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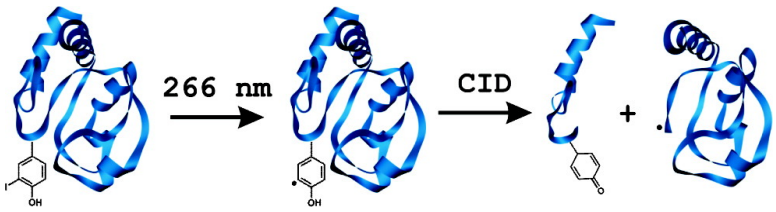

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Residue-Specific Radical-Directed Dissociation of Whole Proteins in the Gas Phase

Tony Ly and Ryan R. Julian*

Department of Chemistry, University of California, Riverside, California 92521

Received August 30, 2007; E-mail: ryan.julian@ucr.edu

Abstract: The rapid identification of proteins from biological samples is critical for extracting useful information in proteomics studies. Mass spectrometry is one among the various methods of choice for achieving this task; however, current approaches are limited by a lack of chemical control over proteins in the gas phase. Herein, it is shown that modification of tyrosine to iodo-tyrosine followed by UV photodissociation of the carbon−iodine bond can be used to generate a radial site specifically at the modified residue. The subsequent dissociation of the protein is largely dominated by radical-directed reactions, including dominant backbone fragmentation at the modified tyrosine. If iodination of the protein is carried out under natively folded conditions, the modification and ultimate fragmentation can typically be isolated to a single tyrosine residue. Some secondary backbone cleavage in the immediate vicinity of the modified tyrosine also occurs, especially if proline is present. In the absence of a reactive tyrosine residue, similar chemistry occurs via iodination at histidine. Possible mechanisms which would lead to the observed a-type fragments at tyrosine and the secondary fragments at proline are discussed. A method for using this type of site-specific information to reduce database searching times in proteomics experiments by several orders of magnitude is outlined.

Introduction

The chemical bonds holding together the naturally occurring amino acids are typically stable, as are the peptide bonds which link them to one another in a protein.[1] From a functional standpoint, it is logical that proteins are stable species; however, sequence identification usually requires at least partial disassembly, meaning that some of these strong bonds must be broken. There are several methods available for doing this in the condensed phase. Selective or semi-selective cleavage can be performed in solution with trypsin or a variety of other enzymes.[2] Site selective chemical digestion is also possible.[3] Importantly, the results are predictable with reasonable reliability in both cases and are used for protein identification. Random fragmentation of the protein by implementing very harsh conditions is not typically performed.

In contrast to solution-phase methods, site-directed dissociation of peptides or proteins in the gas-phase remains a formidable challenge, despite a variety of available techniques. Collision induced dissociation (CID) is the most common method. CID is performed by depositing energy into an ion by many collisions with neutral gases.[4] Typically, this leads to bond fracture at many sites which cannot be easily determined a priori; however, preferred cleavages have been observed under certain conditions. For example, Reid and co-workers used chemical derivatization to direct very selective side-chain fragmentation at methionine residues.[5] In other experiments, dissociation of the backbone at acidic residues or proline has been shown to occur preferentially.[6,7] However, factors such as charge state and sequence are known to influence these backbone fragmentations, and there is no facile method for rationally directing dissociation at these residues, particularly with whole proteins. Electron capture dissociation (ECD)[8] and electron-transfer dissociation (ETD)[9] utilize electrons to facilitate backbone dissociation via complex and incompletely understood mechanisms.[10,11] ECD and ETD are amenable to experiments with whole proteins, but do not typically exhibit site-selective dissociation. This may relate to difficulties associated with trying to dictate the location where an electron will interact with a biomolecule or to conformational heterogeneity.[12] Modest preferential dissociation is observed at disulfide bonds, but this does not yield sequence information.[13] Finally, less commonly

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employed photodissociation experiments using various ultraviolet wavelengths generally produce a large number of fragments in experiments with peptides.[14,15] This type of photodissociation has not been explored with whole proteins, which would probably necessitate the implementation of a multiphoton approach.

Although none of these techniques yield selective dissociation alone, it may be possible to combine the strengths of each method to achieve this goal. For example, experiments with ECD and ETD suggest that the presence of a radical facilitates backbone dissociation. It is also known that CID will follow the lowest energy dissociation pathway (if a single such pathway can be established). In fact, recent experiments employing radical precursors that can be activated by CID to generate odd electron species have yielded some fragment selectivity following re-isolation and further collisional activation, although the initial radical generation also led to many undesirable side products.[16−18] Notwithstanding, these experiments successfully combined aspects of CID and ECD or ETD to yield results not obtainable with either method alone.

