Sites of Hydroxyl Radical Reaction with Amino Acids Identified by ^{2}H NMR Detection of Induced ^{1}H/^{2}H Exchange

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Abstract: Hydroxyl radical reacts with the aliphatic C−H bonds of amino acids by H atom abstraction. Under anaerobic conditions inclusion of a ^{2}H atom donor results in ^{1}H/^{2}H exchange into these C−H bonds [Goshe et al. *Biochemistry* **2000**, *39*, 1761−1770]. The site of ^{1}H/^{2}H exchange can be detected and quantified by ^{2}H NMR. Integration of the ^{2}H NMR resonances within a single spectrum permits the relative rate of H atom abstraction from each position to be determined. Analysis of the aliphatic amino acid spectra indicates that the methine and methylene positions were more reactive than the methyl positions. The ^{2}H NMR spectra of isoleucine and leucine show that H-atom abstraction distal to the α-carbon occurs preferentially. Significant ^{1}H/^{2}H exchange was observed into the δ positions of proline and arginine and into the ϵ-methylene of lysine, indicating that a positive charge on a geminal N does not inhibit the ^{1}H/^{2}H exchange. Comparisons of ^{2}H NMR integrations between amino acid spectra indicated that ^{1}H/^{2}H exchange occurred in the following descending order: L > I > V > R > K > Y > P > H > F >M> T > A > [C, S, D, N, E, Q, G, W]. The extent of ^{1}H/^{2}H exchange into methionine, *N*-glycyl-methionine, and methionine sulfoxide suggests that a prominent solvent exchange pathway involving hydroxyl radical mediated oxidation of methionine exists to account for the large ^{2}H incorporation into the γ-methylene of methionine sulfoxide that is absent for *N*-glycyl-methionine. Analysis of the ^{1}H NMR spectra of the reactions with phenylalanine and tyrosine indicated that hydroxyl radical addition to the phenyl ring under the anaerobic reductive reaction conditions did not result in either exchange or hydroxylation.

The reaction of the hydroxyl radical (•OH) with biological macromolecules has been an area of extensive study due to the deleterious effects it has on biological systems.[1] The reactions of •OH with nucleic acids have also been used to map solvent-accessible surfaces.[2] •OH reactions with amino acids, peptides, and proteins are also of interest and the relative reactivity of the amino acids has been established by determining the second-order rate constants for the reaction of the •OH with amino acids using pulse radiolysis.[3] However, determining the sites of reaction of •OH has been more difficult. ^{1}H NMR,[4−8] EPR radical trapping,[9] and mass spectral characterization[10] of the different •OH-induced products of amino acids generated in aerobic aqueous solution support the conclusion that side chain reactions are the most prominent at physiological pH. In the presence of oxygen, the aromatic and sulfur containing side chains are the most prominent sites of oxidation, with O-atom incorporation being the main reaction.[10,11]

Characterization of the reaction of glycine and glycine derivatives with hydroxyl radical has permitted the reactivity of α-amino, α-carboxylates, and the polypeptide backbone to be studied independently of the side chain reactions. Initial pulse radiolysis studies indicated that the glycine anion is ∼10^3-fold more reactive than the zwitterionic form.[3] More recent results indicate that •OH reacts with the unprotonated amine by both electron transfer and H-atom abstraction to generate aminium and aminyl radicals, respectively,[12] despite the glycine C−H bond having a significantly lower bond dissociation energy than the N−H bond.[13] •OH has been shown by EPR spin trapping to abstract the α-H of glycine and alanine residues in peptides,[9] but for all other amino acids reactions within the side chains appear to predominate.[9,14]

We have developed an anaerobic ^{1}H/^{2}H exchange procedure that detects the sites of hydrogen atom abstraction by •OH.[14,15]

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Previously, detection was provided by mass spectrometric analysis of the altered isotope ratios in peptides or in the individual amino acid residues. Here we report ^2H NMR analyses of the •OH-induced $^1H/^2H$ exchange into amino acids that permit the location of exchange within the side chains of the amino acids to be quantified and identified with atomic, rather than residue, resolution. This procedure should be readily applicable to peptides and other molecules where the reactions with •OH are of interest.

Materials and Methods

Materials. Amino acids and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) were purchased from Sigma, Aldrich, and Fischer Scientific. D_2O (99.9 atom % D) and H_2O (^2H depleted, [^2H] = natural abundance $\times 10^{-2}$) were from Aldrich. Dithiothreitol (DTT) was from Boehringer Mannheim. Semiconductor Grade 4.8 nitrous oxide (N_2O) was from Praxair (Cleveland Ohio).

Preparation of Amino Acid Samples for $^1H/^2H$ Exchange. From 10 mM stock solutions of each of the 19 standard amino acids in H_2O, 5 mL of 2.5 mM solutions was prepared in 13 × 100 mm test tubes and dried on a centrifugal vacuum concentrator. Solubility constraints limited the concentrations of tyrosine to 2 mM. The amino acid residues were redissolved in 5 mL of 5 mM sodium phosphate buffer in D_2O, pD 7.2,[16] and transferred into 12 mL glass vials sealed with Teflon-lined silicone septa. To each amino acid solution were added freshly prepared aliquots of DTT dissolved in D_2O to achieve a final concentration of 250 μM DTT in each vial. With 20 G needles connected to Teflon tubing, the samples were bubbled with N_2O for at least 2 h to saturate the solution with N_2O and to remove atmospheric O_2 through the venting needle. To maintain a slight positive pressure of N_2O, a rubber bulb was inflated with N_2O and secured on the cap of the vial during irradiation.

