Published in final edited form as:

J Phys Chem B. 2007 June 14; 111(23): 6586–6592.

ESEEM Studies of Peptide Nitrogen Hyperfine Coupling in Tyrosyl Radicals and Model Peptides

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Abstract

Tyrosyl radicals are important in long-range electron transfer in several enzymes, but the protein environmental factors that control midpoint potential and electron transfer rate are not well understood. To develop a more detailed understanding of the effect of protein sequence, we have performed ¹⁴N and ¹⁵N electron spin echo envelope modulation (ESEEM) measurements on tyrosyl radical, generated either in polycrystalline tyrosinate or in its ¹⁵N labeled isotopomer, by UV photolysis. ¹⁴N ESEEM was also performed on tyrosyl radical generated in tyrosine-containing pentapeptide samples. Simulation of the ¹⁴N and ¹⁵N tyrosyl radical ESEEM measurements yielded no significant isotropic hyperfine splitting to the amine or amide nitrogen; the amplitude of the anisotropic, nitrogen hyperfine coupling (0.21 MHz) was consistent with a dipole-dipole distance of 3.0 Å. Density functional theory was used to calculate the isotropic and anisotropic hyperfine couplings to the amino nitrogen in four different tyrosyl radical conformers. Comparison with the simulated data suggested that the lowest energy radical conformer, generated in tyrosine at pH 11, has a 76° C_α-C_β-C_{1'}-C_{2'} ring and a -73° C-C_α-C_β-C_{1'} backbone dihedral angle. In addition, the magnitude, orientation, and asymmetry of the nuclear quadrupole coupling tensor were derived from analysis of the tyrosyl radical ¹⁴N ESEEM. The simulations showed differences in the coupling and orientation of the nuclear quadrupole tensor, when the tyrosinate and pentapeptide samples were compared. These results suggest sequence- or conformation-induced changes in the ionic character of the NH bond in different tyrosine-containing peptides.

Keywords

electron transfer; redox-active amino acid; EPR; peptide bond; UV-photolysis; tyrosine

INTRODUCTION

Redox-active tyrosine residues play an important role in enzymatic catalysis in several proteins, including prostaglandin H synthase,¹ galactose oxidase,² ribonucleotide reductase (RNR),³ and photosystem II (PS II).^{4,5} Tyrosine residues may function as electron transfer intermediates or may catalyze proton-coupled electron transfer reactions, due to oxidation-

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induced changes in the pK_A of the phenol oxygen.⁶ The conformational freedom of the tyrosine side chain has been proposed to be a key structural element in regulating these different functions.^{7,8}

Previous model compound studies have described the spectroscopic properties of tyrosyl and phenoxyl radical (see, for example, refs^{9–17}). EPR (electron paramagnetic resonance) studies have shown that tyrosyl radicals *in vivo* and *in vitro* have an odd-alternate spin system, with significant hyperfine splittings to the 3' and 5' ring protons. A $C_1'-C_\beta$ conformation-sensitive coupling to the beta-methylene protons is also observed.^{14,15,18,19}

Recent studies have shown that tyrosine and tyrosyl radical vibrational frequencies are sensitive both to the conformation of the phenol ring with respect to the β -methylene carbon of the tyrosine side chain ($C_\alpha-C_\beta-C_1'-C_2'$ dihedral angle) and to the conformation of side chain with respect to the α -carbon of the protein backbone ($C-C_\alpha-C_\beta-C_1'$).^{20–22} Electronic structure calculations and normal mode analysis have permitted a more detailed understanding of the relationship between vibrational frequencies and changes in tyrosine conformation and redox state.¹⁷

EPR methods provide a means to sample the phenol ring conformation because the cw-EPR lineshape is sensitive to the projection that the β -methylene protons make onto the axis of the ring C_1 p^π orbital.²³ The distribution of conformers has been measured for model tyrosine in glasses and for the redox-active tyrosines of Photosystem II, Y_Z and Y_D , using the cw-EPR spectrum¹⁸ or by deuteration of the β -methylene protons and subsequent simulation of their 2H -ESEEM intensities.^{19,24} Electronic structure calculations have predicted conformation(s) for the phenol ring with respect to the β -methylene carbon in agreement with those determined by EPR and ESEEM measurements.¹⁷

