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Photoactivated H/D Exchange in Tyrosine: Involvement of a Radical Anion Intermediate

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Abstract: The aromatic hydrogen nuclei of tyrosine are photochemically labile and exchange with deuterons in neutral D_2O solution. The site *meta* to the ring hydroxyl substituent is preferentially deuterated, exhibiting a *meta/ortho* deuteration rate of ~4:1. In contrast with acid-catalyzed H/D exchange and with nearly all of the reported photoactivated H/D exchange studies, the UV-induced H/D exchange of tyrosine is optimal at pH 9 and is effectively quenched at acid pH. Photochemical H/D exchange is strongly stimulated by the α-amino group (the aromatic hydrogens of *p*-cresol are far less subject to exchange) and by imidazole or phosphate buffers. On the basis of the results obtained here and on the previously identified cyclohexadienyl radical (Bussandri, A.; van Willigen, H. *J. Phys. Chem. A* **2002**, *106*, 1524−1532), we conclude that the exchange reaction involves a radical intermediate and results from two distinct roles of tyrosine: (1) as a phototransducer of light energy into solvated electrons (e_{aq}^-), and (2) as an acceptor of an electron to create a radical anion intermediate which is rapidly protonated, yielding a neutral cyclohexadienyl radical. Regeneration of the tyrosine can occur via a bimolecular redox reaction of the cyclohexadienyl and phenoxyl radicals to yield a carbocation/phenoxide pair, followed by deprotonation of the carbocation. The oxidation step is pH dependent, requiring the deprotonated form of the cyclohexadienyl radical. The H/D exchange thus results from a cyclic one-electron (Birch) reduction/protonation/reoxidation (by phenoxyl radical)/deprotonation cycle. Consistent with these mechanistic conclusions, the aromatic hydrogens of tyrosine O-methyl ether are photochemically inert, but become labile in the presence of tyrosine at high pH. The deuteration rate of O-methyl tyrosine is lower than that of tyrosine and shows a preference for the *ortho* positions. This difference is proposed to result from a variation in the oxidation step, characterized by a preferential oxidation of a cyclohexadienyl resonance structure with the unpaired electron localized on the oxygen substituent.

Introduction

Tyrosine residues play a significant role in enzyme catalysis and, as such, have been the focus of extensive investigations. Tyrosine radicals have been implicated in photosynthesis,[1] in the activity of monoamine oxidase—an important drug target,[2] and in the mechanism of ribonucleotide reductase.[3] Tyrosine also serves as the precursor to the 2,4,5-trihydroxyphenylalanyl quinone (TPQ) cofactor found in copper amino oxidases[4] and to the fluorophore present in green fluorescent protein.[5] Additionally, tyrosine is a precursor to dopamine, epinephrine, and other neurotransmitters, so that it plays a central role in neurochemistry. These multiple roles of tyrosine have motivated the development of many isotopic labeling strategies.[6] Although photochemical H/D exchange studies of tryptophan[7,8] and of various phenolic compounds have been reported (refs 9−18 and references therein), there is a surprising lack of information on the photoactivated H/D exchange of tyrosine itself. In the case of L-tryptophan dissolved in D_2O, exposure to Pyrex-filtered

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UV light has been shown to result in a remarkably specific and clean deuterated product.[7]

The photochemical behavior of tyrosine is strongly dependent on the medium in which the studies are performed. In nonaqueous environments, degradation to *p*-coumaryl derivatives and phenol ring breakdown have been observed.[19] In aqueous solution in the absence of oxygen, the primary photoproducts are phenoxyl radicals, solvated electrons, and hydrogen atoms,[20] and the stable products that accumulate include 2-amino-4-ethenylhex-4-enic acid and 3,3′-dityrosine.[21] In the presence of oxygen, the phenoxyl radical is oxidized to 3,4-dihydroxyphenylalanine (DOPA) and other hydroxylated products.[22] Although there is an extensive literature on photoactivated isotope exchange (refs 9−18 and additional references therein), we are aware of no reported studies of photoactivated H/D exchange in tyrosine, consistent with the fact that little of this work has been performed in aqueous solution. Additionally, nearly all of the reported photoactivated H/D exchange studies have been performed under acid conditions—a condition which in the present study *inhibits* the reaction. However, FT-EPR studies of tyrosine photochemistry have demonstrated the production of a cyclohexadienyl radical intermediate that could mediate H/D exchange.[23] In the present study, we have investigated photochemical H/D exchange in L-tyrosine and related compounds in D_2O solution, determined the regioselectivity of the reaction, and obtained strong support for a photoactivated Birch reduction/protonation/oxidation/deprotonation cycle as the mechanistic basis for H/D exchange.

Experimental Section

UV irradiation utilized a four-bulb low-pressure mercury lamp system obtained from Ultra Violet Products, Inc. (San Gabriel, CA). Tyrosine and *p*-cresol were from Sigma-Aldrich (St. Louis, MO), and D_2O (99.9 atom %) was obtained from Isotec, Inc. (Miamisburg, OH). All pH values represent uncorrected pH meter readings. Samples were dissolved in D_2O, placed in quartz NMR tubes (Wilmad, Buena, NJ), degassed by bubbling with argon for 6 min, and then placed in the UV lamp for the time periods indicated. All work was done at room temperature. For the larger volume study, a 50 mL quartz beaker (49 mm o.d. × 50 mm height, was obtained from Kimble/Kontes (Vineland, NJ).

Proton NMR spectra were obtained at 25 °C on a Varian INOVA 500 NMR spectrometer using a 5 mm ^{1}H probe. Typical spectra parameters: spectral width of 8 kHz, pulse width of 22°, acquisition time of 3.125 s, relaxation delay of 2 s (to minimize intensity variations due to incomplete relaxation). The VNMR integration software was used to determine resonance intensities.