Radiolysis To Effect $^1H/^2H$ Exchange into Amino Acids. Reductive anaerobic $^1H/^2H$ exchange was achieved at room temperature by exposing the amino acids in the glass vials to γ-rays from a ^{137}Cs irradiator. The dose rate of 3018 rads/min (100 rads = 1 Gy) and the G(•OH) of 5.6 for N_2O saturated solutions[17] allowed the time of exposure to be calculated by using eq 1. The amino acid solutions were

$$\text{time (min)} = \frac{[\text{OH radical}]\ \text{mM}}{\text{dose rate (Gy/min)} \times G(\cdot\text{OH}) \times 1.04 \times 10^{-4}\ \text{mmol/J}} \quad (1)$$

exposed to 500 μM •OH after which they were removed from the irradiator and an additional 100 μM DTT was added to each vial. Further bubbling with N_2O was done for 15 min and the reaction was completed by exposure to an additional 500 μM •OH.

Sample Preparation for NMR Analysis. The irradiated samples were concentrated in a centrifugal vacuum concentrator and exchanged with H_2O three to four times to remove solvent D_2O. In preparation for ^2H NMR, the residues were dissolved in 500 μL of ^2H depleted water and transferred to 5 mm NMR tubes. The concentration of the amino acids, except tyrosine, from 5 mL for irradiation to 0.5 mL for NMR analysis resulted in 25 mM samples which enhanced the ^2H NMR signal.

^1H and ^2H NMR Spectroscopy. A Varian Inova 600-MHz NMR instrument operating at 599.908 MHz (14.1 T) with a broad-band probe was used to collect the ^1H NMR spectra. This equated to a resonance frequency of 92.106 MHz for ^2H. The spectrometer's electronics were tuned for ^2H acquisition with the appropriate 5-point stick inserted into the probe. Prior to tuning, the lock power was turned off and a 1/4 wavelength broadband preamp cable corresponding to a 60−130 MHz range was attached to the wavelength jack. A sample containing an equivalent isotopic mixture of water [^2H(50 atom %)]H_2O was used to obtain the shim file used in the acquisition of both ^1H and ^2H spectra. The data were then acquired on the amino acid samples dissolved in ^2H-depleted H_2O with broadband proton decoupling and solvent (D_2O) presaturation. Typically, the number of scans required per run for each sample was about 10 000. All the spectra were acquired at 25 °C with sweep widths of 6.6 and 1.5 kHz for ^1H and ^2H, respectively. The acquired data were processed with Varian software on an SGI O_2 computer. Processing of the ^1H NMR data was done referencing the chemical shift signals to the methyl resonance of DSS, and the spectra were all processed while maintaining the same vertical scales.

Assignment and Quantitation of Site-Specific Isotope Enrichments in Amino Acids. The ^2H chemical shifts in ppm are identical to the corresponding ^1H chemical shifts, except for small intrinsic isotope shifts. On this premise, the chemical shifts obtained were correlated with those of the ^1H NMR of the corresponding standard amino acids acquired with the same instrument. Any observable scalar coupling between ^1H and ^2H, $J(^1H,^2H)$, which is known to be 6.51 times smaller than the corresponding $J(^1H,^1H)$ coupling, was eliminated by proton decoupling. The relaxation of the ^2H nucleus is dominated by an efficient quadrupolar mechanism accounting for the complete loss of any NOE's. The $J(^2H,^2H)$ scalar couplings are ignored since they characteristically are only 2.4% of the $J(^1H,^1H)$ coupling.[18] These factors simplify the resonances observed in the ^2H NMR spectra to singlets that are characteristic of the various sites in the amino acid side chain and whose relative integration becomes a direct measurement of the relative ^2H enrichment. Each amino acid was dissolved at identical concentrations and exposed for the same time to the ^{137}Cs source. Hence, the integration of each amino acid spectrum should be comparable to each other, permitting the intermolecular propensity for $^1H/^2H$ exchange to be assessed by comparing the integrations of the ^2H NMR spectra of the different amino acids.

Results and Discussion

Elucidating the sites of •OH mediated $^1H/^2H$ exchange into the side chains of amino acids is important for two reasons. Understanding the reactions of the •OH with amino acids will serve in unraveling the reaction pathways of the deleterious effects of •OH-induced oxidation in biological systems. Second, the irreversible $^1H/^2H$ exchanged amino acid side chain modification can serve as a marker to probe solvent accessibility and hence interactions between peptides and proteins. The relative propensity of the different amino acids to undergo •OH-promoted $^1H/^2H$ exchange and the mechanisms of the exchange will be discussed. Shown in Scheme 1 is the previously established mechanism of $^1H/^2H$ exchange into amino acids.[15] At low concentrations, DTT serves more efficiently as an alkyl radical trap by donating a ^2H atom than as a •OH scavenger. DTT is the preferred ^2H atom donor over other thiols for two reasons: (1) the DTT thiyl radical quenches itself by forming a stable six-membered ring disulfide radical anion[20] and (2) the DTT disulfide radical anion disproportionates to oxidized and reduced DTT.[21] This reactivity with C-centered radicals has been shown to occur with second-order rate constants of $\sim 10^7\ M^{-1}\ s^{-1}$.[3] The ^2H NMR results reported here are the first NMR characterization of the often invoked, thiol-mediated "repair reactions" of C-centered radicals.[19]