There are several inherent difficulties associated with directing dissociation of a whole protein at a particular amino acid in the gas phase. First, the chemical complexity of proteins is problematic. The twenty naturally occurring amino acids comprise side chains with acids, bases, hydrocarbons, alcohols, aromatic rings, amides, etc. This list does not include additional chemical groups that can be added post-translationally.[19] Thus, rationally directed dissociation must utilize chemistry capable of functioning in the presence of any combination of these chemical groups. Second, proteins are large molecules. Even ignoring the chemical details, proteins can contain thousands of atoms with $3N - 6$ vibrational degrees of freedom (N = number of atoms). Site-directed dissociation requires that sufficient energy be directed to a particular bond in order to break it without disrupting the remaining bonds. There are two ways to do this: (1) the dissociation energy for one bond must be reduced significantly below the threshold for all other bonds,[20] or (2) sufficient energy to cleave a bond must be delivered to a specific bond which must dissociate prior to intramolecular vibrational energy redistribution (IVR).[21] IVR must be circumvented in the case of a protein because randomization of the amount of energy required to break a single bond over the number of vibrational degrees of freedom in a protein would lead to no bond dissociation.[22]

Previous experiments have demonstrated that radical mediated backbone dissociation is a low-energy process.[23] In order to utilize this chemistry to initiate site-selective protein dissociation, the radical must be generated in a specific location. To facilitate maximum selectivity, radical generation should also be achieved without heating the entire molecule. Radicals can be generated site specifically by photolysis of a carbon-iodine (C−I) bond.[24] C−I bonds of either the alkyl or aryl variety are known to undergo direct dissociation following photoactivation in the ultraviolet.[25] Aryl iodides can also undergo rapid predissociation following conversion of the excited-state localized in the aromatic ring to a dissociative state along the C−I bond.[26] Both processes occur in less than 600 fs.[27] In proteins, there are no C−I bonds in the naturally occurring amino acids; however, tyrosine and, to a lesser extent, histidine can be iodinated under mild conditions at biological pH using the chloramine T method. Iodination under these conditions generates 3-iodotyrosine and 4(5) iodohistidine.[28] This reaction requires only minutes to complete and exhibits a high degree of selectivity, meaning that the most exposed tyrosine residues (or residue) can be selectively iodinated.[29]

Herein, it is shown that 266-nm photoactivation of an iodotyrosine containing protein cleanly leads to the generation of a highly localized radical on the aromatic ring of the modified tyrosine side chain. This radical can then be used to direct selective a-type fragmentation of the protein backbone at the modified residue following re-isolation and further collisional activation. The technique is successfully demonstrated with a variety of proteins. Some iodination at histidine is also observed, leading to backbone cleavage at the modified histidine residues as well. Proline is shown to be a susceptible site of secondary cleavage, leading to backbone fragments when in close proximity to a modified tyrosine or histidine. Furthermore, it is demonstrated that this type of selective dissociation can be used to identify known proteins in proteomics experiments 3 to 4 orders of magnitude faster than that with traditional techniques.

Experimental Section

Materials. All reagents and proteins were used without purification unless otherwise noted. Cytochrome *c* (horse heart), ubiquitin (bovine), lysozyme (chicken egg), myoglobin (horse heart), hemoglobin (human), and dithiothreitol were purchased from Sigma Aldrich (St. Louis, MO). Chloramine-T, sodium metabisulfite, and sodium iodide were purchased from Fisher Chemical (Fairlawn, NJ). Water was purified to 18.2 MΩ resistivity using a Millipore Direct-Q (Millipore, Billerica, MA). Dialysis membranes and clips (MWCO = 3500 Da) were purchased from Spectra Por (Rancho Dominguez, CA).

Chloramine-T Iodination of Proteins. Proteins were iodinated by modification of a previously published procedure.[30] I^- is oxidized by the addition of chloramine-T to I^+, which adds to the ortho position of tyrosine side-chain by electrophilic aromatic addition. After a short reaction period, sodium metabisulfite, a reducing agent, is added to quench the iodination. Stoichiometric quantities of reagent were used to limit the extent and heterogeneity of iodination (1:1:2:4 protein: sodium iodide:chloramine-T:sodium metabisulfite). Reactions were initiated by mixing ∼1−5 mg of protein and sodium iodide in 0.2 mL

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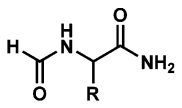
water, followed immediately by addition of chloramine-T. Sodium metabisulfite was added after 10 min to quench the reaction. We found that lysozyme, which has four disulfide bonds, required harsher reaction conditions to produce comparable iodination yield to the other proteins investigated (2× iodination reagents, 30 min reaction time). Iodinated lysozyme and hemoglobin were purified by dialysis against water. This iodination procedure typically yields 30−60% mono-iodinated protein (see Supporting Information).

Reduction/Alkylation of Disulfide Bonds. Disulfide bonds in iodinated lysozyme were reduced and alkylated using dithiothreitol (DTT) and iodoacetamide.[31] Iodinated lysozyme was dissolved in 6 M urea in 0.025 M triethylamine acetic acid (TEAA) buffered at pH 8.5. DTT was added in excess (5X disulfides, 50 °C, 2 h). Reduced lysozyme was then alkylated by addition of iodoacetamide (10× DTT + free thiols, room temperature, 1 h). Reduced, alkylated lysozyme was dialyzed against 1% acetic acid, lyophilized, and reconstituted in water before mass spectrometry.

