RADICAL APPROACHES TO PROBE PROTEIN STRUCTURE, FOLDING, AND INTERACTIONS BY MASS SPECTROMETRY

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This review describes mass spectrometry-based strategies and investigations to determine protein structure, folding dynamics, and protein-protein interactions in solution through the use of radical reagents. The radicals are generated in high flux within microseconds from synchrotron radiation and discharge sources, and react with proteins on time scales that are less than those often attributed to structural reorganization and folding. The oxygen-based radicals generated in aqueous solution react with proteins to effect limited oxidation at specific amino acids throughout the sequence of the protein. The extent of oxidation at these residue markers is highly influenced by the accessibility of the reaction site to the bulk solvent. The extent of oxidation allows protection levels to be measured based on the degree to which a reaction occurs. A map of a protein's three-dimensional structure is subsequently assembled as in a footprinting experiment. Protein solutions that contain various concentrations of substrates that either promote or disrupt dynamic structural transitions can be investigated to facilitate site-specific equilibrium and time-resolved studies of protein folding. The radical-based strategies can also be employed in the study of protein–protein interactions to provide a new avenue for investigating protein complexes and assemblies with high structural resolution. The urea-induced unfolding of apomyoglobin and the binding of gelsolin to actin are among the systems presented to illustrate the approach. © 2002 Wiley Periodicals, Inc. Mass Spec Rev 20:388–401, 2001; Published online in Wiley InterScience (www.interscience.wiley.com).
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I. INTRODUCTION

Investigations of the reactions of radicals with proteins or their amino acid constituents date back almost one hundred years. In the early 1900s, Henry Dakin published several articles that described the oxidation of amino acids with hydrogen peroxide (Dakin, 1906, 1908a,b) in the presence of ferrous sulfate in a classic Fenton system (Fenton, 1894; Fenton & Jackson, 1899). Later, Dakin was the first to report on the physiological effects of these processes (Dakin, 1909, 1922).

The treatment of intact proteins with hydrogen peroxide followed (Breinhl & Baudisch, 1907), and separate studies by Edelbacher (1924) demonstrated the formation of oxidation and degradation products. Edelbacher found that an oxidized protein was more resistant to proteolysis than its unreacted form, and anticipated the observation that such oxidized proteins can accumulate in the body to promote disease and aging. His results also suggested changes in the structural and functional properties of a protein in its oxidized form. Dakin, too, anticipated the oxidative damage to proteins when he intravenously injected rabbits with hypochlorite. This report (Dakin, 1917), in the first issue of the British Medical Journal, described a drop in the ''anti-tryptic'' content of the blood associated with the radical-based oxidative inactivation of proteinase inhibitors.

The same decade saw the publication of work that demonstrated that radium rays could cause the coagulation of protein solutions (Fernau & Pauli, 1915), and Hussey and Thomson reported the decomposition and inactivation of trypsin with X-, beta- and gamma-rays (Hussey & Thomson, 1923a,b). The photo-oxidation of proteins later demonstrated the selective oxidation of aromatic amino acids in 1926 (Harris, 1926).

The extent of oxidation in Dakin's and other early radical-induced oxidation approaches is considerably greater than that achieved in most instances today, and the biological significance of such processes is largely influenced by the degree to which oxidation occurs. The concentration of the protein, the concentrations of other radical scavengers present, the solvent environment, and the nature and source of the radical flux all influence the yield and effect of protein oxidation. In Fenton (Fenton, 1894; Fenton & Jackson, 1899) and other metal-catalyzed oxidation systems, the location of the active metal becomes important, such that the residues histidine, cysteine, and methionine may localize reactions in their vicinity. Even the reaction of hydroxyl radicals in photolysis experiments can result in site selectivity (Harris, 1926). The reasons for this selectivity are not entirely clear, but may include the accessibility of certain sites to radicals, the stability of certain radical intermediates formed, and the occurrence or lack of radical-transfer processes. A comparison of the data from several studies suggests that the products of metal-catalyzed and radiolysis reactions are quite distinct (Hunt, Dean, & Wolff, 1988; Hunt, Simpson, & Dean, 1988).

Because reaction selectivity is apparent in many radical-based studies, it follows that radical reactions may offer a means to investigate features of a protein's structure. In a simple case, the selective oxidation of a single histidine residue may indicate that a previously unknown metal is bound to a protein site. Should the accessibility of reaction sites to radicals play a particularly dominant role in the reactivity of proteins, it follows that radical-based approaches could be applied to investigate protein structure.

This review describes studies that involve the reaction of proteins with a high flux of radicals generated on time scales shorter than those often associated with the structural reorganization or folding of proteins. Beyond the study of protein structure that use several radical sources, the strategies have been extended to investigations of protein-folding dynamics and the preliminary investigation of protein complexes.

II. CASE FOR RADICALS

One advantage of a radical reagent is that radicals can be generated by chemical and biochemical processes through a multitude of different pathways. Radicals are formed in high yields on relatively short time scales, and their subsequent reactions are rapid and usually irreversible in nature. They react with a wide variety of chemical and biochemical substrates across a range of solution conditions.

Radiation sources provide one means of generating radicals. In the case of the irradiation of dilute aqueous solutions, the initial radicals formed are the hydrated electron (e^-_{aq}), hydrogen atoms (H•), and the hydroxyl radical (HO•). The HO• radical is also formed via UV photolysis of hydrogen peroxide and by the reduction of hydrogen peroxide with Fe^{2+}. This latter process is known as the Fenton reaction (Fenton, 1894; Fenton & Jackson, 1899) (Equation 1).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + HO^- \qquad (1)$$

Further reaction of these species with solvated molecules gives rise to secondary radicals by a series of pathways that include hydrogen atom abstraction, electron donation, addition, substitution (addition followed by elimination), and fragmentation processes. The many reaction possibilities open to radical reagents provide an ability to probe many structural features of a molecule.

