Identification of the Sites of Hydroxyl Radical Reaction with Peptides by Hydrogen/Deuterium Exchange: Prevalence of Reactions with the Side Chains†

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ABSTRACT: Hydroxyl radical-effected protium/deuterium ($^1H/^2H$) exchange into the C−H bonds present in peptides has been used to identify the site of hydrogen atom abstraction by hydroxyl radical. Radiolysis of anaerobic, N_2O-saturated D_2O solutions containing peptide and dithiothreitol generates a hydroxyl radical that mediates $^1H/^2H$ exchange into the side chains of peptides of up to 66 atom % excess ^2H. The $^1H/^2H$ exchange is determined by measuring the isotope ratio, $[M + H + 1]^+/[M + H]^+$, of the peptide using electrospray ionization−mass spectrometry. The $^1H/^2H$ exchange within each residue of the peptide was determined by measuring the isotope ratio of each isolated dansyl amino acid following hydrolysis and derivatization. Generation of 0.40 mM hydroxyl radical effected $^1H/^2H$ exchange into each of the five different residues of (Ala^2)-leucine enkephalin (YAGFL). The propensity of the residues to undergo exchange was L > Y > A ≅ F > G, independent of whether they were radiolyzed separately or as the peptide. The minimal exchange into glycine suggests that reaction of hydroxyl radical with the side chain hydrogens predominates over reaction with the polypeptide α-hydrogens. The ability of radiolysis to effect $^1H/^2H$ exchange into a larger peptide, SNEQKACKVLGI, was also demonstrated.

The hydroxyl (OH) radical[1] is known to react with amino acids, peptides, and proteins from pulse radiolysis studies (*1*−*3*). These studies define the rate constants and, hence, the relative reactivity of the amino acids. However, determining where the OH radical reacts has been a far greater problem. One of the predominant modes of OH radical-initiated damage is abstraction of a H atom from a C−H bond to produce a carbon-centered radical (*2*). These carbon-centered radicals have various chemical fates. Reaction with molecular oxygen initially forms a hydroperoxyl species that by reduction or disproportionation results in hydroxylation (*4*). Alternatively, elimination of the hydroperoxyl results in oxidation of the carbon and results in cleavage of the protein, if the initial H atom abstraction occurred at an α-carbon (*5*). Recombination of two carbon-centered radicals to form carbon−carbon cross-links has also been reported (*6*−*8*). Chemical repair by H atom donation, such as that mediated by sulfhydryls (*9*−*11*), is a potential physiologically relevant fate. Thus, the initial site of OH radical attack on a molecule is often obscured by the multiplicity of products.

With amino acid residues in peptides or proteins, the site of OH radical attack can be differentiated based on whether the reaction is with the side chain or the α-carbon. Only abstraction of hydrogen from the α-carbon and subsequent oxidation will lead directly to cleavage of the peptide backbone. H atom abstraction from the side chain carbons leads largely to hydroxylation (*4*) and not cleavage. Consequently assays for hydroxylation that rely on cleavage of the polypeptide backbone may fail to detect the most common reactions of the hydroxyl radical with proteins.

The reaction of OH radical with alkyl C−H bonds is rapid, 10^8 $M^{-1}s^{-1}$ (*3*), a value 10−100-fold less than the diffusion limit. This indicates that a H atom abstraction occurs on average once in every 10−100 collisions of OH radical with an alkyl hydrogen. This high frequency of reaction prevents OH radical generated in bulk solution from diffusing into the interior of macromolecular complexes. The success of DNA footprinting with the OH radical demonstrates that formation of macromolecular complexes protects the residues at the molecular interface from reacting with OH radical (*12*). If the reaction with hydroxyl radical is performed in the absence of O_2 , the carbon-centered radicals that are produced can be isotopically "repaired" by a free radical interceptor as shown in Scheme 1. Spin trapping agents have been used extensively as free radical interceptors in electron spin resonance to study transient free radicals (*13*). Highly reactive and short-lived radical intermediates that are difficult, if not impossible, to detect are "intercepted" and chemically converted to a less reactive and more stable species suitable for electron spin resonance detection. In these studies, we have used dithiothreitol (DTT)[2] in D_2O as the interceptor of the carbon-centered radical because of its ability to donate a ^2H atom and generate a C−^2H bond. In this process, the amino acid side chain is isotopically and irreversibly labeled, thus identifying the residue as being solvent accessible. The

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[1] The term OH radical will refer to both hydroxyl (OH) radical and deuteroxyl (OD) radical for samples irradiated in H_2O and D_2O, respectively.

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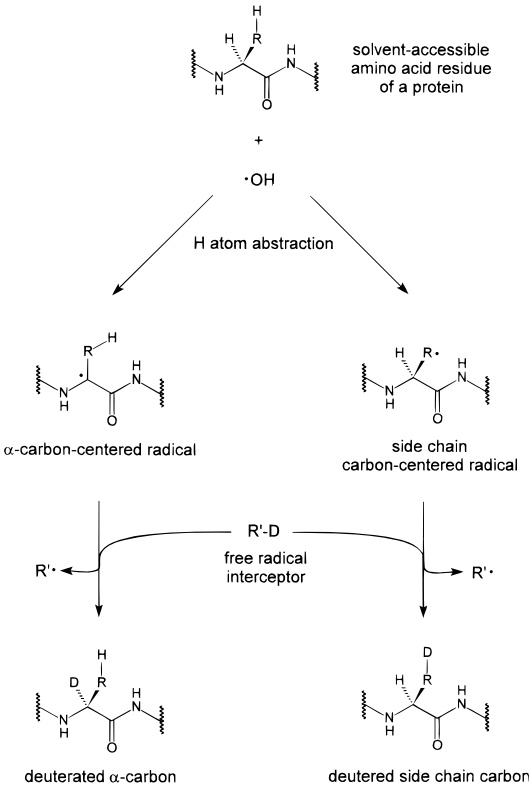
Scheme 1



determination of the amino acid residues containing the isotopic label would provide a means of assigning residues of peptides as solvent accessible and could be employed to study protein conformational changes and protein−protein interactions at the amino acid level as envisioned nearly 30 years ago (*14*).

Previously, we demonstrated ^{1}H/^{2}H exchange into the alkyl side chains of amino acids and the peptide (Ala^2)-leucine enkephalin (YAGFL) induced by anaerobic radiolysis. The ^{1}H/^{2}H exchange was quantified by measuring the amino acid or peptide isotope ratio using ESI−MS (*11*). We extend those studies by determining the relative reactivity of each residue of a peptide toward alkyl ^{1}H/^{2}H exchange. This requires (i) establishing ^{1}H/^{2}H exchange reaction conditions to maximize isotope incorporation while minimizing peptide decomposition, (ii) quantifying the ^{1}H/^{2}H exchange in each residue, and (iii) demonstrating that ^{1}H/^{2}H exchange into larger peptides is practical. This process is shown in Scheme 2 and has permitted us to quantify the site of reaction of OH radical with peptides. The results show that the reactivity of any amino acid side chain is not significantly altered by being incorporated into a peptide and that the α-hydrogens of the peptide are not the primary site(s) of reaction.