Electrospray Mass Spectrometry. Solutions containing ~10 μM of protein in 10% methanol were infused into a standard electrospray source. Ions were then transferred into an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) for mass analysis and/or dissociation experiments. The posterior plate of the LTQ was modified with a quartz window to transmit fourth-harmonic (266 nm) laser pulses from a flashlamp-pumped Nd:YAG laser (Continuum, Santa Clara, CA). Pulses were synchronized to the end of the isolation step of a typical MS^2 experiment by feeding a TTL trigger signal from the mass spectrometer[32] to the laser via a digital delay generator (Berkeley Nucleonics, San Rafael, CA). Photodissociation (PD) of iodinated protein always resulted in loss of iodine as the most abundant product ion (>50% relative abundance). Further MS^3 experiments were performed by re-isolation and collision induced dissociation (CID) of the photodissociation (PD) product. CID experiments were performed by applying an excitation voltage on mass-selected ions using default instrument parameters. Protein fragments were assigned with the aid of UCSF Protein Prospector v.4.0.8. The nomenclature[33] adopted also includes the superscripts "*" and "°" to indicate NH_3 loss and H_2O loss, respectively (e.g., $b^*_{58} \equiv b_{58} - NH_3$). Assignments do not distinguish between closed shell vs radical (e.g., -H·) fragments.

Ab Initio Calculations. All calculations were performed using hybrid density functional theory (B3LYP) at the B3LYP/6-31G* level of theory as implemented in Gaussian 03 Version 6.1 Revision D.01. Unrestricted methods (UB3LYP) were used for all open-shell systems. Spin contamination was minimal for all systems. Candidate structures were built using GaussView 3.0. Transition state (TS) calculations included optimizations and frequency calculations of the reactants, products, followed by a quasi-Newton synchronous transit (QST3) calculation[34] and a frequency calculation on the resulting TS candidates. Visualization of the single imaginary frequency found (TS1: −1575.33 cm^{-1}, TS2: −1635.26 cm^{-1}) verified that the structures are indeed saddle points connecting the reactant and product. Zero point corrected energies were used to calculate activation barriers.



R = Asn, Pro, Trp, Val, Tyr

C−H bond dissociation energies (BDEs) for tyrosine (βH), asparagine (βH), proline (δH), tryptophan (βH), and valine (βH) in the model peptide shown above were calculated using isodesmic reactions[35] with glycine as performed previously by Rauk, et al.[36] The calculations were performed on the trans peptide, with the exception of proline, wherein both conformers were considered. The C_α−H BDE for glycine was used as a reference value, and taken as 331.0 kJ/mol.[36,37]

Results and Discussion

Experimental results obtained with cytochrome *c* (Cyt*c*) are shown in Figure 1. Iodination was carried out under mild conditions which produce mostly mono-iodo species. Electrospray ionization can be used to transfer the protein into the gas phase. The resulting desolvated, multiply protonated ions are collected into a linear ion trap. Photodissociation of isolated $[\text{iodo} - \text{Cyt}c + 10\text{H}]^{10+}$ with 266 nm light yields a single peak corresponding to the loss of I· as shown in Figure 1a. Radical formation via direct dissociation (or fast predissociation) of the C−I bond was confirmed by comparing the results with CID experiments on the same peak. CID in an ion trap is caused by thousands of collisions with gaseous neutrals, ensuring that any observed dissociation follows a statistical process.[20,38] No loss of I· is observed with CID, suggesting that C−I cleavage is not a low-energy dissociation pathway. Therefore, photodissociation of the C−I bond must occur promptly from a dissociative excited electronic state. Photoactivation of the unmodified protein results in no appreciable dissociation, as shown in Figure 1b. This result indicates that after IVR, the energy from a single 266-nm photon absorbed by tryptophan or tyrosine is insufficient to fragment the protein. In contrast, much smaller peptides containing tryptophan or tyrosine can be fragmented by 266-nm light.[15]

These results demonstrate that photoactivation of iodotyrosine is an effective method for generating a radical site selectively, even when attached to an entire protein. Cyt*c* contains one tryptophan and four tyrosines, meaning that at least three tyrosine residues are unmodified. Despite the fact that these competitive chromophores are present in the protein, effective radical generation is still possible. It is likely that some absorption may occur at these residues; however, this leads to fluorescence or internal conversion of the energy. Neither of these outcomes will dramatically affect the protein as demonstrated in Figure 1b, where native chromophore absorption leads to no observable change. The bond dissociation energy for an aromatic C−I bond is ~280 kJ/mol.[39] The energy from a 266-nm photon is 450 kJ/mol, meaning that additional energy will be available following bond breakage. This energy can be dissipated translationally in the departing I· or by internal conversion within the side chain.

The protein radical produced by loss of I· can be re-isolated and fragmented by CID as shown in Figure 1c. This step of the experiment does not produce a single fragment, as observed following photodissociation; however, the number of abundant peaks is small, and examination reveals that all of them result from radical-directed fragmentation. Most importantly, the a_{74} fragment results from cleavage C-terminal to Tyr74 and is the second most abundant fragment, actually appearing twice in two

