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Study of the Triplet State Properties of Tyrosines and Tryptophan in Azurins Using Optically Detected Magnetic Resonance†

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ABSTRACT: Optically detected magnetic resonance (ODMR) signals and phosphorescence spectra were seen of tyrosine in the *P. aeruginosa* and tryptophanless *P. fluorescens* azurins and of tryptophan in the former. This confirmed a conclusion from other experiments that the tryptophan of *P. aeruginosa* cannot effectively quench the singlet energy of both tyrosines. The ODMR signals were all very narrow, additional evidence that the chromophores are buried in the interior of the protein. Accurate values of the zero-field coupling constants D and E lead to a tentative correlation of D values with the red shift of the $0 \rightarrow 0$ peak of the phosphorescence spectrum. The environment of tryptophan in *P. aeruginosa* is the most hydrocarbon like of any tryptophan so far observed. The experiments raise a number of unanswered questions concerning rate processes. The intensities of the $|2E|$ transition of tyrosine and the phosphorescence of both tyrosine and tryptophan are substantially reduced when the copper is oxidized. Nevertheless the phosphorescence lifetimes are unaffected. A hole cannot be burned in the ODMR resonances. The homogeneously broadened lines may conceivably be a result of low-temperature proton tunnelling.

Azurin is a low molecular weight (~14 000) bacterial protein which contains one "blue" (type 1) copper coordination center per molecule (Sutherland and Wilkinson, 1963; Sutherland, 1965; Ambler, 1963; Ambler and Brown, 1967). All of the azurins thus far sequenced (Ambler, 1971) contain tyrosines at positions 72 and 108; all except one contain a single tryptophan residue at position 48.

The fluorescence properties of both "class A" (containing only phenylalanines and tyrosines but no tryptophan) and tryptophan-containing azurins are unique; tyrosine quantum yields in the "class A" *Pseudomonas fluorescens*[1] azurin are unusually high (Ugurbil and Bersohn, 1977), and tryptophan-containing *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* azurins exhibit a fluorescence maximum at 308 nm, an unprecedented blue shift for tryptophan emission (Finazzi-Agrò et al., 1970;[2] Grinvald et al., 1975). In both "class A" and Trp-containing azurins, the fluorescence yields vary with the presence or absence of the copper but are relatively insensitive to its oxidation state (Finazzi-Agrò et al., 1970; Grinvald et al., 1975; Ugurbil and Bersohn, 1977).

In this study, we extend the investigation of the emission properties of these unusual chromophores to their lowest triplet state. Included are phosphorescence lifetime measurements, determination of the zero-field splitting (ZFS[3]) parameters, and hole burning experiments on "class A" *P. fluorescens* (containing tyrosine but no tryptophan), and "class B" (containing tryptophan as well as tyrosines) *P. aeruginosa* azurins. In the following these proteins will be referred to as azurins A and B, respectively.

Materials and Methods

The *P. fluorescens* culture (ATCC 13430) was purchased from the American Type Culture Collection. A culture of *P. aeruginosa* was kindly supplied by Dr. David C. Wharton of the University of Texas, San Antonio, Texas. The organisms were grown in large scale by Grain Processing Corp., Muscatin, Iowa.

Azurin was isolated according to the procedure described previously (Ugurbil and Bersohn, 1977). The highest purity azurin A and azurin B obtained had $A^{ox}_{vis\ max}/A_{280}$ ratios of 1.27 and 0.58, respectively; $A^{ox}_{vis\ max}$ is the absorption at the

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[1] Azurins from at least two different strains of *P. fluorescens* lack the tryptophan residue (Ugurbil and Bersohn, 1977; Ambler, 1971). Sequenced azurins from two other *P. fluorescens* strains possess a tryptophan (Ambler, 1971).

[2] These authors reported using azurin from *P. fluorescens*. This organism was in the past confused with *P. aeruginosa* (Ambler and Wynn, 1973). Therefore, it is not clear which organism was used by Finazzi-Agrò et al. (1970, 1973).