In contrast to phenol ring orientation with respect to the β -methylene group, the cw-EPR spectrum is not sensitive to the conformation of tyrosyl radical side chain with respect to the protein backbone, i.e. to the $C-C_\alpha-C_\beta-C_1'$ dihedral angle. However, one interpretation of previous theoretical work¹⁷ is that a small amount of unpaired electron spin density may delocalize onto the tyrosine amino group. In this interpretation, observed changes in the frequencies of N-H vibrational modes with tyrosine oxidation may result from spin delocalization to the amino nitrogen.²⁵

In this paper, we report ^{14}N and ^{15}N -ESEEM studies of model tyrosyl radical aimed at measuring weak hyperfine couplings to the amine nitrogen. ESEEM studies were also done on model pentapeptides containing a tyrosyl radical to gauge the effects that peptide backbone conformation might have on peptide nitrogen hyperfine coupling.²² EPR provides a direct method for studying tyrosyl radicals in proteins. Therefore, the development of an EPR-based method for extending our capabilities to elucidate this additional information on side chain conformation would provide an important tool for biophysical studies.

MATERIALS AND METHODS

Natural abundance L-Tyrosine and boric acid were purchased from Sigma (St. Louis, MO). The tyrosine-containing pentapeptides, IYPIG, EYPIG, RYPIG, and RSYTH were purchased from Sigma-Genosys (The Woodlands, TX). Pentapeptide samples were purified by the manufacturer on a Discovery Bio Wide Pore C-18 column (mobile phase A, 0.1% TFA/water; mobile phase B, 0.1% TFA/acetonitrile). The purity of each sample was assessed from the 214 nm chromatograms and was reported in the range from 94 to 99% homogeneity. The extinction coefficients of these compounds at 266 nm were similar, with values between 890 and 1000 $M^{-1} cm^{-1}$.²² The sequences of the pentapeptides were chosen to maximize their solubility. The pentapeptide, IYPIG, is the sequence containing the redox-active tyrosine, Y160, in a

cyanobacterial PSII D1 subunit (NCBI entry, NP_68263426). Two other pentapeptides, EYPIG and RYPIG, are variants of the D1 sequence, in which the amino terminal isoleucine has been replaced by a glutamic acid or an arginine. The pentapeptide, RSYTH, is the amino acid sequence containing the redox-active tyrosine, Y122, in the R2 subunit of Class I RNR from *E. coli* (PDB entry, 1MXR_B27). The concentration of tyrosine or model peptide in the samples was 16 mM, and the samples were buffered at pH = 11.0 with 10 mM borate-NaOH. The samples were placed into fused quartz sample tubes and frozen to 85 K under a flow of cooled nitrogen. Tyrosyl radical was generated using the 266 nm output from a Surelite III Nd:YAG laser (Continuum, Santa Clara, CA), as previously described.²²

Cw- and pulsed-EPR data were collected on a Bruker E-680X spectrometer operating at X-band and equipped with a model ER4118X-MD-4-W1 probe that employs a 4 mm dielectric resonator. The sample temperature was maintained at 10 K using an Oxford Instruments liquid helium flow system equipped with a CF-935 cryostat and an ITC-503 temperature controller. ESEEM data were collected using a three-pulse, stimulated echo sequence (90° -t- 90° -T- 90°) with 90° microwave pulse widths of 16 ns (full width half maximum) and peak powers of 250 W. A four-step phase cycling sequence, (+x, +x, +x), (-x, +x, +x), (+x, -x, +x), (-x, -x, +x), together with the appropriate addition and subtraction of the integrated spin-echo intensities served to actively remove the contributions of two-pulse echoes and baseline offsets from the data.²⁶ An integration window of 24 ns was used to acquire spin-echo amplitudes, and data set lengths were 512 points. ESEEM data were tapered with a Hamming window and Fourier transformed.²⁷ ESEEM spectra were obtained by taking the absolute value of the real portion of the transforms.