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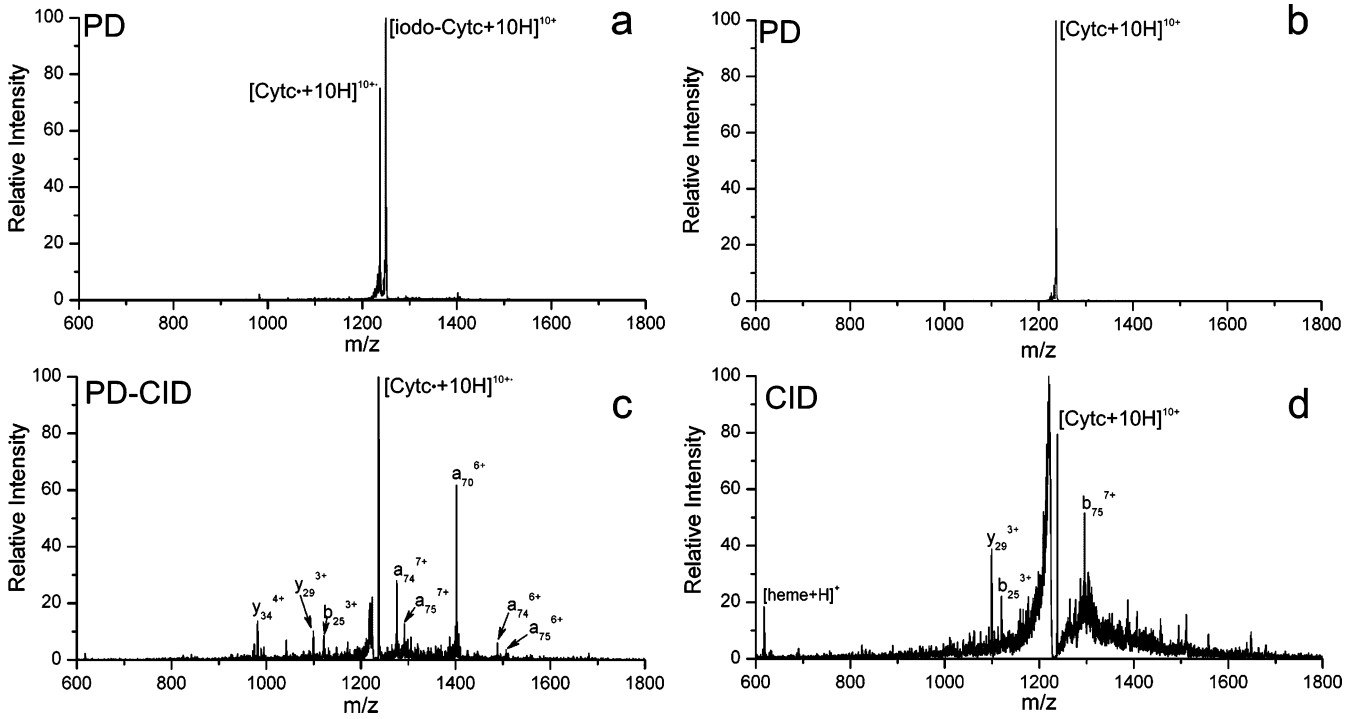

Figure 1. (a) Photodissociation spectrum for the +10 charge state of iodo-Cyt*c*. The only significant observed loss is I• which occurs with an excellent yield. (b) In contrast, photoexcitation of the unmodified protein leads to no significant dissociation. (c) The radical protein generated in (a) is fragmented by CID. All of the labeled diagnostic fragments (except b_{25}^{3+}) represent radical-directed dissociations. Each of these radical-directed fragments is produced within four amino acids of the modified tyrosine residue. (d) CID spectrum of the unmodified protein is provided for comparison. Few fragments are resolved from the bulk of nonselective cleavages. It should be emphasized that all of the spectra shown (a−d) are not magnified in any way.

different charge states. This fragment is not observed in a regular CID experiment performed on the unmodified protein (see Figure 1d for comparison). The a_{70}/y_{34} fragments are also facilitated by the tyrosine radical (as indicated by their absence in normal CID) on the n-terminal side of Pro71, which is in close proximity to Tyr74. For all other charge states studied (+9, +8), a_{74} and a_{70} are the most intense ions observed. These and other secondary fragments are discussed further below.

In addition, the y_{29} and b_{25} ions that are generated in the standard CID experiment are observed but at much lower relative intensity, and the b_{75} ion (the most abundant product by standard CID) is not observed. Overall, these results suggest that the barriers to dissociation are lower for radical-directed cleavages. Furthermore, dissociation is heavily favored in the immediate vicinity where the radical is generated, including a diagnostic backbone cleavage at the modified tyrosine residue. Interestingly, there are four tyrosines in Cytc,[40] but the results in Figure 1 suggest that Tyr74 is the most reactive, allowing for selective derivatization. This is in agreement with previous observations where Tyr74 and (to a lesser extent) Tyr67 were found to be iodinated under harsher conditions.[41] Inspection of the crystal structure[40] reveals that Tyr67 is partially buried, supporting the possibility for preferential iodination of Tyr74 under mild conditions.