EXPERIMENTAL PROCEDURES

Materials. The materials used were obtained from the following commercial chemical sources and were used without further purification: (Ala^2)-leucine enkephalin (YAGFL), BACHEM (King of Prussia, PA); DTT, Boehringer Mannheim; acetonitrile (HPLC grade), Fisher Scientific; D_2O (99.9 atom % D), Aldrich; N_2O (Medipure U.S.P. grade containing 300 ppm O_2), PraxAir (Cleveland, OH). The C-terminal peptide (SNEQKACKVLGI, abbreviated as CTP) of the third intracellular loop of the 5-hydroxytryptamine$_{2A}$ receptor (obtained from Research Genetics) was a generous gift from Dr. Bryan L. Roth at Case Western Reserve University. CTP was further purified by reversed-phase HPLC as described (vida infra).

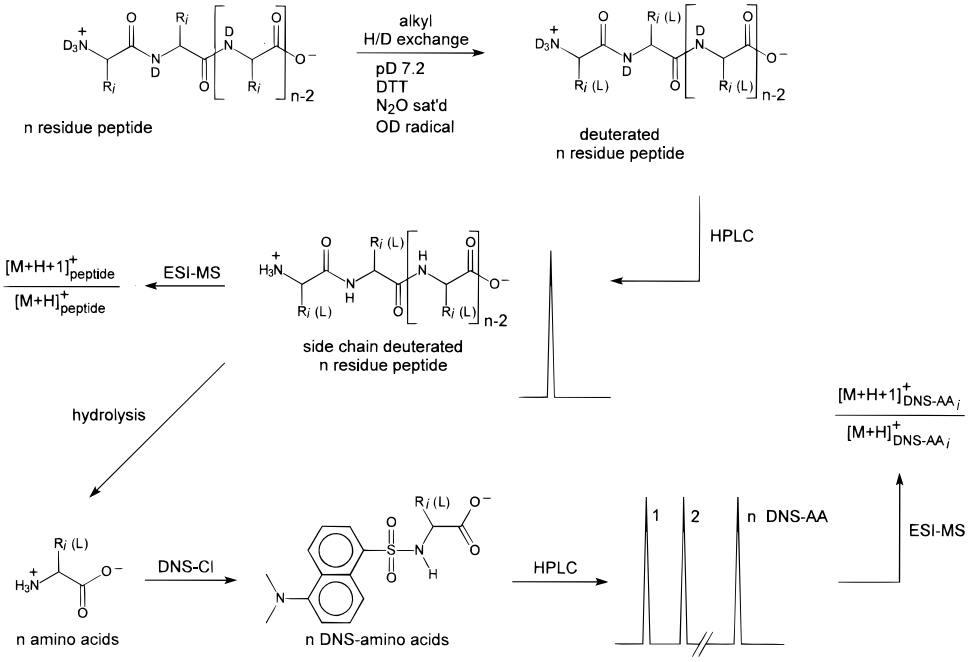
^{1}H/^{2}H Exchange into Amino Acids in the Presence of DTT. The reductive anaerobic reaction in D_2O contained 100 μM amino acid, 33 μM DTT, and 50 μM total OH radical in 10 mM phosphate buffer, pD 7.2 [pD = pH meter reading in D_2O + 0.4 (*15*)] at room temperature (20−25 °C). Each solution was gassed for 30 min with N_2O and irradiated within 30 min. Radiolysis was performed using a ^{137}Cs source for a specified period of time as determined using a dose rate of 0.52 Gy s^{-1} and the G(OH radical) value of 5.6 × 10^{-7} mol J^{-1} for N_2O-saturated solutions (22 mM) (*16*) as previously described (*11*). Two sets of experiments were conducted. One set of reactions contained a total reaction volume of 1.75 mL using Li phosphate buffer. The amino acids were desalted using cation-exchange chromatography as previously described (*11*). The other set contained a total volume of 1.0 mL containing sodium phosphate buffer. The amino acids were derivatized with DNS-Cl and then extracted (vida infra).

^{1}H/^{2}H Exchange into YAGFL at Various Total OH Radical Concentrations. The ^{1}H/^{2}H exchange reactions described above for the amino acids were used several times in succession, concomitant with reduced DTT and N_2O addition, to facilitate ^{1}H/^{2}H exchange into YAGFL. Each reaction (1.75 mL) contained 100 μM YAGFL, 29 μM DTT, and 0.050−1.6 mM total OH radical in 10 mM phosphate buffer (pD 7.2) at room temperature (20−25 °C). Each solution was gassed for 30 min with N_2O and irradiated within 30 min. Each sample was exposed for a total dose equivalent of 0.050 mM OH radical, then 40% of the original amount of reduced DTT present (20 nmol) was added to the reaction, and the sample was gassed for 10 min with N_2O. The samples were then exposed for another total dose equivalent to 0.050 mM OH radical. This process was repeated until the desired total OH radical concentration was achieved. The sample exposed to 1.6 mM OH radical concentration was the exception: the last exposure was equivalent to 0.80 mM total OH radical.

^{1}H/^{2}H Exchange into CTP. The ^{1}H/^{2}H exchange reaction with CTP was performed in a similar manner as conducted with YAGFL. The reaction (0.5 mL) contained 100 μM CTP, 20 μM leucine, 25 μM DTT, and 0.40 mM total OH radical in 10 mM phosphate buffer (pD 7.2) at room temperature (20−25 °C). Leucine serves as an internal reference to normalize the effective OH radical dose. The total of 0.40 mM OH radical was achieved by applying exposures equivalent to 0.050 mM followed by the addition of DTT and N_2O as described above for YAGFL. Isolation of leucine from the reaction mixture was achieved by removing an aliquot equivalent to 1 nmol of leucine, derivatizing with DNS-Cl, and then purifying the DNS-Leu by reversed-phase

[2] Abbreviations: cm-CTP, carboxymethylated C-terminal peptide; CTP, C-terminal peptide, DNS, 5-dimethylaminonaphthalene-1-sulfonyl or -dansyl, DNS-Cl, 5-dimethylaminonaphthalene-1-sulfonyl chloride or dansyl chloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESI−MS, electrospray ionization−mass spectrometry; HPLC, high-performance liquid chromatography; SIM, selected ion monitoring; TFA, trifluoroacetic acid; u, mass-to-charge ratio or atomic mass unit.

Scheme 2



HPLC as described for the peptides. The remaining CTP was carboxymethylated then purified by reversed-phase HPLC.

Quantitative Assays. The quantitation of the sulfhydryl groups of DTT and the CTP was performed using the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) assay (*17*). The quantitation of amines was performed with 2,4,6-trinitrobenzenesulfonic acid according to Fields (*18*) using leucine to generate a standard curve.