[3] Abbreviations used: fwhm, full width at half-maximum; ODMR, optically detected magnetic resonance; ZFS, zero-field splitting; EPR, electron paramagnetic resonance; IR, infrared; UV, ultraviolet; HLAD, horse liver alcohol dehydrogenase; NAG, tri-*N*-acetylglucosamine; EG, ethylene glycol.

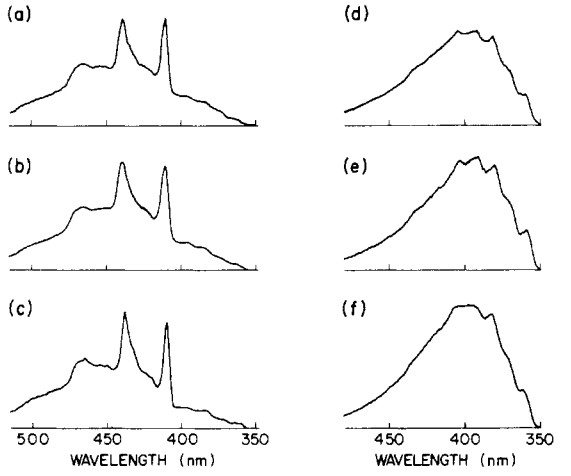

FIGURE 1: Phosphorescence emission of azurin A and azurin B at 4.2 K, in EG–ammonium acetate buffer, 0.1 M, pH 6.5 (1:1). (a) Oxidized azurin B; (b) reduced azurin B; (c) apoazurin B; (d) oxidized azurin A; (e) reduced azurin A; (f) apoazurin A. Excitation λ is 286 nm for the azurin B samples and 275 nm for the azurin A samples.

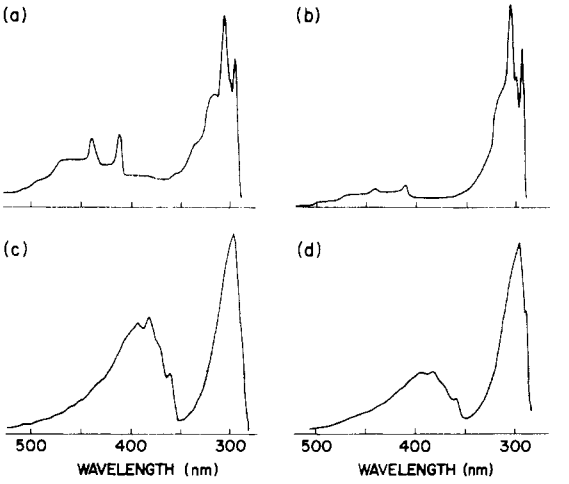

FIGURE 2: Luminescence (phosphorescence plus fluorescence) spectra of azurin A and azurin B at 77 K, in EG–ammonium acetate buffer, 0.1 M, pH 6.0 (1:1). (a) Reduced azurin B; (b) oxidized azurin B; (c) reduced azurin A; (d) oxidized azurin A. Excitation λ is 261 nm for azurin B and 270 nm for azurin A. Emission monitored at 3-nm resolution.

maximum in the visible (622 for azurin A and 625 for azurin B) of a ferricyanide oxidized sample and A_{280} is the absorption at 280 nm of an untreated sample. Apoazurin was prepared by dialysis at 4 °C against 0.5 M KCN in 0.1 M ammonium acetate buffer. Apoazurin preparations were always checked for rebinding with Cu^{2+} to give native azurin spectroscopic properties. Reduced azurin was prepared by addition of a slight excess of solid sodium dithionite and dialysis against buffer.

Total emission (fluorescence plus phosphorescence) spectra at liquid-nitrogen temperatures were recorded on a Perkin-Elmer MPF-3L spectrofluorimeter. The apparatus used for optical detection of magnetic resonance (ODMR) and hole burning experiments at pumped liquid-helium temperatures (~1.2 K) and for obtaining phosphorescence spectra plus lifetimes at 4.2 K have been described elsewhere (Maki and Co, 1976). The slow passage ODMR spectra were recorded at 1.2 K once by sweeping from high to low microwave frequencies and a second time by sweeping from low to high microwave frequencies; the average of the two frequencies obtained in this way for the zero-field transition maxima were used to calculate the D and E values. Hole burning experiments were performed at 1.2 K by applying continuous wave microwave power at a fixed frequency while a second microwave frequency was swept simultaneously over the signals as in slow-passage ODMR.

