Protein Damage by Reactive Electrophiles: Targets and Consequences

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It has been 60 years since the Millers first described the covalent binding of carcinogens to tissue proteins. Protein covalent binding was gradually overshadowed by the emergence of DNA adduct formation as the dominant paradigm in chemical carcinogenesis but re-emerged in the early 1970s as a critical mechanism of drug and chemical toxicity. Technology limitations hampered the characterization of protein adducts until the emergence of mass spectrometry-based proteomics in the late 1990s. The time since then has seen rapid progress in the characterization of the protein targets of electrophiles and the consequences of protein damage. Recent integration of novel affinity chemistries for electrophile probes, shotgun proteomics methods, and systems modeling tools has led to the identification of hundreds of protein targets of electrophiles in mammalian systems. The technology now exists to map the targets of damage to critical components of signaling pathways and metabolic networks and to understand mechanisms of damage at a systems level. The implementation of sensitive, specific analyses for protein adducts from both xenobiotic-derived and endogenous electrophiles offers a means to link protein damage to clinically relevant health effects of both chemical exposures and disease processes.

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1. Introduction

The covalent modification of proteins by xenobiotics was first reported 60 years ago by Elizabeth and James Miller, who observed a yellow pigment irreversibly bound to liver proteins in rats treated with methylaminoazobenzene carcinogens (*1*). Figure 1 depicts visible spectra of the chromophores of the carcinogens 3-methyl-4-dimethylaminoazobenzene and its N-monomethyl derivative in solution and of the yellow pigments bound to protein following administration in vivo—the first covalent binding analysis (*2*). This initial observation was followed by several reports that characterized protein binding by polycyclic aromatic hydrocarbons and other azo dye carcinogens, as described in an early review by the Millers (*3*). Soon thereafter, characterization of the structure and function

of DNA as genetic material and the discovery of carcinogen–DNA adducts in the early 1960s (*4, 5*) established a DNA damage/mutation/carcinogenesis paradigm that dominated the field for the next 40 years. Interest in protein covalent binding by carcinogens gradually disappeared and did not resurface until 20 years later, when studies by Ehrenberg, Tannenbaum, and Farmer employed adducts to hemoglobin as biomarkers of carcinogen exposure (*6–9*).

The idea that protein covalent binding contributed to the toxicity of drugs and other chemicals emerged in the early 1970s from the laboratories of Brodie, Gillette, and Reynolds who measured covalent binding of radiolabeled hepatic toxicants to liver proteins following treatment of rats and mice in vivo (*10, 11*). A classic series of papers published in 1973 by Jollow, Mitchell, Gillette, and colleagues described the role of metabolism and covalent binding in acetaminophen hepatotoxicity (*12–15*). Key findings from this work were that microsomal enzymes metabolized acetaminophen to a reactive metabolite that covalently bound to microsomal proteins in vitro and that in vivo metabolism and covalent binding of the drug were tightly correlated. Moreover, covalent binding of the acetaminophen metabolite was blocked by reaction with hepatic glutathione, in what emerged as a critical detoxication process. These studies established the key elements of the covalent binding/toxicity paradigm, which has been extended to account for the toxicity of diverse xenobiotics.

The covalent binding hypothesis of chemical toxicity offered an appealing, broadly applicable mechanism to explain the toxicities of structurally diverse xenobiotics. However, the covalent binding hypothesis had two major problems. First, not all covalent binding necessarily leads to toxicity, as studies with nontoxic acetaminophen analogues were able to demonstrate covalent binding in the absence of toxicity (*16, 17*). The lack of a clear causal relationship between binding and toxicity complicates the interpretation of covalent binding data (*18, 19*).

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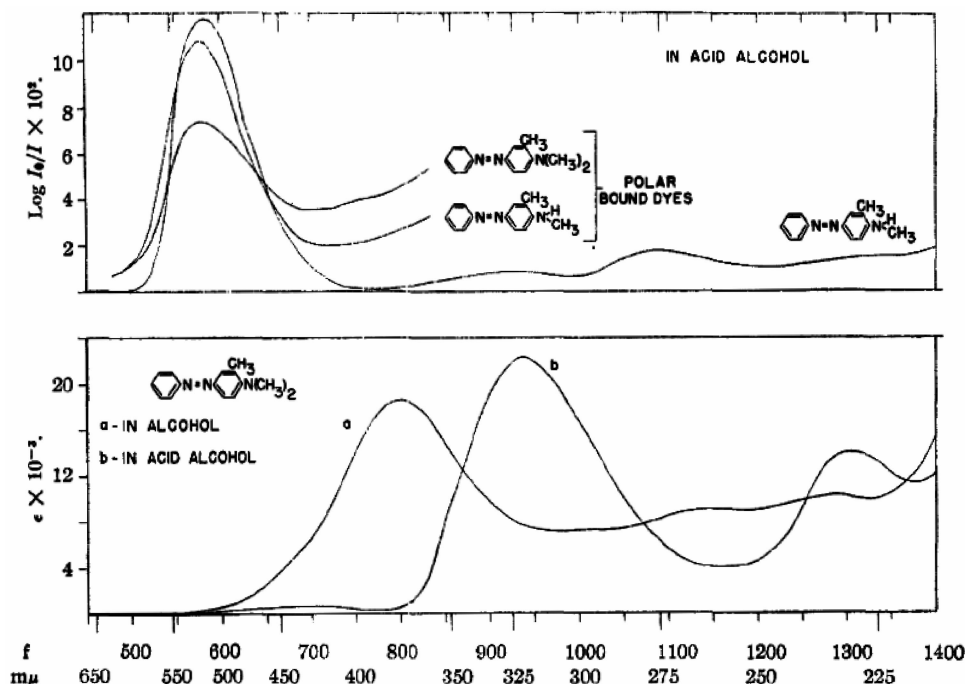
Figure 1. Visible spectra of the carcinogens 3-methyl-4-dimethylaminoazobenzene, its N-monomethyl derivative, and the protein-bound chromophores formed in liver following administration of the same compounds to rats in vivo. Reprinted with permission from ref *2*. Copyright 1948 American Chemical Society.