^2H NMR is a unique tool to detect the sites of modification by $^1H/^2H$ exchange because the observed peaks are necessarily singlets due to negligible $^1H/^2H$ and $^2H/^2H$ coupling. The chemical shifts of ^2H NMR can be compared directly to those of the corresponding ^1H NMR, making the assignments straightforward. In addition, integration of the peaks provides

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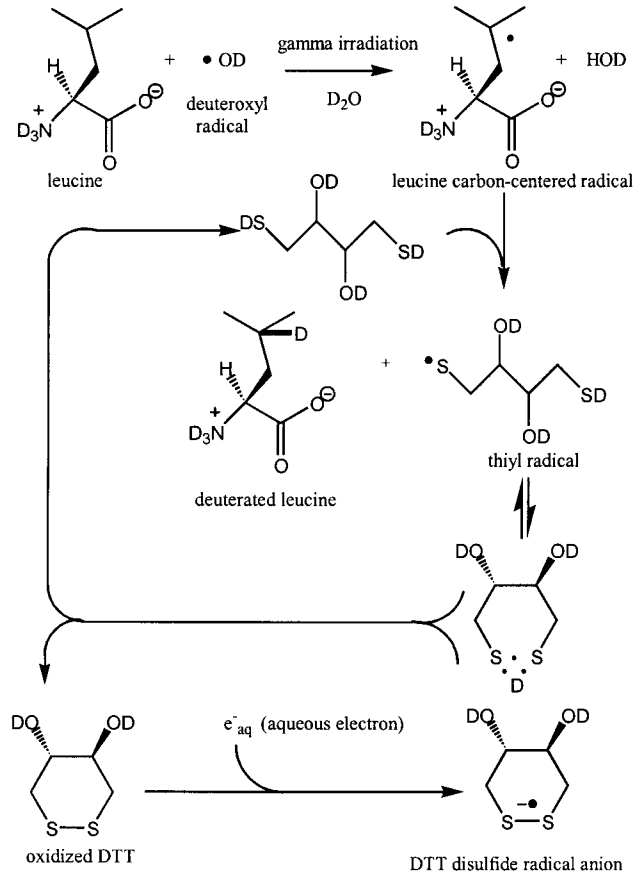
Scheme 1



the relative reactivities of the different sites of incorporation and their propensities toward exchange.

The chemical shift assignments shown in Table 1 summarize the sites of enrichment of the amino acids susceptible to $^1H/^2H$ exchange. The relative propensity to undergo the $^1H/^2H$ exchange reaction is indicated by being listed in descending order of enrichment. The absence of the polar amino acid residues, glycine, cysteine, and tryptophan, does not indicate that $^1H/^2H$ exchange is absent in these residues, only that with the less sensitive ^2H NMR detection method, this exchange could not be unequivocally detected. We estimate the limit of detection to be between ~0.25 and 0.5 mM ^2H. Because of the concentration step, the effective •OH concentration generated was 10 mM.

$^1H/^2H$ Exchange into Aliphatic Amino Acids. The aliphatic amino acids have the greatest susceptibility to $^1H/^2H$ exchange with incorporation being effected into the side chain C-centers, illustrating separable reactivities of the primary, secondary, and tertiary sites. The rate constants for the reactions of the •OH with aliphatic amino acids range from 7.7×10^7 M^{-1} s^{-1} at the lower end for alanine to 20-fold greater, 1.3×10^9 M^{-1} s^{-1}, for leucine.[3] At physiological pH, the α-H atoms of the amino acids are less reactive to the •OH than the side chain C−H bonds due to inductive deactivation by both the ammonium and carboxylate groups present in the zwitterionic state.[22] This is demonstrated by the lack of ^2H incorporation into glycine and is a feature of $^1H/^2H$ exchange into peptides as well.[9,14]

Figure 1 shows $^1H/^2H$ exchange into L-alanine, L-valine, L-leucine, and L-isoleucine. ^2H incorporation can be identified into each of the alkyl carbons of the aliphatic amino acids. There is a direct relationship between the amount of $^1H/^2H$ exchange and the number of aliphatic C−H bonds. The most prominent result is that $^1H/^2H$ exchange was increased, the greater the distance from the α-carbon. The incorporation into the methyl groups decreased in the order Leu > Ile > Val > Ala. These results suggest that the ionized ammonium and carboxylate groups either have an inductive or steric effect that diminishes as the C−H bonds become further removed from the α-carbon. These results are consistent with our previous determination of $^1H/^2H$ exchange by mass spectrometry.[14]