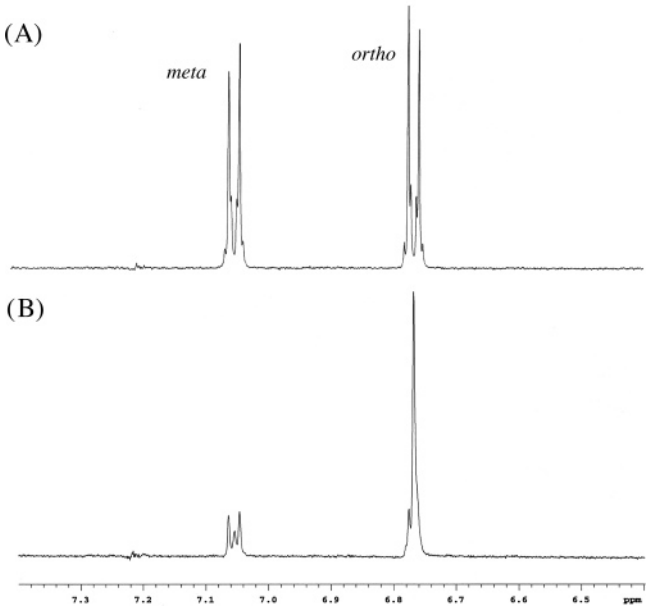

Figure 1. Aromatic resonances of tyrosine. (A) The region of the ^{1}H spectrum containing the aromatic resonances of 5 mM tyrosine dissolved in 400 mM phosphate, pH 7.0. (B) Sample A after a 15 min period of UV exposure. The *ortho* resonances of the A_2B_2 spin system are at 6.77 ppm, and the *meta* resonances are at 7.05 ppm. The sample was prepared in D_2O, treated for 6 min with argon to remove dissolved oxygen, and the stated pH value represents an uncorrected meter reading.

Results

Exposure of a 5 mM solution of tyrosine in neutral D_2O to 254 nm UV produced by a low-pressure mercury lamp results in a ^{1}H NMR spectrum that is identical with that of the starting material, except that the intensities of the aromatic 3,5 (*ortho* to the hydroxyl) and 2,6 (*meta* to the hydroxyl) resonances are reduced in a time-dependent manner, with the *meta* position preferentially deuterated. Inspection of the NMR spectra (Figure 1) reveals that the *meta* protons appear as superposed doublet (J = 9.01 Hz) and singlet resonances, with the latter showing a small upfield displacement from the center of the doublet (δ = 1 ppb) due to the γ-isotope effect of the deuteron present in the *ortho* position (Figure 1). The structure of the *ortho* proton resonance is analogous, except that the singlet/doublet ratio is much greater due to the greater level of deuteration at the *meta* position. In addition, only the downfield component of the doublet resonance for the *ortho* protons is resolved from the large singlet, again consistent with an upfield isotope shift of the singlet resulting from deuteration of the *meta* position. There is no significant loss of proton resonance intensity from the α or β proton positions, indicating that the UV irradiation does not significantly affect the amino acid backbone. The lack of significant deuteration at the β positions also eliminates the importance of a quinone methide intermediate in explaining the observed UV-induced chemistry.[17]

The photoactivated H/D exchange reaction exhibits a pronounced pH dependence (Figure 2), and exponential fits of the $I/I\alpha$ intensity ratio versus time give a mean ratio for the *meta*/*ortho* deuteration rate constants of 4.3 (Figure 3). As is apparent from the data in Figure 2, the exchange rate increases with pH up to ∼9, after which there is a marked decrease. Since the

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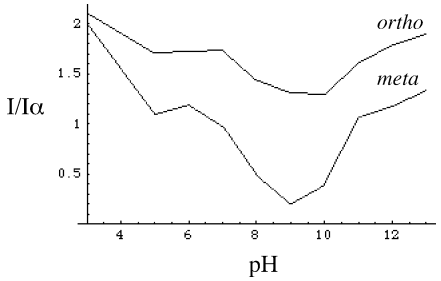

Figure 2. pH dependence of photoactivated deuterium incorporation. The pH dependence of the intensity of the *ortho* and *meta* resonances of tyrosine, normalized by the intensity of the *I*α proton resonance, after UV exposure for a period of 1 h. Samples were prepared in D_2O, treated for 6 min with argon to remove dissolved oxygen, and the stated pH values represent uncorrected meter readings.

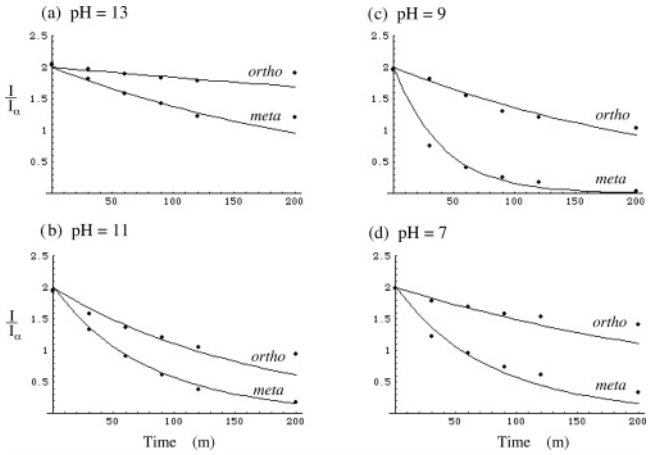

Figure 3. Time-dependent *Im*/*I*α and *Io*/Iα intensity ratios for the pH values indicated. Samples were prepared in D_2O, treated for 6 min with argon to remove dissolved oxygen, and the stated pH values represent uncorrected meter readings.

tyrosine hydroxyl group has a pK of 10, one implication of the pH profile shown in Figure 2 is the preferential reactivity of tyrosine with a nonionized hydroxyl group. Surprisingly, no significant deuteration of the aromatic positions is observed at pH 2, while the appearance of additional resonances indicates partial degradation of the tyrosine. This result was unexpected since most of the reported tyrosine deuteration studies,[6] as well as most of the photochemical H/D exchange studies in related phenolic compounds,[9−18] have been performed under acid conditions. The fact that deuteration under acid conditions is selective for the *ortho* position[6] and the absence of significant photochemical H/D exchange at low pH indicates that the photodeuteration process for tyrosine in aqueous media proceeds by a different mechanism than acid-catalyzed H/D exchange. One exception to this generalization is the rhodium-catalyzed deuteration of 4-hydroxybenzoate, which preferentially deuterates *meta* to the hydroxyl group.[24]