This does not necessarily mean that binding is unimportant but simply that the relationship between binding and toxicity mechanisms is not simple. Second, the covalent binding hypothesis was untestable at the level of specific biochemical and chemical mechanisms, because it was impossible to identify protein targets of reactive electrophiles with the analytical technologies of the 1970s and early 1980s. Protein covalent binding was measured (and still is in most cases) as chemical-derived radiolabel bound to bulk tissue or cell protein (*18*). This analysis only allows overall trends in bioactivation and covalent binding to be estimated across all affected proteins but indicates nothing about specific targets. In contrast, the technology for chemical characterization of specific nucleic acid adducts was much more advanced and contributed substantially to the validation of the DNA damage hypothesis of chemical carcinogenesis. This disparity in analytical methodology inevitably contributed to widespread ambivalence regarding the importance and relevance of protein adduction in toxicity and carcinogenesis. Nevertheless, analysis by relatively crude radiochemical assay of covalent binding to proteins in both in vitro and in vivo models has been widely used to identify compounds with significant potential to exert toxicity via bioactivation (*18*).

The above synopsis describes the understanding of protein covalent binding and its significance in chemical toxicity 20 years ago, when *Chemical Research in Toxicology* was founded. Since then, advances in analytical technology for immunochemistry, affinity labeling, MS[1]-based proteomics, definition of

specific cell-signaling pathways associated with stress, and the integrative tools of bioinformatics have revolutionized the field. The following sections describe the major developments in the analysis and significance of protein damage by reactive electrophiles.

The terms "protein covalent binding" and "protein adduction" have been widely used to denote protein covalent modifications generated by electrophiles, typically from xenobiotics. However, the scope of this phenomenon is broader than its original context of protein adducts formed by toxic drugs and carcinogens and is now understood to encompass modifications by endogenously generated electrophiles resulting from oxidative stress, glycation, and related processes. Moreover, the changes induced may include the formation of adducts with electrophiles but also protein oxidations produced by reactive oxygen and nitrogen species. In addition, stress may perturb the distribution of endogenous regulatory modifications to proteins, including phosphorylation, acylation, methylation, ubiquitination, and sumoylation (*20–23*). The modification of proteins by endogenously generated reactive intermediates is now thought to be a key element in the molecular pathology of diseases involving inflammation and oxidative damage, including atherosclerosis, neurodegenerative diseases, diabetes and associated metabolic diseases, asthma and related pulmonary diseases, and cancer. Thus, damage by xenobiotic-derived electrophiles associated with toxicity and endogenous electrophiles and oxidants associated with disease represents essentially the same phenomenon. This mechanistic link between the molecular toxicology and the molecular pathology of disease underscores the potential of fundamental research in toxicology to impact human health. In this article, I use the term "protein damage" to refer broadly to covalent protein adduction and oxidation and the more specific term "protein adduction" to refer to electrophile-derived covalent modifications.

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[1] Abbreviations: 15-deoxy-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin-J$_2$; AMS, accelerator mass spectrometry; ARE/ERE, antioxidant response element/electrophile response element; ATF6, activating transcription factor 6; BiP/GRP78, immunoglobulin heavy chain-binding protein/glucose-regulated protein of molecular mass 78 kDa; BMCC, 1-biotinamido-4-(4′-[maleimidoethylcyclohexane]carboxamido)butane; ER, endoplasmic reticulum; HNE, 4-hydroxy-2-nonenal; keap1, Kelch-like ECH-associated protein 1; MALDI-MS, matrix-assisted laser desorption–ionization mass spectrometry; MS-MS, tandem mass spectrometry; Nrf2, nuclear factor-E2-related factor 2;

IAB, N-iodoacetyl-N-biotinylhexylenediamine; IRE1, inositol-requiring enzyme 1; LC-MS-MS, liquid chromatography–tandem mass spectrometry;

PEO-IAB, (+)-biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine; PERK, PKR-like ER protein kinase; eIF2α, eukaryotic translation initiation factor 2, α-subunit, kinase; PIC, phenylisocyanate; ONE, 4-oxo-2-nonenal.
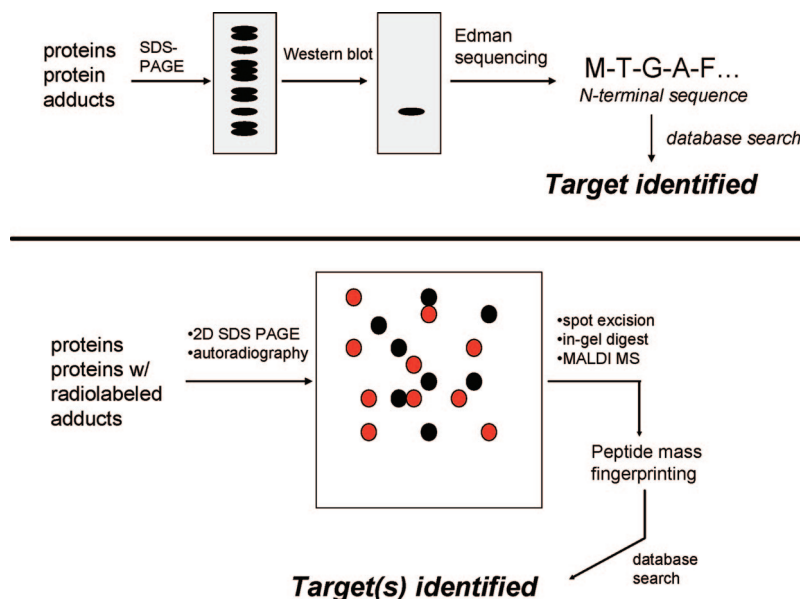
Figure 2. Detection and identification of protein adducts using SDS-PAGE and Western blotting with antibodies against adducts (top). Application of 2D SDS-PAGE to resolution and detection of protein adducts (bottom). The example depicts use or radiolabel to locate adducts, although Western blotting with antibodies against adducts is also used (see the text for a discussion).

