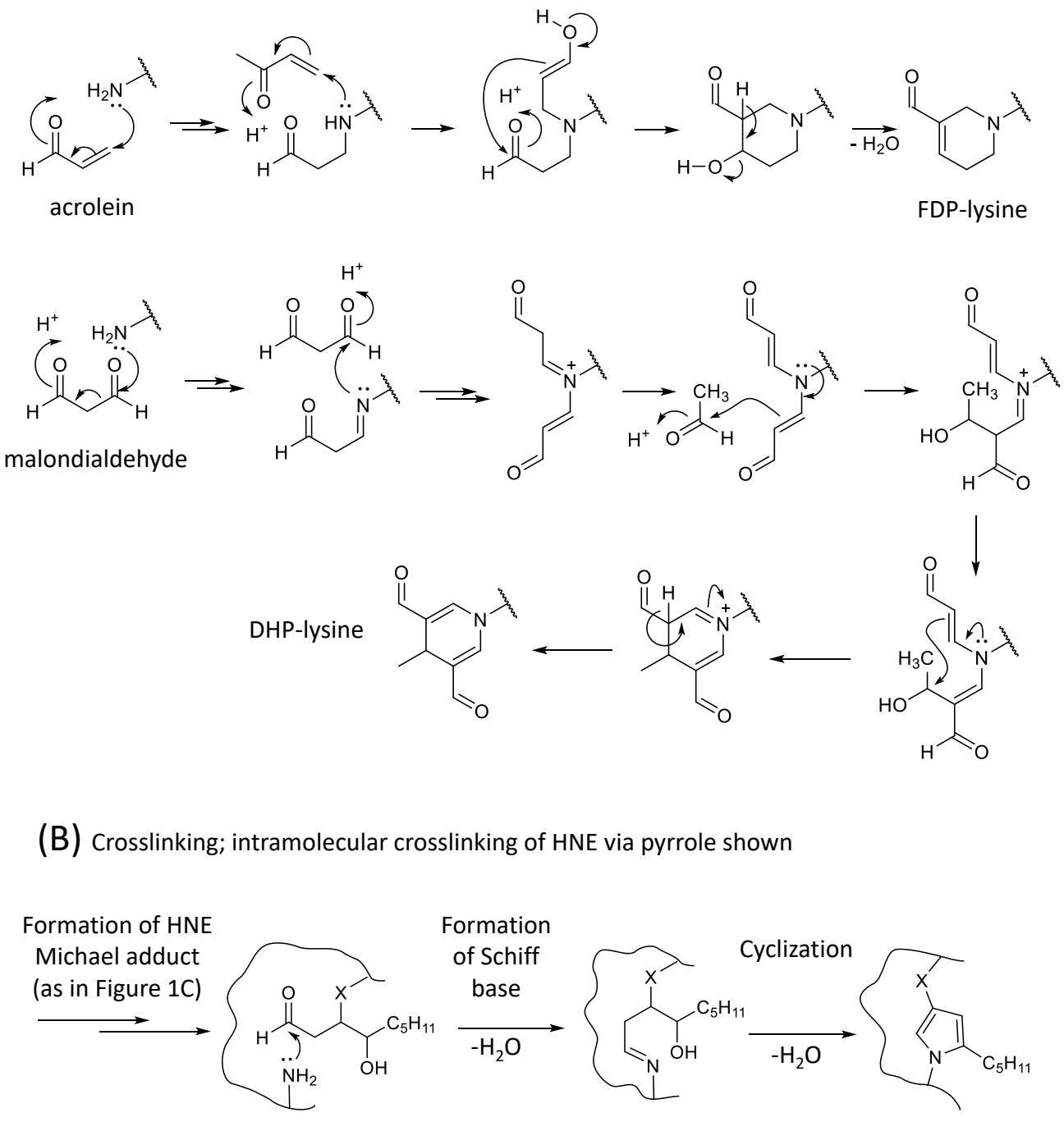

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