In Figure 2, the results for applying this technique to four additional proteins are shown. In these spectra, the relative contributions for fragments that appear only after CID of the protein radical are shown as a function of sequence. These plots emphasize only radical-directed fragments (which typically dominate the spectrum). The contributions for related fragment types[33] (such as a and x, b and y, and c and z, as shown in Figure 3a) are summed, although the contributions from x-type fragments are very minor. The results for ubiquitin are shown in Figure 2a. Cleavage on the C-terminal side of Tyr 59 produces the second most abundant fragment, a_{59}. Additionally, a radical-mediated secondary fragmentation on the C-terminal side of Asn60 is observed, and several fragments occur at Arg54-Thr55. All of the abundant radical reactivity is localized in the vicinity of Tyr59 (ubiquitin has only one tyrosine). Examination of the crystal structure reveals that Arg54 is held in close proximity to Tyr59.[42] This may suggest that portions of the crystal structure remain intact in the gas phase,[43] although this issue will require further examination.[44] Cleavage at His68 suggests some secondary iodination at this residue. Histidine iodination is discussed in further detail (vide infra). The identified modifications are in excellent agreement with previous iodination experiments where Tyr59 and His68 were found to be the only residues iodinated in ubiquitin.[45]

In Figure 2b, the results for the A-chain of human hemoglobin, a substantially larger protein, are shown. The most intense peak results from a-type fragmentation on the C-terminal side of Tyr140. There are no other fragments observed in the vicinity of Tyr140, indicating that secondary fragments are sequence- or structure-dependent and will not always be observed. In fact,

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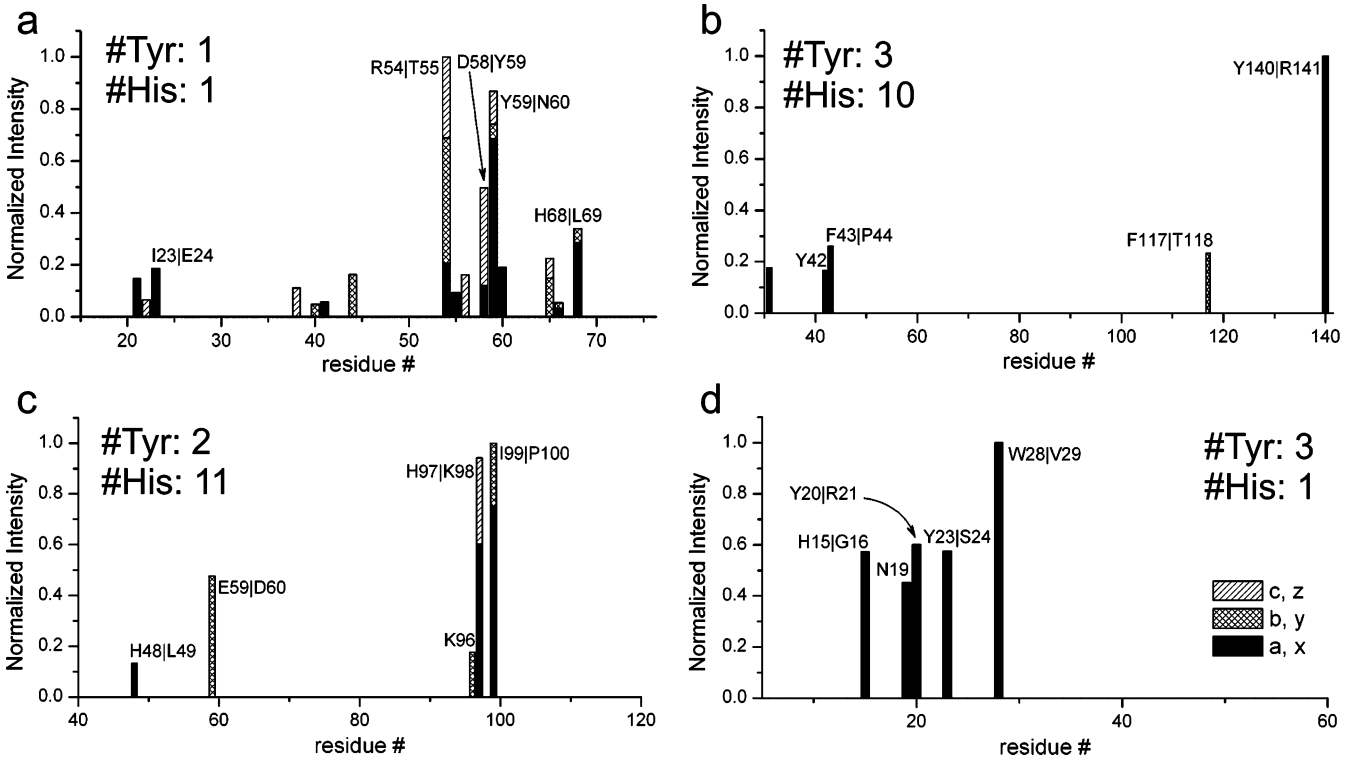

Figure 2. Stack plots showing the relative contributions from different radical-directed fragments and the number of tyrosine and histidine residues for each protein. In general, fragmentation is localized around a modified tyrosine or histidine residue, producing dominant a-type fragment ions on the C-terminal side of the modified residue. X-type fragments are not common, meaning that most of the black bars represent intensity from a-type fragments. C and z and b and y type fragments are more equally distributed. Results are for (a) ubiquitin (6+), (b) hemoglobin (9+), (c) myoglobin (9+), and (d) lysozyme (10+).