Carboxymethylation of CTP. The carboxymethylation of the cysteine sulfhydryl group of CTP was performed in a similar manner as previously described (*19*). After the $^1H/^2H$ exchange reaction, approximately 5 mg of iodoacetate (sodium salt) was added to the reaction mixture containing CTP. Since the solution was at neutral pH, only the sulfhydryl groups were carboxymethylated. Within 5 min of iodoacetate addition, the extent of sulfhydryl carboxymethylation was monitored by using the DTNB assay. An additional amount of iodoacetate was added if required to complete the carboxymethylation.

Reversed-Phase HPLC Purification of Peptides. All peptides were purified by reversed-phase HPLC using a Macrosphere C_{18} column (4.6 mm × 250 mm, 7 μm) (Alltech) and a Perkin-Elmer series 400 liquid chromatograph equipped with an inline flow-through cell (Helma Cells, Inc.). The peptides were monitored by measuring the absorbance between 200 and 340 nm using a Hewlett-Packard 8452A diode array spectrophotometer. The solvents used were 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B) at a flow rate of 1 mL/min. The column was equilibrated for 10 min with 100% of solvent A. After loading the sample, the column was washed for 10 min with 100% of solvent A, followed by a 60-min linear gradient from 0 to 100% of solvent B. The column was washed for 10 min with 100% of solvent B, then a 5-min step gradient followed by a 10-min wash with 100% of solvent A was used to equilibrate the column for the next injection. On the basis of the chromatograph monitored at 220 nm, the appropriate 1-mL fractions were pooled, and the solvent was removed via vacuum centrifugation. Several 1-mL additions of water were added and then removed prior to storing the samples at −20 °C until needed.

Peptide Hydrolysis. Peptides were hydrolyzed into their constituent amino acids by acid hydrolysis. The reversed-phased HPLC purified peptide was dissolved in 0.5 mL of distilled−deionized H_2O, and then 0.5 mL of concentrated HCl was added. The sample was placed into a 1-mL glass ampule, gassed with N_2 for 30 min, and then immediately flame sealed. The sealed ampule was placed into a heating block at 110 °C for 12 h. Acid-catalyzed proton exchange does not occur with side chain C−H bonds and only to a limited extent at the α-carbon position (*20*).

Amino Acid Derivatization Using DNS-Cl. Amino acids were dansylated as previously described by Gray (*21*). Typically, a sample containing 1.0 μmol of amino acid was added to a test tube, and the solvent was removed via vacuum centrifugation. The resulting residue was dissolved in 100 μL of 0.5 M $NaHCO_3$. For the amino acids produced by peptide hydrolysis, usually 200−300 μL of 0.5 M $NaHCO_3$ was required to dissolve the residue. To this solution was added 100 μL of a DNS-Cl solution containing 2 μmol excess over the total of all reactive groups. A solution containing the appropriate amount of DNS-Cl in acetone was prepared. Material not dissolving in the acetone was removed by centrifugation at 15000*g* for 1 min. The supernatant was removed and used for the derivatization reaction. The reaction was incubated for 1 h at 37 °C and allowed to cool to room temperature, followed by the addition of 800 μL of 10% (v/v) formic acid. The DNS-amino acids did not decompose under these storage conditions.

Extraction of DNS-Amino Acids. The amino acids subjected to $^1H/^2H$ exchange were derivatized with DNS-Cl as described above. Since DTT was also present in the reaction, the DNS-DTT and diDNS-DTT derivatives were extracted prior to the DNS-amino acid derivatives. After the DNS-Cl derivatization reaction was complete, the volume of the solution was increased to 0.4 mL by the addition of H_2O.

Then, 3 equal vol of water-saturated ethyl acetate was used to extract the DTT adducts. The aqueous layer was acidified using 800 μL of 10% (v/v) formic acid. The DNS-amino acid derivatives were extracted using 3 equal vol of water-saturated ethyl acetate. The extracts were pooled, and the ethyl acetate was removed under a stream of dry N_2. The remaining residue was stored at −20 °C until ESI−MS analysis.

Reversed-Phase HPLC Purification of DNS-Amino Acids. The purification of DNS-amino acid derivatives was performed using reversed-phase HPLC as described by Grego and Hearn (*22*) using a Macrosphere C_{18} column (4.6 mm × 250 mm, 7 μm) (Alltech) and a Perkin-Elmer series 400 liquid chromatograph equipped with an inline flow-through cell (Helma Cells, Inc.). The DNS-amino acids were monitored by measuring the absorbance between 200 and 400 nm using a Hewlett-Packard 8452A diode array spectrophotometer. Solvent A contained 0.2 M NH_4HCO_3 in acetonitrile:H_2O (10:90, v:v), and solvent B contained 0.2 M NH_4HCO_3 in acetonitrile:H_2O (45:55, v:v). The column was equilibrated for 10 min with 100% of solvent A. After loading the sample, the column was washed for 10 min with 100% solvent A, followed by a series of linear gradients: 25-min linear gradient from 0 to 30% of solvent B; 20-min linear gradient from 30 to 45% of solvent B; and 15-min linear gradient from 45 to 100% of solvent B. The column was washed for 15 min with 100% of solvent B, then a 5-min step gradient followed by a 10-min wash with 100% of solvent A was used to equilibrate the column for the next injection. On the basis of the chromatograph monitored at 340 nm, the appropriate 1-mL fractions were pooled, and the solvent was removed via vacuum centrifugation. Then, 0.5 mL of 10% (v/v) formic acid was added to each residue, and the solvent was removed via vacuum centrifugation. The samples were stored in the dark at −20 °C until ESI−MS analysis.

Isotope Ratio Measurements Using ESI−MS. For all the $^1H/^2H$ exchange experiments conducted, the amount of ^2H incorporated into the amino acid or peptide was determined using electrospray ionization−mass spectrometry (ESI−MS) as previously described (*23*). For each reaction, the isotope ratio $[M + H + 1]^+/[M + H]^+$of the standard was determined prior to the $^1H/^2H$ exchange reaction sample. Isotope ratio measurements of amino acids, DNS-amino acids, and YAGFL were performed on an Extrel Benchmark mass spectrometer equipped with a prototype electrospray interface (ABB Extrel, formerly Extrel, Pittsburgh, PA) and a 20-cm (4 mm inscribed radius) quadrupole mass filter with a range of 0−2000 u. Samples were dissolved in an appropriate amount of 2% acetic acid and diluted with an equal volume of methanol. Mass spectral analysis of carboxymethylated CTP (cm-CTP) and the internal reference (as the DNS-Leu derivative) were performed on a Micromass Quattro II triple quadrupole mass spectrometer with an *m*/*z* range of 0−4000 u. Samples were dissolved in an appropriate amount of 0.6% formic acid and diluted with an equal volume of acetonitrile. The selected ion monitoring (SIM) acquisitions used for isotope ratio measurements of the amino acids, DNS-amino acids, and YAGFL consisted of a 1-s scan (with an 8-ms scan delay) of the following positive ions and their dwell times: $[M + H - 1]^+$ (50 ms), $[M + H]^+$ (400 ms), $[M + H + 1]^+$ (400 ms), $[M + H + 2]^+$ (100 ms), and $[M + H + 3]^+$ (50 ms). The SIM acquisitions for cm-CTP and the internal reference DNS-Leu used a 100-ms dwell time for each ion.