2. Analysis of Protein Damage: An Analytical Challenge

The analysis of protein damage is complicated by two major problems. First, many different proteins are modified by reactive intermediates in cell and tissue proteomes. This was evident in the first reported electrophoretic studies of liver proteins modified by yellow pigment in azo-dyed treated rats and has been observed by similar approaches with many other protein modifications. Second, modified proteins apparently make up only a small fraction of proteins present in cell and tissue extracts. This reflects both the relatively low stoichiometry of protein modification (in most cases) and the diversity of protein targets. Often described as a "needle in a haystack" problem, analyzing protein damage is really more like looking for dozens of needles in thousands of haystacks. Thus, global analysis of protein covalent binding requires tools for affinity enrichment of specific adducted or modified forms and identification methods capable of resolving and detecting anywhere from dozens to thousands of different modified species. The evolution of approaches to detect and identify protein adducts is described in the following sections. The examples cited are illustrative of the approaches and their applications but do not constitute a comprehensive literature review. Unfortunately, there is no comprehensive database resource for all data on protein modification by various reactive intermediates. However, Hanzlik and colleagues have implemented a useful database restricted to xenobiotics and their reported protein targets (see http://tpdb.medchem.ku.edu:8080/protein_database/) (*24*).

3. Immunochemical Detection and Analysis of Protein Adducts

Initial progress in the molecular characterization of protein damage came from the application of immunochemical methods in the 1980s and early 1990s. Satoh, Pohl, and colleagues first reported that antibodies raised against Nε-trifluoroacetyllysine recognized hepatocytes and specific hepatocyte proteins from rats treated with the inhalation anesthetic halothane (*25, 26*). Trifluoroacetylation is a covalent modification produced by a reactive metabolite of halothane. Sera from humans with

halothane-induced hepatitis recognized multiple ER proteins, including a trifluoroacetylated carboxylesterase (*27*). The antibodies against trifluoroacetylated proteins were used in Western blot analyses to identify numerous proteins modified by halothane or other trifluoroacetylating chemicals in vivo and in vitro (*28–30*). This approach was extended to analysis of other protein adducts by the laboratories of Hinson, Cohen, Pumford, and several other groups to identify putative protein targets of covalent adduction by xenobiotic metabolites (Figure 2). Approximately 30 putative targets of about a half dozen electrophiles were identified in this manner (*31–41*). This body of work represented tremendous efforts by these groups in antibody development and target identification. Nevertheless, there were drawbacks to the approach. The antibodies used lacked the affinity for efficient immunoenrichment of adducts and were used primarily for detection by Western blotting. Proteins in immunoreactive bands were identified by Edman sequencing or peptide mass fingerprinting by MS. The problem with this approach is that these analyses did not actually detect adducts but instead identified proteins that comigrated with immunoreactivity. The use of one-dimensional SDS-PAGE to resolve complex protein mixtures also meant that immunoreactive bands inevitably contained multiple proteins, thus adding further ambiguity to the identification process. Moreover, it is nearly impossible to distinguish the detection of truly adducted proteins from nonspecific reactivity of the antibodies.

4. Proteomics Approaches To Identify Protein Targets of Electrophiles: Initial Studies and Two-Dimensional (2D) Gel-Based Proteomics Approaches

A major advance in the analysis of protein adducts was the introduction of proteomics methods in the late 1990s. Qiu et al. employed 2D SDS-PAGE to analyze liver proteins extracted from mice treated with [^{14}C]acetaminophen (*42*). Autoradiography of the gels revealed the locations of adducted proteins, and these spots were excised, the proteins were digested with trypsin, and the peptides were analyzed by matrix-assisted laser desorption–ionization mass spectrometry (MALDI-MS) to identify the proteins (Figure 2). This study identified 23 putative targets of acetaminophen in a single analysis, several of which

were also identified in earlier studies in which antibodies were used to detect acetaminophen-derived adducts. This approach was replicated to identify protein targets of reactive metabolites of naphthalene (*43–46*), monocrotaline pyrrole (*47, 48*), and bromobenzene (*49, 50*). These studies also provided some degree of cross-validation, as many of the proteins identified were targets of multiple electrophiles. Williams et al. (*51*) applied highly sensitive accelerator mass spectrometry (AMS) to detect ^{14}C enrichment in proteins from liver and bone marrow of mice after treatment with [^{14}C]benzene. The overall approach was analogous to that described above, except that AMS analysis detected attomolar levels of ^{14}C. The detection of radiolabels associated with histones in bone marrow samples led them to analyze proteolytic digests by AMS, and ^{14}C label was found to be widely distributed across the protein sequences analyzed. The authors concluded that benzene metabolite adduction was "promiscuous" with respect to the protein nucleophile targets modified.

Strictly speaking, analyses based on identifying proteins that comigrate with radioactivity do not detect adducts. This approach was nevertheless a significant advance over the immunochemical approach to adduct detection and analysis, as the increased resolution of 2D gel separations and MS-based analyses increased both the numbers and the reliability of the identifications. However, the 2D gel-based approach using radiolabeled compounds is biased toward detection of more abundant protein targets of the electrophiles. Indeed, many of the proteins detected are expressed at high abudance in cells (e.g., cytoskeletal proteins, chaperones, enzymes of intermediary metabolism). The dynamic range of the approach depends on the specific activity of the radiochemical, and only protein spots containing significant amounts of radiolabel are detected by autoradiography. Despite these limitations, the application of 2D gel-based proteomics together with radiolabeling provided the first method for global discovery of the protein targets of electrophiles.

A more recently applied variation of the 2D gel-based approach involves the use of antibodies directed against adducts to detect protein targets (i.e., 2D Western blotting). Petersen and colleagues reported adduction of the heat shock proteins Hsp72 and Hsp90 by the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) (*52, 53*). These studies were undertaken in the context of a rat model of alcoholic liver disease, in which HNE and other lipid oxidation products are generated in vivo. Adduction of both proteins by HNE in vivo was detected by 2D SDS-PAGE followed by immunoblotting with anti-HNE Michael adduct antibodies. HNE-adducted retinal proteins in aging rats were identified in a similar study (*54*). Immunochemical detection of adducts has been combined with 2D SDS-PAGE and MS to identify adducts formed from 2,6-di-*tert*-butyl-4-methylphenol (*55, 56*).