Integration of the ^2H NMR peaks shows enrichment of the methyl sites to be 1.4 times greater than the methine and methylene hydrogens of leucine. The sites of enrichment in L-isoleucine for the β-methine, γ-methylene, γ-methyl, and δ-methyl groups are in the ratio 1.3:1:1.5:2, respectively. After normalizing to the number of hydrogens under each peak the yields showed a 4:2:1.5:2 ratio for the four sites, respectively. This implies that the β-methine hydrogens are more prone to exchange relative to the methylene and methyl hydrogens. Similarly in valine, the incorporation of ^2H is 2.85 times greater into the methine position when compared to the methyl groups. These results are qualitatively consistent with previous experimental and computational studies that have shown that the methylene hydrogens of propane are ~10-fold more reactive than the methyl hydrogens.[23,24] In leucine the β-methylene and γ-methine ^2H resonances are not resolved. The methyl groups

Table 1. Assignment of Observed ^2H NMR Resonances[a]

amino acid	position	^2H resonance assignment (ppm)
L-leucine	methyls	δ 0.90
L-leucine	methine and methylene	δ 1.63
L-isoleucine	δ-methyl	δ 0.89
L-isoleucine	γ-methyl	δ 1.20
L-isoleucine	γ-methylene	δ 1.40
L-isoleucine	β-methine	δ 1.99
L-valine	γ-methyls	δ 0.95
L-valine	β-methine	δ 2.19
L-arginine	δ-methylene	δ 3.18
L-arginine	β-methylene	δ 1.85
L-arginine	γ-methylene	δ 1.60
L-lysine	ϵ-methylene	δ 2.96
L-lysine	γ-methylene	δ 1.45
L-lysine	β-methylene	δ 1.85
L-lysine	δ-methylene	δ 1.74
L-tyrosine	β-methylene	δ 3.16
L-tyrosine	phenyl 3H and 5H	δ 6.86
L-proline	γ-methylene	δ 1.96
L-proline	β-methylene	δ 2.29
L-proline	δ-methylene	δ 3.22
L-histidine	β-methylene	δ 3.24
L-histidine	imidazole 2H	δ 7.10
L-histidine	imidazole 4H	δ 7.87
L-phenylalanine	β-methylene	δ 3.22
L-methionine[b]	ϵ-methyl	δ 2.09
L-methionine[b]	γ-methylene	δ 2.58
methionine sulfoxide[c]	γ-methylene	δ 3.06
L-threonine	γ-methyl	δ 1.28
L-alanine	β-methyl	δ 1.42

[a] The amino acid order is indicative of relative incorporation into the entire amino acid. The intramolecular order also represents the relative integration of each position. [b] Methionine data obtained from unoxidized methionine. [c] Methioninesulfoxide data are for methionine sulfoxide, not for the methionine sulfoxide formed during irradiation of methionine.

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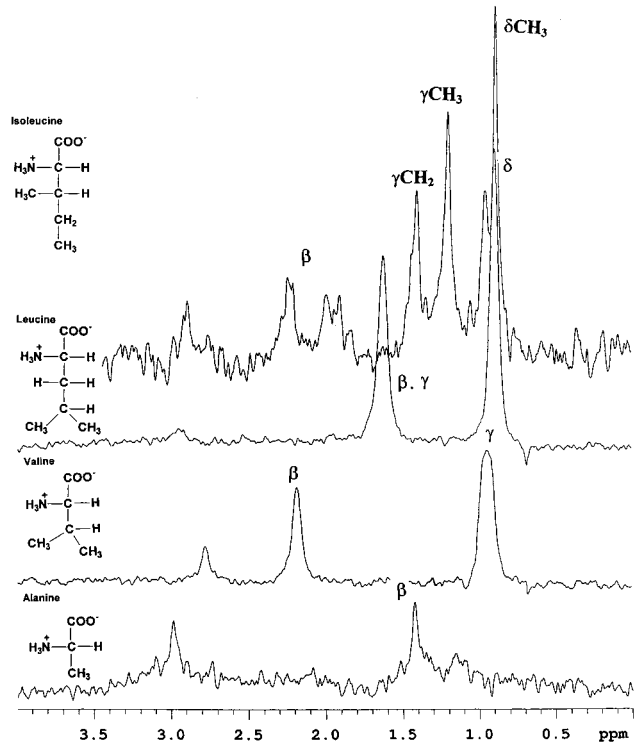

Figure 1. The ^2H NMR spectra of alanine, valine, leucine, and isoleucine illustrating the sites of hydroxyl radical induced alkyl $^1H/^2H$ exchange. The ^2H NMR chemical shifts were correlated with those of the ^1H NMR of the same amino acid samples referenced to DSS. The relative ratios of incorporation determined by integration of the peaks demonstrate an increase in $^1H/^2H$ exchange with the increase in the number of side chain alkyl groups.

