JOURNAL

OF THE AMERICAN CHEMICAL SOCIETY

VOLUME 107, NUMBER 12 JUNE 12, 1985

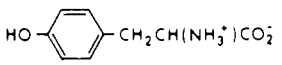
The Electron Spin Resonance Spectrum of the Tyrosyl Radical

Roger C. Sealy,*‡ Laura Harman,† Paul R. West,† and Ronald P. Mason†

Contribution from the National Biomedical ESR Center, Department of Radiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226, and the Laboratory of Molecular Biophysics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709. *Received July 6, 1984*

Abstract: The electron spin resonance spectrum of the tyrosyl radical in aqueous solution at pH 9.5 has been studied over a range of temperatures. Above 60 °C the high-temperature limit for all rotational processes is achieved, allowing complete characterization of the tyrosyl radical spectrum for the first time. The magnetic inequivalence expected for the diastereotopic methylene protons adjacent to the amino acid moiety has been observed. At room temperature selective broadening of half the lines is ascribed to restricted rotation about the $ArCH_2$–$CHNH_2CO_2^-$ bond. It is concluded that the spin on the aromatic ring reflects conformational changes in the side chain, even though rotation next to the ring remains above the fast limit. The conformation of the tyrosyl radical has been compared with those of related radicals: Dopa semiquinones and immobilized tyrosyl radicals in ribonucleotide reductase. It is suggested that charge repulsion effects are important in determining the conformation of Dopa semiquinones.

The tyrosyl radical and related species are of considerable importance in biochemistry. Tyrosine (**1**) is involved in many protein oxidations, being one of the most easily oxidized amino acids. Oxidation to the tyrosyl radical can occur by photolysis[1]

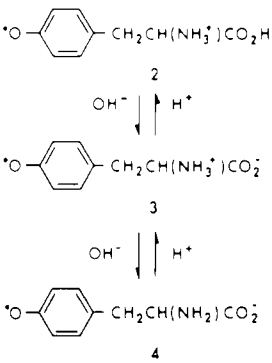


1

and one-electron oxidations by metal ions,[2] oxidizing radicals,[3,4] and oxidizing enzymes.[5] Migration of oxidation from a primary oxidation site to a tyrosyl residue has been detected in peptides and proteins.[6] Molecular products of tyrosine oxidation include dityrosyl units (from combination of tyrosyl radicals), which are involved in protein cross-linking,[5] and dopaquinone units, which have been shown to be formed in some peptide systems.[4] Apart from its involvement in the oxidation of proteins, a tyrosyl radical is believed[7] to play an important role in the action of ribonucleotide reductase from *E. Coli*.

Electron spin resonance (ESR) spectra of radicals from the oxidation of tyrosine have been obtained in fluid[2,8] and frozen[9] solutions, in single crystals,[10,11] and in proteins.[12] One-electron oxidation is expected to give the tyrosyl radical, possibly together with other oxidation products.[11] The tyrosyl radical can exist in three forms (**2–4**) that differ with respect to the protonation state of the amino acid side chain. For tyrosine itself, pK_as of the carboxyl and amino groups are 2.2 and 9.1, respectively.[13] Corresponding pK_as for the tyrosyl radical side chain are not known, but it seems unlikely that they differ markedly from those of the parent compound.

In fluid solution, ESR spectra attributed to the tyrosyl radical have been observed by Borg and Elmore[2] and by Tomkiewicz et al.[8] The data reported from these studies are given in Table I. Borg and Elmore[2] obtained spectra from the chemical oxidation



2 3 4

‡Medical College of Wisconsin.
†National Institute of Environmental Health Sciences.

(1) J. F. Baugher and L. I. Grossweiner, *Photochem. Photobiol.*, **28**, 175–184 (1978).
(2) D. C. Borg and J. J. Elmore, Jr., In "Magnetic Resonance in Biological Systems", Pergamon Press, Oxford, pp 341–349, 1967.
(3) W. A. Prütz, F. Siebert, J. Butler, E. J. Land, A. Menez, and T. Montenay-Garestier, *Biochim. Biophys. Acta*, **705**, 139–149 (1982).
(4) S. Dukler, M. Wilchek, and D. Lavie, *Tetrahedron*, **27**, 607–614 (1971).
(5) P. Tressel and D. J. Kosman, *Biochim. Biophys. Res. Commun.*, **92**, 781–786 (1980).
(6) J. Butler, E. J. Land, W. A. Prütz, and A. J. Swallow, *Biochim. Biophys. Acta*, **705**, 150–162 (1982).
(7) L. Thelander and P. Reichard, *Annu. Rev. Biochem.*, **48**, 133–158 (1979).
(8) M. Tomkiewicz, R. D. McAlpine, and M. Cocivera, *Can. J. Chem.*, **50**, 3849–3856 (1972).
(9) N. K. King, F. D. Looney, and M. E. Winfield, *Biochim. Biophys. Acta*, **88**, 235–236 (1964).
(10) E. L. Fasanella and W. Gordy, *Proc. Natl. Acad. Sci. U.S.A.*, **62**, 299–304 (1969).