Exposure of *p*-cresol solutions to UV for 1 h failed to produce significant deuteration and resulted only in low levels of deuteration even after 16 h, as judged from the ^{1}H NMR spectrum. This result was unexpected since previous FT-EPR studies have not found significant differences in the photochemical behavior of these two molecules.[23] The dramatic difference between the response of tyrosine and *p*-cresol to UV

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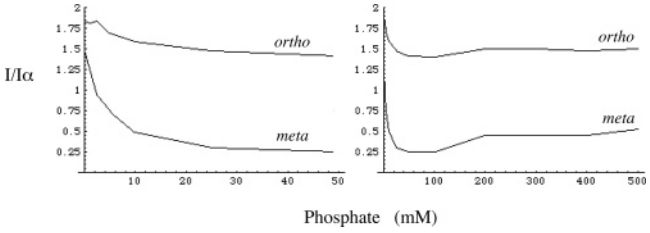

Figure 4. Intensity ratio of the *meta* and *ortho* ^{1}H resonances relative to that of Hα after exposure to UV for 15 min, as a function of the phosphate buffer concentration. The sample contained 5 mM tyrosine at an uncorrected pH meter reading of 7. Note that, even in the absence of phosphate, the 15 min irradiation period gives $I_o/I_\alpha = 1.84$ and $I_m/I_\alpha = 1.48$ compared with a value of 2.0 in the absence of UV exposure. Samples were prepared in D_2O, treated for 6 min with argon to remove dissolved oxygen, and the stated pH values represent uncorrected meter readings.

irradiation as judged by the degree of deuteration suggested the importance of an intramolecular protonation step involving the α-amino group, analogous to the process proposed for the photodeuteration of L-tryptophan.[7,8] We therefore investigated the possibility that buffers could significantly enhance the H/D exchange rate by providing a more abundant source of D^+ ions. Consistent with this hypothesis, photodeuteration of *p*-cresol is readily observed in phosphate buffer. On the basis of the resonance multiplet structures, the fractional protonation of the *ortho* and *meta* sites is 0.67 and 0.15 after 1 h of UV exposure (25 mM *p*-cresol, 0.3 M phosphate, pH = 7.3), again indicating a *meta*/*ortho* deuteration ratio of ∼4:1. Similarly, phosphate concentrations up to ∼50 mM significantly enhance the deuterium substitution rate at both the *ortho* and *meta* positions of tyrosine (Figure 4), with the *meta*/*ortho* deuteration ratio remaining similar to that observed in the absence of buffer. Interestingly, the phosphate buffer effect becomes limiting at ∼50−100 mM. In contrast with these results, the deuteration rate was unaffected by 0.5 M concentrations of the nonbuffering salts NaCl, CsCl, or $LiClO_4$. However, imidazole buffer was also effective as a UV H/D exchange catalyst (data not shown).

To further evaluate the role of an intramolecular protonation step, we performed similar studies on 3-hydroxyphenylalanine (*m*-tyrosine) (Figure 5). The resulting spectrum demonstrates that the major determinant of photochemical H/D exchange is the position relative to the hydroxyl substituent since the C-5 position *meta* to the C-3 OH is clearly the most heavily deuterated ($I_5/I_\alpha = 2.2\%$ after 5.5 h, where the intensity of the H-5 resonance, I_5, has been normalized relative to the intensity of the Hα proton resonance). In the deuterated species, the H-4 and H-6 resonances, which initially appear as overlapping doublets, are observed as singlets ($I_4/I_\alpha = 29\%$, $I_6/I_\alpha = 24\%$ after 5.5 h). The H-2 position shows the lowest level of deuteration, although there is a significant loss of intensity at this position relative to Hα ($I_2/I_\alpha = 37\%$ after 5.5 h). Hence, although the presence of an amino group optimally positioned for intramolecular protonation/deuteration at the site undergoing H/D exchange is beneficial, the results for *m*-tyrosine indicate that it is not an absolute requirement. Presumably, the role of the α-amino group in this case is similar to the role of the phosphate or imidazole buffer described above.

Involvement of a Radical Anion Intermediate. In FT-EPR studies of phenol in D_2O subjected to UV radiation at a wavelength of 266 nm, Bussandri and van Willigen have observed a cyclohexadienyl radical, in which the deuterated

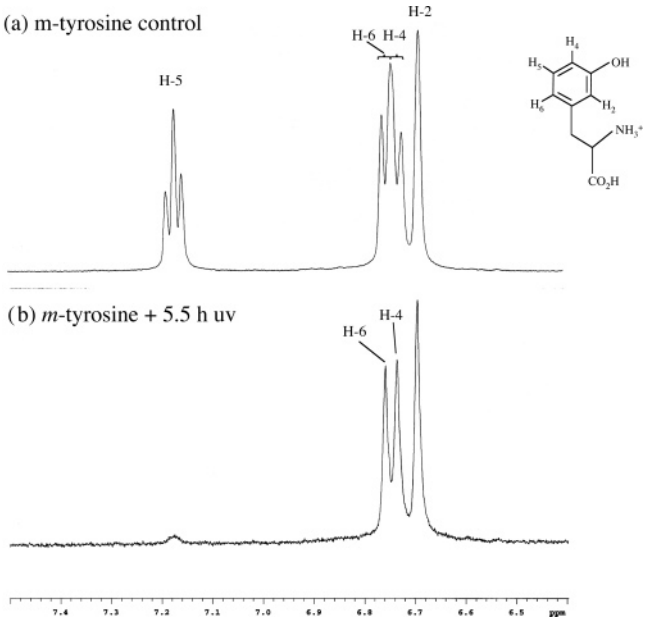

Figure 5. Aromatic resonances of *m*-tyrosine (3-hydroxyphenylalanine): (a) 15 mM *m*-tyrosine in 5 mM phosphate, pH 7.1; (b) above sample after 5.5 h UV exposure. The structure and position identification of *m*-tyrosine are indicated in the inset. The two overlapping doublets corresponding to H-4 and H-6 in (a) become primarily singlets in (b). The sample was prepared in D_2O, treated for 6 min with argon to remove dissolved oxygen, and the stated pH value represents an uncorrected meter reading.