Phosphorescence decays obtained for lifetime determination were signal averaged to improve signal-to-noise ratios. When monitoring tyrosine emission, an excitation pulse of 10 s and observation time of 25 s were employed. For tryptophan decays, the sample was excited for 25 s and the decays were monitored for 40 s. Phosphorescence emission both for lifetime measurements and ODMR experiments were monitored at 3.2-nm resolution, at the specified wavelengths. Electron paramagnetic resonance (EPR) spectra of the azurin-bound triplets were obtained using a Varian E-line EPR spectrometer. Samples were excited using a 1000-W mercury–xenon lamp. Most of the visible and IR output of the excitation source were filtered out using a filter solution ($NiCl_2$ (0.3 M) and $CoSO_4$ (0.1 M) in a 2.5-cm long cell) and a Corning 7-54 filter.

Results and Discussion

Phosphorescence spectra at 4.2 K of apo-, oxidized, and reduced azurins A and B are shown in Figure 1. The tyrosine contribution to the phosphorescence of the azurin B emission is unmistakable. This confirms our earlier prediction based on the high tyrosine yields observed in azurin A and information about the spatial distribution of the three chromophores (Ugurbil and Bersohn, 1977; Ugurbil et al., 1977). Previous workers (Finazzi-Agrò et al., 1970; Grinvald et al., 1975) had ruled out significant tyrosine contribution to the fluorescence of Trp-containing *P. fluorescens* and *P. aeruginosa* azurins. Assuming a tyrosine phosphorescence-to-fluorescence ratio similar to that of azurin A (Figure 2), we obtain for the reduced azurin B a tyrosine contribution of ~20 and ~36% of the overall emission intensity (77 K) at 306 and 296 nm, respectively. Under these conditions, the conclusion that the variations observed in the emission anisotropy factor of reduced azurin B stem totally from a heterogeneous tryptophan population (Grinvald et al., 1975) may be questionable.

The positions of the tryptophan phosphorescence maxima in azurin B are independent of the presence of or oxidation state of the copper ion. The first maximum, presumably the 0 → 0 transition, is at 411 nm; the second principal maximum appears at 438.5 nm. These are to the red of what is observed for free tryptophan in polar solvents and comparable to phosphorescence originating from buried residues (Purkey and Galley, 1970; also see Table II).

The spectral shape and the emission wavelength of azurin A phosphorescence is similar to that of *N*-acetyltyrosine amide. The tyrosine phosphorescence in azurin B, although not different in its structure from that of azurin A, is red shifted by 4 nm with respect to it. The difference is not completely unexpected because the pK_a of one of the two tyrosines is greater by ~0.6 unit in azurin B than it is in azurin A (Ugurbil et al., 1977; Ugurbil and Bersohn, 1977). The phosphorescence emission wavelength and spectral shape exhibited by the tyrosines in the two azurins are distinctly different from those of tyrosines near disulfide bridges (Longworth, 1971). Thus, we conclude that the two tyrosines are not adjacent to the single disulfide bridge in either azurin. This conclusion assumes that both of the tyrosines contribute to the overall phosphorescence. That this is indeed the case has been shown for azurin A only (discussed further on). Small differences in the tyrosine phosphorescence are detectable between the apo-, oxidized, and reduced forms; the structure in the oxidized azurin phos-

phorescence from both organisms is less well defined. The relative tryptophan contribution to the overall phosphorescence is larger in apoazurin B than it is in the reduced and oxidized forms of the haloazurin B. This may stem from a larger increase, upon removal of the copper ion, in the steady-state population of the tryptophan fluorescent state compared with that of tyrosine(s). It has already been shown using Trp-containing azurins and azurin A (Finazzi-Agrò et al., 1970; Grinvald et al., 1975; Ugurbil and Bersohn, 1977) that the fluorescence of both the tryptophan and the tyrosines is quenched in the presence of the copper.