0002-7863/85/1507-3401$01.50/0

Table I. Electron Spin Resonance Data Reported for the Tyrosyl Radical in Solution

radical[b]	t, °C	hyperfine splittings,[a] G $a^H_{2,6}$	$a^H_{3,5}$	$a^H_{\beta_1}$	$a^H_{\beta_2}$	a^H_{γ}	g[c]	ref
2	23	6.6	1.8	7.8	7.8			2
4	23	6.6	1.6	15.70[d]	0[d]			2
4	RT	6.15	1.5	7.7[e]	7.7[e]	0.4	2.0041	8
4	5.5	6.25	1.50	14.80[f]		0.35	2.0046	this work
4	23	6.20	1.50	15.05[f]		0.35	2.0046	this work
4	60	6.18	1.50	7.13	8.53	0.38	2.0046	this work

[a] Estimated accurate to ±0.05 G based on simulation. [b] For structure, see text. [c] ±0.0001. [d] Values assumed based on the observation of an apparent doublet splitting of 15.7 G. [e] Values assumed for identical β-protons based on an apparent doublet splitting of 15.4 G. [f] Sum of inequivalent β-protons.

of tyrosine at extremes of pH. In acidic solution (where the radical is presumably in protonation state **2**), splittings of 6.6 (2 H), 1.8 (2 H), and 7.8 (2 H) G were reported and assigned to protons at ortho, meta, and side chain methylene positions, respectively. In alkaline solution (where tyrosyl radical **4** should predominate), data for ring protons were similar to those obtained at low pH, but a triplet splitting from the methylene protons was not observed. Instead an apparent doublet of magnitude 15.7 G was observed, which was assigned to one of the methylene protons, with the suggestion that the splitting from the other was too small to measure (i.e., ≈0 G). It was pointed out that a large difference between the hyperfine splittings of the methylene protons could occur as a result of restricted rotation.

In later work Tomkiewicz et al.[8] obtained similar spectra from the UV photolysis of tyrosine at pH 11. In addition to the hyperfine lines detected by Borg and Elmore, they resolved a small splitting of 0.4 G attributable to the γ proton in the side chain. However, they interpreted the apparent coupling of 15.4 G differently, suggesting that it in fact reflected the sum of 7.7 G splittings from two *equivalent* methylene protons, with the center lines of the triplet pattern broadened beyond detection because of restricted rotation.

While the assignments of the small splittings to aromatic protons are consistent with data for other aryloxyl radicals,[14] the issue of the hyperfine splittings associated with the methylene protons in the side chain has not been resolved. However, a factor not previously considered in this system is the expected magnetic inequivalence of the methylene protons, which are adjacent to a chiral center. This environment renders the protons inequivalent *irrespective of* any restricted rotation that may be present.[15-18] The typical spectral feature associated with the methylene protons is then[17] a 1:1:1:1 pattern, even at elevated temperatures. At lower temperatures in the presence of restricted rotation, the two central resonances are selectively broadened. We previously[19] observed this phenomenon in the ESR spectra of semiquinones derived from the related molecule 3,4-dihydroxyphenylalanine (Dopa). The inequivalence resulted in a hyperfine pattern from the methylene protons that consisted of a doublet of doublets rather than a triplet. However, at ambient temperature, the center lines were broadened because of restricted rotation, so that an *apparent* coupling equal to the sum of the non-identical methylene proton couplings was observed. We therefore tested the possibility that a similar situation exists in the spectrum of the tyrosyl radical, since this might account for the previous observations on this species.

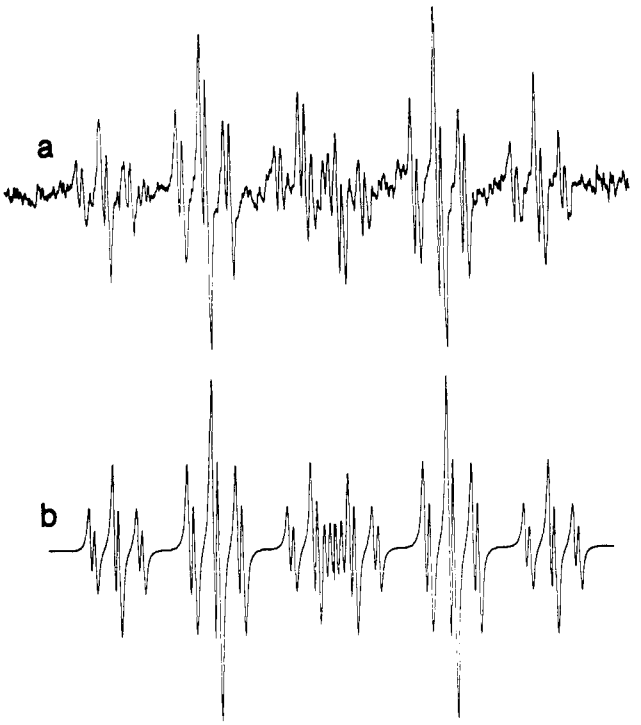


Figure 1. (a) Experimental electron spin resonance spectrum of the tyrosyl radical at 5 °C. Other conditions: 10 mM tyrosine + 1 mM $KMnO_4$ (oxidant) in borate buffer, pH 10.5, modulation amplitude 0.17 G, microwave power 5 mW. Flow rate = 200 mL/min. (b) Spectrum simulated by using the parameters given in Table I.

Experimental Section

Tyrosine (Sigma Chemical Co.), 3,4-dihydroxyphenylalanine (Sigma), potassium permanganate, and sodium borate (Fisher) were reagent grade and were used as supplied.