position is *meta* to the hydroxyl substituent.[23] This is the presumed intermediate species for the *meta*-deuteration product observed in the present studies and supports a free radical mechanism for the photochemical H/D exchange reaction. The EPR studies were interpreted in terms of a bimolecular abstraction of a hydrogen radical, H•, to yield a phenoxyl/cyclohexadienyl radical pair. However, as noted in this study, this reaction requires a nondissociated phenolic proton and so becomes inoperative above the phenolic pK. In contrast, the H/D exchange reaches a maximum near pH 9, and significant exchange is observed at pH 13 (Figures 2 and 3). Thus, even if the H• abstraction process contributes to the H/D exchange reaction at low pH, another mechanism would need to become operative at higher pH. Since photolysis of tyrosine at lower pH yields H• radicals and since the reaction of H• with tyrosine has been reported,[25] addition of H• to the ring could produce the cyclohexadienyl radical. However, no signal arising from this reaction pathway could be identified in the FT-EPR study.[23] Photoexcitation of tyrosine also produces e_{aq}^- ions, and these have also been shown to react with tyrosine at rates not too far below the diffusion limit.[20] Reaction rate constants of 2.8×10^8 M^{-1} s^{-1} (pH 6.6) and 9.6×10^7 M^{-1} s^{-1} (pH 12.5) were determined from pulse radiolysis studies.[20] Thus, formation of a tyrosine radical anion resulting from the addition of e_{aq}^- produced by a second tyrosine molecule, followed by a protonation step, provides a likely pathway for formation of the cyclohexadienyl radical intermediate (Scheme 1).

As indicated in Scheme 1, the deuteration step might involve an intramolecular deuteron transfer from the α-amino group or an intermolecular transfer from the amino group of another tyrosine molecule or from the buffer. Consistent with the

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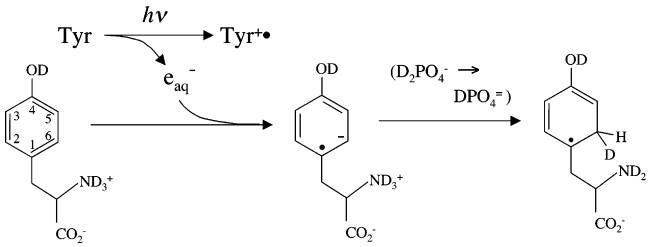
Scheme 1



conclusion of a radical-based mechanism, addition of N_2O, an agent that reacts with free electrons to yield hydroxyl ions and hydroxyl radicals, produces effects somewhat analogous to lowering the pH, quenching the H/D exchange, and resulting in extensive chemical transformations. Thus, H^+ and N_2O, which both scavenge electrons, inhibit the photochemical H/D exchange of the aromatic protons in tyrosine. Surprisingly, the rate reported for the reaction of e_{aq}^- with tyrosine only decreases by a factor of ∼3 above the hydroxyl pK, indicating that ionization does not significantly reduce the reaction rate. On the basis of the absorption spectra obtained, it was concluded that reaction at the high pH resulted in the same product(s) that are produced at lower pH.[20]

In the pulse radiolysis study noted above, the reaction rate of e_{aq}^- with *p*-cresol was determined to be 4.2×10^7 M^{-1} s^{-1}, or 15% of the reaction rate with tyrosine. Hence, both a lower reaction rate and the lack of an intramolecular protonation mechanism would be expected to reduce the rate of formation of the cyclohexadienyl radical, consistent with the lower rate of photochemical H/D exchange observed for this analogue.

A rough estimate of the rate of formation of the tyrosine radical anion, $k_{Tyr^{\bullet -}}$, can be made based on the electron concentration, the fraction of tyrosine in the protonated/unprotonated forms, and the rate constants for reaction in these two forms given by Feitelson and Hayon:[20]

$$k_{Tyr^{\bullet -}} \approx [e_{aq}^-](k_1 f_{Tyr-OH} + k_2 f_{Tyr-O^-}) \approx pH^3 \times \left(\frac{2.8 \times 10^8}{1 + 10^{pK-pH}} + \frac{9.6 \times 10^7 (10^{pK-pH})}{1 + 10^{pK-pH}}\right) \quad (1)$$

where f_{Tyr-OH}/f_{Tyr-O^-} is the fraction of tyrosine in the protonated/unprotonated form (pK = 10), the rate constants k_1 and k_2 (in M^{-1} s^{-1}) are given by Feitelson and Hayon,[20] and the electron concentration has been approximated by a cubic dependence on the pH. The latter approximation is suggested by data given by Bussandri and van Willigen, who report that the EPR signal arising from e_{aq}^- decreases by 77% on going from pH 11 to 7.[23] This behavior is most simply modeled by the pH^3 dependence shown above. Clearly, the actual variation of e_{aq}^- with pH would be represented by a much more complex function, with the primary dependence resulting both from the pH-dependent generation of e_{aq}^- by tyrosine photoexcitation and the scavenging of the electron by H^+ described by the reaction: $e_{aq}^- + H^+ \rightarrow H\bullet$. A plot of eq 1 using an arbitrary scaling factor gives a qualitatively reasonable description of the 1 h deuteration levels at the *meta* position, determined from the resonance intensities as $2 - Im/I\alpha$, although there is a large divergence above pH ∼ 11 (Figure 6). Undoubtedly, the deprotonation of the α-amino group and the reduction in $[H^+]$ at high pH are related to the difference between the theoretical and experimental data at high pH. A better fit is obtained by