The most striking feature of the triplet state emission from both azurins is that its yield varies with the oxidation state of the azurin bound "blue" copper (Figure 2). Although the fluorescence yield decreases only 10% with the reduction of the Cu^{2+} in azurin A (Ugurbil and Bersohn, 1977), the ratio of 382 nm emission intensity to that at 296 nm at 77 K is 0.29 and 0.6 for the oxidized and reduced forms, respectively. Similarly, in azurin B, we have found the fluorescence yield (280 nm excitation, 25 °C) to be independent of the copper ion oxidation state; however, the ratio of the 411-nm emission intensity to the intensity of the fluorescence maximum (306 nm) is 0.37 for the reduced protein and decreases to 0.11 in the oxidized protein. For the azurin A, the apoprotein as well as the apoprotein complex with Hg^{2+} gives phosphorescence-to-fluorescence ratios equal to that of the reduced native azurin. When the tyrosine $\Delta m = 2$ transition of azurin A was monitored with EPR at 77 K, the signal intensity of the reduced azurin A, under identical conditions, was twice that of the oxidized azurin A. This further confirms that the increase in phosphorescence yields upon reduction of the Cu^{2+} indeed represents an increase in the steady-state populations of the triplets.

In marked contrast to our results, Finazzi-Agrò et al. (1973) had reported that the phosphorescence intensities of a Trp-containing *P. fluorescens* azurin is unaffected by the copper oxidation state. The azurin luminescence spectra published by these authors also show much larger phosphorescence-to-fluorescence ratios and lack the well-defined structure, especially the 306- and 293.5-nm fluorescence peaks, observed in the azurin B emission (Figure 2). A less pronounced but similar structure is evident in azurin B fluorescence at ambient temperatures (Grinvald et al., 1975). Very few proteins possess such a structured fluorescence emission. The lone tryptophan of ribonuclease T_1 at 77 K exhibits a fine structure (Longworth, 1971) very similar to that of reduced azurin; however, azurin fluorescence emission is shifted ~9 nm to the blue of the ribonuclease T_1 emission and is narrower, especially in the oxidized form. As with ribonuclease T_1 (Longworth, 1971), the fine structure is attributable to a pure hydrophobic environment of the tryptophan residue in azurin B. The fluorescence fine structure at 77 K is slightly different in the oxidized than it is in the reduced azurin (Figures 2a and 2b). This may stem from a change in the tryptophan environment when the oxidation state of the copper ion is altered.

Phosphorescence Lifetimes

Phosphorescence lifetimes (Table I) were measured in order to clarify the triplet state quenching which occurs in oxidized azurin. The tryptophan lifetime is 6.0 ± 0.1 s at 4.2 K independent of the presence of copper or of its oxidation state. The tyrosine phosphorescence in both azurins exhibits more than one lifetime. The nonexponential nature of the decay is not apparent unless the phosphorescence intensity is followed over about three orders of magnitude. One may easily be led to believe that there are two decay lifetimes, each of which corresponds to one of the two tyrosines. However, careful measurement of the decay kinetics of simpler tyrosine containing systems such as tyrosine itself in 50:50 EG–H_2O (pH 7) buffer have shown nonexponential decay at 4.2, 77, and at 1.2 K with microwave saturation of all sublevel populations during the decay (S. Siegel and A. H. Maki, 1976, to be published). Although we do not understand the complex time dependence of the phosphorescence, it is the same in the apo-, oxidized, and reduced forms of both azurins.