Radicals were generated by chemical oxidation of tyrosine by potassium permanganate in a rapid-flow system. Solutions of tyrosine (10 mM) and permanganate (1 mM) in borate buffer (pH 9.5–10.5) were mixed just before entry into a quartz TM flat cell (Scanlon) contained within the ESR cavity. The total flow rate was 200 mL/min. Oxidation of 3,4-dihydroxyphenylalanine (2 mM) at pH 10.5 used 0.25 mM $KMnO_4$.

Solutions were preheated or cooled by passage through a 10 ft glass coil immersed in a constant temperature water bath. Temperatures were recorded by a platinum resistance thermometer, the probe of which was inserted into the mixing cell as far as the head of the flat portion.

ESR spectra were obtained with a Varian E-109 spectrometer operating at X-band (9.5 GHz) with a TM cavity. A 100-kHz field modulation was employed. The field scan was calibrated with Fremy's salt. Hyperfine splittings were obtained from spectral simulations and are believed accurate to better than ±0.05 G. In variable temperature studies relative values of a^H_β were optimized to 0.025 G intervals. Fremy's salt (g = 2.0055)[14] also was used as a reference to determine (to ±0.0001) the g value of the tyrosyl radical.

(11) H. C. Box, E. E. Budzinski, and H. G. Freund, *J. Chem. Phys.*, **61**, 2222–2226 (1974).
(12) B.-M. Sjöberg, P. Reichard, A. Gräslund, and A. Ehrenberg, *J. Biol. Chem.*, **253**, 6863–6865 (1978).
(13) J. A. Dean, Ed., "Lange's Handbook of Chemistry", 12th ed., McGraw-Hill, New York, p 5–41, 1979.
(14) H. Fischer and K.-H. Hellwege, Ed., "Landholt-Boernstein New Series", Group II, Vol. 9, Springer-Verlap, Berlin, 1977–1979.
(15) R. W. Kreilick, J. Becher, and E. F. Ullman, *J. Am. Chem. Soc.*, **91**, 5121–5124 (1969).
(16) R. J. Weinkam and E. C. Jorgensen, *J. Am. Chem. Soc.*, **93**, 7028–7033 (1971).
(17) B. C. Gilbert, J. P. Larkin, and R. O. C. Norman, *J. Chem. Soc., Perkin Trans. 2*, 1272–1279 (1972).
(18) G. P. Laroff, R. W. Fessenden, and R. H. Schuler, *J. Am. Chem. Soc.*, **94**, 9062–9073 (1972).
(19) C. C. Felix and R. C. Sealy, *J. Am. Chem. Soc.*, **103**, 2831–2836 (1981).

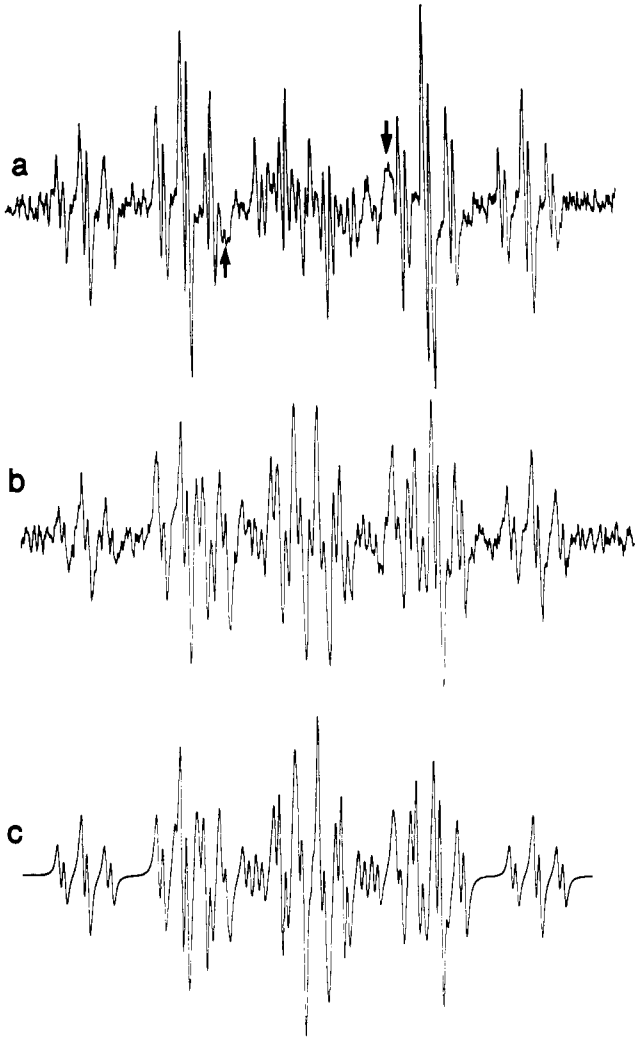
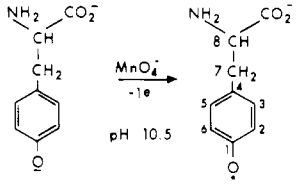


Figure 2. (a and b) Experimental electron spin resonance spectra of the tyrosyl radical at (a) 23 °C and (b) 61 °C. Other conditions were the same as in the legend to Figure 1. (c) Spectrum simulated by using the parameters given in Table I.