Calculating the Extent of $^1H/^2H$ Exchange. The extent of $^1H/^2H$ exchange expressed as atom % excess ^2H at low levels of exchange (Δ % ^2H) was calculated using

$$\Delta\ \%\ ^2H = (\text{mean isotope ratio of the } ^1H/^2H \text{ exchange sample} - \text{mean isotope ratio of the standard}) \times 100 \quad (1)$$

The concentration of deuterated amino acid or peptide resulting from $^1H/^2H$ exchange was determined using

$$[[^2H]\text{product}] = \Delta\ \%\ ^2H \times [\text{reactant}] \quad (2)$$

with the efficiency defined as

$$\text{efficiency (\%)} = \frac{[[^2H]\text{product}]}{[\text{total OH radical produced}]} \times 100 \quad (3)$$

For the ESI−MS analysis of cm-CTP and the internal reference leucine (analyzed as DNS-Leu), the relative intensity (%) of each ion is determined using

$$[M + H + i]^+_{\text{relative intensity}} = \frac{[M + H + i]^+_{\text{intensity}}}{\sum_{i=0}^{3}[M + H + i]^+_{\text{intensity}}} \times 100 \quad (4)$$

The resulting values for the relative intensities were used to calculate the atom % excess ^2H at high levels of exchange (% ^2H) using

$$\%\ ^2H = ([M + H + 1]^+_{^1H/^2H} - [M + H + 1]^+_{CNT}) + 2([M + H + 2]^+_{^1H/^2H} - [M + H + 2]^+_{CNT}) + 3([M + H + 3]^+_{^1H/^2H} - [M + H + 3]^+_{CNT}) \quad (5)$$

where $^1H/^2H$ and CNT denote the relative intensity of the $^1H/^2H$ exchange sample and unirradiated control, respectively.

RESULTS

$^1H/^2H$ Exchange into YAGFL and Its Constituent Amino Acids in the Presence of DTT. The isotope ratios of each amino acid standard and irradiated sample were determined using ESI−MS and appear in Table 1. The isotope ratios presented in Table 2 are for the DNS-amino acids resulting from the derivatization of the free amino acids before and after $^1H/^2H$ exchange. Thus, Tables 1 and 2 are two parallel $^1H/^2H$ exchange experiments with ^2H incorporation determined by ESI−MS of free and DNS-amino acids, respectively.

Dansylation has several favorable properties in addition to the obvious aid in detection during chromatography. The derivatives favored formation of the monoprotonated ion enhancing the ESI−MS isotope ratio measurements, and the 233 u increase in the amino acid $[M + H]^+$ ion elevated the ions above the mass region contaminated with background solvent ions while retaining the unit mass resolution required for precise isotope ratio determinations (Figure 2). This advantage is experimentally demonstrated when comparing

Table 1: Hydroxyl Radical-Induced Hydrogen/Deuterium Exchange into Amino Acids Tyr, Ala, Gly, Phe, and Leu: Free Amino Acid Isotope Ratio Measurements

amino acid[a] (μM)	DTT[a] (μM)	total OH radical produced (μM)	mean isotope ratio ± SD (n)[b]	efficiency[c] (%)
50 μM l-Tyr standard[d]			0.291 ± 0.024 (3)	
100 (155)	33 (20)	50	nd[e]	
50 μM L-Ala standard			0.0434 ± 0.0011 (4)	
100 (121)	33 (18)	50	0.0461 ± 0.0017 (4)	0
50 μM Gly standard			0.0351 ± 0.0019 (3)	
100 (108)	33 (18)	50	0.0338 ± 0.0014 (4)	0
50 μM L-Phe standard			0.117 ± 0.002 (5)	
100 (116)	33 (20)	50	0.101 ± 0.003 (4)	0
100 μM L-Leu standard			0.0816 ± 0.0006 (4)	
100 (89)	33 (18)	50	0.192 ± 0.006 (4)	22

[a] The value in parentheses is the amount of amino acid or DTT remaining after radiolysis. [b] The number of consecutive injections used in the ESI−MS analysis. [c] The efficiency was calculated using eq 3 in the Experimental Procedures. [d] The isotope ratio for each standard was determined prior to the $^1H/^2H$ exchange sample. [e] The isotope ratio was not determined because of high $[M + H + 1]^+$ background ion current.

Table 2: Hydroxyl Radical-Induced Hydrogen/Deuterium Exchange into Amino Acids Tyr, Ala, Gly, Phe, and Leu: Dansyl Amino Acid Isotope Ratio Measurements

dansyl amino acid[a] (μM)	DTT[a] (μM)	total OH radical produced (μM)	mean isotope ratio ± SD (n)[b]	efficiency[c] (%)
100 μM DNS-Tyr standard[d]			0.278 ± 0.003 (4)	
100 (150)	33 (20)	50	0.387 ± 0.009 (4)	22
50 μM DNS-Ala standard			0.209 ± 0.001 (4)	
100 (112)	33 (17)	50	0.207 ± 0.004 (4)	0
50 μM DNS-Gly standard			0.196 ± 0.001 (4)	
100 (112)	33 (17)	50	0.187 ± 0.002 (4)	0
50 μM DNS-Phe standard			0.277 ± 0.001 (4)	
100 (116)	33 (20)	50	0.266 ± 0.002 (4)	0
100 μM DNS-Leu standard			0.242 ± 0.004 (4)	
100 (103)	33 (17)	50	0.356 ± 0.008 (4)	23

[a] The value in parentheses is the amount of amino acid or DTT remaining after radiolysis. [b] The number of consecutive injections used in the ESI−MS analysis. [c] The efficiency was calculated using eq 3 in the Experimental Procedures. [d] The isotope ratio for each standard was determined prior to the $^1H/^2H$ exchange sample.

the data presented in Tables 1 and 2. The isotope ratio of the free amino acid tyrosine in Table 1 could not be determined because of the high background ion current overlapping with the tyrosine $[M + H + 1]^+$ ion. The increase from 182.2 u (Tyr) to 415.2 u (DNS-Tyr) permitted an isotope ratio determination. In addition the data in Tables 1 and 2 show that isotope ratio determinations of the free amino acids and DNS-amino acids are comparable, indicating that converting the amino acids to their dansyl counterparts did not compromise the precision of the isotope ratio measurements.