Reactions, however, are preferred at sites where the resulting radical is stabilized by neighboring functional groups, such as double bonds, aromatic rings, and heteroatoms (Fig. 1, Table 1). Although, the HO• radical is a powerful oxidizing species, it does show selectivity in the types of secondary species and products that are generated. HO• will react with most C–H bonds by hydrogen atom abstraction, but addition reactions to unsaturated systems, such as aromatic rings often predominate. These reactions have rate constants in the range of 10^7–10^{10} $M^{-1}\,sec^{-1}$ (Buxton et al., 1988), where a rate constant that exceeds 10^9 $M^{-1}\,sec^{-1}$ has been found to be

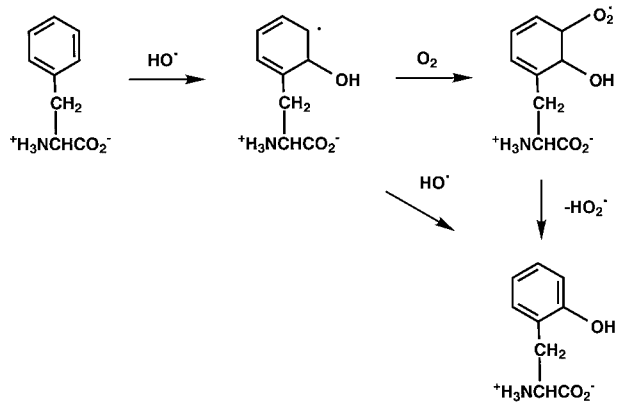

FIGURE 1. Mechanism for the oxidation of phenylalanine with hydroxyl radicals.

required for oxidation to be effected in radiolysis and discharge studies (Table 1). This preference for electron-rich aromatic rings and sulfur centers is due to the electrophilic nature of the HO• radical. This reaction selectivity (Buxton et al., 1988) has been observed and exploited in the context of our studies of protein structure and folding (Maleknia, Chance, & Downard, 1999; Maleknia et al., 2000, 2001a,b; Maleknia & Downard, 2001; Maleknia, Chance, & Downard, 2001; Maleknia, Kiselar, & Downard, 2002). The small size of the HO• radical facilitates its use as a structural probe of proteins, and enables changes in solvent accessibility within sites of a protein to be monitored at a single amino acid resolution.

TABLE 1. Rate constants for the reaction of common amino acids with HO•

Amino acids	Rate constant ($M^{-1}\,sec^{-1}$)[a]	Product detected[b]
Glycine	1.7×10^7	No
Alanine	7.7×10^7	No
Valine	7.6×10^8	No
Leucine	1.7×10^9	Yes[c]
Serine	3.2×10^8	No
Threonine	5.1×10^8	No
Cysteine	3.4×10^{10}	Yes
Methionine	8.3×10^9	Yes
Aspartic Acid	7.5×10^8	No
Glutamic Acid	2.3×10^8	No
Lysine	3.5×10^7	No
Arginine	3.5×10^9	No
Asparagine	4.9×10^7	No
Phenylalanine	6.5×10^9	Yes
Tyrosine	1.3×10^{10}	Yes
Histidine	1.3×10^{10}	Yes
Tryptophan	1.3×10^{10}	Yes
Proline	1.2×10^{10}	Yes

[a]Data sourced from Buxton et al. (1988) except for proline (Masuda, Nakano, & Kondo, 1973).

[b]In synchrotron radiolysis and discharge studies (Maleknia, Brenowitz, & Chance, 1999; Maleknia, Chance, & Downard, 1999, 2001; Maleknia et al., 2000, 2001a,b; Maleknia & Downard, 2001; Maleknia, Kiselar, & Downard, 2002).

[c]Minimal levels of leucine oxidation were evident in some radiolysis studies (Maleknia, Brenowitz, & Chance, 1999; Maleknia et al., 2001a).

III. RADIATION AND DISCHARGE RADICAL SOURCES

A disadvantage of generating radicals by chemical means (Fenton, 1894; Fenton & Jackson, 1899; King et al., 1992) or low-flux radiation sources (Garrison, 1987) is the length of time required to produce sufficient concentrations of radicals for subsequent reaction. HO• radicals are formed from peroxonitrous acid (King et al., 1992) over several seconds, whereas the use of Fe-EDTA as a reaction catalyst (Heyduk & Heyduk, 1994) is effected on time scales of the order of several minutes. These time scales are incompatible with kinetic studies that seek to monitor structural changes and folding events in proteins that can occur over microseconds to several seconds *in vivo* and *in vitro* (Eaton et al., 2000).

Two sources of oxygen radicals have been employed in our studies of protein structure and folding events (Maleknia, Brenowitz, & Chance, 1999; Maleknia, Chance, & Downard, 1999, 2001; Maleknia et al., 2000, 2001a,b; Maleknia & Downard, 2001; Maleknia, Kiselar, & Downard, 2002) that achieve the production of HO• radicals in high flux within microseconds. The first uses synchrotron radiation in the X-ray region of the electromagnetic spectrum. The synchrotron-based experiments described in this review were performed at the National Synchrotron Light Source that operates an X-ray ring at 2.5 GeV with a beam current of approximately 200 mA. The synchrotron delivers between 10^{14} and 10^{15} photons/sec of a continuous spectrum of X-rays in the range of 3–30 keV. Two beryllium windows cap the beam pipe that extends over several meters from the X-ray ring to the experimental hutch. Radiation emitted from the beam pipe passes through air over a path of approximately 10 cm and irradiates the entire solution volume. When aqueous solutions are irradiated with synchrotron light, hydroxyl radicals are generated according to Equation 2:

$$H_2O + h\upsilon \rightarrow H_2O^+\bullet + e^-_{dry} \xrightarrow{H_2O} H_3O^+ + HO\bullet + e^-_{aq} \quad (2)$$

When the reaction is performed in the presence of oxygen, superoxide anions and hydroperoxyl radicals are generated, according to Equation 3:

$$O_2 + e^-_{aq} \rightarrow O_2\bullet^- \xrightarrow{H_2O} HO_2\bullet + HO^- \quad (3)$$

The hydroxyl radicals are produced in a high flux of approximately 300 radicals for every 10 keV thermalized in solution within 100 msec (Klassen, 1987). These radicals either react with the low concentration of protein in solution or recombine to form hydrogen peroxide with a second-order rate constant of 5×10^9 $M^{-1}sec^{-1}$. The concentration of HO• reaches a steady state of 0.2 μM estimated from the photon flux (Sclavi et al., 1998). An electronic shutter impervious to the X-ray beam facilitates the exposure of protein solutions for between 10 and 50 msec.