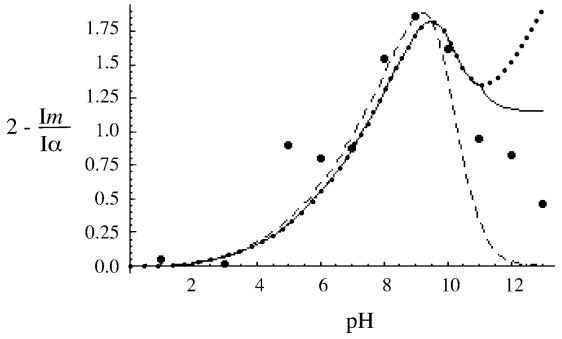


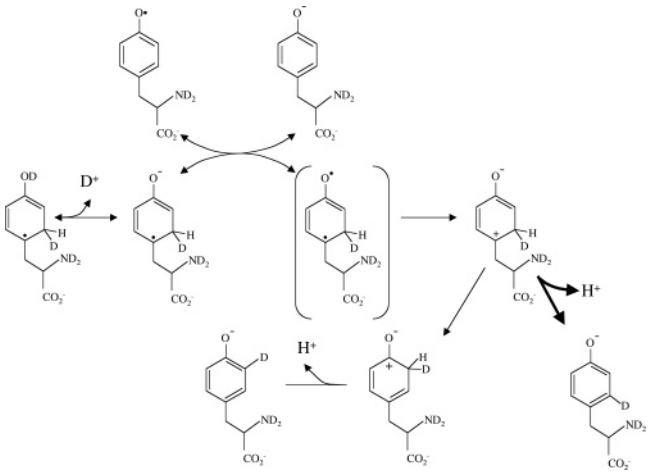
Figure 6. Comparison of deuteration level at the *meta* position of tyrosine after a 1 h period of UV exposure (•), calculated as $2 - Im/I\alpha$, with calculated rate constant given by eq 1: (1) assuming $[e_{aq}^-] \sim pH^3$ (· · ·); (2) assuming $[e_{aq}^-] \sim pH^3$ up to pH 11, and constant thereafter (—); (3) assuming $[e_{aq}^-] \sim pH^3$, and no electron addition to the tyrosine (phenolic) anion ($k_2 = 0$) (– – –). The scale of the calculated curves was set arbitrarily to approximate the deuteration level.

assuming that, after building up to a high level, $[e_{aq}^-]$ remains constant above pH 11, which would be consistent with the maximum yield of electrons after the tyrosine hydroxyl group is fully deprotonated (Figure 6). A more accurate approximation of the H/D exchange data would require a more complete description of all of the reactions that produce and consume e_{aq}^-, and particularly the rates for protonation of the tyrosine radical anion and for the subsequent chemical transformations of the cyclohexadienyl radical. Nevertheless, in addition to providing a qualitatively reasonable description of the exchange, the fit is consistent with the high pH results obtained. A model that assumes no electron addition to the phenoxide (i.e., $k_2 = 0$ in eq 1 above) predicts that the exchange rate falls to near zero above pH ~12, in contrast with the observations (Figures 3 and 6). Although eq 1 also lacks rate constants for the oxidation/deprotonation steps described in Scheme 2 (see below), such steps may not be rate limiting if formation of the cyclohexadienyl radical via the pathway shown in Scheme 1 is irreversible.

On the basis of these observations, a reaction between e_{aq}^- and tyrosine, as has been observed in pulse radiolysis experiments,[20] appears to be central to the H/D exchange reaction. The reaction shown in Scheme 1 also is equivalent to the initial step of a Birch reduction,[26,27] in which electrons derived from alkali metals react with aromatic substrates to yield a radical anion which is subsequently protonated to produce a cyclohexadienyl radical intermediate. For the Birch reduction, however, the strongly reducing conditions result in a second electron addition/protonation step to yield a reduced product. Interestingly, Birch reduction of the tyrosine O-methyl ether has been used as a starting point for the synthesis of aeruginosins.[28]

Regeneration of Tyrosine. Regeneration of tyrosine can, in principle, be accomplished by reversing the steps involved in its formation, that is, a deprotonation followed by dissociation of the electron from the tyrosyl radical anion or by a one-electron oxidation followed by loss of a proton. Regeneration of phenylalanine by oxidation of the cyclohexadienyl radical[29] and oxidative regeneration of tyrosine from a related intermediate by the phenoxyl radical[30] have previously been proposed. Das and co-workers have discussed the redox chemistry of tyrosine and related phenolic compounds and note that above the hydroxyl pK, a facile redox reaction, can occur.[31] Under the conditions of the present study, deprotonation of the cyclohexadienyl radical will allow it to be oxidized by the photochemically generated phenoxyl radical according to Scheme 2.

Scheme 2



According to Scheme 2, oxidation of the cyclohexadienyl radical anion (a structurally distinct species relative to that formed from by e_{aq}^- addition to tyrosine depicted in Scheme 1) would be expected to yield a biradical species which would be expected to adopt the lower energy, zwitterionic resonance form indicated in Scheme 2. This should rapidly deprotonate, but the carbocation can also tautomerize, providing one possible avenue for deuteration of the *ortho* position. A particularly attractive feature of Scheme 2 is the consistency with the EPR observation of the cyclohexadienyl radical formed in the FT-EPR studies reported by Bussandri and van Willigen.[23] In that study, the EPR signal for this radical is approximately constant between pH 2 and 7, but is absent in basic solution. This behavior is consistent with Scheme 2, in which deprotonation of this radical facilitates its oxidative decomposition.

Photochemistry of Amino Acid Mixtures. Scheme 1 postulates two separate roles for tyrosine in the photochemical H/D exchange reaction. Initially, a tyrosine molecule that absorbs light energy serves as a source for the photoejected electron, which then reacts directly with a second tyrosyl ring to produce a radical anion. Therefore, the critical role of the tyrosine is as a transducer of light energy into an aquated electron radical, while the role of acceptor theoretically could be played by another ring system. To evaluate this possibility, we studied two mixtures containing equimolar concentrations of tyrosine and phenylalanine, or tyrosine and tyrosine-O-methyl ether (O-methyl tyrosine: OMT). Exposure of either phenylalanine or OMT to UV light under the conditions of the above studies does not result in significant deuteration of the ring positions, but in chemical transformations, as judged by the appearance of new proton resonances (spectra not shown).