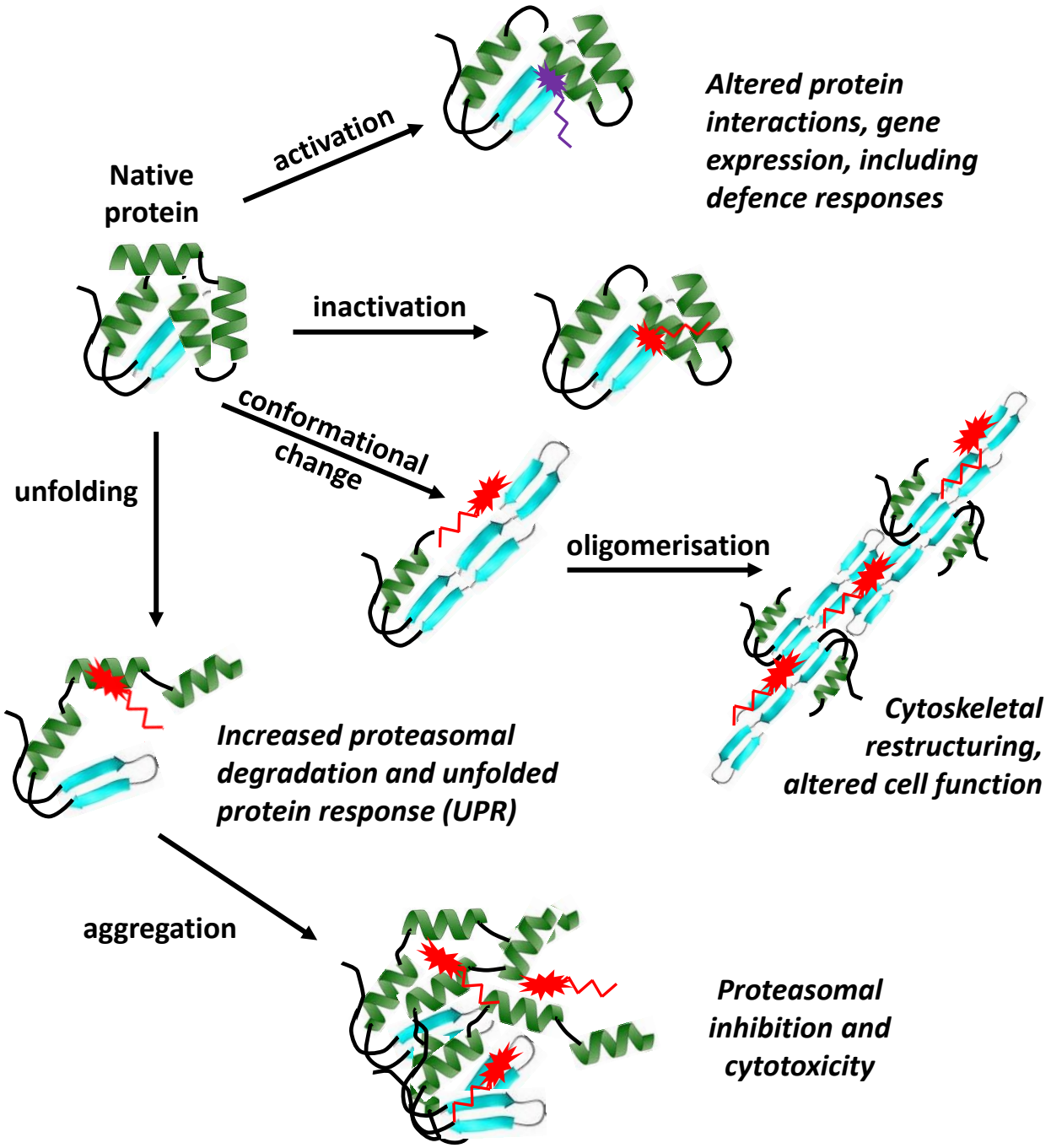

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