The YAGFL peptide was subjected to a series of $^1H/^2H$ exchange reactions at various total OH radical concentrations. After the $^1H/^2H$ exchange reactions were complete, the peptides were purified using reversed-phase HPLC. Each sequential exposure resulted in an increase in the detected $^1H/^2H$ exchange. However, based on the reversed-phase HPLC chromatographs monitored at 220 nm, the amount of peptide remaining after each OH radical dosage was diminished. At 400 μM OH radical, the peptide was decomposed by approximately 40%, and at 800 μM and 1.6 mM, the amount of unaltered peptide was further reduced (data not shown). This decomposition has been attributed to peptide oxidation, resulting from O_2 introduction by gassing with U.S.P. grade N_2O (containing 300 ppm O_2). Decreased degradation of peptides has been more recently achieved by employing semiconductor grade N_2O and maintaining a positive N_2O pressure during radiolysis. The isotope ratios of the YAGFL standard and $^1H/^2H$ exchange samples appear in Table 3. By increasing the $^1H/^2H$ exchange into peptides by sequential exposure to radiolytic doses equivalent to 50 μM OH radical, 66 atom % excess ^2H was introduced into YAGFL.

$^1H/^2H$ Exchange into YAGFL: Isotope Ratio Determination of Amino Acid Residues. After determining the YAGFL peptide isotope ratio, the remaining material (approximately 50 nmol) was used to determine the extent of $^1H/^2H$ exchange into each amino acid residue. The peptide was hydrolyzed into its constituent amino acids, which were then derivatized with DNS-Cl. The resulting DNS-amino acids were separated using reversed-phase HPLC. The isotope ratios of each DNS-amino acid standard and $^1H/^2H$ exchange residue were determined by ESI−MS and are presented in Table 4.

$^1H/^2H$ Exchange into CTP. The CTP was subjected to the $^1H/^2H$ exchange reaction at 0.40 mM total OH radical produced in the presence of leucine (internal reference). After the $^1H/^2H$ exchange reaction, the peptide was carboxymethylated, purified using reversed-phase HPLC, and then analyzed by ESI−MS. The mass spectra of cm-CTP before and after exchange appear in Figure 1. The relative intensity of each ion monitored during the SIM acquisition of the cm-CTP standard and $^1H/^2H$ exchange sample were determined and appear in Table 5. The internal reference leucine was converted into DNS-Leu, purified by reversed-phase HPLC, and then analyzed by ESI−MS. The mass spectra of the DNS-Leu samples appear in Figure 2, and the relative intensity of each ion monitored during the SIM acquisition is reported in Table 5.

DISCUSSION

$^1H/^2H$ Exchange into C−H Bonds of Peptides. Determining the site of the reaction of OH radical with biological molecules is an important problem for two separate reasons: first, it impacts on the pathological role that OH radical may play in oxidative stress, and second, the reactions of OH radical can be used to probe solvent-accessible surfaces. The ability to label side chains of free amino acids in solution by alkyl $^1H/^2H$ exchange was previously established (*11*). The data in Table 3 indicate that it is possible to effect $^1H/^2H$ exchange into a peptide that contains both aliphatic and aromatic amino acids. Acid hydrolysis of the peptide, derivatization with DNS-Cl, followed by HPLC separation

Table 3: Hydroxyl Radical-Induced Hydrogen/Deuterium Exchange into YAGFL: Peptide Isotope Ratio Measurements

YAGFL[a] (μM)	DTT (μM)	total OH radical produced(mM)	mean isotope ratio ± SD (n)[b]	Δ % ^{2}H[c]	efficiency[d] (%)
50 μM standard[e]			0.344 ± 0.001 (3)		
100 (100)	29	0.050	0.403 ± 0.002 (3)	5.9	12
100 (117)	29	0.10	0.456 ± 0.004 (3)	11.2	11
100 (130)	29	0.20	0.542 ± 0.003 (3)	19.8	9.9
100 (122)	29	0.40	0.692 ± 0.011 (3)	34.8	8.7
100 (83)	29	0.80	0.878 ± 0.006 (3)	53.4	6.7
100 (61)	29	1.6	1.00 ± 0.05 (3)	65.6	4.1

[a] The value in parentheses is the amount of peptide remaining after radiolysis. [b] The number of consecutive injections used in the ESI−MS analysis. [c] Δ % ^{2}H was calculated using eq 1 in the Experimental Procedures. [d] The efficiency was calculated using eq 3 in the Experimental Procedures. [e] The isotope ratio for the standard was determined prior to the ^{1}H/^{2}H exchange sample.

Table 4: Isotope Ratio Measurements of DNS-Amino Acid Residues of YAGFL Subjected to Hydroxyl Radical-Induced ^{1}H/^{2}H Exchange

total OH radical produced (μM)	mean isotope ratio ± SD (n)[a]				
	DNS-Tyr	DNS-Ala	DNS-Gly	DNS-Phe	DNS-Leu
0[b]	0.268 ± 0.002 (4)	0.201 ± 0.002 (3)	0.181 ± 0.004 (3)	0.254 ± 0.001 (3)	0.220 ± 0.005 (4)
0.050	0.281 ± 0.003 (3)	0.205 ± 0.007 (3)	0.188 ± 0.003 (2)	0.256 ± 0.002 (3)	0.270 ± 0.007 (3)
Δ % ^{2}H[c]	1.3	0	0	0	5.0
0.10	0.300 ± 0.004 (3)	0.206 ± 0.005 (3)	0.177 ± 0.003 (3)	0.250 ± 0.006 (3)	0.315 ± 0.015 (3)
Δ % ^{2}H	3.2	0	0	0	9.5
0.20	0.308 ± 0.005 (4)	0.215 ± 0.006 (3)	0.181 ± 0.004 (3)	0.261 ± 0.002 (3)	0.397 ± 0.028 (3)
Δ % ^{2}H	4.0	1.4	0	0.7	17.7
0.40	0.336 ± 0.009 (2)	0.224 ± 0.004 (4)	0.199 ± 0.004 (3)	0.259 ± 0.001 (3)	0.496 ± 0.011 (3)
Δ % ^{2}H	6.8	2.3	1.8	0.5	27.6

[a] The number of consecutive injections used in the ESI−MS analysis. [b] The isotope ratio for each standard was determined prior to the ^{1}H/^{2}H exchange sample. [c] Δ % ^{2}H was calculated using eq 1 in the Experimental Procedures. Differences not statistically significant were assigned zero.