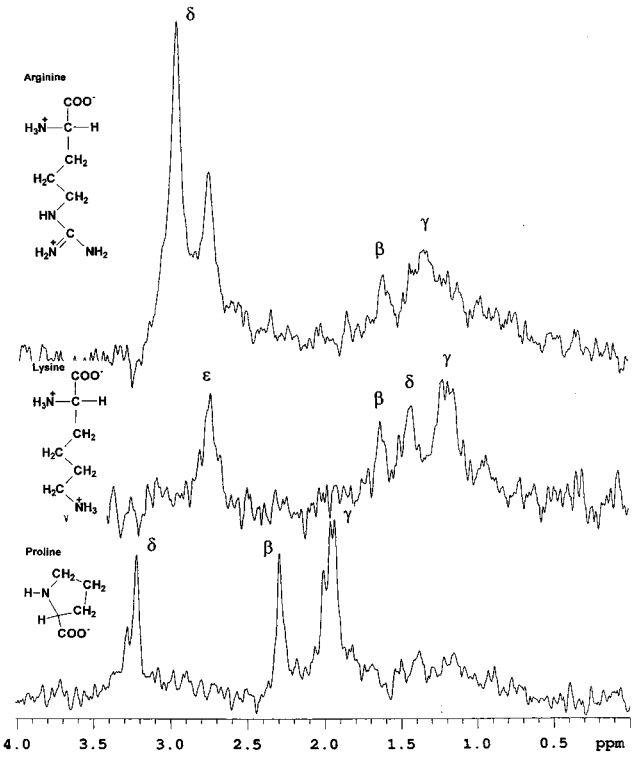

Figure 2. ^2H NMR spectra of proline, lysine, and arginine illustrating the sites of hydroxyl radical induced alkyl $^1H/^2H$ exchange. The ^2H NMR chemical shifts were correlated with those of the ^1H NMR of the same amino acid samples referenced to DSS. Incorporation into the δ-methylene group of arginine and the ϵ-methylene group of lysine suggests that the side chain guanidinium ion of arginine and the ϵ-ammonium ion of lysine do not deactivate these alkyl carbon centers. Similarly for proline, there was minimal deactivation toward the •OH by the amino group on the neighboring δ-methylene carbon.

showed a 2.1-fold greater ^2H incorporation than the methine and methylene groups, corresponding to an equal reactivity on a per H basis. These results indicate that the preferential abstraction of methine and methylene hydrogens in the aliphatic amino acids is reduced by their closer proximity to the deactivating effects of the α-amino and carboxylate groups. Because significant $^1H/^2H$ exchange is observed into the thermodynamically less stable methyl positions, we infer that the DTT radical trap is sufficient to prevent either inter- or intramolecular migration of the radical. Knowledge of the reactivities of the amino acid C−H bonds with •OH is required to predict sites of biological oxidative damage to proteins. These results further establish the direct abstraction of hydrogen atoms by •OH and subsequent replacement with ^2H donated by DTT as the mechanism of •OH-induced $^1H/^2H$ exchange.

Influence of Positive Charge on $^1H/^2H$ Exchange. Incorporation of ^2H into L-arginine, L-lysine, and L-proline is shown in the ^2H NMR spectra of Figure 2. L-Arginine shows incorporation into all the methylene side chain carbons, the ratio of enrichment into the δ-CH_2, γ-CH_2, and β-CH_2 sites being 11:3:1, respectively. As in L-arginine, L-lysine incorporates ^2H into the side chain methylenes with the ϵ- and γ-methylenes showing 3-fold more incorporation than the δ- and β-methylenes. The significant incorporation into the methylenes adjacent to the positively charged functional groups was unanticipated based on the dogma that the protonated α-amino group deactivates the reaction of the α-C−H.[9] However, previous EPR spin trapping studies with protonated amines indicate that the vicinal C−H bonds are subject to H-atom abstraction by •OH and that the guanidinium group is particularly activating.[25] This activation of H-atom abstraction by the guanidinium group is also reflected in the observed large rate constant of 3.5×10^9 M^{-1} s^{-1} for the reaction of arginine with •OH[3]. The predominant $^1H/^2H$ exchange into the δ-methylene of arginine is consistent with the greater rate constant for the reaction being attributable to the presence of the guanidinium group. The γ-hydrogens of proline were most prone to exchange, showing a 2.7-fold greater ^2H incorporation than the β-hydrogens, while the δ-hydrogens showed exchange 1.4 times greater than the β-hydrogens. These data may all be taken to indicate that the presence of a formal positively charged N atom does not deactivate the vicinal C−H bonds from •OH-induced $^1H/^2H$ exchange. These results corroborate the observation that the δ-methylenes of arginine and proline are preferentially oxidized to carbonyl groups by Fe-EDTA catalyzed oxidation of polyamino acids.[26]

$^1H/^2H$ Exchange into Sulfur-Containing Amino Acid Residues. Figure 3 shows the results of the $^1H/^2H$ exchange into methionine. The ^1H NMR spectrum following the $^1H/^2H$ exchange reaction shows a small but detectable amount of methionine sulfoxide being formed, estimated by relative integration of the ϵ-methyl hydrogens of methionine sulfoxide at 2.73 ppm as 4.0% or 1 mM. In the ^2H NMR, the γ-methylene resonance of methionine sulfoxide at 3.1 ppm represents at least 2.5 mM ^2H (corresponding to 250 μM in the irradiated solution). Because no other amino acids demonstrated any signs of oxidation in the ^1H NMR, the formation of this amount of methionine sulfoxide cannot be attributed to oxidation by