Powder ESEEM simulations to obtain ^{15}N -, ^{23}Na - and ^{14}N -hyperfine couplings were accomplished by using software written in FORTRAN (Absoft). Calculations used the density matrix formalism of Mims to simulate the time domain ESEEM data.^{28,29} MATLAB scripts were then used to assemble the simulations according to the product rule and to complete the Fourier analysis to obtain simulated ESEEM spectra. The processing and Fourier transformation procedure was identical to that outlined above for the Bruker software. The spherical model approximation to the product rule was used for simulations of ESEEM patterns for multiple nuclei.³⁰

Electronic structure calculations were performed on gas-phase models¹⁷ of tyrosine and tyrosyl radical with Kohn-Sham density-functional theory (DFT) using the hybrid exchange functional of Becke^{31,32} and the Lee, Yang, and Parr correlation functional³³ (B3LYP). All electronic structure calculations were performed with the GAUSSIAN98 and GAUSSIAN03 suites of programs.³⁴ The potential energy surface over the $\text{C}_\alpha\text{-C}_\beta\text{-C}_{1'}\text{-C}_{2'}$ and $\text{C-C}_\alpha\text{-C}_\beta\text{-C}_{1'}$ dihedral angles was scanned using the 6-31G(d) basis set.³⁵ Minima on the B3LYP/6-31G(d) surface were optimized at the B3LYP/6-311++G(d,p) level of theory. The energies of the minima were then refined with single-point calculations at the B3LYP/6-311++G(2df,2p) level of theory. For further details, see ref¹⁷ and references therein. Single point calculations using the B3LYP/EPR-III level of theory were used to calculate hyperfine coupling constants for each atom in each radical conformation.

RESULTS

Cw-EPR spectra of natural abundance L-tyrosine (a), ^{15}N -labeled L-tyrosine (b) and two different pentapeptides, EYPIG (c) and RSYTH (d), are shown in Figure 1. The samples were in borate buffer at pH 11, and tyrosyl radical was generated by UV photolysis. These spectra are typical for a neutral tyrosyl radical and are all centered at $g = 2.005$.¹⁵ The spectra obtained for ^{14}N -tyrosine (Figure 1a) and ^{15}N -tyrosine (Figure 1b) are essentially identical, commensurate with the 0.065 mT upper bound placed on the isotropic hyperfine coupling of

the amino nitrogen by Ayala and coworkers.²⁵ The EPR spectra of EYPIG (Figure 1c) and RSYTH (Figure 1d) differ slightly from each other and from the model tyrosine spectra (Figures 1a,b) reflecting modest differences in phenol ring conformation.

Figure 2a shows the stimulated echo ESEEM spectrum obtained for the ^{15}N -labeled tyrosine radical at 347.0 mT. The ESEEM peaks centered at 1.5 and 3.9 MHz occur at the Larmor frequencies of ^{15}N and ^{23}Na , respectively, and are assigned accordingly. The data of Figure 2a were collected under conditions in which matrix protons also contribute to the modulation function. Overall, the modulation depth of the pattern that gives rise to these data is in the range of 10 – 15%. If one weights the relative contributions of ^{15}N , ^{23}Na and ^1H by the amplitudes of their respective ESEEM peaks, 2:3:4.5 for ^{15}N : ^{23}Na : ^1H , the modulation depth for the ^{15}N amino nitrogen ranges from 2–3%. The ESEEM spectrum of Figure 2b was obtained for ^{14}N -labeled tyrosine radical under identical resonance and pulse timing conditions as the data of Figure 2a. The sharp peak at 3.9 MHz is assigned to ^{23}Na as before, while the new features at 0.7, 1.8 and 3.5 MHz are assigned to ^{14}N of the amino group. A portion of the ESEEM intensity at 0.2 MHz is likely a result of the data processing procedure.

ESEEM spectra for ^{14}N -tyrosyl radical, and the tyrosyl radicals of EYPIG and RSYTH are shown in Figure 3a, 3b and 3c (solid lines), respectively. The data were collected using slightly different pulse timing parameters from those of Figure 2. All three spectra show the peak at 3.9 MHz, which we have assigned to ^{23}Na , and a complex pattern of 3 peaks that arise from the amino or peptide ^{14}N . For tyrosyl radical (Figure 3a), ^{14}N ESEEM modulations are observed at 0.7, 1.8 and 3.5 MHz. For EYPIG (Figure 3b), these peaks have shifted to 0.6, 1.6 and 3.1 MHz, while, for RSYTH (Figure 3c), ^{14}N ESEEM features were detected at 0.6, 1.6 and 2.7 MHz. Data were also collected for two additional pentapeptides, IYPIG and RYPIG, and found to be nearly identical to the data shown above for EYPIG (data not shown). The ^{14}N modulation depths for all of these data sets are approximately 3%, comparable to that measured for the ^{15}N -tyrosyl radical.