The second source of hydroxyl radicals utilizes an electrical discharge within an atmospheric pressure electrospray ion source (Maleknia, Chance, & Downard, 1999, 2001). Discharge sources are used widely in mass spectrometry (Riciputi et al., 1995; Barshick et al., 1996; Barshick, 2000; Itoh, Hasegawa, & Amano, 2000) and the phenomenon contributed to the birth of the field (Thomson, 1913). When a high-voltage difference (~8 keV) is applied between an electrospray needle and a sampling orifice to the mass analyzer, typically either a conical lens or metal capillary, radicals can be produced according to Equation 2. If oxygen is used as a nebulizer sheath gas, then the formation of HO• radicals is enhanced by the process shown in Equation 4.

$$O_2 - e^-_{aq} \rightarrow O_2\bullet^+ \xrightarrow{2H_2O} H_3O^+ + HO\bullet + e^-_{aq} \quad (4)$$

The production of these radicals has been shown in high-pressure mass spectrometry and flowing afterglow experiments to occur with rate constants of the order of 10^{-9} cm^3 mol^{-1} sec^{-1} (Good, Durden, & Kebarle, 1970; Fehsenfeld, Mosesman, & Ferguson, 1971). The rapid production of radicals permits their reaction with protein ions as the ions are emitted from the needle tip. This, in turn, allows the products of the reaction to be determined by direct mass spectrometric analysis or after the subsequent condensation of the electrosprayed droplets (Maleknia, Chance, & Downard, 1999).

Both radical sources facilitate the production of radicals on time scales that are less than those usually attributed to protein folding and reorganization. The latter approach has the advantage that it can be effected with minimal modification to an electrospray ion source, and allows protein ions to be studied as they emerge from solution to the gas phase; an environment of considerable interest in fundamental and applied mass spectrometry.

IV. REACTION PRODUCTS: NATURE OF AMINO ACID MODIFICATIONS

Three processes are open to reactions of radicals with proteins. These processes are amino acid side-chain modification, backbone cleavage, and cross-linking. The last reaction can be minimized when the concentration of protein in solution is kept relatively low. Pulsed radiolysis studies reveal that the rate of reaction of amino acid side-chains is significantly faster (5×10^9–10^{10} M^{-1} sec^{-1}) than the rate at which radicals react with the α-carbon hydrogen atoms (of 10^9 M^{-1} sec^{-1}); the latter leads to backbone cleavage (Garrison, 1987). This reaction preference is in accord with synchrotron and discharge-based studies (Maleknia, Brenowitz, & Chance, 1999; Maleknia, Chance, & Downard, 1999, 2001; Maleknia et al., 2000, 2001a,b; Maleknia & Downard, 2001; Maleknia, Kiselar, & Downard, 2002) described in this review.

The structural diversity of the amino acid side chains of the twenty common amino acids results in a variety of possible reaction sites and products. The nature of the reaction products that result from the treatment of amino acids and proteins has been extensively characterized. The amino acids found to react with hydroxyl radicals in synchrotron studies (Maleknia, Brenowitz, & Chance, 1999) are shown in Table 1 are grouped according to the nature of their side chains.

The sulfur-containing amino acids cysteine and methionine are found to be the most reactive amino acid residues when reacted with HO• produced by either synchrotron irradiation or electrical discharges (Maleknia, Brenowitz, & Chance, 1999; Maleknia, Chance, & Downard, 1999). Reaction of cysteine with hydroxyl radicals is known to occur rapidly and almost exclusively at the sulfur center to yield radical RS• (von Sonntag, 1987, 1990). In the presence of oxygen, these radicals react to form RSOO• and lead to the formation of sulfonic acid. The thioether group of methionine also reacts rapidly with HO• at the sulfur center. In the presence of oxygen, methionine sulfoxide and methionine sulphone are produced in radiolysis experiments (Kopoldova, Liebster, & Gross, 1967).

As described above, aromatic residues react with HO• by the initial rapid addition of the radical to the ring. Hydrogen atom abstraction is reported to be a minor process. The resulting radical can undergo a number of subsequent reactions. The addition of HO• to the phenyl ring of phenylalanine has little selectivity with a mixture of positional isomers formed (Dizdaroglu & Simic, 1980) (Fig. 1). In contrast, the addition of HO• to tyrosine is much more selective due to the strong directing effect of the hydroxyl substituent (Steenken & O'Neill, 1977).

Rapid addition reactions to the aromatic rings of tryptophan and histidine are also observed. In the case of

tryptophan, HO• is known to add to the benzene and pyrrole rings (Armstrong & Swallow, 1969), and the relative ratio of rates of attack at each ring system have been estimated to be 1:1.5, respectively (Solar, 1985). The former process results in the formation of 5- and 7-hydroxy-tryptophan in the absence of oxygen. The peroxyl radicals formed on the phenyl ring by the addition of molecular oxygen also can eliminate water to yield 5 and 7-hydroxy-tryptophan. When HO• addition occurs on the pyrrole ring at position C-3, the formation of a peroxyl radical at position C-2 leads to the formation of the N-formyl-kynurenine in oxygenated solutions by ring opening (Davies & Dean, 1997). The products from the addition of HO• to the imidazole ring of histidine are less well-characterized, but include 2-oxo-histidine (Uchida & Kawakishi, 1986, 1993), asparagine, and aspartic acid (Dean, Wolff, & McElliott, 1989).

V. PROTEIN INTEGRITY VERSUS DAMAGE

A search of the scientific literature with the chemical abstract service of the American Chemical Society returned over 1,400 articles that referred to protein damage and oxidation. Many of these reports detail oxidative alterations to a protein's structure or function that are induced by radical reactions at either a limited or large number of sites (Davies & Dean, 1997).

Against this literature tide, a growing number of studies have shown that the structural and functional characteristics of proteins can be preserved after their limited oxidation. For example, the limited oxidation of glutamine synthetase (Nakamura & Stadtman, 1984) gave rise to some products that were fully active—as judged by their interaction with substrates. In the case of this and several other enzymes, it has been shown that limited modifications to an enzyme's structure do not bring about dramatic changes in conformation. Little change to either the size or hydrophobicity of the enzyme glycerol dehydrogenase was reported upon its treatment with hydrogen peroxide *in vivo* (Chevalier, Lin, & Levine, 1990). Several other *in vitro* studies have reported that under physiological conditions, proteins are not appreciably damaged when treated with radicals. When bovine serum albumin was treated with hydroxyl radicals in the presence of oxygen, only low levels of molecules of a reduced size are detected by HPLC under non-denaturing conditions (Wolff & Dean, 1986).