Another strategy combines biotin-labeled electrophile probes with 2D SDS-PAGE. The endogenous electrophile 15-deoxy-$\Delta^{12,14}$-prostaglandin-J$_2$ (15-deoxy-PGJ$_2$) has garnered considerable interest as a contributor to the effects of oxidative stress and inflammation (*57*). Work by Uchida and colleagues employed a biotin-tagged analogue of this electrophile to identify adducts with thioredoxin 1 (*58*), γ-actin (*59*), the electrophile sensor protein Kelch-like ECH-associated protein 1 (keap1) (*60*), and the DNA damage response mediator ATM (*61*). The biotinylated 15-deoxy-PGJ$_2$ probe detectably adducted at least a dozen proteins in SH-SY5Y neuroblastoma cells (*59*) and nine proteins in cultured MC mesangial cells (*62*).
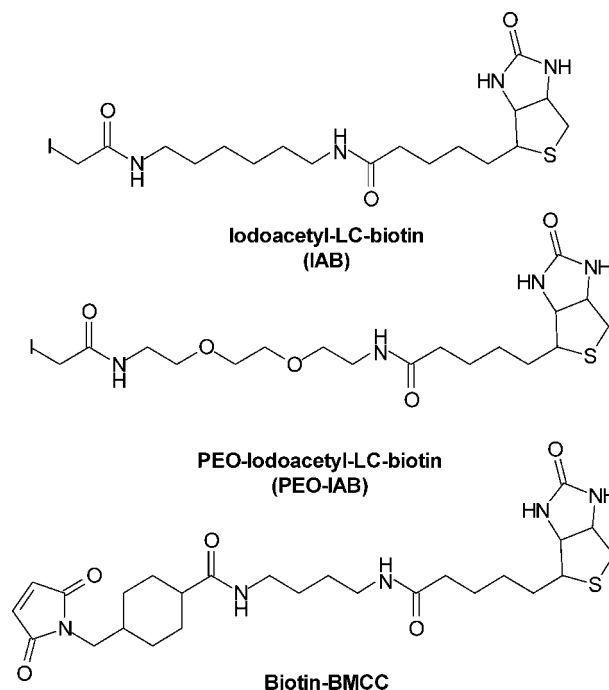

Figure 3. Biotin electrophile probes used for shotgun LC-MS-MS analysis of cellular protein targets.

Although the 2D gel-based approach increased the numbers of targets identified, the absolute numbers of targets and the information contained in these lists still was quite limited. Discussion of the results typically included a rundown of the target list and speculation about the links, if any, between the identified targets and the mechanisms of toxicity. Although only a small fraction of the proteome comprised the detected electrophile targets, greater numbers of protein targets presumably went unidentified due to the limitations of the technologies employed, especially the dynamic range for immunoblotting from 2D gels. Overcoming these limitations requires much more efficient affinity capture tools and the application of shotgun proteomics technology, as described in the next two sections.

5. Shotgun Proteomics and Affinity Capture

The most basic problem in analyzing protein targets of electrophiles is that the adducts represent a small fraction of all proteins present. If adducts can be selectively captured with minimal contamination by nonadducted proteins, then shotgun proteomics approaches can identify the peptides and proteins captured. It appeared to us from previous work (see above) that antibodies directed against adducts had significant limitations in both affinity and specificity. We decided instead to employ commercially available protein biotinylating agents as model electrophiles for studies with cultured cells. The three compounds that we have studied, *N*-iodoacetyl-*N*-biotinylhexylenediamine (IAB), (+)-biotinyl-iodoacetamidyl-3,6-dioxaoctanediamine (PEO-IAB), and 1-biotinamido-4-(4′-[maleimidoethylcyclohexane]carboxamido)butane (BMCC), are shown in Figure 3. Our initial experiments indicated that both probes penetrate cells and react with proteins in multiple subcellular compartments. The biotin tag on alkylated proteins makes it possible to efficiently capture either alkylated proteins or peptides for shotgun liquid chromatography–tandem mass spectrometry (LC-MS-MS) analysis. This label and capture strategy is similar to the activity-based proteome profiling approach developed by Cravatt and colleagues (*63, 64*). However, their probes contained functional groups recognized
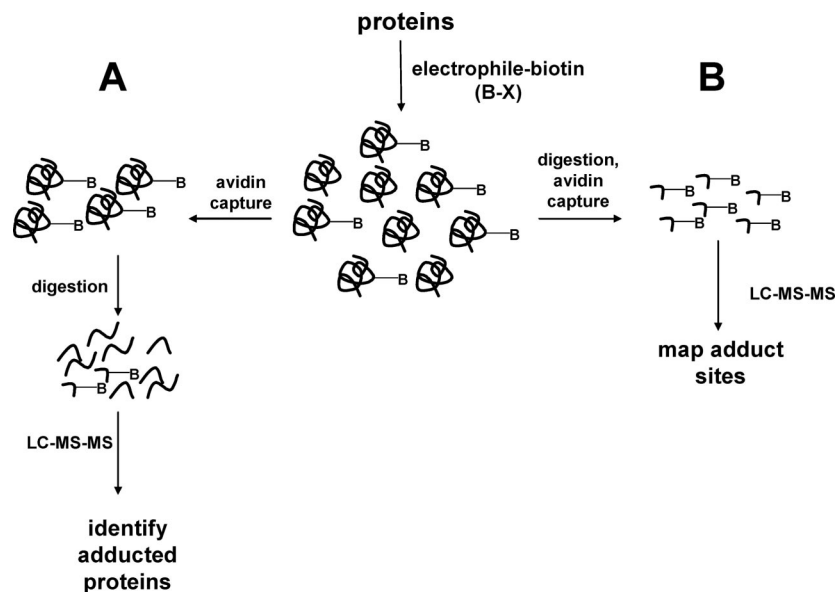
Figure 4. Strategies for analyses of proteins and peptide sequence targets modified by biotinylated electrophile probes. The capture of intact protein adducts (A) followed by digestion and LC-MS-MS analysis primarily yields unmodified peptides, which enables identification of protein targets but results in few adduct identifications. The capture of adducted peptides following proteolytic digestion (B) results in an enriched adduct population for LC-MS-MS and identifies greater numbers of specific adduct sites.

by specific enzyme classes and latent electrophiles that alkylated the bound enzymes, whereas our electrophile probes simply contain the electrophile, a linker, and biotin. It is worth noting that the linker and biotin may determine the targeting of protein alkylation by these probes in some cases.