The data presented in Figures 2 and 3 show differences between the ^{14}N -ESEEM arising from the amino nitrogen of tyrosyl radical and the amide nitrogen of tyrosyl radical-containing pentapeptides. To understand the differences in magnetic parameters that give rise to these observations, spectral simulations were undertaken. Our analysis began with simulations of the ^{15}N -ESEEM pattern shown in Figure 2a. The simulations used analytical expressions developed by Mims and others for an $S = 1/2$, $I = 1/2$ coupled spin system, in which the spin Hamiltonian for the ^{15}N nucleus consists only of nuclear Zeeman and electron-nuclear hyperfine terms.^{28,30} The data (Figure 2a) show a single ESEEM peak centered at the ^{15}N Larmor frequency that is substantially broader than the ^{23}Na matrix peak at 3.9 MHz.

For our simulations, we considered the ^{15}N hyperfine anisotropy to be of axial symmetry, yielding hyperfine coupling tensor principal values of the form, $A_{XX} = A_{YY} = a - T$ and $A_{ZZ} = a + 2T$, where “a” represents the isotropic hyperfine coupling and T is the axial anisotropic portion. We found that the experimental modulation depths of 2–3% gave rise to $|T|$ values of $0.3 \pm .05$ MHz. The maximum value of the isotropic hyperfine coupling, $|a|$, was limited by the lineshape of the ^{15}N -ESEEM peak and, therefore, constrained to be less than 0.2 MHz and of the same sign as T. Larger values of the isotropic hyperfine coupling led to simulated ^{15}N ESEEM lineshapes with pronounced shoulders or a resolved frequency separation about 1.5 MHz (results not shown).

For the ^{14}N ESEEM spectra of Figure 3, simulations were done numerically and employed a nuclear spin Hamiltonian that included nuclear Zeeman, electron-nuclear hyperfine and nuclear quadrupole interactions. For tyrosyl radical (Figure 3a), the hyperfine coupling determined for the ^{15}N -tyrosyl radical was scaled to the appropriate values for ^{14}N and fixed in our

calculations. Five parameters are needed to describe the nuclear quadrupole interaction (NQI): e^2qQ , the quadrupole coupling constant; η , the asymmetry parameter; and α , β and γ , the Euler angles that describe the orientation of the NQI principal axis system with respect to the principal axis system of the hyperfine tensor.³⁶ Because the hyperfine tensor is of axial symmetry, only the first two Euler angles, α , a positive rotation about the NQI z-axis, and β , a positive rotation about y' (the y-axis after the α -rotation), affect the simulations. At pH = 11, the amino nitrogen of tyrosinate will be tricoordinate, and can be viewed as having a lone pair of electrons in a p-orbital perpendicular to the plane that contains the sigma bonds to the two amine hydrogens and the α -carbon of the amino acid. The lone pair of electrons will define the principal axis of the NQI tensor and lead to an e^2qQ value of about 3.5 MHz. The sigma bonding arrangement will cause the asymmetry parameter to be > 0 .³⁷