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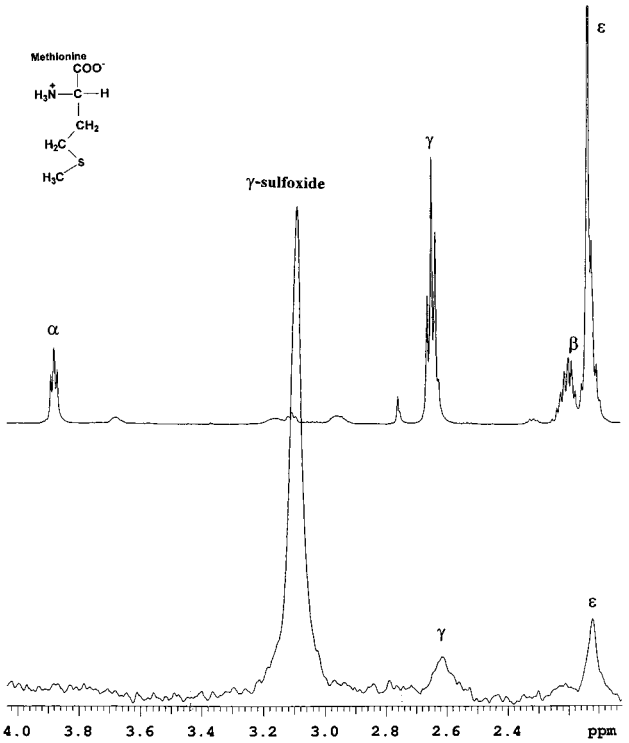


Figure 3. The ^{1}H (top panel) and ^{2}H NMR (bottom panel) spectra of methionine subjected to hydroxyl radical mediated ^{1}H/^{2}H exchange. The ^{2}H NMR spectrum indicates minimal incorporation into the γ-CH_2 and the ϵ-CH_3 groups of methionine and a larger amount of exchange into the γ-CH_2 group of methionine sulfoxide. The ratio of the ϵ-CH_3 group of methionine to the ϵ-CH_3 group of methionine sulfoxide (2.73 ppm) shown in the ^{1}H NMR spectrum indicates the absence of nonexchanged methionine sulfoxide hydrogens.

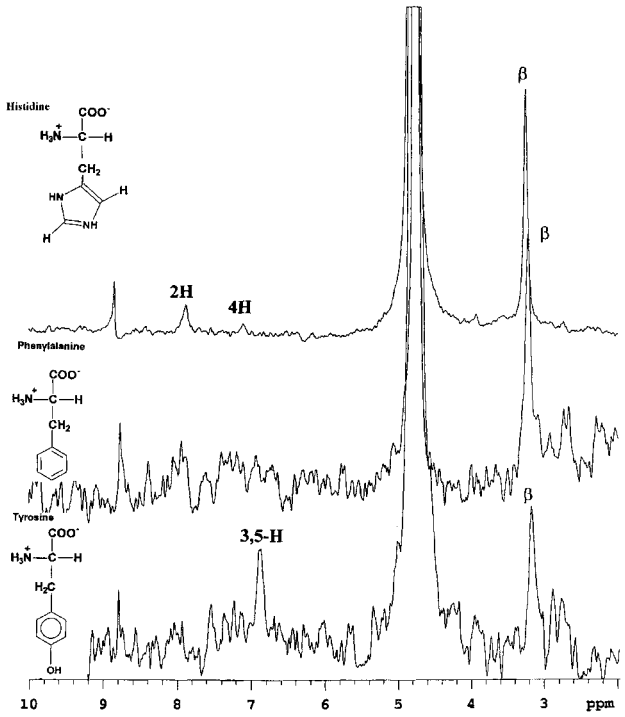
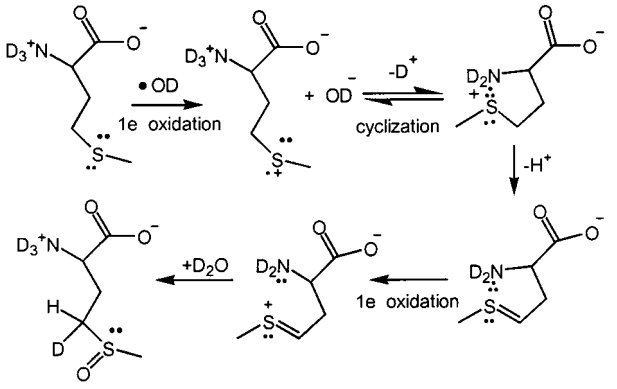


Figure 4. The ^{2}H spectra of the aromatic amino acid tyrosine, phenylalanine, and histidine. The ^{2}H NMR chemical shifts were correlated with those of the ^{1}H NMR of the same amino acid samples referenced to DSS. Exchange occurred predominantly into the β-methylene hydrogens of all three amino acids. The low signal-to-noise ratio of the tyrosine spectrum is due to its low solubility at 25 °C. (The concentration of tyrosine used was approximately half that of all the other amino acids.) Incorporation into the aromatic rings is minimal except for tyrosine where ^{1}H/^{2}H exchange occurs at C3 and C5. The peak at 8.6 ppm is a contaminant.