Shotgun proteomics is the analytical strategy in which complex protein mixtures are first digested to peptides and the peptides are then analyzed by LC-MS-MS to generate tandem mass spectrometry (MS-MS) spectra (65). A database search of the MS-MS spectra assigns them to peptides, and reassembly of the information indicates the proteins present in the original mixture. If biotin-tagged proteins generated with the probes are analyzed, the result is an inventory of adducts—either peptides or proteins, depending on how the analysis is done (Figure 4). If a mixture of adducted and unadducted intact proteins is passed over an avidin column, adducted proteins are captured. Elution and digestion of this fraction yield mostly unadducted peptides and a few peptide adducts. LC-MS-MS analysis of this sample identifies peptides from the captured proteins, but few of these are adducts (Figure 4A). This indicates proteins that are most likely targets of the electrophile, but unless adducted peptides are detected, there is no absolute proof. The alternative approach is to digest the proteins first and then capture the adducted peptides with avidin. Analysis of the eluted peptides by LC-MS-MS primarily yields MS-MS spectra of adducted peptides. If the sequences of the peptides and the position of adduction can be established with confidence, this approach offers the benefit of unambiguously identifying both the protein target and the sequence position of the modification (Figure 4B). However, the matches of spectra to sequence must be of very high quality to establish the protein identifications, as the unmodified proteins are lost during avidin enrichment of the adducts, and corroborating unadducted peptide sequences thus are not detected. Nevertheless, because even abundant protein targets are represented by only their adducted peptides, lower abundance peptide adducts are more likely to be detected, and this approach is most likely to yield a broader range of high and low abundance targets.

We used the latter approach with biotin electrophile probes to label proteins in isolated subcellular fractions from human

cells (66). This approach of labeling complex subproteomes under native conditions enabled adduction of a broad range of cell proteins that could be susceptible to damage in cells. Affinity capture of the adducted peptides following tryptic digestion yielded a sample highly enriched in peptide adducts for LC-MS-MS analysis (see the strategy depicted in Figure 4B). Analysis of cytoplasmic and nuclear proteins alkylated by the probes indicated that 897 specific cysteine residues on 539 proteins were labeled by either PEO-IAB or BMCC. Surprisingly, only about 20% of the identified proteins were adducted by both PEO-IAB and BMCC, which indicates that two different thiol alkylation chemistries could target distinctly different populations of proteins.

This study represented the first large-scale characterization of proteins and protein sites targeted by electrophiles. The results provided several interesting observations. First, protein alkylation was selective and reproducible—Replicate analyses indicated that the electrophile probes reproducibly targeted the same residues, thus indicating selective chemistry as opposed to random alkylation. Second, alkylation by the Michael acceptor BMCC, but not by the S_N2 alkylator PEO-IAB, was favored at Cys residues adjacent to Lys or Arg residues, which suggests that local structural features favor certain alkylation chemistries. Third, over 80% of all protein targets were modified at only a single Cys residue. Fourth, some protein domain and motif structures were highly susceptible to alkylation, including RNA recognition motifs and K-homology domains of RNA-binding proteins. Finally, a core group of approximately 20% of proteins targeted by both electrophiles represented functions known to be highly susceptible to alkylation damage (e.g., regulation of cytoskeletal structures).

Similar studies with human liver microsomes identified 376 peptides in 263 proteins modified by the iodoacetamide IAB and the N-alkylmaleimide BMCC (67). As with cytoplasmic and nuclear protein targets, there was only a ~20% overlap in the protein and sequence targeting by the two electrophiles. An interesting feature of this study was that the two electrophile probes were shown to differ in their ability to trigger an endoplasmic reticulum (ER) stress response—only IAB induced the accumulation of the ER chaperone BiP. This observation
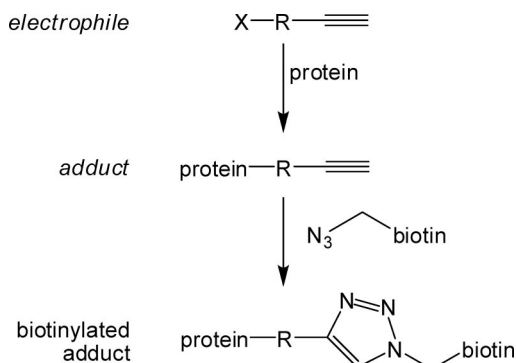
Figure 5. Click chemistry for postreaction tagging of alkynyl-labeled adducts with biotin.

indicated that differences in protein alkylation patterns produce different biological effects. The concomitant observation that Cys50 of microsomal glutathione-*S*-transferase is targeted by both the probes and the quinoneimine metabolite of acetaminophen suggests that a subset of protein targets may be modified by multiple electrophile chemistries and that such alkylations may provide selective screens for reactive metabolites. Liu et al. developed a similar biotin-tagged probe containing a quinone methide structure derived from the selective estrogen receptor modifying drug raloxifene (*68*). This probe was used to label human microsomal proteins, and the alkylated proteins, rather than peptides, were captured and subjected to MS analysis. This study identified several targets, all of which were also targeted in microsomes by the IAB and BMCC probes.