Results and Discussion

Temperature Dependence of the Tyrosyl Radical. Figure 1a shows the ESR spectrum obtained from permanganate oxidation of tyrosine at 5.5 °C and pH 10.5 under rapid-flow conditions. Reaction presumably involves direct one-electron oxidation of the tyrosine dianion (reaction 1). A total of 36 spectral lines are clearly visible, and the spectrum can be simulated (Figure 1b) with hyperfine splittings to ring protons of 6.25 and 1.5 G, a γ-splitting of 0.35 G, and an apparent splitting to a single β-hydrogen of 14.80 G. The g value is 2.0046, consistent with values for other aryloxyl radicals in aqueous solutions[14] although higher than that (2.0041) previously reported for this radical.[8]



(1)

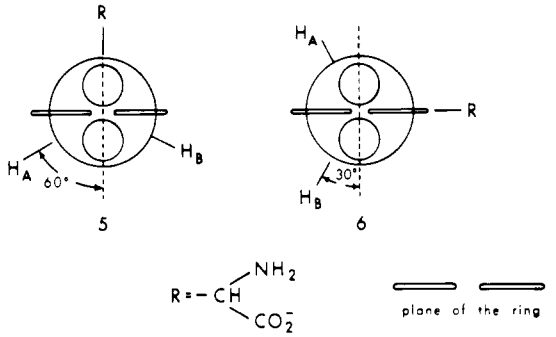
As the temperature is increased additional spectral features appear. At ambient temperature (23 °C) these features are most apparent in the center of the spectrum and adjacent to the most intense triplets of doublets (Figure 2a, arrows). The overall β-splitting is increased to 15.05 G. Increasing the temperature still further (Figure 2b) leads to a quite dramatic change in the spectrum, in which all the additional hyperfine structure associated with two inequivalent methylene protons is now observed. At 60 °C an excellent simulation is obtained with hyperfine splittings to ring protons of 6.18 and 1.50 G, a γ-splitting of 0.38 G, and splittings to *non-identical* methylene protons of 7.13 and 8.53 G. At this temperature there is little, if any, selective broadening of the 36 hyperfine lines that were unobservable at 5.5 °C. Evidently rotation is now sufficiently rapid that the effects of restricted rotation are no longer apparent on the ESR time scale.

We conclude from these experiments that the methylene protons are intrinsically inequivalent and that the complexity of the spectra is due to a combination of this phenomenon and hindered rotation about single bonds in the radical. The effects of restricted rotation become more apparent as the temperature is lowered.

Temperature-dependent line-broadening effects have been shown to occur in many π-radicals, including nitro radical anions,[20] semiquinones,[21,22] and phenoxyl radicals.[23] These effects result from a restricted rotation in the radical which modulates one or more of the hyperfine splittings at a frequency related to the change in the hyperfine splitting expressed in frequency units.

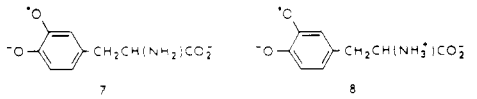
In the tyrosyl radical spectra, it is the $m_I = 0$ (central) lines of doublets due to the diastereotopic β-hydrogens at C-7 that are broadened. Therefore, conformational analysis must focus on factors that affect the hyperfine splittings of these methylene hydrogens.

Hyperfine coupling to β-hydrogens is usually related to the spin density at the α-carbon and to the dihedral angle between the C–H bonds and the 2p orbital through an expression of the form $a_\beta = B\cos^2\theta$. The existence of potential energy minima for certain values of θ is inferred from the temperature-dependent average (fast limit) hyperfine couplings. In organic radicals two limiting conformations, **5** and **6**, have been identified. For $\theta = 60°$ (**5**),



a_β^H has the low value of $0.25B$. For $\theta = 30°$ (**6**), a_β^H increases to $0.75B$ and higher couplings are observed than for $\theta = 60°$. If rotation is unrestricted, i.e., if the substituent is a methyl group, $a_\beta^H = 0.5B$. For the 4-methylphenoxyl radical in aqueous solution, $a_{CH_3}^H$ is 12.5 G,[24] giving a value for B of 25 G. In tyrosyl radical **4**, taking $a_\beta^H = 7.5$ G from the room temperature data where $a_\beta^H(1) + a_\beta^H(2) = 15.05$ G gives $a_\beta^H \simeq 0.30B$. We conclude that the dihedral angle is near 60° and that the most favored conformation resembles **5**.

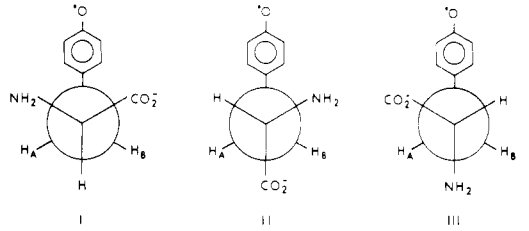
These results can be compared with those for radicals of related structure, Dopa semiquinones **7** and **8**. At high pH, where the ionization state of the side chain in the semiquinone is identical with that in **4**, the mean hyperfine splitting for the two methylene hydrogens is $0.28B$.[19] At neutral pH, where the amino group is protonated (i.e., as in **8**), the value is decreased slightly to $0.27B$.[19] Again, in each case the implication is that the mean dihedral angle made by the methylene hydrogens is close to 60°.