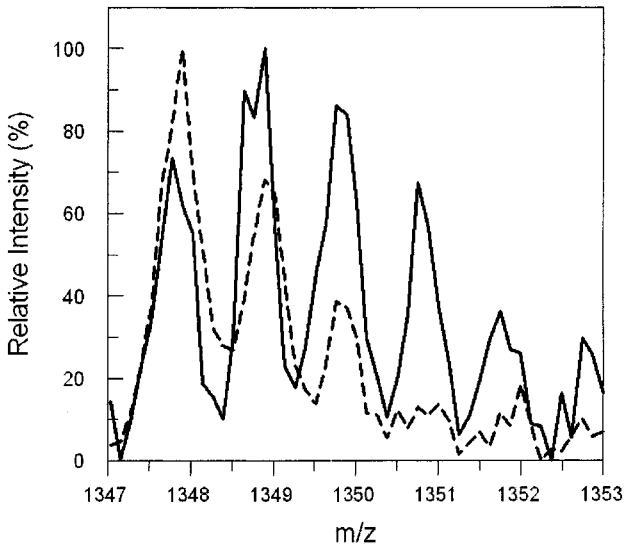


FIGURE 1: ESI−MS spectra of carboxymethylated C-terminal peptide: evidence of alkyl ^{1}H/^{2}H exchange. The ESI−MS spectra (normalized to the most abundant ion in the monoprotonated isotopic envelope) are of the C-terminal peptide standard (dashed line) and the peptide subjected to ^{1}H/^{2}H exchange (black line). The peptides were derivatized with iodoacetate and analyzed as the carboxymethylated derivative. The data were acquired using a profile acquisition with 1/8 u step size, 100 ms scan delay with a scan speed of 300 u/s. The relative ion intensities before and after ^{1}H/^{2}H exchange were determined by ESI−MS using selected ion monitoring and appear in Table 5.

and collection of the individual amino acid derivatives permitted each amino acid to be examined separately for ^{1}H/^{2}H exchange. The data presented in Table 4 indicate that leucine and tyrosine are most susceptible to ^{1}H/^{2}H exchange. As the amount of OH radical is increased, ^{1}H/^{2}H exchange becomes detectable in the other residues.

Table 4 shows that the susceptibility of the five amino acids present in YAGFL toward alkyl ^{1}H/^{2}H exchange is L > Y > A ≅ F > G. This trend in reactivity for the alkyl amino acids corresponds to the second-order rate constants for reaction of OH radical with the *N*-glycyl derivatives.[3] The ratio of ^{1}H/^{2}H exchange being 15:1.3:1 (based on the $[M + H + 1]^+/[M + H]^+$ isotope ratio using 400 μM OH radical) and the ratio of rate constants being 19:1.4:1 (*24*). This correlation has been extended to 13 additional glycyl-amino acids, indicating that the ^{1}H/^{2}H exchange can be detected in every type of amino acid with only asparagine, aspartate, and glycine less reactive than alanine (*25*).

Comparing the data in Table 2 with that in Table 4 indicates that incorporation of the amino acids into a peptide does not alter their relative reactivity. This is most important with regards to glycine. Stable glycyl radicals have been identified in enzyme active sites and have been suggested to be stabilized by the captodative effect (*26*). From these observations, it might be anticipated that α-hydrogens in general and glycyl residues, in particular, may be more reactive. However, our data indicate that the α-hydrogens of peptides are not unusually reactive as shown most dramatically by the minimal ^{1}H/^{2}H exchange that was detected into glycine. Consistent with this observation, the second-order rate constant for the reaction of OH radical with glycyl-glycine is 10-fold slower than the reaction of OH radical with glycyl-leucine. Similar conclusions were reached in a spin trapping study of small aliphatic peptides reacted with Ti(III) and H_2O_2 (*27*) and very recently in a study of oxidative radiolysis (*28*).

[3] The *N*-glycyl derivative is used as the standard of comparison because the protonated α-amino group effectively eliminates the reactivity of the α-hydrogens of the glycine and permits the α-hydrogen of the amino acid to be included in determining the relative reactivity.

Table 5. Hydroxyl Radical-Induced Hydrogen/Deuterium Exchange into the C-Terminal Peptide in the Presence of the Internal Reference Leucine

	relative intensity[a] (%)				
compound	$[M + H]^+$	$[M + H + 1]^+$	$[M + H + 2]^+$	$[M + H + 3]^+$	% ^2H[b]
standard[c]					
DNS-Leu	71.7	16.2	9.8	2.4	
cm-CTP	40.9	31.0	17.8	10.3	
$^1H/^2H$ exchange					
DNS-Leu	52.1	31.6	12.5	3.8	25.0
cm-CTP	25.0	27.6	25.4	22.0	67.5

[a] The relative intensity (%) was calculated by using eq 4 in the Experimental Procedures. [b] % ^2H was calculated using eq 5 in the Experimental Procedures. [c] The isotope ratio for each standard was determined prior to the $^1H/^2H$ exchange sample.

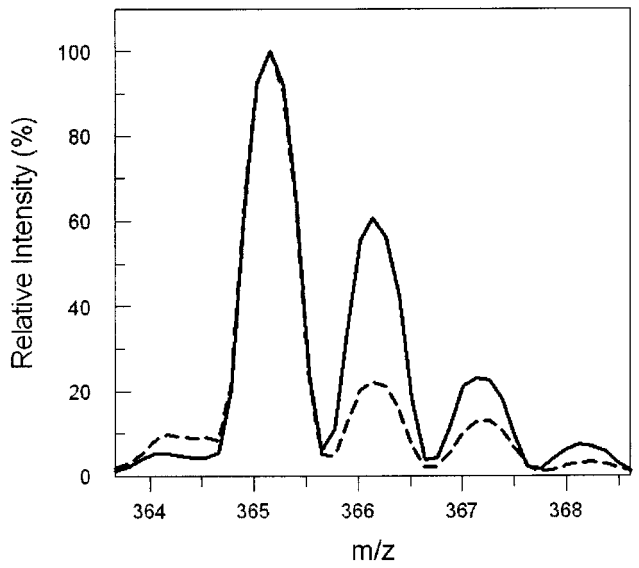


FIGURE 2: ESI−MS spectra of DNS-leucine: the effect of $^1H/^2H$ exchange into the leucine internal reference. The ESI−MS spectra (normalized to the $[M + H]^+$ ion at 365.2 Th) are of the leucine standard (dashed line) and leucine resulting from $^1H/^2H$ exchange in the presence of the C-terminal peptide (black line). The data were acquired using a profile acquisition with 1/8 u step size, 100 ms scan delay with a scan speed of 300 u/s. The relative ion intensities before and after $^1H/^2H$ exchange were determined by ESI−MS using selected ion monitoring and appear in Table 5.

The only deficiency of the acid hydrolysis protocol occurs when the peptide contains two or more residues of the same amino acid. In this case the $^1H/^2H$ exchange measured will be an average for the residues within the peptide. If $^1H/^2H$ exchange occurred, the extent of reaction with each residue will be ambiguous. The ambiguity could be removed either by selectively cleaving the peptide between the two residues and separating the product peptides prior to hydrolysis or by subjecting the peptide to Edman degradation and determining the isotope ratio of each successive phenylthiohydantoin derivative of the amino acids. The potential to identify the site of $^1H/^2H$ by either ^2H NMR or by tandem mass spectrometry exists, but NMR is about 1000-fold less sensitive, and preliminary investigations indicated that the isotope ratio of the product ions could not be determined with sufficient precision.