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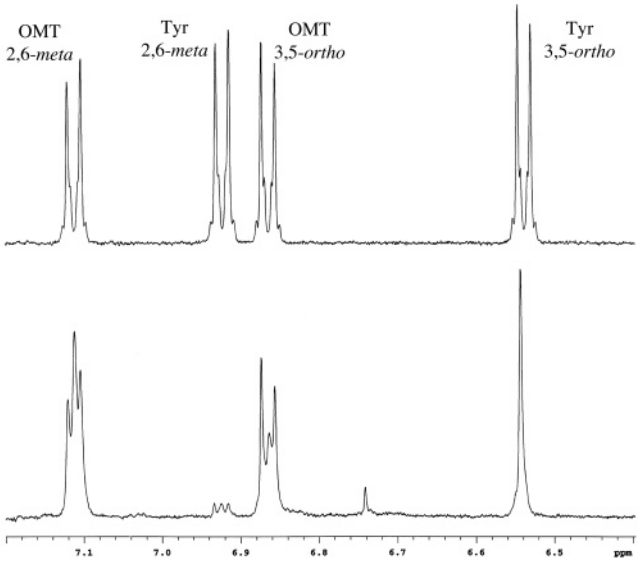


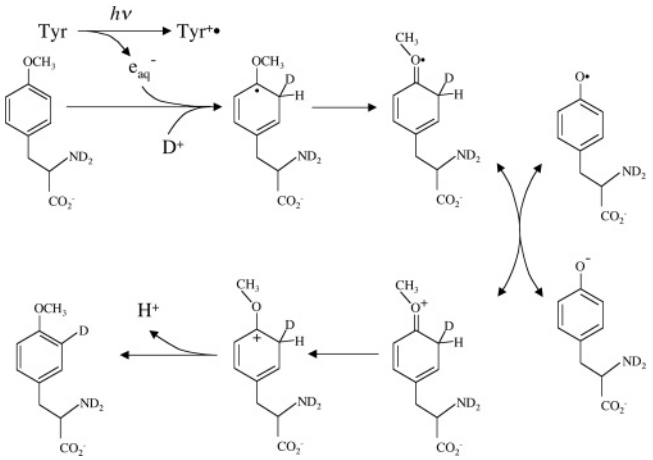
Figure 7. Aromatic resonances of a solution containing 3 mM tyrosine, 3 mM O-methyl tyrosine in 50 mM phosphate, pH 11: (a) prior to UV irradiation; (b) after a 60 min exposure to UV. The sample was prepared in D_2O, treated for 6 min with argon to remove dissolved oxygen, and the stated pH value represents an uncorrected meter reading.

The presence of tyrosine confers some protection from the degradative effects of the UV, and in the case of OMT, a significant level of ring deuteration is observed (Figure 7). Interestingly, the deuteration pattern observed in OMT under these conditions differs from that of tyrosine, with a lower level of deuteration and a relatively greater incorporation at the 3,5-*ortho* position than at the 2,6-*meta* positions. In this experiment, tyrosine itself exhibits the same deuteration pattern observed in solutions not containing the phenylalanine or OMT (Figure 1). Additionally, although a complete pH-dependent study was not performed, a negligible level of OMT deuteration was observed at pH 7, in contrast with the results shown in Figure 7, which were obtained at pH 11. Thus, the greater photochemical yield of electrons from tyrosine at higher pH is correlated with a greater degree of H/D exchange of the OMT. The dramatic difference between the effects of UV exposure on OMT in the presence or absence of tyrosine provides strong support for the general features of Scheme 1, which postulates two separate roles for tyrosine. In this case, the tyrosine is the primary transducer of light energy producing free electrons, while either molecule can act as the electron acceptor. As noted above, Birch reduction of OMT to produce a synthetic intermediate has previously been described.[28]

The basis for the significant difference in the regioselectivity of the H/D exchange reaction between tyrosine and OMT is unclear. However, since the oxidation pathway outlined in Scheme 2 is not available for OMT, a modification of this pathway is required. We postulate that the different regiospecificity of OMT deuteration is related to a variation in the oxidation chemistry. One such pathway is shown in Scheme 3.

According to Scheme 3, the more likely deuteration of the *ortho* position of OMT results from the availability of a resonance structure with the radical localized on the oxygen substituent. In this form, a redox reaction with the phenoxyl radical can take place, ultimately leading to a carbocation which deprotonates as in the case of tyrosine, resulting in deuteration at C-3. Oxidation/deprotonation via Scheme 3 will lead to a

Scheme 3



greater level of deuteration at the *ortho* positions. Specifically, radical localization at C-4 will result in *ortho* deuteration as shown, while radicals formed at C-1 will need to tautomerize to the oxygen-centered structure in order to become oxidized. Neglecting any kinetic isotope effect, this would lead to equal labeling at the *ortho* and *meta* positions. Consistent with the chemistry shown in Scheme 3, studies of the chemical behavior of the anisole radical cation have been interpreted in terms of localization of the radical on the carbon bearing the methoxy substituent.[32]

Using pulse radiolysis, Mittal and Hayon find that the rate constant for the reaction of phenylalanine with e_{aq}^- is 1.6×10^8 M^{-1} s^{-1}, more than half the rate of reaction of e_{aq}^- with tyrosine under similar conditions.[29] Although the above rate should be sufficient to allow significant ring deuteration if electrons are generated by the photolysis of tyrosine in the mixture, the regeneration pathway shown in Scheme 2 will not be operative in the absence of the hydroxyl substituent. Another limitation on the reaction with phenylalanine results from the greater symmetry of the ring, which would be expected to result in a more even distribution of the negative charge in the radical anion. Lack of charge buildup at specific ring positions would in turn be expected to reduce the rate of protonation and hence formation of the cyclohexadienyl radical. Thus, it is not surprising that no significant deuteration of phenylalanine was observed even in the presence of tyrosine.