(20) E. W. Stone and A. H. Maki, *J. Chem. Phys.*, **27**, 1326–1333 (1962).
(21) J. Pilař, *J. Phys. Chem.*, **74**, 4029–4037 (1970).
(22) C. C. Felix and R. C. Sealy, *Photochem. Photobiol.*, **34**, 423–429 (1981).
(23) T. J. Stone and W. A. Waters, *J. Chem. Soc.*, 213–218 (1964).
(24) W. T. Dixon, M. Moghimi, and D. Murphy, *J. Chem. Soc., Faraday Trans. 2*, **70**, 1713–1720 (1974).

A mean dihedral angle of 60° has been found in many systems involving magnetically equivalent methylene protons.[20-23] However, whereas the dihedral angles for magnetically equivalent methylene protons are *equal*, i.e., there is a symmetrical distribution relative to the plane of the unpaired electron, the mean angle of the diastereotopic methylene protons in the tyrosyl radical and Dopa semiquinones is slightly removed from the symmetrical position[25] as revealed by two distinct a_β^H values.

The origin of the displacement can be understood by first considering the possible orientations of the chiral center of L-tyrosine or L-Dopa generated by rotation about C_7–C_8, i.e., the C_β–C_γ bond relative to the para ring position as C_α. The staggered rotamers (I–III) are shown below: I is expected to be destabilized relative to II and III because of the more bulky amine and carboxylate substituents adjacent to the aromatic moiety. The

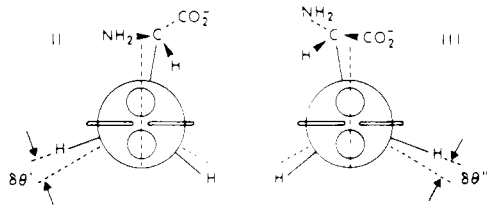


average fast limit values of the two β hyperfine splittings (a^{H_A}, a^{H_B}) are approximated by eq 2 and 3, where C_I, C_{II}, and C_{III} are

$$a^{H_A} = C_I a^{H_A}(H,NH_2) + C_{II} a^{H_A}(CO_2^-,H) + C_{III} a^{H_A}(NH_2,CO_2^-) \quad (2)$$

$$a^{H_B} = C_I a^{H_B}(H,CO_2^-) + C_{II} a^{H_B}(CO_2^-,NH_2) + C_{III} a^{H_B}(NH_2,H) \quad (3)$$

the relative contributions of each conformer and the conformers are labeled by the nearest neighbors of H_A and H_B, respectively. The more stable conformers II and III can be viewed from the same perspective as **5**, i.e., along C-4, C-7, as shown below:



Different values of H_A and H_B are observed because $|\delta\theta'| \neq |\delta\theta''|$. If one assumes that the ratio of the methylene proton hyperfine couplings is given by $\cos^2\theta/\cos^2(\theta + 120°)$, the displacement of the mean dihedral angle from the symmetrical position is about 1.4° for tyrosyl radical **4**, 2.5° for Dopa semiquinone **7**, and 4.6° for Dopa semiquinone **8**.

When the temperature is lowered, restricted rotation around the two C–C single bonds in radical **4** will be observed in turn, depending on their relative rotational energy barriers. For rotation about "ethane-like" C_{sp^3}–C_{sp^3} bonds (e.g., C_7–C_8), barriers are on the order of 30–40 kJ/mol, whereas the C_{sp^2}–C_{sp^3} bond to the ring, C_4–C_7, is much less hindered, with a barrier of 4–10 kJ/mol. Thus, in the spectra of para-substituted phenoxyl radicals, alternating line widths, originating from restricted rotation at the ring, typically occur only at low temperatures (≈220 K).[26] Also, such restriction is characterized by magnetically inequivalent *o*-hydrogens, resulting in a broadening of the center line of their triplet (26).

We therefore conclude that it is the rotation about C-7,C-8 that is restricted in the 278–334 K range and which gives rise to the observed line broadening, Figure 1a. The available "frozen limit"

(25) H. B. Stegmann, H. U. Bergler, and K. Scheffler, *Angew. Chem., Int. Ed. Engl.*, **20**, 389–390 (1981).
(26) W. J. Van den Hoek, W. G. B. Huysmans, and M. J. C. van Gemert, *J. Magn. Reson.*, **3**, 137–145 (1970).

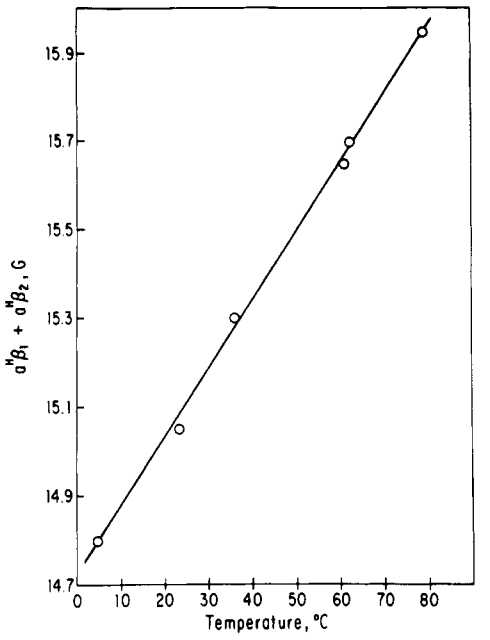


Figure 3. Temperature dependence of the sum of the *p*-methylene hydrogen hyperfine splittings, $a^{H_A} + a^{H_B}$, in tyrosyl radical **4**.