The ability of an affinity capture and shotgun proteomics strategy to identify hundreds to thousands of protein targets clearly indicates the ability of this approach to advance the global characterization of protein damage. Development of biotinylated analogues of other electrophiles could be envisioned. However, the influence of the linker and the biotin moiety, as opposed to electrophile chemistry per se, on protein target selectivity is unknown. An alternative to bulky, linker/biotin probes is the application of new postlabeling strategies utilizing Click chemistry (*69*) (Figure 5) and the Staudinger ligation (*70*) to attach biotin probes to azido- or alkynyl-electrophiles. This approach has the advantage of adding the affinity tag during sample workup. The azido and alkynyl tags are not bulky and display little reactivity toward normal cellular constituents. This approach has been used successfully in an activity-based proteome profiling strategy with probes directed against serine hydrolase enzymes (*71*). Although this strategy requires the synthesis of azido or alkynyl electrophiles or their metabolic precursors and the complementary alkynyl- or azido-biotin tags, the power of the approach could nevertheless be realized with studies of a limited number of well-chosen electrophile probes.

A final approach to adduct affinity capture is the use of a biotin-linked capture reagent that targets a specific feature of certain adducts for covalent capture. Maier et al. have reported the use of *N'*-aminooxymethylcarbonylhydrazino-d-biotin, which covalently captures the residual carbonyls on adducts formed by HNE and related lipid oxidation products (*72, 73*). The reaction forms a Schiff base that can be stabilized by borohydride reduction and captured for MS analysis. This interesting approach offers a means to capture broad classes of adducts (e.g., lipid oxidation electrophile-derived adducts) for analyses of the global impact of oxidative stress.

A final point worth emphasizing in global analysis of protein targets is that many adducts may be chemically unstable, which

may complicate their analysis. For example, quinone adducts are often difficult to analyze because reoxidation of the initial addition products allows the formation of crosslinks and other unexpected structures (*74, 75*). Other adducts, such as those formed from acyl halides and some epoxides, may form esters that easily undergo hydrolysis, as was observed with protein adducts formed from trichloroethylene oxide (*76*). Finally, secondary reactions of adducts can affect their detection, as was observed by Burcham et al., who reported that reaction of acrolein adducts with the amino group of Tris buffers prevented antibody-based adduct detection (*77*). Other work by these same authors actually showed that trapping of the aldehyde group in acrolein Michael adducts with the hydrazine-containing drug hydralazine prevented the formation of protein adduct oligomers, thus offering the novel prospect of adduct detoxication in situ (*78*).

6. Protein Adduction Selectivity and Its Determinants: Studies with Individual Proteins

MS analyses have been used to characterize protein modifications by dozens of electrophiles. In most cases, incubation of the target protein with electrophiles was done to generate adducts, which were then analyzed by MS of either the intact protein or a proteolytic digest. Mapping of adducts to specific sequence locations was achieved by MS-MS or by peptide mass fingerprinting and chemical inference. Such studies have almost invariably revealed that protein modification is selective and specific—Most proteins are reproducibly adducted at a subset of the sites that would appear to be available for reaction. The reactivity of solvent-exposed nucleophiles (e.g., cysteine, histidine, and lysine) is expected, but solvent exposure per se is not necessarily the dominant factor in adduction site selectivity.

Several interesting studies have linked sequence-specific modifications to functional changes in proteins. In the following examples, the proteins of interest were found to be targets of the electrophiles in cell and tissue analyses by 2D SDS-PAGE with biotinylated electrophiles or by immunoblotting for adducts. Follow-up studies in vitro were directed at identifying sequence targets and establishing effects on enzymatic activities or other functions. As discussed above, heat shock proteins were identified by the Petersen group as targets of HNE generated by oxidative stress in vivo (*52, 53*). The reaction of the purified Hsp72 and Hsp90 proteins with HNE, followed by LC-MS-MS analysis, revealed adduction of Hsp72 at Cys267 and of Hsp90 at Cys572. However, the adduction at Cys267 of Hsp72 apparently accounted for the inhibition of ATPase activity of the protein, whereas the Cys572 adduct on Hsp90 was not confirmed as responsible for the inhibition of Hsp90 activity, although the activity was highly sensitive to the thiol modifier NEM. Adduction of thioredoxin 1 was detected using a biotinylated 15-deoxy-PGJ$_2$ probe in cells, and the adduct then was mapped to Cys35 and Cys69 by MS analysis when the protein was treated with 15-deoxy-PGJ$_2$ in vitro (*58*). Cys35 is part of the functional thiol–disulfide redox couple of the enzyme. Similar studies in vitro identified the major actin target of 15-deoxy-PGJ$_2$ and other α,β-unsaturated aldehydes such as Cys374, which led to dysfunction in actin polymerization (*59, 79*).

Among the most interesting studies of protein adduction in vitro reveal features of protein structure that confer susceptibility to damage. Person et al. reported a novel adduct formed by benzoquinone and a benzoquinone–glutathione thioether conjugate with adjacent lysine residues on cytochrome *c* (*74, 75*). Both quinones formed cross-linking adducts as Lys25–Lys27

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and Lys86–Lys87 on the protein. The initial formation of an addition product of one of the lysine ε-amino groups was followed by reoxidation of the quinone ring and then attack of the second lysine ε-amino nucleophile in a reaction favored at elevated pH. This case illustrates the potential importance of adjacent nucleophiles in protein targets generating cross-links with electrophiles capable of sequential oxidation–adduction cycles (in the case of the quinones) or with bifunctional electrophiles, as in the case of the lipid oxidation products HNE and 4-oxo-2-nonenal (ONE) (*80, 81*).

We previously characterized electrophile modifications of serine-threonine phosphatase PP2A, a three-subunit protein that serves as a key regulator of signal transduction (*82*). Reaction with the biotinylated electrophile probes PEO-IAB and BMCC (see above and Figure 3) labeled the structural (A), regulatory (B), and catalytic (C) subunits, as analyzed by affinity capture and Western blotting. However, only BMCC inhibited the phosphatase activity of the holoenzyme. LC-MS-MS-based adduct-mapping studies revealed that the two electrophile probes differed only in the labeling pattern for the C subunit, in which BMCC labeled four cysteine residues not labeled by PEO-IAB. Two of these BMCC-specific adduct sites placed the electrophile side chain in direct contact with critical catalytic residues in the enzyme active site, which would account for the inhibition of activity.