It could be postulated that the decrease in the tyrosine phosphorescence yields is due to a selective and total quenching of one of them. (The same argument could not apply to tryptophan as there is only one Trp residue in azurin B.) However, the following experiment disproves the postulate. The two tyrosines in azurin A have different pK_a's, 10.75 and 12.78, but both contribute approximately equally to the overall fluorescence at 22 °C (Ugurbil and Bersohn, 1977); at pH 11.9, the 22 °C emission comes from a tyrosine and a tyrosinate ion. The total luminescence spectra (77 K) of this azurin at pH 11.9 also show a tyrosinate and tyrosine contribution to the fluorescence and phosphorescence for both reduced and oxidized forms (Figure 3). At pH 11.9, the overall phosphorescence-to-fluorescence ratio of the reduced protein remains approximately twice that of the oxidized protein. Because the phosphorescence-to-fluorescence ratio of the tyrosinate ion is very dif-

TABLE I: Phosphorescence Lifetimes at 4.2 K of the Tryptophan and the Tyrosines in Azurins A and B.

Sample[a]	Excitation λ[b]	Monitoring λ[c]	α_i[d]	τ_i[d] (s)
Azurin A				
Apo	275	360	2.8	3.98
			1	2.71
Apo	275	395	3.2	3.99
			1	2.36
Reduced	275	359	3.3	3.98
			1	2.66
Reduced	275	395	3.4	3.96
			1	2.46
Oxidized	275	360	3.2	3.97
			1	2.68
Oxidized	275	380	3.4	3.98
			1	2.57
Azurin B				
Apo	280	395	3.0	4.0
			1	2.07
Apo	280	411	2.3	6.01
			1	3.85
Reduced	286	395	3.26	4.01
			1	1.39
Reduced	286	411.5	6.6	5.98
			1	1.67
Oxidized	280	365	2.9	3.92
			1	1.84
Oxidized	280	411	8.0	6.02
			3.7	4.03
			1	1.63

[a] Azurin samples were ~10^{-3} M in EG–ammonium acetate buffer, pH 6, 0.05 M (1:1). [b] Resolution of the excitation monochromator was set at 5.8 nm. [c] Emission was monitored at the specified wavelength at 3.2 nm resolution. [d] Decay was fitted to the equation, $I(t) = \Sigma\alpha_i e^{-t/\tau_i}$, where $I(t)$ is phosphorescence intensity in time. The error in the τ_i and α_i of the component which dominates the decay is ± 0.1 and ~10%, respectively. The α_i and τ_i of the shorter lifetime component are less accurate.

TABLE II: Tryptophan Triplet State Data (T = 1.2 K).

Sample[a]	Phosphorescence Peaks (nm)	$D-E$ Transition[b] Frequency (GHz)	$D-E$ Transition[b] Width (MHz)	$2E$ Transition[b] Frequency (GHz)	$2E$ Transition[b] Width (MHz)	Zero-Field Splitting[c] $\|D\|/hc$ (cm^{-1})	Zero-Field Splitting[c] $\|E\|/hc$ (cm^{-1})
Apoazurin B	411, 438.5	1.605	40	2.780	60	0.0999 (3)	0.0464 (3)
Native azurin B[d]	411, 438.5	1.605	40	2.790	60	0.1000 (3)	0.0465 (3)
Tryptophan[e]	407.5, 433.5	1.736	165	2.449	345	0.0988 (6)	0.0408 (6)
HLAD, trp 1[e]	406, 436	1.793	125	2.490	175	0.1013 (4)	0.0415 (3)
HLAD, trp 2[e]	412.5, 441.0	1.667	75	2.521	125	0.0977 (3)	0.0421 (2)
α-Chymotrypsin[f]	413	1.595	42	2.71	110	0.0985	0.0450
Lysozyme[e]	415.4, 443.6	1.604	145	2.565	435	0.0963 (7)	0.0428 (6)
Lysozyme-tri-NAG[e]	415.6, 430.4, 444.2	1.559	85	2.667	225	0.0965 (4)	0.0445 (3)

[a] Azurin B samples were in EG–ammonium acetate buffer, pH 6.5, 0.1 M (1:1). Lysozyme and lysozyme-tri-NAG were crystals (Zuclich et al., 1973). α-Chymotrypsin was in 1:1 EG–potassium phosphate buffer, 0.02 M, pH 6.8 (Maki and Co, 1976). The rest was in EG–H_2O (1:1) (Zuchlich et al., 1973). [b] Phosphorescence intensity was monitored at the first phosphorescence peak (0 → 0 band maxima). Widths are fwhm. [c] Uncertainty in the last figure is given in the parentheses. [d] Both the reduced and the oxidized protein give the same results. [e] From Zuclich et al. (1973). [f] From Maki and Co (1976).