Clearly, the extent and site of oxidation as well as the nature of the protein and its environment all contribute to its ability to resist structural and functional damage. Although the complete oxidation of a single amino acid residue that plays a key role in a protein's structural stability or function can dramatically alter or inactivate the protein, limited oxidation at this same site or oxidation elsewhere in the protein may not substantially change either its structure or activity.

It is worthwhile to consider the level and type of modification that can take place before a protein's structure, and consequently its function, is irreversibly damaged. From the studies cited and reported in this review and published elsewhere, it seems it is not possible to answer this question conclusively. Indeed, there may be no general answer given the unique structural features that are inherent to individual proteins and the influences of the environment on a protein's structural and functional integrity. Yet, it can be argued that, just as the treatment of proteins with radicals and their subsequent oxidation can afflict different levels of structural damage, conditions can be found in which a large number of proteins are essentially resilient to such treatment. The experiments described in this review effect limited protein oxidation using a high-flux of oxygen-based radicals on time scales (several milliseconds) that are less than those usually attributed to protein perturbations (Eaton et al., 2000). Under these conditions, no detectable levels of degradation products were observed by either mass spectrometry or gel electrophoresis.

The ESI mass spectrum of hen egg lysozyme after 30 msec exposure to the synchrotron beam is shown in Figure 2 (Maleknia, Kiselar, & Downard, 2002). The spectrum shows no evidence of protein dissociation during irradiation because no ion signals above the background were detected at lower mass-to-charge ratios than those associated with the unmodified protein. The only additional peaks evident arise at intervals of + 16/n units (where n equals the charge of the ion) above mass-to-charge of the ions for the unmodified protein. These peaks are due to the oxidized forms of the protein.

Analysis of the irradiated protein solution by gel electrophoresis similarly shows that minimal protein degradation occurs (Fig. 3). Major bands are evident for the unmodified form of the protein, in addition to closely spaced bands at higher molecular weight that correspond to unresolved forms of the oxidized protein. Material below the molecular weight of the protein is evident, although these cleavage products appear in low amounts. It is of note that some cleavage products appear in higher relative abundance over others. These data are consistent with observations exploited in classical footprinting experiments (Price & Tullius, 1992; Sclavi et al., 1997, 1998). Some trace levels of high molecular weight material associated with cross-linked products are detected in apomyoglobin solutions irradiated for 60 and 80 msec. After 80 msec of exposure, it also becomes apparent from the gel data that the level of unreacted protein and any cleavage products diminish. This result suggests that they

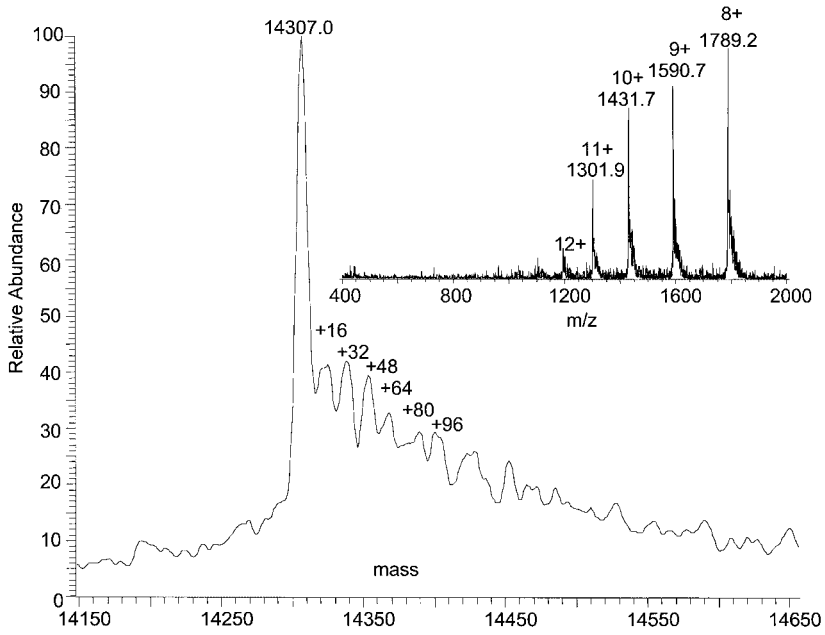

FIGURE 2. Deconvoluted mass spectrum of hen egg lysozyme after radiolysis for 30 msec. Insert shows the full electrospray mass spectrum.

are further degraded. For these reasons, and those rationalized above, radiolysis experiments are best performed with exposure times up to 50 msec under the present beam conditions.

Subsequent mass spectrometry studies of the proteolysis products of irradiated lysozyme reveal that the extent of oxidation at amino acid side chains correlates with their accessibility to the bulk solvent in the native protein structure (Maleknia et al., 2001a; Maleknia, Kiselar, & Downard, 2002). As an example, tandem mass spectrometric analysis of the tryptic peptide that comprises residues 62–68 shows that oxidation occurs exclusively at tryptophan at position 62 over the same residue at position 63 (Fig. 4). All y-type ions detected (y_2–y_6) remain at the same mass-to-charge ratios in the dioxidized peptide as the unmodified form, whereas the b_5 ion appears + 32 units higher in the spectrum of the oxidized form (Fig. 4). These data indicate that oxidation occurs exclusively on tryptophan at position 62, and is in accord with the accessibility of the residue side chains. Residue Trp-62 is over five-times more exposed to the bulk solvent than Trp-63, based on the NMR structure of the protein (Schwalbe et al., 2001).

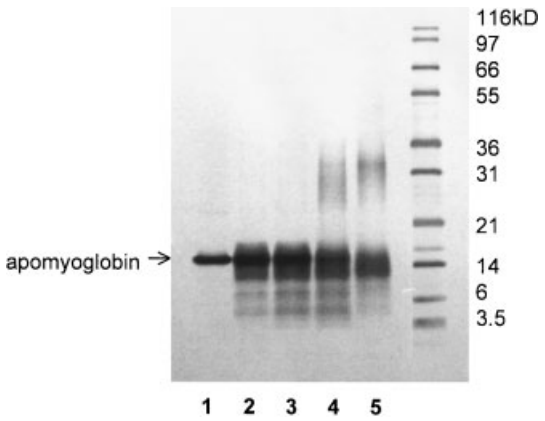

FIGURE 3. Polyacrylamide gel (silver stained) of apomyoglobin (1) and products from radiolysis with synchrotron X-rays for 20 (2), 40 (3), 60 (4), and 80 (5) msec.