(low temperature) conformations will be I–III, each with a discrete set of β-hyperfine parameters. With coalescence at 5 °C, these spectra are unobtainable in aqueous solutions. However, in principle, one or more frozen conformations may be observable, with their relative intensities dependent on their relative stability. With regard to rotation about C_4–C_7, it should be noted that the sum $a^{H_A} + a^{H_B}$ increases linearly with temperature (Figure 3). The positive temperature dependence ($\partial a_\beta/\partial T = +9.0$ mG/K) is consistent with $\theta = 60°$ conformations, as a_β^H increases from $0.25B$ toward $0.5B$.

In the accessible high-temperature range (60–80 °C) where $\Delta a_\beta^H = a^{H_A} - a^{H_B}$ can be accurately measured, it remains constant at 1.40 G. Our model, in which II and III are principal energetically comparable conformations, is consistent with this observation.

pH Dependence. With use of the permanganate generating system, it was difficult to obtain ESR spectra below pH 9.5 with sufficient signal-to-noise for a detailed extended temperature study of the expected zwitterionic tyrosyl radical **3**. At pH 9.5, measured ESR parameters of **4** were the same as those obtained at pH 10.5, implying that the pK_a for the amino group in the tyrosyl radical is probably less than 9.0. Changes in temperature over the pH range investigated similarly did not reveal changes suggestive of shifts in acid–base equilibria.

It is interesting to note that the spectrum obtained by Borg and Elmore[2] under acid conditions does not show the resolved inequivalence of the methylene protons that was observed at high pH. While this is likely, in part, to be due to the low resolution of their spectra, it may also partly reflect different conformational preferences in radical **2** which possesses a side chain that is doubly protonated relative to **4**. Thus the difference between the methylene splittings could be less than 1.4 G in **2**.

Temperature Dependence of Dopa Semiquinone. Our observations on tyrosyl radical **4** prompted us to examine the temperature dependence of the ESR spectrum of the analogous Dopa semiquinone **7** generated at pH 10.5. In this case individual a_β^H splittings can be examined over a wider temperature interval. Splittings (Figure 4) were determined by computer simulation of 10 G scans at each temperature (including provision for broadening of the $m_I = 0$ components of the β-H doublets at lower temperatures). The spectra were recorded during flow, employing low permanganate and Dopa concentrations. Figure 5 shows experimental spectra at 36 and 87 °C.

Interestingly, while the sum ($a^{H_A} + a^{H_B}$) still increases smoothly, it reflects an increase in only one of the splittings, i.e., both a_β^H and Δa_β^H change with temperature. This suggests that the in-

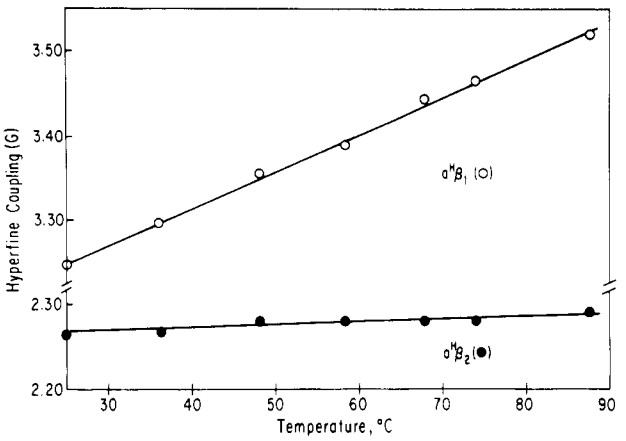
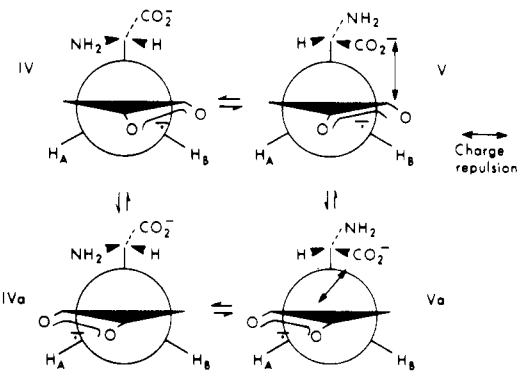


Figure 4. Temperature dependence of the *p*-methylene hydrogen hyperfine splittings a^{H_A} and a^{H_B} in Dopa semiquinone **7**.

Scheme I



teraction of the two rotations in Dopa semiquinone is more complex than that in the tyrosyl system, consistent with the presence of an asymmetric negatively charged ring in the semiquinone (Scheme I).

In this case each of the $\theta = 60°$ conformations has two potential orientations of the *o*-semiquinoid ring. Charge repulsion in V and Va will play a role in (i) destabilizing V and Va relative to both IV and IVa, (ii) favoring Va over V, and (iii) raising a_β^H for H_B relative to H_A in Va. These factors could serve to enhance a_β^H for H_B in *all* populated conformations.