The dashed curve in Figure 3a is a simulation for the tyrosyl radical ESEEM spectrum (solid line) that best accounts for the frequencies, amplitudes and lineshapes of the ^{14}N peaks. To facilitate comparison between experiment and theory, a simulation of the ESEEM spectrum expected for a single ^{23}Na nucleus at a dipole-dipole distance of 6.8 Å from the paramagnetic center was combined with the ^{14}N simulation and gives rise to the peak at 3.9 MHz. The ^{14}N simulation shows broad features at 0.5 MHz, 1.7 MHz and 3.5 MHz. The Hamiltonian values used in this calculation were: $a = 0.0$ MHz, $T = 0.21$ MHz, $e^2qQ = 3.4$ MHz, $\eta = 0.25$, and $[\alpha, \beta] = [57^\circ, 34^\circ]$. The value of e^2qQ primarily determines the position of the highest frequency peak at 3.5 MHz, and the β angle, which approximately describes the relative orientation of hyperfine and NQI principal axes, controls the lineshape of this peak. At β values less than 40° , this peak is skewed towards higher frequency. For β values from 40 to 60° , this higher frequency peak is split and shows maxima at both high and low frequency extremes. The overall appearance of the ^{14}N -ESEEM spectra, with three broad lines at frequency positions <1 , 1.5 – 2.0 , and 2.5 – 3.5 MHz, requires that the asymmetry parameter, η , be in the range from 0.2 to 0.4. ESEEM simulations for tyrosine radical in the EYPIG and RSYTH pentapeptides are shown as dashed traces along with the experimental spectra (solid traces) in Figures 3b and 3c, respectively. Each simulation includes a ^{23}Na contribution equivalent to that used in Figure 3a. The Hamiltonian parameters for all three ^{14}N ESEEM simulations are listed in Table 1 for ready comparison. For each simulation, the hyperfine coupling to the amine or amide nitrogen is identical to that determined for ^{15}N -tyrosyl radical. The differences in these three ^{14}N -ESEEM spectra can be attributed to differences in the NQI coupling constants and the orientation of the NQI principal axis system relative to the hyperfine principal axes. The Hamiltonian parameter values listed in Table 1 for tyrosine provide an estimate of the uncertainty in the parameters that also applies to the calculations done for EYPIG and RSYTH.

DFT was used to calculate the isotropic and anisotropic hyperfine couplings for tyrosyl radical conformers (Table 2). The singlet tyrosine was modeled by an anionic tyrosine (with a proton on the phenolic oxygen), and the tyrosyl radical was modeled by an anionic tyrosyl (with a deprotonated phenolic oxygen). These models were chosen for the singlet and radical structures because they correctly reproduced previous experimental FTIR results (see section 3 of reference ¹⁷ for details). Table 2 also shows the zero-point energies for each predicted radical and singlet conformer. Under the experimental conditions employed here, the distribution of conformers should reflect the singlet energies and conformations, because the samples are frozen before UV photolysis is used to generate the radical. In Table 2, the A conformer is predicted to be the lowest energy state, but the B singlet state is less than 1 kcal/mol higher in energy, when compared to A. As shown in Table 2, the A and B conformers are predicted to be distinguishable on the basis of their isotropic hyperfine coupling. Comparison of calculated parameters (Table 2) and the isotropic hyperfine couplings, derived from ESEEM simulations (Table 1), suggests a predominance of the B conformer at pH 11. This can be rationalized if solvent interactions favor the B conformer, which has a large dipole moment (7.2 Debye), compared to the A conformer (6.3 Debye). A predominance of the B conformer is consistent

with observed tyrosyl radical FT-IR frequencies, which are known to exhibit conformation dependence.¹⁷

DISCUSSION

The ¹⁵N-ESEEM detected for the amino nitrogen of tyrosyl radical places an upper bound on the isotropic hyperfine coupling of 0.2 MHz. For ¹⁴N, this limiting value scales to 0.14 MHz. In our simulations (Figure 3), we found that a modest increase in the isotropic hyperfine coupling from 0 to .1 MHz caused the feature at 1.6 MHz to split into two components. Therefore, simulations with $a = 0.0$ MHz were favored. Our finding that the ¹⁴N or ¹⁵N hyperfine coupling for tyrosyl radical can be modeled as purely anisotropic agrees with our DFT calculations for conformer B (Table 2) and is in agreement with the computational work of Qin and Wheeler.³⁸