One of the hazards of MS-based analysis of protein adducts is the determination of relative reactivities of different nucleophile targets. The inference of reactivity from MS spectral or signal intensities is often complicated by the different ionization efficiencies of different peptide sequences bearing the same adduct. It may seem that a particular sequence is highly reactive based on a strong MS signal or MS-MS spectrum of the peptide adduct, but this may reflect only highly favorable ionization properties in MS. The most rigorous way to assess competing reactivities is to measure the rates of reaction at the sites and to compare the kinetic constants. However, the measurement of adduction kinetics presents a challenging problem, largely because of the difficulty of absolutely quantifying individual adducts. We recently reported an approach that employed differential stable isotope tagging of peptides in digests of adducted proteins (*83*). Derivatization of the N-termini of tryptic peptides with $^{12}C_6$- or $^{13}C_6$-PIC (phenylisocyanate) forms light and heavy derivatives that are easily distinguished by MS and MS-MS. To extract kinetic parameters from a time–course study with electrophile-treated protein, the sample from the last time point is digested and labeled with the heavy label ($^{13}C_6$-PIC) and all other time points are digested and labeled with the light label ($^{12}C_6$-PIC) (*84, 85*). Each light-labeled sample is spiked with an equal portion of the heavy-labeled sample as a reference standard, and then, the ratios of light to heavy signals extracted from MS-MS data are plotted as a function of reaction time. The plots are fitted to appropriate first-order exponential curves to derive k_{obs} values, which can be used directly to compare reactivities and different protein sites. Application of this approach to measure k_{obs} for HNE adduction of several histidine and lysine residues in human serum albumin revealed a range of three orders of magnitude in the measured constants (*84*). The most reactive target of HNE was His242, which lies in a hydrophobic binding pocket in the IIa subdomain known to bind fatty acids and lipophilic xenobiotics. The reactivity of the His242 imidazole was further enhanced by electrostatic effects of nearby lysine residues, which reduce the estimated pK_a of the imidazole nitrogen to less that 1.0.

7. Protein Damage and Signaling: Mechanisms of Toxicity and Adaptation

The first studies linking covalent binding to toxicity in the early 1970s established the dominant paradigm in the field: Xenobiotic metabolism yields reactive electrophiles, which form adducts with certain critical targets, which leads to toxicity and cell death. However, low-dose exposures to electrophilic chemicals can protect against subsequent, larger doses that would induce toxic or carcinogenec responses (*86, 87*). These seemingly paradoxical consequences of exposure to electrophiles can be explained in part through the induction of protective responses due to low levels of protein damage. Two of the protective systems that appear to contribute to this effect are the ER stress response and the induction of genes regulated by the antioxidant response element/eletrophile response element (ARE/ERE). In both cases, stress leads to the induction of genes and proteins that express chaperone, antioxidant, xenobiotic detoxication, and protein degradation functions.

The ER stress response is the term given to the coordinated response triggered by binding of the ER resident chaperone immunoglobulin heavy chain-binding protein/glucose-regulated protein of molecular mass 78 kDa (BiP/GRP78) to unfolded or misfolded proteins in the ER lumen, thereby dissociating BiP from and activating the proteins PKR-like ER protein kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). BiP is a member of the Hsp70 family of protein chaperones, and increases in levels of unfolded proteins are thought to lead to BiP dissociation from these mediators, which then down-regulate protein translation and up-regulate transcription of ER stress response genes and activate complementary stress pathways (*88*). Studies by Stevens and colleagues in the mid-1990s established that activation of the ER stress response in LLC-PK1 cells blocks apoptosis in response to an otherwise lethal dose of iodoacetamide (*89, 90*).

Our recent studies with the electrophile probes IAB and BMCC revealed that ER stress induction differed with different patterns of protein alkylation in microsomes (*67*). The iodoacetamide probe IAB induced ER stress in HEK293 cells, whereas the *N*-alkylmaleimide BMCC did not. Interestingly, both probes alkylated BiP, as was also shown for the biotinylated raloxifene quinone methide probe used by Liu et al. (*68*). Direct alkylation of the critical BiP chaperone could provide a direct triggering mechanism for alkylation-induced ER stress. Alternatively, alkylation of other ER proteins could cause their misfolding and induce activation of ER stress through essentially the same mechanism as thiol reductants or modifiers of protein glycosylation (*20, 88*). At present, neither hypothesis has been adequately tested.

The induction of glutathione-*S*-transferase and antioxidant enzymes by phenolic antioxidants has now come to be understood as a broadly based induction of over 200 genes by prooxidants, electrophiles, and cancer chemopreventive agents through the ARE/ERE (*91, 92*). Expression of these ARE/ERE-regulated genes is controlled by the transcription factor nuclear factor-E2-related factor 2 (Nrf2) and its regulator, the thiol-rich sensor protein keap1 (*93*). Talalay and Yamamoto originally proposed that keap1 tethers Nrf2 in the cytoplasm until electrophiles and oxidants promote dissociation of the complex and translocation of Nrf2 to the nucleus (*94, 95*). Both the Hannink and the Hayes groups subsequently demonstrated that keap1 is an adapter protein for the ubiquitination of Nrf2 by the E3 ubiquitin ligase Cul3 (*96–98*). Electrophile and oxidant inducers of the ARE/ERE disrupt the ubiquitination of Nrf2 by

modifying the adapter protein (keap1), thus leading to stabilization of Nrf2 and activation of the ARE/ERE (95, 99–102).