Plancherel and von Zelewsky[27] have calculated rates of exchange between two extreme conformations of the methylene protons in Dopa semiquinone **8**. Lacking suitable low-temperature limits for tyrosyl **4** and for Dopa semiquinone **7**, we have not carried out an analogous calculation. However, it is clear from Figures 2 and 5 that the nature of the line broadening for these species at ambient temperature is similar to that for zwitterionic Dopa semiquinone **8**. Thus it seems likely that exchange rates and activation energies are of the same order of magnitude as those estimated for **8**, i.e., ca. 10^8 s^{-1} at 30 °C and 37 kJ mol^{-1}, respectively.[27]

Line broadening in both the tyrosyl radical and Dopa semiquinones is thus attributed to a modulation of the β-splitting by C-7,C-8 rotation. However, both a_β^H and $\sum a_\beta^H$ *can* vary with temperature as observed in the semiquinone. Therefore, the use in calculations of a_β^H values derived from a fixed (crystal) orientation of the phenoxyl ring (i.e., a frozen limit of C-4,C-7 rotation) requires further extrapolation before application to the higher temperature C-7,C-8 rotation. Moreover, the local environment of the aromatic ring in the crystal may influence the conformational equilibrium, as is the case in frozen enzymatic systems (below).

Tyrosyl Radicals in Ribonucleotide Reductase Enzymes. The tyrosyl radical at the active site of ribonucleotide reductase has

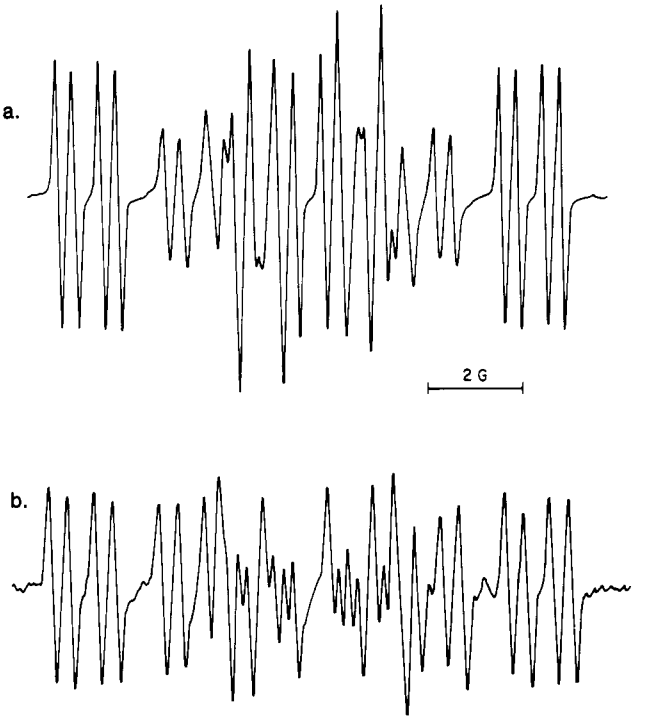
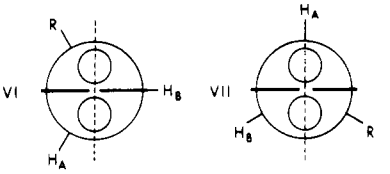


Figure 5. Experimental electron spin resonance spectra of the Dopa semiquinone **7** at (a) 36 °C and (b) 87 °C. Other conditions: 2 mM 3,4-dihydroxyphenylalanine + 0.25 mM $KMnO_4$ (oxidant) in borate buffer, pH 10.5, modulation amplitude 0.17 G, microwave power 1 mW.

one unusually high a_β^H value: 18.8 G for *E. coli* and 18.2 G for *E. coli* induced by bacteriophage T4.[12,28] On the basis of a *B* value of 23.72 G estimated from data for 4-methylphenoxyl in a hydrophobic environment (benzene solution[29]), we would calculate the contributing single hydrogen, H_A, to lie at 27.1° and 28.84° of dihedral angle, respectively, in the two enzymes, conformation VI. It follows that the dihedral angle of the second



hydrogen, H_B, should be near 90° in both these cases, with $|\theta| = 92.9°$ for *E. coli* and 92.1° for T4-induced enzyme. The smaller β-hydrogen coupling would then be much less than 1 G and too small to resolve on the basis of conformation VI and the solution *B* values, as originally suggested for the *E. coli* system.[12] With $B = 25$ G (the value for a phenoxyl radical in aqueous solution; see above), the smaller splitting would be higher but still less than 1 G.

Gräsland, Sjöberg, and co-workers have observed a second, smaller splitting (7.5 G) in the spectrum from the bacteriophage T4 induced enzyme.[28] However, their analysis, in this case based on conformation VII, requires a value of *B* (18.8 G) much lower than is normally encountered with 4-alkylphenoxyl radicals in order to accommodate the near zero dihedral angle required for H_A.

Both conformations VI and VII are expected to be energetically unfavorable for the tyrosyl radical per se. It is likely that protein tertiary structure constrains the phenyl ring to one or more fixed plane orientations relative to the peptide backbone. While such conformations are not required when the tyrosyl radical is free in solution, values of *B* are determined by the spin density distributions and ordinarily apply in all cases. However, it may be

(27) D. Plancherel and A. von Zelewsky, *Helv. Chim. Acta*, **65**, 1929–1940 (1982).

(28) M. Sahlin, A. Gräsland, A. Ehrenberg, and B.-M. Sjöberg, *J. Biol. Chem.*, **257**, 366–369 (1982).

(29) S. A. Weiner, *J. Am. Chem. Soc.*, **94**, 581–584 (1972).

possible that the iron center present in the enzyme decreases B to 18.8 G.