Effect of Tyrosine Concentration on H/D Exchange. The photochemical H/D exchange reactions outlined in Scheme 1 is inherently multi-molecular and would be expected to show a positive correlation with tyrosine concentration. Similarly, a bimolecular reaction of the cyclohexadienyl and phenoxyl radicals is proposed to be important for converting the former back to tyrosine (Scheme 2). The concentration dependence of the photoactivated H/D exchange reaction was investigated at pH 11, due to the limited solubility of tyrosine at neutral pH, and the absence of a deuteration reaction at low pH (Figure 2). As shown in Figure 8, the resonance intensities of the tyrosyl ring protons show a large dependence on tyrosine concentration, such that the deuteration reaction is nearly eliminated at 50 mM tyrosine. One limitation on the reaction rate is the inner filtering of the UV light by the tyrosine itself.[33] The significance of this

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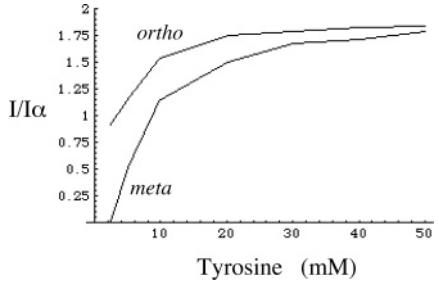


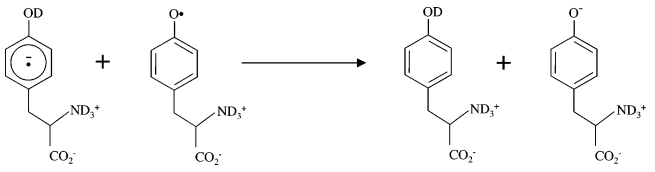
Figure 8. Dependence of $Im/I\alpha$ and $Io/I\alpha$ ^{1}H intensity ratios on tyrosine concentration. Samples contained the tyrosine concentration indicated at pH 11 and were subjected to a 60 min period of UV irradiation. Note that under these conditions, at the lowest tyrosine concentration evaluated (2.5 mM), $I_o/I_\alpha = 0.91$, $I_m/I_\alpha \sim 0.05$.

effect was confirmed by studies of a 5 mM tyrosine solution (100 mM phosphate, pH 11.0 in D_2O) that were performed in a 49 mm diameter quartz beaker. In this case, the larger volume had the same effect as higher concentration in limiting the observed H/D exchange reaction (data not shown).

An additional limitation on the H/D exchange reaction is expected to result from the formation of small amounts of more absorptive tyrosine oligomers. Tyrosine radicals formed under anoxic conditions are known to form oligomeric species, particularly 3,3′-dityrosine, as well as 3,3′,5′,3″-trityrosine and pulcherosine.[34] These adducts are fluorescent and will absorb UV radiation and emit longer wavelength light, thus inhibiting UV-dependent H/D exchange. Although the ^{1}H NMR spectra generally indicate that such side reactions are of limited quantitative significance, the small singlet at 6.74 ppm observed in the OMT−tyrosine experiment (Figure 7) most probably arises from the H-2 protons in 3,3′-dityrosine (the resonance is shifted ∼0.4 ppm upfield relative to the reported shift of 3,3′-dityrosine,[34] probably due to the pH difference). The presence of some colored material, barely detectable in the NMR tubes, was readily apparent in the larger diameter beaker. Nevertheless, while these products significantly influence the light absorption characteristics of the sample, the NMR analysis indicates that in most of the studies, they do not represent a significant fraction of the available tyrosine molecules (e.g., Figure 1). Most probably, the accumulation of these products is related to the "reaction fatigue" that can be observed at the longest time points of the studies (Figure 3).

A final consideration relevant to the effect of tyrosine concentration is a potential redox reaction between the initially formed radical anion and a phenoxyl radical. It is likely that the phenoxyl radical can oxidize this radical, perhaps involving a resonance form in which the radical is localized on the oxygen (Scheme 4).

Scheme 4



Analogous reactions have been discussed by Lynn and Purdie[30] and, for *p*-aminobenzoate, by Nakken.[35]

Discussion

An understanding of the photochemistry of tyrosine is central to an appreciation of its many roles in enzyme biochemistry, photosynthesis, and neurochemistry, as well as to preparative methods for introducing deuterium or tritium labels. Additionally, the lability of deuterium and tritium labels to photochemical H/D(T) exchange makes this phenomenon of practical importance for the analysis of deuterated or tritiated biological phenols. The isotopic labeling pattern that is produced as a result of physical or chemical manipulations provides information about the chemical history of the molecule. The regiospecificity and pH dependence of photochemically activated H/D exchange in tyrosine are unusual and differ from the widely used acid-catalyzed deuteration pattern which favors *ortho* substitution. Despite an extensive literature on photochemical H/D exchange and excited-state proton transfer (ESPT), we found essentially no reported study of photoactivated H/D exchange in tyrosine, consistent with the fact that little of the reported work was done in aqueous solution. More importantly, nearly all of the reported work has been performed under acid conditions—which in the present study *inhibit* the H/D exchange reaction. In general, most of the reported deuteration studies of aromatic systems—both chemical and photochemical—have been performed under considerably more acidic conditions at which cationic species are formed.[6,8−15] Interestingly, a 1994 study of UV-induced H/D exchange in the *o*-methoxyphenol ring of the antibiotic tomaymycin suggested that this might be the first example of photoactivated H/D exchange in an aromatic ring at neutral pH.[16]