A requirement for the success of radical-based strategies for protein structure elucidation is control of the oxidation process. It has been found in the radical-based oxidation approaches reported in this review that total oxidation levels of up to 30–50% can be achieved without measurable damage to a protein's structure. This level of oxidation is controlled in the case of the synchrotron studies by the time the protein solution is exposed to radiation. Irradiation times of between 0–50 msec at beam energies of 2.5GeV and currents of approximately 200 mA are found to produce this level of oxidation. Where electrical discharges are employed, the voltage difference across the electrospray source and the flow rate of the oxygen sheath gas both contribute to the level of oxidation achieved (Maleknia, Chance, & Downard, 1999, 2001).

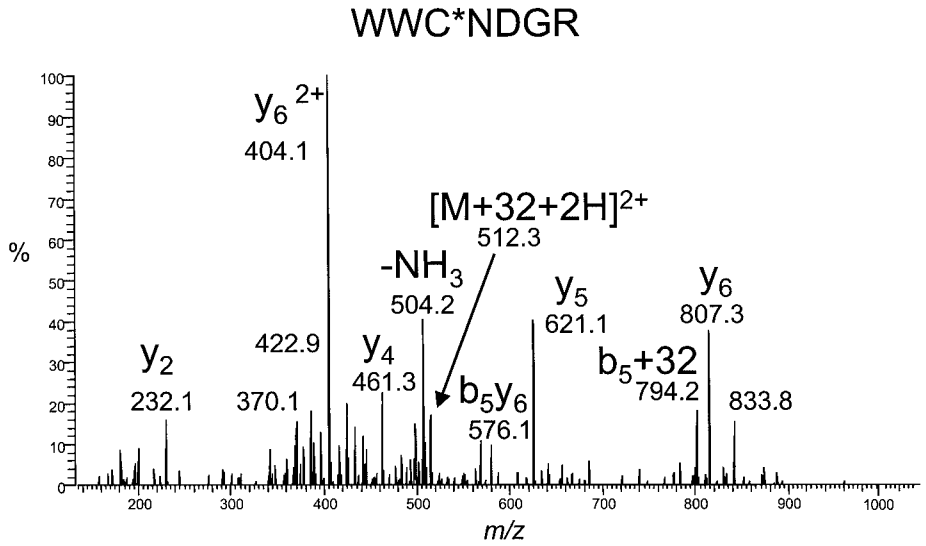

FIGURE 4. Electrospray tandem MS/MS spectrum of the deoxidized tryptic peptide 62–68 of irradiated hen lysozyme. C* corresponds to carboxyamido-cysteine.

VI. REACTION KINETICS AND PROTEIN STRUCTURE ELUCIDATION

During the first hundreds of microseconds of exposure of aqueous solutions to radiation or an electrical discharge, the concentration of HO• steadily increases until the radicals undergo recombination to hydrogen peroxide. At this stage, hydroxyl radicals are consumed either by their reaction with protein or by recombination (Buxton, 1987). Where the concentration of hydroxyl radicals reaches a steady state (Sclavi et al., 1998), the rate of HO• formation (k_f) can be defined by Equation 5.

$$k_f = k_{recombination} + k_{reaction} \quad (5)$$

At high radical-to-protein concentrations, the rates of HO• formation (k_f) and recombination will significantly exceed the rate of reaction with protein, and Equation 5 can be approximated to $k_f \approx k_{recombination}$. This approximation ignores the bias toward the reaction of HO• with protein due to a protein's large physical size and cross-section. Dose-response profiles for the oxidized protein and proteoytic peptides are subsequently fit to a first-order rate equation, independent of the concentration of HO•, where y is the fraction of unmodified protein or peptide at time *t*, and k is a first-order rate constant (Equation 6).

$$y = A \cdot e^{-kt} \quad (6)$$

The amount of oxidation is measured by mass spectrometry, and is based on the relative ratio of the area under the ion signal peaks for the oxidized and unoxidized forms (Maleknia et al., 2001a; Maleknia, Kiselar, & Downard, 2002). The level of oxidation is calculated from a set of mass spectral data as a function of exposure time to radicals. When plotted on a logarithmic scale, these data produce a dose-response profile (Klassen, 1987) where the rate of oxidation is measured from the slope of the line of best fit.

Two dose-response profiles for the tryptophan-containing tryptic peptides of lysozyme are shown in Figure 5. The first-order rate constants for oxidation at the reactive tryptophan residues in peptides 62–68 and 117–125 are measured from the slope of the line of best-fit to be 12.7 ± 0.8 and $7.7 \pm 0.7\ s^{-1}$, respectively. Thus, the tryptophan residue at position 62 is estimated to be 1.6 times more reactive than the tryptophan residue at position 123. This ratio is in accord with the solvent accessibility of the side chain of each residue, based upon the NMR structure of the protein. The ratio of the sum of surface area of the reaction centers in each residue side chain is measured to be 1.4 (Richards, 1974; Richmond, 1984). No oxidation is detected at the remainder of the tryptophan residues throughout the protein sequence—consistent with the inaccessibility of their side chain groups to the bulk solvent. As shown in Figure 4, the tryptophan residue at position 63 remains unoxidized. This result is in accord with the solvent accessibility of its side chain, which is less than 20% of that of neighboring Trp-62. A similar correlation between the reaction rates and the accessibility of the reactive residue side chains is seen across the entire protein (Maleknia, Kiselar, & Downard, 2002) and in studies in which radicals were generated by an electrical discharge (Maleknia, Chance, & Downard, 1999, 2001).

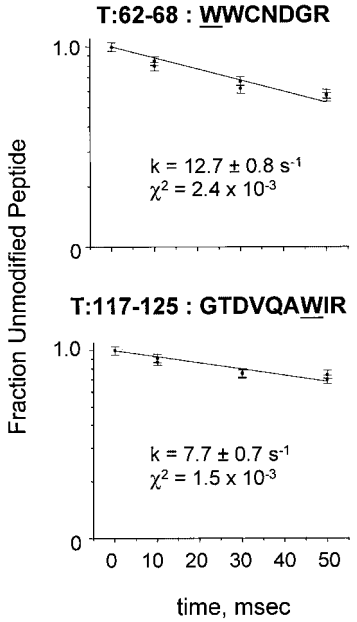

FIGURE 5. Dose-response profiles following oxidation in tryptophan-containing segments of hen lysozyme as a function of X-ray exposure time. Data from two separate measurements are shown to illustrate the reproducibility of the experiments.