fewer radical-directed peaks are generated for hemoglobin than for ubiquitin, confirming that selectivity is not a function of protein size. Importantly, this should allow the technique to be extended to even larger proteins, with the ultimate limitation being mass resolution. Although no substantial secondary backbone fragmentation is observed in the vicinity of Tyr140, there are fragments in the vicinity of Tyr42 that suggest some iodination occurs at this residue. Hemoglobin contains 3 tyrosine residues in total and 10 histidines. Tyr24 is substantially buried (precluding iodination), yet many of the histidine residues are fully exposed. Thus, observation of radical fragmentation only in the vicinity of tyrosine residues confirms the selectivity for tyrosine iodination over histidine if both residues are chemically available.[46]

Myoglobin is similar in size to hemoglobin but yields slightly different results from the other proteins, as shown in Figure 2c. For myoglobin, the second most abundant fragmentation is observed on the C-terminal side of His97 (a_{97}). A dominant secondary fragment (a_{99}) occurs in close proximity, N-terminal to Pro100. Typically, tyrosine will be iodinated 30−100 times faster than histidine.[46] However, if no tyrosine residue is accessible, then histidine can be iodinated. Our results indicate that the two tyrosine residues in myoglobin must be inaccessible in the natively folded protein. Examination of the crystal structure reveals that Tyr146 is clearly buried, whereas the alcohol in Tyr103 reaches the surface but is crowded on all sides, which may explain the lack of reactivity.[47] Importantly, selective fragmentation at the modified histidine is still observed, suggesting that any histidine residues that are iodinated will behave similarly to iodo-tyrosine (as is also observed with His68 in ubiquitin). It follows that proteins with no chemically available tyrosine residues may still be targeted via histidine.

The results for lysozyme are shown in Figure 2d. This protein contains disulfide bonds which must be reduced and alkylated prior to analysis. For lysozyme, a-type fragmentation on the C-terminal side of two tyrosine residues (Tyr20 and Tyr23) is observed.[48] Iodination is performed on the folded protein prior to alkylation, suggesting that these two residues have similar solvent accessibility. Indeed, previous reports have identified both Tyr20 and Tyr23 as sites of iodination.[49] Examination of the crystal structure supports these results.[50] Abundant cleavage is also observed on the C-terminal side of a proximal tryptophan residue. Tryptophan should not be iodinated by our procedure, indicating that this is a secondary fragmentation.

Cleavage at Iodinated Residues. It is interesting to consider why cleavage occurs specifically at modified tyrosine or histidine residues for every protein that we have examined. In both cases, the radical that is initially produced by the loss of iodine should be very reactive. This prediction can be evaluated more quantitatively in terms of relative C−H bond dissociation energies. A high C−H bond dissociation energy suggests that hydrogen abstraction from sites with lower C−H bond dissociation energies will be thermodynamically favorable. The C−H bond dissociation energy for either radical center (tyrosine or histidine) is ∼490 kJ/ mol.[51,52] This is higher than most other

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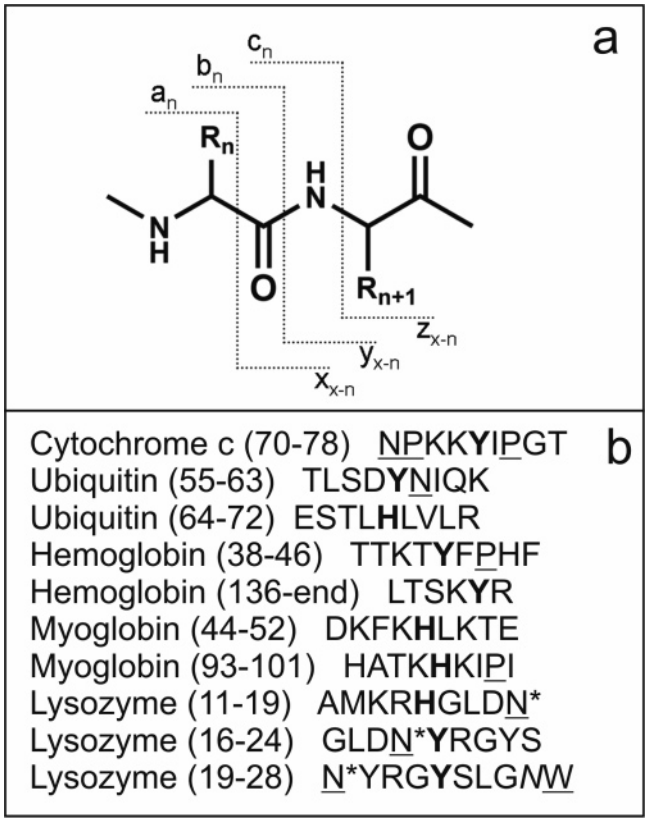

Figure 3. (a) Representative types of fragments typically observed in mass spectra. a/ x, b/y, c/z form complementary pairs. X = total number of amino acids in protein. (b) Local sequence around iodinated residues (in bold) for each protein. Proline, asparagine, or tryptophan residues that undergo secondary fragmentation are underlined. *Italicized* residues represent sites where secondary fragmentations are expected but not observed. Numbers in parentheses represent amino acid numbers for the sequence shown. The "*" represents all the same Asparagine.

nonaromatic hydrogens present in a protein by 80−160 kJ/mol, making virtually all possible subsequent hydrogen abstractions exothermic. Given that either radical should abstract the first hydrogen that it comes in contact with, specificity would not be expected unless subsequent reactions all led to the same product (which would seem unlikely). For tyrosine, there is a further difficulty because the initially formed radical cannot interact with the protein backbone where cleavage is observed. Previous work has shown that a-type fragments are typically produced in radical systems by abstraction of the β hydrogen, followed by cleavage of the carbon−carbon bond in the backbone.[18] Due to steric constraints caused by the rigid nature of the tyrosine side chain, the initially produced radical and the β hydrogen cannot interact. In fact, they are separated by >4 Å, with the radical pointed away from the hydrogen. Therefore, the selective reactivity and the direct a-type cleavages observed at tyrosine residues suggest that a fast rearrangement of the initially very reactive radical may occur.