The simulations of figure 4 were accomplished with an axial hyperfine tensor with $T = 0.21 \pm 0.05$ MHz. Because the nuclear spin Hamiltonian for ¹⁴N is dominated by the nuclear quadrupole interaction (NQI), the intensities of the modulations are not as sensitive to the hyperfine anisotropy as they are for the $I = 1/2$ case presented by ¹⁵N. Therefore, the limits on “a” and T given for ¹⁴N were derived from the ¹⁵N tyrosyl radical ESEEM analysis and not varied significantly, while developing the ¹⁴N simulations of Figure 3. If we apply the dipole-dipole approximation to the value of T obtained from our ¹⁵N ESEEM study of tyrosyl radical, we obtain a dipolar distance of 3.0 Å. Analysis of the structure of conformer B from the DFT calculations (Table 2) shows that the distance from the amine nitrogen to the C₁ carbon of the phenol ring is 3.042 Å. Because the spin density is distributed about the tyrosyl phenol ring in an odd-alternate fashion, this “agreement of distances” is fortuitous. However, only conformers A and B from the DFT analysis yield nitrogen-C₁ carbon distances in the 3.0 Å range. The C and D conformers show this distance at 3.83 and 3.71 Å, respectively. The anisotropic hyperfine couplings derived from our DFT calculations also show near-axial symmetry with $2 \cdot T_X \cong -T_Z$, and feature absolute values, $|T_X|$, that are on the same order of magnitude as the $|T|$ value derived from the ESEEM analysis.

The NQI parameters that dominate the ¹⁴N-ESEEM spectra of tyrosyl radical in borate/NaOH glass and of tyrosyl radical in EYPIG and RSYTH are in agreement with data from NQR measurements on amino acid powders. In our study, we found the quadrupole coupling constant, e^2qQ , to be 3.4 MHz for tyrosyl radical, 3.1 MHz for EYPIG and 3.4 MHz for RSYTH. These values are indicative of tricoordinate nitrogen with a lone pair of electrons, and the variation we observed in our measurements is in line with what has been found in the few NQR studies that have been reported. NQR experiments on glycyl-glycine and GGG powder have shown e^2qQ values for peptide nitrogens of 3.0 MHz and asymmetry parameters of 0.41 to 0.48.³⁹ For glycyl-glycine hydrochloride hydrate, NQR measurements showed the peptide nitrogen e^2qQ to be 3.4 MHz with $\eta = 0.40$.⁴⁰ The differences in these measured e^2qQ values for the peptide nitrogen of glycyl-glycine point to the importance of the environment of the dipeptide in the solid state. An additional study of glycyl-alanine powder yielded a peptide nitrogen e^2qQ of 3.25 MHz, also with $\eta = 0.40$.⁴⁰ Although NQR studies of peptides are few in number, there is enough information in these two previous reports to indicate that changes in e^2qQ of 0.3 MHz, commensurate with what we observed in our study of Y•, EY•PIG and RSY•TH, are within the realm of changes that can be realized by formation of a peptide bond, changes in hydrogen bonding, or alterations in the placement of ionic species in the vicinity of the peptide nitrogen.

One of the goals of this study was to evaluate ¹⁴N-ESEEM as a spectroscopic tool to augment cw-EPR and ²H-ESEEM in evaluating amino acid conformations for tyrosyl radical. The differences in the ¹⁴N-ESEEM spectra between tyrosyl radical (Figure 3a) and the radical in

EYPIG and RSYTH are subtle. All three spectra show weak modulations (approximately 2–3% of the electron spin echo amplitude) with three broad peaks, which have frequencies determined primarily by the nuclear quadrupole and Zeeman interactions. The hyperfine coupling serves to broaden these peaks, while the relative orientations of the NQI and hyperfine tensors contribute to their relative amplitudes and lineshapes. The ^{14}N -ESEEM spectrum of the tyrosyl radical amino nitrogen of Figure 3a is very similar to that measured for the amide nitrogen in the EYPIG pentapeptide, Figure 3b. The differences in frequencies of the three broad peaks, resolved for these samples, reflect the 0.3 MHz decrease in e^2qQ determined for EYPIG, as compared to the amino acid. Both spectra show relative peak amplitudes for the nitrogen coupling that are nearly the same, so it was not surprising to find that the Euler angles describing the relative orientation of the NQI tensor to the hyperfine tensor were close to one another. For the peptide nitrogen of the tyrosine radical in RSYTH, the higher frequency peak is skewed to the low frequency side, 2.7 MHz, and the relative amplitudes of the three ESEEM components are such that the center frequency peak at 1.6 MHz is half the amplitude of those at 0.6 and 2.7 MHz.