Conclusions

The effect of a chiral carbon center in rendering adjacent methylene protons inequivalent is well-known for nitroxides, but few examples have been reported in other radical systems. The effect has now also been observed in the spectrum of the tyrosyl radical. One would anticipate that examples of this phenomenon should be apparent in the ESR spectra of many other radicals, in particular those from other amino acids and their derivatives. For example, sulfinyl radicals derived from several thiols[30,31] show inequivalent methylene protons.[32] Presumably ESR spectra of radicals from tyrosyl esters and peptides[2] show behavior similar to that found for tyrosyl, i.e., reflecting the effects of the chiral center and restricted rotation.

Acknowledgment. This work was supported by NIH Grant GM-29035. We thank Drs. P. Smith and K. K. Karkustis (Duke University) for carrying out preliminary ESR experiments on the flow oxidation of tyrosine and Dr. V. Fischer for helpful comments regarding the conformational analysis.

(30) J. C. Kertesz, W. Wolf, and H. Hayase, *J. Magn. Reson.*, **10**, 22-23 (1973).

(31) J. C. Kertesz and W. Wolf, *Intra-Sci. Chem. Rep.*, **5**, 371-374 (1971).

(32) B. C. Gilbert, H. A. H. Laue, R. O. C. Norman, and R. C. Sealy, *J. Chem. Soc., Perkin Trans. 2*, 892-900 (1975).

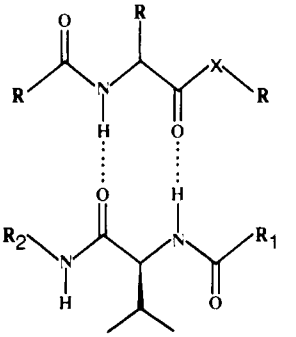
Extended Scope of Chiral Recognition Applying Hydrogen Bond Associations in Nonaqueous Media: (*R*,*R*)-*N*,*N*′-Diisopropyltartramide (DIPTA) as a Widely Applicable Resolving Agent

Yasuo Dobashi and Shoji Hara*

Contribution from the Tokyo College of Pharmacy, Horinouchi, Hachioji, Tokyo 192-03, Japan.
Received December 6, 1984

Abstract: The addition of (*R*,*R*)-*N*,*N*′-diisopropyltartramide (DIPTA) to the nonaqueous mobile-phase liquid of a silica gel column made possible the chiral recognition of a wide range of enantiomers containing α- or β-hydroxycarboxylic acid, β-hydroxy ketone, β-amino alcohol, α-amino acid, α-hydroxy ketoxime, 1,2-diol derivatives, and bi-β-naphthol. The enantioselections observed here were based on diastereomeric associations between the chiral additive and enantiomer to be resolved in the column. These associations were ascribed to the hydrogen bonds of the additive with the enantiomer. The steric environment of chiral centers and hydrogen bond sites of the solute enantiomers were found to influence the degree of enantioselectivity. For acyclic 1,2-diols, the dependence of the separation factors on the steric environment of the hydrogen bond sites is discussed on the basis of preferential conformations.

Recent investigations on chiral recognition by chromatography clearly demonstrate this technique to hold promise for the expeditious determination of optical purity, prediction of absolute, configuration, and preparative-scale resolution of enantiomers.[1] We recently reported that enantiomers of α-amino acid derivatives (I) could be readily resolved on a silica gel surface by using the L-valine derivative IIa[2] as a chiral additive to a nonaqueous mobile-phase liquid as well as on chiral silica gel surface IIb[3] to which L-valine diamide was covalently bound. In such a case, the diastereomeric associations responsible for the observed enantioselectivities were attributed to dual NH···O=C hydrogen bonds of the resolving agents with solutes. Our observations proved that the association mode applying dual hydrogen bonds as the maximum number of bonding interactions can elicit a considerable degree of enantioselectivity, but the application of these systems to chiral recognition is limited to the α-amino acid family.



(I) X=O, NH

(II-a) R_1 = CH_3, R_2 = tert-Bu

(II-b) R_1 = H, R_2 = $(CH_2)_3Si$≡Si

However, functionalities which can serve as hydrogen bond sites are fairly common among many kinds of enantiomers of interest in the fields of synthetic and biological chemistry. Therefore, attempts to extend the scope of application of such association mode to chiral recognition through development of other types of resolving agents are deemed worthwhile. In this paper, it is shown that the scope of chiral recognition through the application of hydrogen bond associations in nonaqueous media can be extended to a wide range of enantiomers by using (*R*,*R*)-*N*,*N*′-di-

(1) Schurig, V. In "Asymmetric Synthesis"; Morrison, J. D., Ed.; Academic Press: New York, 1983, Vol. 1, p 59. Pirkle, W. H.; Finn, J. In "Asymmetric Synthesis"; Morrison, J. D., Ed.; Academic Press: New York, 1983, Vol. 1, p 87 and reference cited therein.

(2) Dobashi, A.; Hara, S. *Tetrahedron Lett.* **1983**, *24*, 1509. Dobashi, A.; Hara, S. *J. Chromatogr.* **1983**, *267*, 11. Dobashi, A.; Hara, S. *Anal. Chem.* **1983**, *55*, 1805.

(3) Hara, S.; Dobashi, A. *J. Chromatog.* **1979**, *186*, 543. Dobashi, A.; Oka, K.; Hara, S. *J. Am. Chem. Soc.* **1980**, *102*, 7122.

0002-7863/85/1507-3406$01.50/0