$^1H/^2H$ Exchange into a Peptide in the Presence of an Internal Reference. The $^1H/^2H$ exchange reaction was applied to a 12 residue peptide (SNEQKACKVLGI), the C-terminal peptide (CTP) of the third intracellular loop of the 5-hydroxytryptamine$_{2A}$ receptor, in the presence of leucine (internal reference). Alkyl $^1H/^2H$ exchange was shown to occur in both molecules and is clearly demonstrated in Figures 1 and 2 and Table 5. For the monoprotonated form of cm-CTP, the most abundant ion shifted from $[M + H]^+$ at 1347.9 u (before exchange) to $[M + H + 1]^+$ at 1348.9 u (after exchange). In fact, a shift toward higher mass for all ions in the monoprotonated isotopic envelope is observed as a result of $^1H/^2H$ exchange. Complete analysis of the isotopic envelope indicated the incorporation was 68 atom % excess ^2H. For DNS-Leu the most abundant ion remained the $[M + H]^+$ species at 365.2 u, and the $^1H/^2H$ exchange generated a 25 atom % excess ^2H. Since the extent of $^1H/^2H$ exchange has been shown to be a function of the OH radical generated, the concentration of DTT (*11*), and the presence of other competing substrates present in the irradiated volume, a method of normalizing the amount of $^1H/^2H$ exchange must be developed.

On the basis of our experience with leucine, this amino acid was chosen to serve as an internal reference for the exchange reaction. As suggested by Scheme 3, the following equations describe the time course for the $^1H/^2H$ exchange:

$$d[^2H]Leu/dt = k_{OH-L}[Leu][^{\bullet}OH]\, f_{D-L} \quad (6)$$

$$d[^2H]R_i/dt = k_{OH-R_i}[R_i][^{\bullet}OH]\, f_{D-R_i} \quad (7)$$

where k_{OH-L} and k_{OH-R_i} are the second-order rate constants for the reaction of OH radical with the reference leucine and the *i*th residue of the peptide, respectively, and f_{D-L} and f_{D-R_i} are the fraction of the generated radicals that are quenched by ^2H atom incorporation for the reference leucine and the *i*th residue, respectively. According to Scheme 3, this fraction is determined by the rate constant for ^2H incorporation divided by the sum of the effective first-order rate constants for all other decomposition processes. Hence, the fractions for the reference leucine and the *i*th residue would be given by

$$f_{D-L} = k_{D-L}/(k_{D-L} + k_{nonex-L}) \quad (8)$$

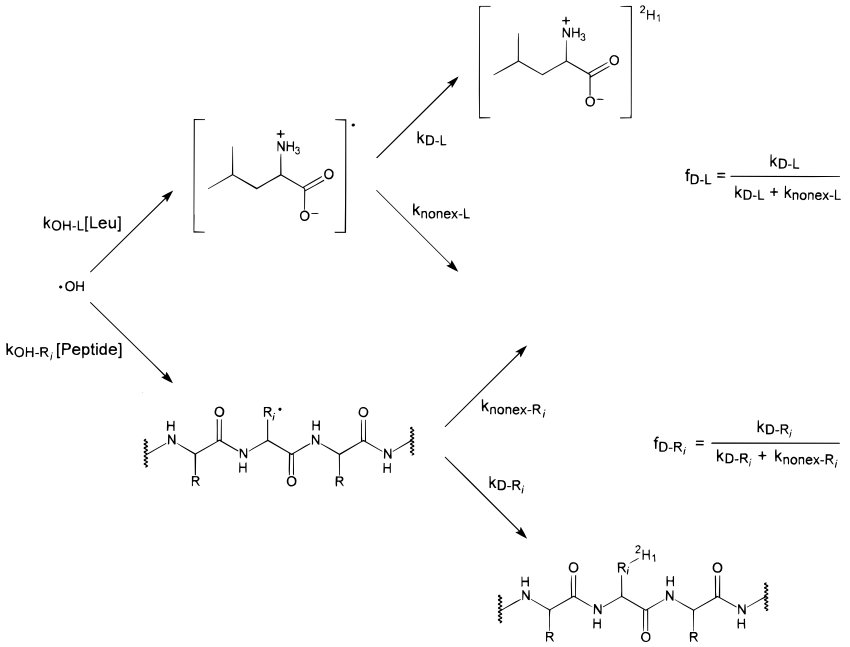
$$f_{D-Ri} = k_{OH-R_i}/(k_{OH-R_i} + k_{nonex-R_i}) \quad (9)$$

Assuming that only the concentration of OH radical varies with time, integration of eqs 6 and 7 yields