These changes are caused by differences in the orientation of the NQI principal axis system (PAS) relative to the PAS of the hyperfine tensor and reflect a change in peptide conformation. To visualize this shift in conformation, one can consider the β Euler angles summarized in Table 1, to reflect the angle between a vector that connects the amide or amino nitrogen with the C_1 carbon of the phenol ring (the principal axis of the hyperfine tensor), and a vector that is normal to the sigma bonding plane of the amide or amino nitrogen (the principal axis of the NQI). Given the observed similarities in the relative peak amplitudes and EPR lineshapes for Y and EYPIG, we can average their β angles and label this conformer as one described by a relative orientation between hyperfine and NQI principal axes of 37° . The ^{14}N -ESEEM spectrum of RSYTH shows that a conformational change has shifted the relative orientations of these two vectors to 57° .

^{14}N -ESEEM of tyrosyl peptide nitrogen shows some promise in providing a technique for measuring peptide conformation relative to the amino acid side chain. However, a primary concern is the shallow amplitudes of the modulations. For these model systems, in which the only other sources of ESEEM are ^{23}Na and ^1H from the matrix and the shallow ESEEM from the phenol ring protons, these peptide ^{14}N modulations can be resolved. Because these samples featured concentrations of tyrosyl radical in the 1 mM range, it is not clear what one will be able to detect for protein samples that are typically 1–2 orders of magnitude less concentrated. Some improvement in modulation depth and ^{14}N ESEEM resolution should be realized if these experiments are done at lower microwave frequencies. ESEEM simulations done assuming a microwave excitation frequency of 2 GHz and the Hamiltonian parameters of Table 1 show modulation depths of 6% and improved frequency resolution. Another concern is that the information on orientation comes from ESEEM spectral simulations, in which Hamiltonian parameters were restricted for the sake of efficiency. Specifically, we took the similarity of the Y, EYPIG and RSYTH spectra in terms of ESEEM frequencies and amplitudes to indicate that the hyperfine coupling was the same for all three molecules and that the coupling was of axial symmetry. Finally, in interpreting the results of our ^{14}N -ESEEM analyses to describe the conformational differences for the tyrosyl peptide nitrogen of RSYTH with respect to its counterpart in either EYPIG or Y, we used a crude point dipole-dipole picture of electron-nuclear hyperfine interaction and a qualitative Townes-Dailey interpretation of the NQI.⁴¹ Because the literature contains virtually no discussion or in-depth analysis of the NQI for amide or amino ^{14}N , this useful, but over-simplified picture used to interpret our ESEEM results should be viewed with caution.

Previous reaction-induced FT-IR studies of tyrosine and tyrosine-containing peptides detected an oxidation-induced change in the amino NH or amide II (CN stretch/NH bend) frequency.

^{21,22} Oxidation-induced changes in amide I (C=O) and amide II frequencies are also observed when tyrosine is oxidized in photosystem II.⁴² Because the observed changes in NH force constant, which accompany tyrosyl radical formation, are small (2% change) in model compounds, it is possible that the vibrational frequency change is a result of a small amount of unpaired spin delocalization, which cannot be detected here. However, another possible explanation is that the oxidation-induced change in force constant is due to an orientation-dependent Stark effect⁴³ on the vibrational bands of the peptide bond. An oxidation-induced Stark effect could be driven by a change in charge distribution in the aromatic ring when tyrosyl radical is formed. Electrostatic maps have been calculated for tyrosine and tyrosyl radical, and changes in the distribution of charge have been predicted.⁴⁴ This change in electrostatics has been proposed to drive a pK_A change in a pi-pi interacting histidine in a model peptide.⁴⁴

Previous reaction-induced FT-IR studies of tyrosine-containing dipeptides and pentapeptides have also shown that the frequencies of the oxidation-perturbed amide I and II bands depend on sequence.^{21,22} This previous FT-IR result is consistent with our ESEEM results if the change in conformation, which results in a reorientation of the NQI, also changes the amide I and II frequencies. For example, rotation around the amide bond changes the electronegativity of the amide nitrogen, the ionic character of the CN bond, and the force constants for the CN stretching modes.^{45,46} Therefore, small deviations in the planarity of the peptide bond may occur in peptides and proteins of different sequence and be detected by the reaction-induced FT-IR technique.