The cyclohexadienyl radical that has been observed in FT-EPR studies of photochemically activated tyrosine and related phenolic compounds is the presumed intermediate in the photoactivated H/D exchange reaction.[23] However, there are fundamental questions regarding the predominant synthetic route, the mechanism for regeneration of tyrosine from the radical intermediate, and the reason for the poor correlation between the pH dependence of the H/D exchange reaction and of the EPR observation of this radical intermediate. We have considered three likely pathways for the formation of the cyclohexadienyl radical: (1) direct addition of a hydrogen atom; (2) a bimolecular H• abstraction that yields the cyclohexadienyl/phenoxyl radical pair, as proposed by Bussandri and van Willigen;[23] (3) direct addition of e_{aq}^- generated by tyrosine photolysis to the ring of a second tyrosine molecule, followed by protonation. Although the direct reaction of H• with tyrosine has been reported,[25] Bussandri and van Willigen were unable to assign an EPR signal to this reaction pathway.[23] In addition, the pH dependence of the photoactivated H/D exchange observed here appears to be inconsistent with a significant contribution from a reaction involving H•, the concentration of which varies inversely with pH due to the reaction: $e_{aq}^- + H^+ \rightarrow H\bullet$. Thus, while H• addition may be occurring, it does not appear to make a significant contribution to the observed exchange rate. The bimolecular H• abstraction reaction proposed by Bussandri and van Willigen exhibits an apparent inconsistency with the concentration dependence of the H/D exchange reaction (Figure 8). Nevertheless, as discussed above, there are several other factors that may mask the concentration dependence of the reaction. Alternatively, the observation of a significant rate of H/D exchange at pH values up to 13 appears to be inconsistent with this reaction mechanism, and indeed, these authors explain the disappearance of the EPR signal of

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the cyclohexadienyl radical in basic solution as a consequence of the elimination of this reaction pathway resulting from deprotonation of the phenolic hydroxyl substituent.[23]

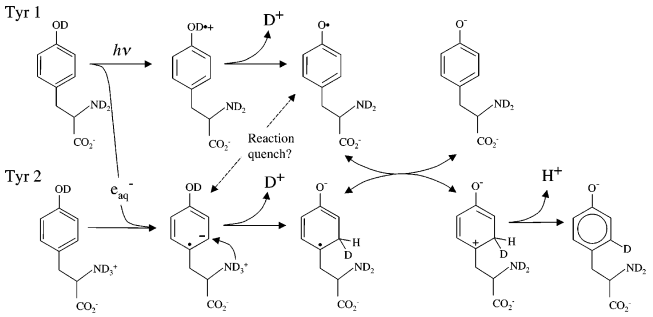
In contrast to the problems with pathways 1 and 2 summarized above, the reaction of e_{aq}^- with the tyrosine ring followed by protonation, as shown in Scheme 1, forms the basis for known Birch reduction chemistry.[26−28] A "photo-Birch reduction", which requires the addition of a reducing agent to drive the reaction toward two-electron reduction, has also been described in the literature.[36] The EPR spectra of several aromatic radical anions formed by reaction with e_{aq}^- have been observed.[37] The increase in the observed deuteration level parallels the large increase in the e_{aq}^- signal with pH that has been observed in EPR studies.[23] The observation of a significant rate of H/D exchange at pH 13 is consistent with the data of Feitelson and Hayon, showing that the reaction rate of e_{aq}^- with tyrosine falls off by only a factor of 3 above the phenolic pK.[20] Alternatively, since this observation is made well above the amino pK, there is no optimal candidate for the protonation reaction shown in Scheme 1. Most probably, the solution to this problem is that the much higher level of e_{aq}^- at high pH[23] compensates for the lack of an optimal protonation reaction, as is qualitatively indicated by the calculations shown in Figure 6.

The regeneration of tyrosine from the cyclohexadienyl radical is proposed to result from a redox reaction involving the cyclohexadienyl and phenoxyl radicals, which becomes optimal upon deprotonation of the former (Scheme 2). This mechanism provides an attractive explanation for the unobservability of the cyclohexadienyl radical at high pH noted by Bussandri and van Willigen,[23] that is, it results from rapid oxidation rather than from reduced formation. Continued formation of the cyclohexadienyl radical at high pH is required to explain the observed photoactivated H/D exchange. Possibly, the coupled redox reaction shown in Scheme 2 may be related to the negatively correlated polarization between the EPR signals of the phenoxyl and cyclohexadienyl radicals observed in the FT-EPR study, which has been interpreted in terms of a bimolecular H• extraction process.[23] As noted above, related redox reactions involving phenolic compounds have been reported by Das and co-workers.[31]

As noted above, the rate constant given by eq 1 neglects the pH-dependent effect of the oxidation reaction described by Scheme 2. However, the observation of deuterated tyrosine requires both the formation of the radical intermediate as well as the regeneration of the tyrosine. Interestingly, the results for OMT may provide a more unequivocal interpretation of the relative importance of the contributions of H• and e_{aq}^-/H^+ reaction pathways for the synthesis of the cyclohexadienyl radical. For the proposed oxidation of the OMT cyclohexadienyl radical, no deprotonation step is involved (Scheme 3). Hence, the fact that the H/D exchange rate at pH 11 is much greater than the rate at pH 7 indicates that formation of the cyclohexadienyl radical is much more rapid at the higher pH. This observation unambiguously favors the e_{aq}^-/H^+ reaction pathway over the H• pathway, due to the correlation of e_{aq}^- with pH.

Although, as discussed above, the H/D exchange data generally do not support the importance of a bimolecular H• transfer reaction, a bimolecular electron-transfer reaction might form the initial radical anion, and in fact, the entire process could, in principle, involve a pair of tyrosine molecules, as illustrated in Scheme 5.

Scheme 5



Although the possible significance of the oxidation of the tyrosine anion radical by the phenoxyl radical is unclear, this reaction would lead to a strong inhibitory effect of tyrosine concentration, as is observed in these studies (Figure 8). However, the bimolecular complex illustrated above would be strongly destabilized above pH $\sim$ 11, at which the tyrosine is dianionic and the tyrosine radical anion (for which the pK values are unknown) could be trianionic. Hence, there appears at this point to be no compelling basis to invoke such a complex. In this context, we note that Stevenson and co-workers have observed that the aromatic hydrogens of adjacent, stacked fluorene molecules can exchange if activated via electron addition.[38] This process might have some common features with the mechanism of photochemical H/D exchange in tyrosine described here.

The apparent ease with which the tyrosine radical anion can be generated suggests that it may play a more general biochemical role. The ability of a protonation step to trap the anion radical species would appear to have interesting implications for electron-transfer reactions in proteins.

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