All of the results can be rationalized with a charge-remote mechanism, given in Scheme 1 (note that in all cases, protein ions are protonated and multiply charged). In this mechanism, the initial radical undergoes intra-side chain hydrogen abstraction from the tyrosine OH. The calculated barrier to this rearrangement is ∼137 kJ/mol; however, there is excess energy (up to

Scheme 1

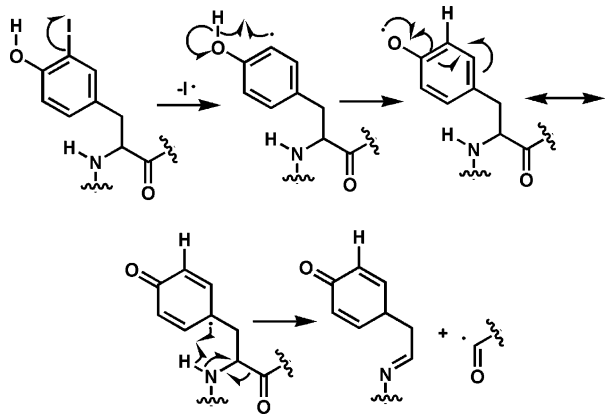

Table 1. Calculated Bond Dissociation Energies[a]

amino acid	BDE (kJ/mol) @ B3LYP 6-31G*
Tyr βC−H	381.8
Pro δC−H *cis*	393.7
Pro δC−H *trans*	391.5
Trp βC−H	350.9
Val βC−H	400.6
Asn βC−H	418.6

[a] See Materials and Methods for calculation details.

170 kJ/mol) available from the absorbed photon. If the hydrogen transfer occurs rapidly with respect to energy redistribution, then this excess energy may help facilitate the rearrangement. Transfer of the OH hydrogen is followed by rapid electronic rearrangement, delocalizing the radical into the aromatic ring. The delocalized radical is more stable by ∼140 kJ/mol and is also capable of directly producing an a-type fragment at the modified tyrosine by the mechanism shown in Scheme 1.[53] Abstraction of the amide N−H is likely to be endothermic; however, energy is being added to the system by collisions at this point in the reaction.[54] The most likely alternative to N−H abstraction would involve transfer of the β hydrogen to the rearranged radical, but this route is deemed unlikely because the transition state is ∼246.5 kJ/mol high. A competing dissociative pathway is loss of even-electron p-quinomethide. This pathway often dominates in radical peptides,[55] but does not explain the observed a-type fragments in this study. The present experiments cannot unequivocally determine whether secondary fragments are generated by the initial radical or the stabilized form, but the observed selectivity suggests that the stable form may dominate. Iodohistidine is capable of following a dissociation pathway analogous to that shown in Scheme 1 without rearrangement, directly producing the observed backbone cleavages (see Supporting Information).

Secondary Fragments. The local sequence for each tyrosine or histidine which exhibited an a-type fragment resulting from cleavage on the C-terminal side of the residue (i.e., residues that were likely iodinated) is shown in Figure 3b. In addition to the diagnostic a-fragment produced at the modified residue, the proximity of proline also appears to facilitate secondary fragments. Secondary cleavages on the N-terminal side of

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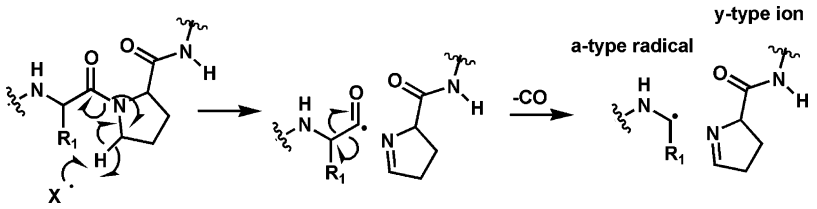
Scheme 2



proline (producing y and a fragments) are observed when proline is within four amino acids of the radical site. It should also be noted that fragmentation on the C-terminal side of asparagine to produce a-type fragments is observed several times in Figure 3. However, one of these cleavages is in conjunction with a proline fragmentation, and one asparagine (Asn27 in Lysozyme) fails to produce any fragmentation; therefore, it is unclear whether asparagine facilitates secondary dissociations. The abundant a-type fragment observed at Trp28 in lysozyme, five residues from Tyr23, is most likely a secondary fragment as well, but there are insufficient tryptophan residues in these proteins to draw any general conclusions. For proline, which represents ~5% of the amino acids in proteins, there is a 44% chance that it will be within ±4 residues of a tyrosine. Therefore, it is likely that secondary cleavages will be observed frequently, potentially making them suitable for obtaining additional sequence information after initial identification of tyrosine or histidine cleavage sites.