Acknowledgements

The National Institutes of Health through grants GM43272 (BAB) and GM-54065 (JLM) provided support for this research. Support for the purchase of the pulse EPR spectrometer came from the NIH National Center for Research Resources (S10-RR15880) and the Michigan Economic Development Corporation.

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FIGURE 1.

Continuous wave EPR spectra of tyrosyl radical found in UV irradiated borate/NaOH glasses containing (a) L-tyrosine, (b) ^{15}N -labeled L-tyrosine, (c) EYPIG peptide, and (d) the RSYTH peptide. Conditions common to these measurements were: microwave frequency, 9.72 GHz; microwave power, 6.3 μW ; field modulation frequency, 10 kHz, field modulation amplitude, 0.2 mT; and sample temperature, 10K.

FIGURE 2.

Stimulated echo detected ESEEM spectra of (a) ^{15}N -labeled tyrosyl radical and (b) ^{14}N -labeled tyrosyl radical in borate/NaOH, pH=11.0. Conditions common to both measurements were: microwave frequency, 9.72 GHz; 90° microwave pulse width, 16 ns; t value, 120 ns; starting T , 40 ns; timing increment, 16 ns; magnetic field strength, 347.0 mT; and sample temperature, 10K.

FIGURE 3.

Stimulated echo detected ESEEM spectra (solid lines) of tyrosyl radical derived from (a) L-tyrosine, (b) EYPIG and (c) RSYTH. Spectra were taken under conditions identical to those of Figure 2 except that $\tau = 160$ ns for all three spectra. The dashed lines for each spectrum show corresponding ^{14}N -ESEEM simulations. Parameters common to the simulations were: $g_{\text{N}} = 0.40349$, electron-nuclear hyperfine coupling principal values, -0.21 , -0.21 , 0.42 MHz; $\tau = 160$ ns; starting $T = 40$ ns; time increment, 20 ns/pt; time domain array length, 256 pts. The NQI parameters were unique to each simulation and are given in Table 1. Spectra were obtained by removing the DC component of the simulation followed by application of a Hamming window and Fourier transformation. Absolute value spectra are shown.

Table 1

¹⁴N-ESEEM simulation parameters

Sample	e^2qQ (MHz)	η	α	β	a (MHz)	T (MHz)
Y	3.4 ± 0.1	0.30 ± 0.1	$57^\circ \pm 3^\circ$	$34^\circ \pm 3^\circ$	0.0 ± 0.1	$0.21 \pm .05$
EYPIG	3.1	0.30	57°	40°	0.0	0.21
RSYTH	3.4	0.30	48°	57°	0.0	0.21

Table 2

Predicted ^{14}N Hyperfine couplings in tyrosyl radical^a

	ring ^b	bb ^c	Newman ^d	A _{iso} ^e	T _y ^f	T _z ^g	E _s ^h	E _r ⁱ
A	85	165		1.1	—	1.3	0.0	0.0
B	76	-73		-0.2	-0.7	1.2	0.5	2.7
C	65	63		0.1	-0.4	0.7	1.6	2.9
D	103	95		4.1	-0.6	1.0	1.6	3.0

^a All hyperfine couplings are in MHz. All energies are in kcal/mol and computed at the B3LYP/6-311++G(3df,2p)/B3LYP/6-311++G(d,p) level of theory. Ty = - (Tx + Tz).

^b C_α-C_β-C1'-C2' dihedral angle in degrees

^c C-C_α-C_β-C1' dihedral in degrees

^d Newman projections of conformers looking down the C_α-C_β bond.

^e Isotropic hyperfine coupling

^f Anisotropic hyperfine coupling along the x-axis in the principal axis system

^g Anisotropic hyperfine coupling along the z-axis in the principal axis system

^h Relative zero-point corrected energy of the parent singlet conformation Boltzmann averaged at 298K

ⁱ Relative zero-point corrected energy of the radical conformation

^j This minima is unique to the radical surface. Therefore, the reported energy is for the closest singlet conformer.