An extension of the method is to study the equilibrium and time-resolved folding transitions of proteins by monitoring their reactivity with hydroxyl radicals, following the addition of substrates that catalyze or disrupt protein folding. Metal ions such as calcium or magnesium play an important role in catalyzing protein folding (Tainer, Roberts, & Getzoff, 1992), whereas the addition of denaturants such as urea can induce protein unfolding by disrupting the interactions between a protein and its environment (Schiffer & Dotsch, 1996).

VII. PROTEIN FOLDING: EQUILIBRIUM AND TIME-RESOLVED STUDIES

During a folding process, the amino acid side chains of proteins undergo substantial changes in their exposure to the bulk solvent. This change is reflected in the significant increases in the extent of oxidation observed in protein radiolysis studies for apomyoglobin as a function of denaturant concentration. The proteolysis of the limited oxidation products enables accessibility changes in regions of the protein to be monitored as the concentration of denaturant is varied.

The equilibrium urea-induced unfolding of apomyoglobin at physiological pH was monitored following its limited oxidation by synchrotron irradiation (Maleknia & Downard, 2001). At a fixed exposure time of 30 msec, the level of oxidation of apomyoglobin is shown as a function of urea concentration in Figure 6. It is immediately apparent that the extent of oxidation increases at a higher urea concentration.

Proteolytic digestion of total protein treated with urea over a range of concentrations enables unfolding profiles to be plotted for regions throughout the protein structure. The profiles for three endoproteinase Lys-C peptides that contain residues 1–16, 17–42, and 103–118 are shown in Figure 7, together with an isotherm generated based on fluorescence spectroscopy measurements for the protein as a whole (Maleknia & Downard, 2001). The Lys-C segments span four helices designated A (residues 4–19), B and C (residues 21–42), and G (residues 101–118). Each segment contains a number of reactive amino acid residues denoted in Figure 7.

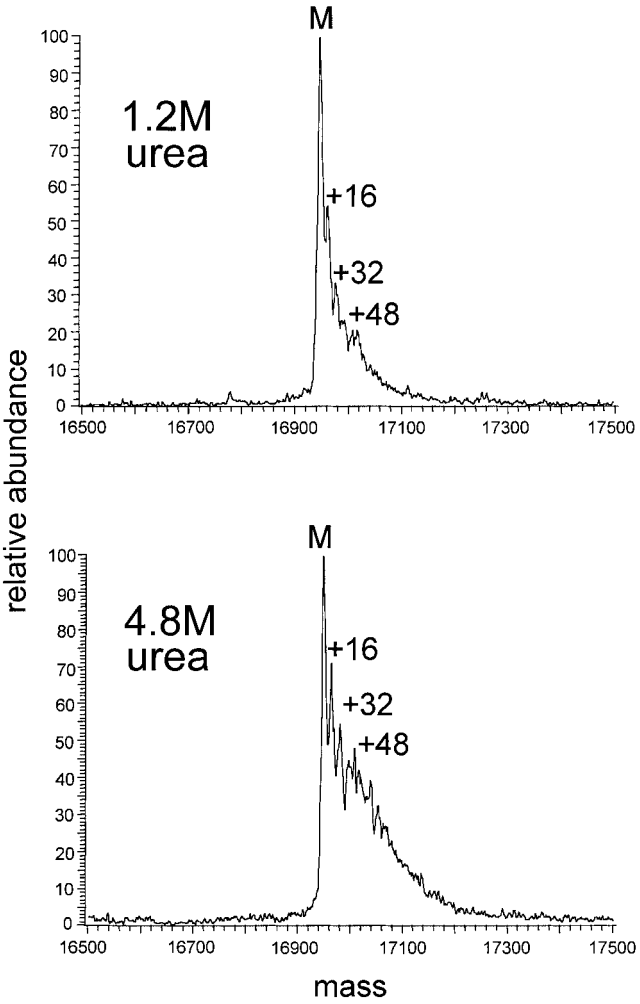

FIGURE 6. Deconvoluted electrospray mass spectra of apomyoglobin irradiated for 30 msec in solutions that contain 1.2 and 4.8 M urea.