adventitious O_2. A similar O_2-independent oxidation of dimethyl sulfide has been reported.[27] The large preference for incorporation of ^{2}H in the γ-methylene of the methionine sulfoxide relative to the ϵ-methyl suggests that the mechanism for forming the sulfoxide involves exchange of the methylene hydrogens from an intermediate generated during the oxidation with solvent D_2O. The radical cation of methionine is known to form a cyclic structure with a 2-center 3-electron bond between the amino N and thioether S.[28−30] Deprotonation of the γ-methylene would form the preferred endocyclic double bond whereas deprotonation of the ϵ-methyl would yield the exocyclic double bond. The oxidant required for the second one-electron oxidation is unidentified but may possibly be derived from disproportionation of the radical cation as suggested for the anaerobic oxidation of dimethyl sulfide[27] and shown in Scheme 2.

To confirm the role of the cyclic intermediate, the ^{1}H/^{2}H exchange into *N*-glycyl-methionine and into preformed methionine sulfoxide was investigated. *N*-Glycyl-methionine was less prone to oxidation as minimal *N*-glycyl-methionine sulfoxide was detected in the ^{1}H NMR. However, ^{1}H/^{2}H exchange into both the γ-methylene and ϵ-methyl groups of *N*-glycyl-methionine was readily detected. Importantly for future studies with peptides and proteins, methionine incorporated into this

Scheme 2



model peptide underwent ^{1}H/^{2}H exchange in preference to oxidation. In *N*-glycyl-methionine, the γ-hydrogens at δ 2.60 and the ϵ-methyl hydrogens at δ 2.10 of methionine showed a 1:1 ratio for ^{1}H/^{2}H exchange. Minimal ^{1}H/^{2}H exchange directly into methionine sulfoxide was detected, indicating the ^{2}H present in the methionine sulfoxide formed from methionine was incorporated during the oxidation reaction and not after oxidation to the sulfoxide. The decreased incorporation relative to methionine suggests that the thioether of *N*-glycyl-methionine promotes the ^{1}H/^{2}H exchange reaction.

^{1}H/^{2}H Exchange into Aromatic Amino Acids. The ^{1}H/^{2}H exchange into aromatic amino acids is shown in Figure 4. The β-methylenes of L-histidine, L-phenylalanine, and L-tyrosine were the major target sites for incorporation. In histidine 81% of the total incorporation occurred in the β position, 13% in

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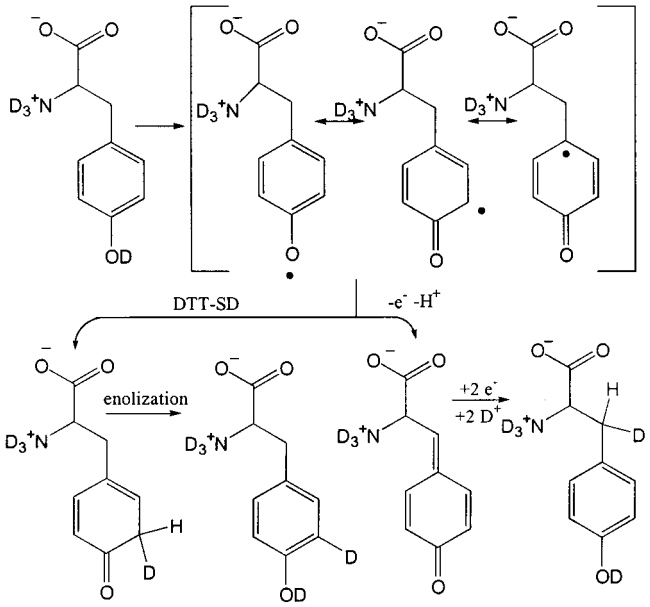
Scheme 3

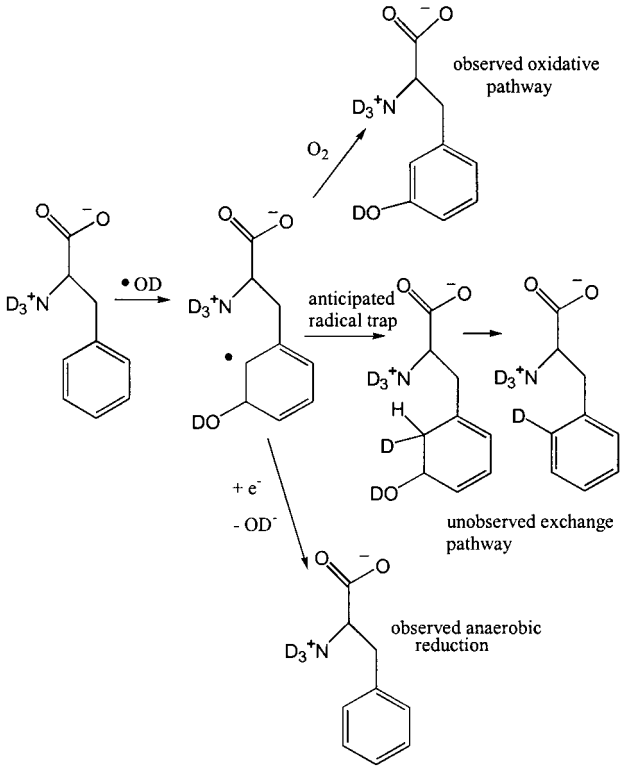


the C2 position of the ring, and only a trace in the C4 position of the ring. Exchange into the C2 position may be mediated by acid−base exchange from a histidyl radical as observed during anaerobic photolysis of histidine in the presence of ^3H_2O.[31] Phenylalanine shows incorporation only into its β position while 63% of the total ^2H incorporated into tyrosine is found in its β position with the remaining 37% contained in the C3 and C5 positions of the aromatic ring.