A previously proposed mechanism invoking hydrogen abstraction by a radical can be used to rationalize the secondary fragments at asparagine and tryptophan highlighted in Figure 3b.[18] In this mechanism, hydrogen abstraction from the β-carbon is followed by β-elimination to produce an a-type fragment. Therefore, amino acids with weakly bound β-hydrogens should be more susceptible to backbone dissociation. Previous experiments have demonstrated that abstraction of the α-hydrogen from asparagine occurs with a lower barrier than any other amino acid.[36] Furthermore, abstraction of the β-hydrogen from asparagine by OH radical is preferred over abstraction of the α-hydrogen,[56] suggesting that loss of the β-hydrogen should be facile. In contrast to these results, calculated bond dissociation energies do not suggest that asparagine should be susceptible to this type of attack. The results in Table 1 suggest that abstraction of the β-hydrogen from asparagine should not be facile relative to other amino acids. Further experiments will be required to resolve this apparent contradiction.

For proline, experiments in solution suggest that abstraction of a δ-hydrogen is preferred over the β-position.[57,58] A proposed mechanism which accounts for the secondary fragmentations observed in our experiments is shown in Scheme 2.[59] Abstraction of a δ-hydrogen leads to homolytic cleavage of the peptide bond.[60] The resulting y-type ion is stable; however, the b-type counterpart is not stable and will rapidly lose CO.[61] CO loss yields a radical a-type ion. Therefore, if this mechanism is correct, complimentary ions produced by radical-directed dissociation at proline will yield a and y ions, not b and y ions. Indeed these are the only type of fragments detected for dissociations at the prolines underlined in Figure 3b. Furthermore, CID of unmodified Cyt*c* yields complimentary b_{75} and y_{29} ions on the N-terminal side of Pro76 due to the proline effect (see Figure 1d).[7] CID of radical Cyt*c* yields a_{75} and y_{29} ions; the b_{75} ion is not observed despite being the most abundant fragment for the unmodified protein. The complete absence of the b_{75} ion is best rationalized by the radical-directed dissociation mechanism in Scheme 2 where b-type ions are not allowed. These observations also offer further proof that radical-directed dissociations occur with lower energy barriers than even favorable non-radical dissociation processes such as those observed at proline residues.

Applications to Database Searching. Experimental methods currently employed in proteomics research are generally considered to be more advanced than the bioinformatics tools which have been developed to interpret the results.[62] Thus, one might conclude that the principle barrier to further progress in proteomics research is bioinformatics. However, the difficulty is also due, in part, to the type of data that is collected in the experiments. As discussed in the introduction, bond breakage with all mass spectrometric dissociation techniques used for protein identification typically occurs randomly. This leads to the production of vast amounts of raw data, but the information content of this data is frequently low.[63] Thus, the nature of the experimental results is currently creating an unduly large burden which bioinformatics has been unable to resolve. An alternative strategy is to acquire better data with high information content that can be more easily interpreted. Radical-directed dissociation can be used to obtain specific (rather than random) information from peptide or protein fragmentation by dictating where fragmentation occurs. As a result, it is possible to significantly reduce the amount of time needed for data analysis.

Either the most intense or second most intense observed peak in each of these experiments results from dissociation on the C-terminal side of tyrosine. The one exception to this occurs for myoglobin where tyrosine is not modified, but in this case, the most intense cleavage is C-terminal to histidine. Given this information, four out of five proteins can be positively identified by checking two experimental peaks against all possible C-terminal a-type cleavages at tyrosine residues. There are 13 tyrosines present out of a total of 603 residues in the 5 proteins studied herein. Therefore, the numbers dictate that 10 experimental peaks would be checked against 13 possible matches for a total of 130 required calculations (assuming the protein is known). In contrast, standard techniques using dissociation results produced by random processes would require checking

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significantly more peaks. For example, if ECD or ETD were used and full sequence coverage was obtained, then 1196 potential peaks (assuming c and z ions only) would need to be checked against 1196 possible matches for a total of ~1.4 million calculations. The database search using the standard approach would therefore require ~4 orders of magnitude more time to perform. If the sequence coverage were reduced to only 50%, then 598 peaks would be checked leading to ~700 000 calculations. The potential gain would still be a factor of 5000. Inclusion of histidine residues in the radical-directed approach would require 390 calculations and lead to the identification of all five proteins. Furthermore, the secondary fragments which are generated at proline should be present ~44% of the time, potentially allowing for independent confirmation of the identification. It should be mentioned that the presence of post-translational modifications will complicate data analysis; however, this problem will scale equally for the traditional and radical-directed approaches.

Conclusions

The results presented here demonstrate that proteins can be efficiently and selectively cleaved at tyrosine or histidine residues in a gas-phase experiment. This abiotic approach is achieved in milliseconds by replacing a single atom in an entire protein. This capability will enable faster bioinformatics that will greatly speed the interpretation of proteomics data. Furthermore, a side benefit of this research is rapid new method for identifying sites of iodination in a protein. Iodination is frequently performed for protein structure analysis, and native iodotyrosines represent essential components in thyroid proteins such as thyroglobulin.[64]

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Supporting Information Available: All raw mass spectra and the full sequence stack plots. This material is available free of charge via the Internet at http://pubs.acs.org.

JA076535A

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