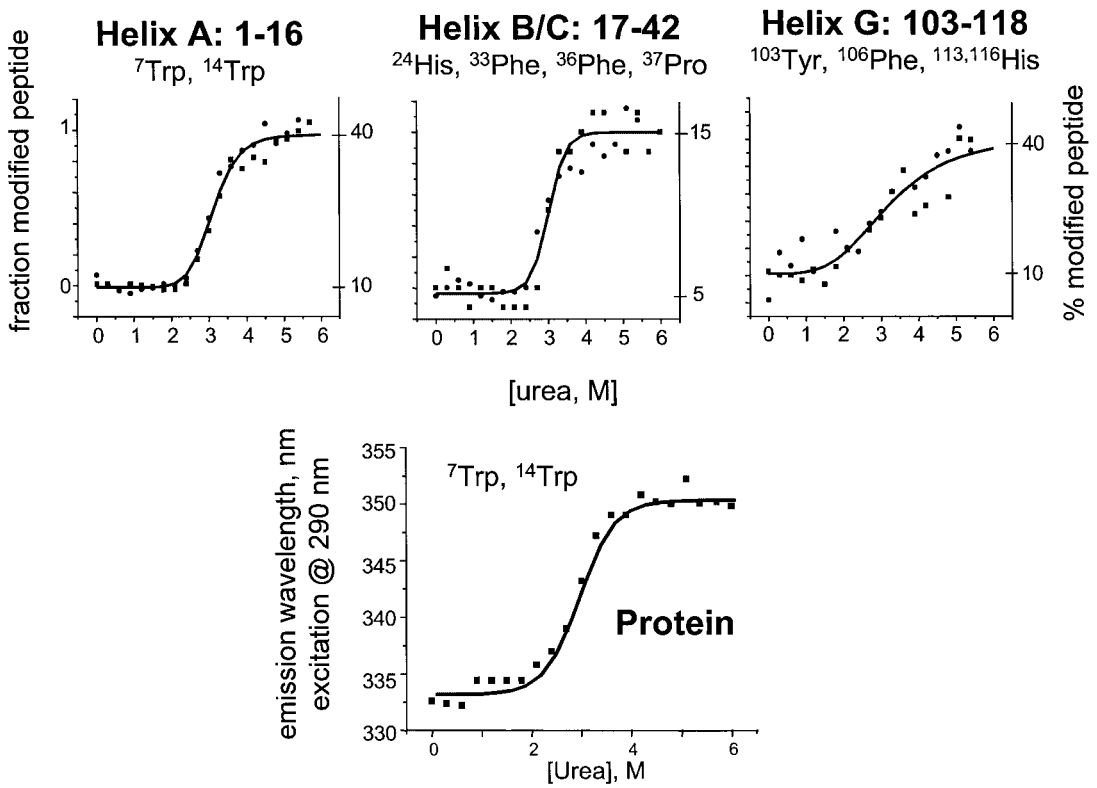

FIGURE 7. Unfolding profiles for segments of apomyoglobin associated with helices A, B/C, and G after irradiation of the protein for 30 msec in solutions of increasing urea concentration. The lower profile shows unfolding of the intact protein in urea as followed by fluorescence spectroscopy.

Thermodynamic midpoint, free energy, and *m*-values were calculated from each profile, using a two-state folding model previously described (Santoro & Bolen, 1988). The urea-induced unfolding of apomyoglobin was investigated by fluorescence spectroscopy under conditions identical to those employed in the radiolysis experiments. The thermodynamic data for all experiments are shown in Table 2. The data for helices A and B/C show a highly co-operative, two-state unfolding behavior that, within experimental error, are similar to those that reflect the unfolding of apomyoglobin as a whole obtained by fluorescence spectroscopy. However, the level of oxidation monitored at the reactive residues in helix G, namely Tyr103, Phe106, His113, and His116, indicates a local unfolding behavior dissimilar to that for the globular protein. The calculated *m*-value for helix G (0.6 ± 0.1 kcal mol^{-1} M^{-1}) is one-third of that measured from the fluorescence data. These data indicate that a small, but significant, fraction of the total surface area of the protein is unfolded during the urea-induced transition. Based on the *m*-value for this domain, the G-helix is 300-fold less stable than the protein as a whole. This observation is in accord with previous reports on the stability of apomyoglobin (Loh, Kay, & Baldwin, 1995; Chi & Asher, 1998), and highlights the ability of this radical-based approach to resolve folding mechanisms within a small protein like apomyoglobin. The method has since been extended to study the hydrophobic core of the protein expressed by the TFAR19 gene that is associated with apoptosis of tumor cells (Maleknia et al., 2001b).

TABLE 2. Thermodynamic data of midpoint, free energy, and *m*-value for segments of apomyoglobin that contain helices A, B/C, and G and the intact protein obtained from extrapolation of the plots generated in synchrotron radiolysis and fluorescence spectroscopy experiments

	Helix A	Helix B/C	Helix G	Protein[a]
$k_{midpoint}$ (M)	3.1 ± 0.1	3.0 ± 0.1	3.2 ± 0.1	2.9 ± 0.1
$G^{o}_{N\text{-}U}$ (kcal/mol)	5.4 ± 0.7	7.6 ± 1.6	2.5 ± 0.5	4.7 ± 0.6
m (kcal/mol/M)	1.7 ± 0.2	2.0 ± 0.2	0.6 ± 0.1	1.7 ± 0.2

[a]Based on fluorescence spectroscopy measurements at tryptophan residues.

Time-resolved kinetic studies of protein-folding or -unfolding transitions can also be performed in a similar manner to that applied to ribonucleic acids (Sclavi et al., 1997, 1998). The rapid mixing of protein solutions with solutions that contain metal ions or denaturants is achieved ahead of the exposure of the combined solution to a radiation or discharge source. Where a continuous radiation source or discharge is maintained, the level of exposure to radiation can be controlled by the time it takes for the mixed solution to pass through the exposure chamber or electrospray needle tip. The collected solutions or condensates can be quenched and analyzed by mass spectrometry either directly or following proteolysis.

VIII. PROTEIN COMPLEXES AND ASSEMBLIES

The topography of protein surfaces is important to many biological processes and events, and influences the association of enzyme-substrate, protein-ligand, and antigen-antibody complexes. Such systems are difficult or impossible to study by conventional spectroscopic and crystallographic approaches. This difficulty is due to their large physical size, their susceptibility to dissociation versus the constituent molecules and, in some cases, the low relative levels of complex present.

Although mass spectrometry techniques have been applied directly to the study of protein–protein interactions (Loo, 1997; Moniatte et al., 1997; Farmer & Caprioli, 1998; Kiselar & Downard, 1999, 2000; Downard, 2000), such approaches are complicated by issues associated with ionization and detection effects, and efficiencies. It is critical, though often difficult, to establish whether the sample preparation and ionization protocols may contribute to the association or disruption of protein complexes. Furthermore, because protein ions are studied within mass spectrometers essentially free of solvent, the nature of the interactions between proteins of a complex may or may not mirror that in solution. In addition, ion detectors typically exhibit non-linear detection characteristics across the mass-to-charge range so that protein constituents may be detected preferentially at the expense of any complex present.

The radical-based strategies described in this review are of immediate value for the study of protein–protein associations under a range of solution conditions provided that such complexes are preserved during the production and reaction of radicals (Maleknia et al., 2000). To demonstrate that protein complexes can indeed be preserved and studied, the synchrotron-based radical approach was applied to the study of the binding of gelsolin to actin (Goldsmith et al., 2000). Gelsolin is an actin regulatory protein composed of six homologous domains denoted G1-6. It is a member of a family of actin-binding proteins that bind to and sever actin filaments. This ability to break actin filaments, results in gelsolin playing a key role in cellular processes such as motility and differentiation, both of which rely on dramatic reorganizations of the actin cytoskeleton (Burtnick, Robinson, & Choe, 2001).

Of the conserved 15-kDa domain, segment G1 binds to a molecule of actin with nanomolar affinity (McLaughlin et al., 1993). A central actin-binding region of this segment of gelsolin consists of residues 96–108 that contains a single oxidizable phenylalanine at position 104. The surface area of this residue's side chain is considerably shielded from the bulk solvent upon binding with a molecule of actin (Table 3). In the presence of actin, the solvent accessibility of the side chain of Phe104 decreases from 28.8 to 0 $Å^2$. A second segment of gelsolin, comprising residues 66–83, is not involved in the binding of actin—affording no protection to the oxidizable tyrosine amino acid side chains at positions 68 and 83 contained within it (Table 3). The non-binding control peptide serves to distinguish site-specific protections that result from the interaction of gelsolin with actin from rate variations associated with actin serving merely as a radical sink.