The incorporation of ^2H into the *ortho* position of the phenyl ring of tyrosine suggests abstraction of the phenoxyl hydrogen of tyrosine. The phenoxyl radical has significant radical character at the *ortho* positions, such that spin traps are incorporated at this position,[32] and *o*-dityrosine is an identified reaction product of tyrosine oxidation.[33] ^2H atom donation to the *ortho* position results in the formation of the keto tautomer of tyrosine. Enolization rearomatizes the phenol ring, resulting in the potential incorporation of ^2H as shown in Scheme 3. A primary kinetic isotope effect on enolization would preferentially retain the ^2H label at the *ortho* position. The tyrosyl radical may alternatively be transiently oxidized to the quinone methide. Reduction of the quinone methide would result in ^2H incorporation into the β-methylene. The significantly greater ^2H incorporation into the tyrosine β-methylene than observed in phenylalanine is suggestive of this mechanism, although a resonance effect of the *p*-hydroxyl group may also be responsible for the enhanced $^1H/^2H$ exchange at the tyrosine β-methylene.

The absence of $^1H/^2H$ exchange into the phenylalanine aromatic ring was unexpected. Pulse radiolysis studies[34] and the characterization of the aerobic reaction products of •OH with phenylalanine and tyrosine both indicate that the •OH readily adds to the aromatic ring to form a 1-hydroxy-2,4-cyclohexadienyl radical.[35] Under aerobic conditions, these radicals are oxidized, forming hydroxylated aromatic rings. The anticipated

Scheme 4



reaction of the •OH adduct under our anaerobic reaction conditions was for DTT to trap the radical by ^2H atom donation. Subsequent rearomatization of the 1-hydroxy-2,4-cyclohexadiene [6-^2H] intermediate by dehydration would result in partial ^2H atom incorporation into the ring, as shown in Scheme 4. However, the stability of the hydroxy-cyclohexadienyl radical has been shown to preclude trapping by thiols.[36] Additionally, the absence of observed exchange excludes this route of reactivity. The absence of any detectable hydroxylated aromatic rings in the ^1H NMR requires that the hydroxy-cyclohexadienyl radical be reduced by electron transfer with concomitant loss of hydroxide in the reducing environment. The reductant may be hydrated electrons, DTT disulfide radicals, or the DTT thiols. Similar dehydration has been previously observed from *o*-hydroxy tyrosyl radicals.[34] These results agree with the mass spectral isotope ratio measurements of $^1H/^2H$ exchange into these aromatic amino acids.[14]

$^1H/^2H$ Exchange into Acidic and Polar Amino Acids. The absence of exchange into the α-carbon of the amino acids has been attributed to the deactivation by the protonated amine. The results of our studies suggest that the carboxylate and carboxamide are also functional groups that deactivate H atom abstraction from vicinal C−H bonds. This can be seen directly in the $^1H/^2H$ exchange into proline, where both the δ-methylene and α-C−H are bonded to the amino group, but exchange is observed only into the δ-methylene, implying that the carboxylate must be deactivating the abstraction of the α-H. The deactivating character of the carboxylate and carboxamide groups is reinforced by the absence of $^1H/^2H$ exchange detected by ^2H NMR into glutamate, glutamine, aspartate, and asparagine in these studies and by the minimal exchange into these residues detected by the more sensitive mass spectral isotope ratio measurements.[15] These results correlate well with the very low

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rate constants for the reaction of these polar amino acids with •OH, ranging from (5 to 10) $\times$ 10^7 M^{-1} s^{-1}.[3]

Conclusion

The ^{2}H NMR has permitted the sites of modification by •OH-induced ^{1}H/^{2}H exchange into the amino acids to be detected and quantified, demonstrating the relative propensities of these amino acids toward exchange. The aliphatic residues show differential rates of exchange into the different alkyl sites. Incorporation into the methine and methylene carbons was preferred on a per H basis but by smaller factors than predicted by studies with alkanes. The ^{1}H/^{2}H exchange was also limited by proximity to the α-amino and α-carboxylate groups. Exchange into the basic amino acids indicated that the guanidinium group promoted the ^{1}H/^{2}H exchange while protonated amino groups were minimally deactivating. The aromatic residues show exchange prominently into their β-carbons, but only tyrosine and histidine have radical mechanisms that permit ^{1}H/^{2}H exchange into the ring positions. •OH addition to the aromatic rings is reversed under the reductive reaction conditions. Identification of the preferred sites of H-atom abstraction will now permit a better assessment of the reactive surface of the amino acid in peptides and proteins, and will aid in the interpretation of planned macromolecular protection experiments that employ ^{1}H/^{2}H exchange.

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