$$[^2H]Leu/Leu = k_{OH-L}\, f_{D-L} \int_t^0 [OH]dt \quad (10)$$

$$[^2H]R_i/R_i = k_{OH-R_i}\, f_{D-R_i} \int_t^0 [OH]dt \quad (11)$$

Scheme 3



So

$$[^2H]R_i/R_i/[^2H]Leu/Leu = k_{OH-R_i} f_{D-R_i}/k_{OH-L} f_{D-L} \quad (12)$$

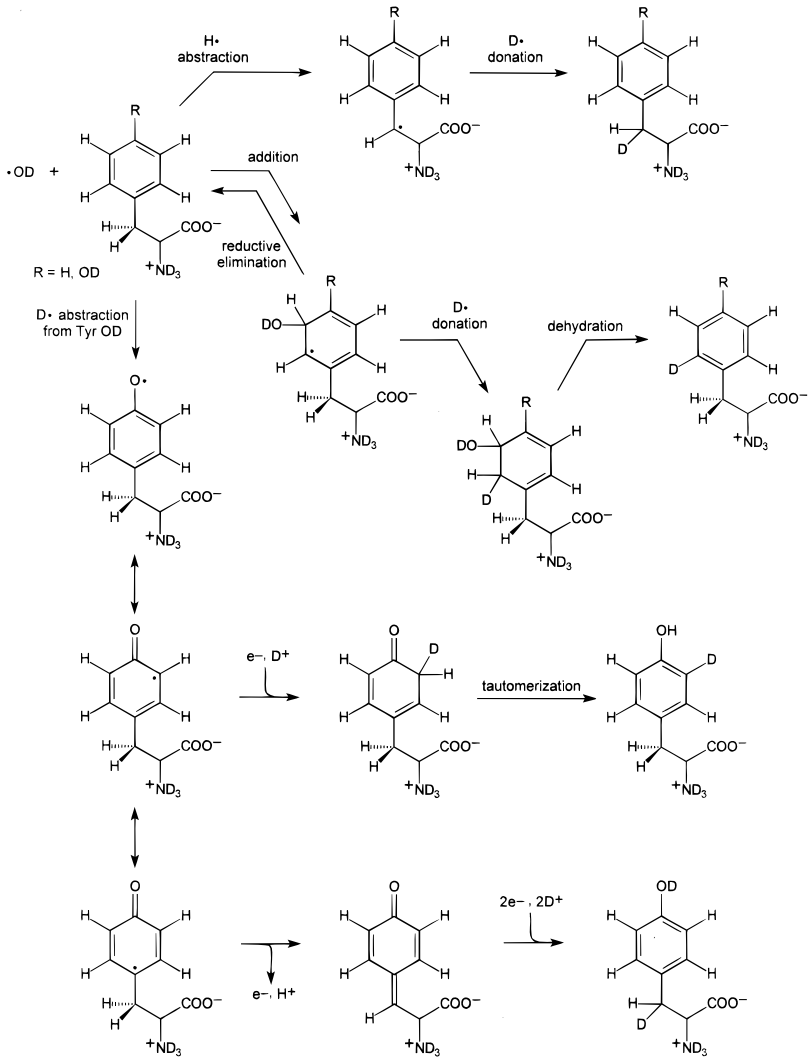
The virtue of the internal reference, as shown by eq 12, is that the ratio of ^2H incorporation into each residue eliminates the dependence on the variability of OH radical with time. An additional virtue of using leucine as an internal reference is that previous results (*11*) strongly suggest that f_{D-L} is near unity. Therefore, k_{OH-L} f_{D-L} can be closely approximated as the second-order rate constant for the reaction of OH radical with the zwitterion of leucine determined by pulse radiolysis studies to be 1.7×10^9 M^{-1} s^{-1} (*3*). This permits the $^1H/^2H$ exchange observed into a peptide of a given sequence to be predicted, based on the known rate constants for OH radical reaction with the given amino acids, assuming that f_{D-AA} is unity for aliphatic amino acids except cysteine, where f_{D-Cys} is assumed to be 0 because the rate constant reflects abstraction of the solvent exchangeable S−H hydrogen (*29*). This prediction is born out for CTP: the sum of the rate constants for the individual residues predicts that there should be 3.1-fold greater $^1H/^2H$ exchange into CTP than for leucine which is in accordance with the observed ratio of 2.7 (Table 5). This corroborates the conclusion drawn from the exchange into YAGFL that the incorporation of an amino acid into a peptide does not significantly alter its reactivity with OH radical. We predict, however, that peptides obtained from proteins could demonstrate a significant reduction in the observed $^1H/^2H$ exchange if the residues that have a significant propensity to undergo $^1H/^2H$ exchange, e.g. leucine, isoleucine and valine, are excluded from the solvent-accessible surface.

Reaction of OH Radical with Aromatic Amino Acids under Reductive Conditions. The aromatic residues phenylalanine and tyrosine behave in a different manner than the aliphatic residues. Radiolysis of phenylalanine and tyrosine under aerobic conditions results in ring hydroxylation, and radiolysis of YAGFL under aerobic conditions results largely in comparable hydroxylation of the phenylalanine and tyrosine residues (unpublished data). However, under the anaerobic conditions used in these $^1H/^2H$ exchange reactions (Table 2), tyrosine exhibited $^1H/^2H$ exchange but phenylalanine did not. The diminished $^1H/^2H$ exchange efficiency of phenylalanine relative to tyrosine was also observed in YAGFL (Table 4). Pulse radiolysis studies suggest that the OH radical reacts with phenylalanine and tyrosine 4- and 10-fold faster than with leucine (*3*).

The decreased $^1H/^2H$ exchange into the aromatic amino acids indicates that the partition factors, f_{D-AA} in eq 8, for phenylalanine (f_{D-F}) and tyrosine (f_{D-Y}) must be 0.02 and 0.1, respectively. The small f_{D-Y} can be readily understood since abstraction and repair of the phenoxyl hydrogen would be an unobserved event. However, the small value for phenylalanine was unexpected but strongly implies that the initial product of the reaction of OH radical with phenylalanine must be reversed under the reductive conditions employed for the $^1H/^2H$ exchange, as shown in Scheme 4. This is precedented by the observation that the cyclohexadienyl radical (*30*), Tl atoms, and Cu^+ (*31*) are sufficiently reducing to effect this elimination.

Phenylalanine and tyrosine share two potential mechanisms of undergoing $^1H/^2H$ exchange: abstraction of the α-H or the benzylic β-H, resulting in exchange similar to that observed for the aliphatic amino acids (Scheme 4). Addition of the OH radical to the aromatic ring could result in exchange if ^2H atom donation results in formation of a 1-hydroxy-[6-^2H]cyclohexadiene, which would re-aromatize by dehydration. Tyrosine could undergo $^1H/^2H$ exchange via two reactions of the tyrosyl radical: either ^2H-atom donation to the ortho position followed by enolization to regenerate tyrosine or by a 1 e^- oxidation to the quinone methide and subsequent 2 e^- reduction back to tyrosine. These pathways are precedented by the trapping of the phenoxyl radical by spin traps at the ortho carbon (*32*) and the observed 1 e^- oxidation of alkyl phenoxyl radicals to quinone methides (*33*). These potential pathways of $^1H/^2H$ exchange are shown

Scheme 4



in the lower half of Scheme 4. Since f_{D-Y} is greater than f_{D-F}, it suggests that at least one of the tyrosine-specific pathways is contributing to the observed exchange.

Protein Footprinting Ramifications. OH radical generated by γ-radiolysis has been used experimentally to identify the DNA bases that make contact with the protein in DNA−protein complexes (*34*). The application of the footprinting technique to examine the protein residues involved in macromolecular interactions is attractive but experimentally difficult. Fenton chemistry has been used to probe solvent-accessible regions in protein−nucleic acid complexes (*35*, *36*). Although this "protein footprinting" methodology permits mapping contact regions of protein domains involved in macromolecular assemblies, the ability to specifically identify the sites involved in recognition at the amino acid residue level has not been possible.

The ability to obtain single residue resolution in DNA footprinting and the lack of similar single residue resolution in protein footprinting can also be understood by examining the difference in the reactivity of OH radical with proteins and DNA. For duplex DNA, the OH radical reacts by abstracting a H atom from solvent-accessible C−H bonds of the deoxyribose ring along the DNA backbone, producing a carbon-centered radical that subsequently leads to strand scission (*37*). While cleavage of globular proteins by similar mechanisms may be detected (*38*, *39*), the results with YAGFL clearly demonstrate that abstraction of the α-H by OH radical is a rare event when compared to reaction with the side chain. For almost all of the amino acids, there is a higher probability that the OH radical will react with the side chain rather than with the α-carbon. This problem will be compounded in proteins where the majority of solvent-accessible C−H bonds present on the protein's solvent-accessible surface are not comprised of the backbone (αC−αH) but those of the side chains. Thus, in protein footprinting that is dependent on cleavage, the major pathway of OH radical reactivity with proteins is not exploited. The necessary inference must be that most proteins cleaved by reaction with OH radical under aerobic conditions will have suffered significant oxidation in the side chains of other residues prior to being cleaved. The use of an assay based on susceptibility to $^1H/^2H$ exchange effected by OH radical demonstrated in this manuscript can provide an alternative method of mapping solvent-accessible surfaces.

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