Dose-response profiles were recorded for the oxidation of each of the proteolytic peptides after solutions of gelsolin-G1 alone and gelsolin-G1 with actin were

TABLE 3. Sequence and accessibility data for regions of gelsolin segment G1 used in binding experiments with actin

Proteolytic fragment (AspN)	Peptide sequence	Oxidizable residue(s)	Solvent accessibility in gelsolin[a](in $Å^2$)	Solvent accessibility in gelsolin-actin complex[a](in $Å^2$)
66–83	DAYVILKTVQLRNGNLQY	Y68	5.0	5.0
		Y83	10.9	10.9
96–108	DESGAAAIFTVQL	F104	28.8	0.0

[a]Calculated (Richards, 1974; Richmond, 1984) based on X-ray crystal structures for gelsolin and the gelsolin-actin complex (McLaughlin et al., 1993).

irradiated with synchrotron light. Profiles for peptides 96–108 and 66–83 are shown in Figure 8. Rate constants were derived from the dose-response profiles in the same manner to that described above. In peptide 96–108, the rate of oxidation decreases from 0.57 to 0.02 s^{-1}, after the addition of actin to the solution of the gelsolin G1 segment. In contrast, the rate of oxidation in peptide 66–83 remains relatively unaffected. Thus, the oxidation rate is essentially unaffected by the presence of actin in segment 66–83, although it is substantially decreased in peptide 96–108, in accord with the shielding of the reactive residue Phe104 upon the binding of actin (Fig. 9). The results illustrate that phenylalanine at position 104 is protected from hydroxyl radical-induced modification in the presence of actin.

These data demonstrate that radical reagents can be used to probe protein-protein interactions provided that the lifetime of the complex is longer than its exposure to radicals. Therefore, the complex should have a slow off-rate and/or the components should exhibit a high binding affinity. At this stage, the lower limit to the binding affinity is unknown and awaits further investigations. In addition, the change in solvent accessibility of residues within the binding site should be significant enough to exceed the measurement errors associated with these experiments. Nonetheless, the approach has considerable merit for the study of protein complexes and assemblies with high structural resolution that are difficult or impossible to investigate by other spectroscopic or crystallographic means.

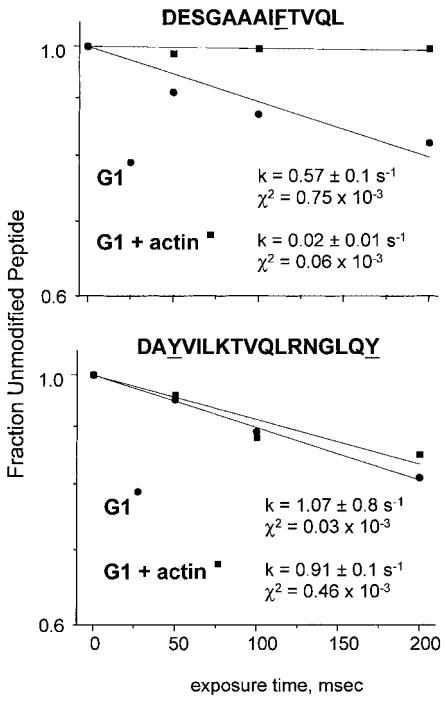

FIGURE 8. Dose-response profiles following oxidation in regions of the gelsolin G1 domain from the irradiation of the protein in the presence and absence of actin.

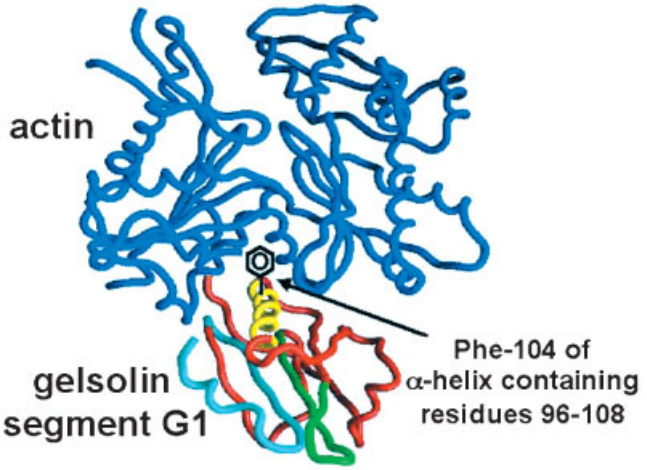

FIGURE 9. Backbone representation of the binding of actin to the gelsolin G1 domain with shielding of the side chain of Phe104 (annotated Phe104 side chain not drawn to scale). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

IX. CONCLUSIONS

The viability of a powerful new approach for studying the structure and dynamics of proteins and their interactions with other macromolecules has been demonstrated. Although radical-based methods for studying proteins are still under development, the data presented in this review suggest that the strategies will find widespread utility for solvent-accessibility measurements of proteins under a wide variety of solution conditions with high structural resolution.

As with any new approach, there are many areas for improvement. These areas include the automation of the methodologies to achieve high sample throughput. It can be easily envisaged that protein solutions could be irradiated or treated with an electrical discharge within a cell of a mixed-flow device, and the exposed solution subsequently passed either directly, or following automated post-treatment, to a mass spectrometer.

Importantly, the results presented in this review demonstrate that radical reagents are a sensitive probe of protein structure and dynamics under appropriate experimental conditions. This observation contrasts the widely held perception that such species only impart structural damage. Although concerns regarding a method that involves the limited chemical oxidation of proteins and the influence that this modification has on the biological

properties of the molecule are well-founded, it is demonstrated here that meaningful biophysical data can be generated for the study of protein structure, folding, and association.

These radical-based approaches appear to offer a number of significant advantages over existing technologies for the study of such protein systems and processes that are difficult to achieve in present spectroscopic and crystallographic methods and strategies, including proteins that are in an impure state. Radical-based studies of proteins are predicted to be of value in solving many important biological problems that are now emerging with the birth of functional genomics and proteomics.

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