Keap1 is perhaps the best example of a specific protein target of electrophiles whose covalent adduction leads to a specific biological effect—the stablization of Nrf2 and the induction of ARE/ERE-regulated genes. However, the keap1 sensor protein contains 27 cysteine residues and has not yet been structurally characterized (except for the Kelch domain (103)). A key question is which of the cysteines in keap1 are critical to its sensor function. Several reports have identified cysteine residues targeted by different electrophiles upon reaction with the bacterially expressed protein in vitro (95, 100–102, 104). However, these studies collectively reported that 25 of the 27 cysteines in human keap1 are adducted by at least one of the electrophiles studied to date. Among these, the most frequently reported targets include Cys151, Cys257, Cys273, Cys288, Cys297, and Cys613, although there are notable differences between laboratories studying mouse vs human keap1 or different electrophile probes, reaction conditions, sample work-ups, and MS analysis methods. Studies with different electrophile probes under the same conditions and using the same analyses can be revealing, as the IAB and BMCC probes formed almost completely different patterns of keap1 adducts (101). This difference corresponded to a difference in the ability of the electrophiles to activate ARE/ERE gene transcription in HepG2 cells (IAB activated, whereas BMCC was without effect).

Despite suggestions that three keap1 residues are particularly reactive (Cys151, Cys273, and Cys288), no rigorous kinetic analyses of the reactions of any electrophiles with keap1 cysteines have been reported. As noted above, assessments of the relative reactivities of different residues based on relative intensities of peptide adducts in MS analyses are unreliable.

Mutagenesis studies indicate that Cys273Ser and Cys288Ser mutations in the central linker domain blocked keap1-dependent ubiquitination of Nrf2 (96) and that Cys273Ala and Cys288Ala mutations blocked keap1-dependent repression of an ARE/ERE reporter (100). Zhang et al. also reported that Cys151 in the keap1 BTB domain is required for inhibition of keap1-dependent degradation of Nrf2 by sulforaphane and oxidative stress, although this mutant does function as a constitutive repressor of Nrf2 (96, 97). Consideration of both the adduct data and the mutagenesis data focuses attention on Cys151, Cys273, and Cys288 as potentially important sensor residues in keap1. Nevertheless, the issue of which keap1 cysteine is a critical target or whether a single critical cysteine residue mediates activation by diverse electrophile chemistries remains to be resolved.

Other regulators of transcription factors and signaling networks are sensitive to oxidants or covalent modification, including several phosphotyrosine and phosphoserine/threonine phosphatases (82, 105–111), IkappaB kinase beta (112–117), the thiol–disulfide redox regulatory enzymes thioredoxin (58, 66, 118), protein disulfide isomerase (43, 46–49, 66, 68, 119–122), and the DNA damage response protein ATM (61). Many more proteins that directly or indirectly regulate signaling networks and pathways are targets of electrophiles (66, 67), and damage inventories using affinity capture and current shotgun proteomics methods most likely will range into the hundreds to thousands of protein targets. Interpretation of cellular outcomes of damage by different electrophiles thus will depend on (i) the combination of signaling-related targets modified, (ii) the extent to which the adducts alter functions of the proteins, and (iii) the interplay between the affected pathways. Given our current ability to

inventory targets of different electrophile chemistries and the degree of selectivity displayed by different electrophiles (e.g., 20% overlap in targets between the iodoacetamido- and the *N*-alkylmaleimido electrophile probes), it should be possible to use systems "damage maps" to hypothesize effects on different pathways.

Studies with model organisms exposed to alkylating agents, oxidants, and arsenic have integrated transcriptome profiling, genomic phenotyping, and systems modeling to reveal agent-specific effects on signaling and metabolic networks (123–129). None of these studies have yet identified protein damage targets of the agents studied in these systems. Identification of protein targets in such well-characterized models could link the genomic and systems responses to initial damage events. Targeted assays of key intermediates and pathway outputs could enable tests of individual hypotheses regarding pathway effects of damage. This problem represents a fascinating challenge and opportunity that is certainly within the reach of presently available tools for proteomics, transcriptomics, metabolomics, and systems modeling.

8. Conclusion

The most widely used assay of protein damage by chemicals still is the binding of radiolabel to bulk protein (18). Although this assay provides a useful basis for identifying molecules likely to cause toxicity via metabolic activation, the analytical toolkit for analyzing protein damage has advanced tremendously in the last 20 years. We are now on the brink of a comprehensive understanding of protein damage and its biological effects. Emerging evidence from studies with electrophile affinity probes and shotgun proteomics shows clearly that different binding patterns lead to distinct biological outcomes. Indeed, it seems reasonable to hypothesize that the binding pattern encodes the biology. The convergence of analytical technology, pathway analysis, and computational systems biology tools will provide the means to interpret these codes.

The synthesis of probes representing diverse structures and chemistries is actually a fairly straightforward proposition, and the application of shotgun proteomics analyses and bioinformatics analyses to identify the targets can now be standardized and implemented in reasonably high throughput. One of the most interesting challenges will be to define the molecular, cellular, and tissue injury phenotypes that characterize different exposures. With these tools in hand, the most important task is to define the relationships between specific protein modifications and changes in function related to toxicity. This will constitute a significant challenge but ultimately can offer the greatest insights into mechanisms.

It is important to emphasize that the problem of protein damage is not limited to the effects of exogenous chemical exposures. Endogenous electrophiles associated with oxidative stress and metabolic imbalances result in protein modifications that contribute to diverse diseases, and understanding these mechanisms of injury represents an important opportunity for toxicology to impact human health (130).

The mapping of specific protein damage targets to pathway effects or injury phenotypes will lead to specific assays for these targets. The use of targeted analyses of specific adducts may offer clinically useful methods of characterizing adverse drug reactions or disease processes. Recent reports described the analysis of acetaminophen–cysteine adducts in serum and tissue protein hydrolysates to detect covalent acetaminophen adducts in both adults and children with liver failure (131, 132). Such studies take the analysis of protein damage from discovery science to the clinic and raise important issues about not only

the analytical validity of the methods applied to measure adducts in clinical settings but also the application of such markers in a clinical context. It seems reasonable to expect that the next decade will bring increasing focus on the analysis of damaged proteins as markers for chemical toxicity and disease.

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TX700235T