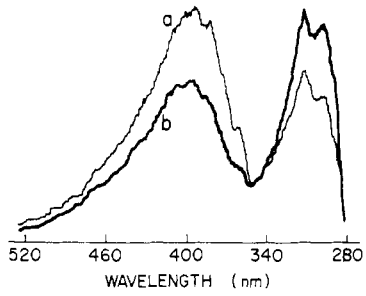

FIGURE 3: Luminescence spectra of (a) reduced azurin A and (b) oxidized azurin A, at pH 11.9 at 77 K, in EG–H_2O (1:1). Vertical scale for the oxidized spectrum is approximately 1.5 times that of the reduced spectrum. Samples excited at 270 nm; emission monitored at 5-nm resolution.

ferent from that of tyrosine (Longworth, 1971), the above observation is inconsistent with the postulate that the phosphorescene but not the fluorescence of one of the two tyrosines is completely quenched in the oxidized azurin A. Therefore we conclude that the fractional contribution of both tyrosines to the phosphorescence of the oxidized form near neutral pH must be approximately the same as in the reduced form. This is a puzzling observation because the higher pK_a tyrosine is expected to be well separated from the copper (Ugurbil et al., 1977) and the distance between the two tyrosines in this protein has been estimated to be ≳20 Å (Ugurbil and Bersohn, 1977).

The decrease in the phosphorescence yield without a corresponding change in the phosphorescence lifetimes could result from (a) a decrease in the intersystem crossing rate; an increase in the singlet–singlet interconversion rate is necessitated in this case because the fluorescence lifetimes (Grinvald et al., 1975) and yields remain approximately independent of the copper ion oxidation state; (b) heterogeneous population of chromophores some of which do not phosphoresce in oxidized azurin. Present experimental data, however, are not sufficient to elucidate the mechanism by which the tyrosine and tryptophan triplet states are quenched in the presence of the Cu^{2+}.

ODMR

Azurin B Tryptophan. Triplet state properties of tryptophan residues in various proteins have already been studied using ODMR (Zuclich et al., 1973; von Schütz et al., 1974; Maki and Co., 1976). Narrower line widths reflecting a more homogeneous environment as would be expected in an interior site of a protein and larger $|E|$ values were detected for the buried tryptophans or horse liver alcohol dehydrogenase (HLAD) and of lysozyme-tri-*N*-acetylglucosamine (NAG) complex.

The triplet state data collected in Table II show an approximate correlation between D values and the red shift of the 0 → 0 phosphorescence band. The zero-field splitting parameters D and E of the spin Hamiltonian

$$\mathcal{H} = D\left(S_z^2 - \frac{1}{3}S^2\right) + E(S_x^2 - S_y^2)$$

are

$$D = \frac{3}{4}g^2\beta^2 \langle\Psi(1,2)|(r_{12}^2 - 3z_{12}^2)/r_{12}^5|\Psi(1,2)\rangle$$

$$E = \frac{3}{4}g^2\beta^2 \langle\Psi(1,2)|(x_{12}^2 - y_{12}^2)/r_{12}^5|\Psi(1,2)\rangle$$

where $\Psi(1,2)$ is the triplet wave function, r_{12} is the distance between the two electrons, and the z direction is the normal to the aromatic plane (Zuclich, 1970a,b). The triplet chromophore within the protein is surrounded by a polarizable medium and/or polar groups. The excited state dipole moment is different from that of the ground state; thus, given a sufficient excited state lifetime, the solvent environment will relax in such a manner as to decrease the electrostatic energy of the combined solvent–chromophore system. The relaxation leads to a red shift of the luminescence (Purkey and Galley, 1970). As far as a triplet chromophore is concerned, the unrelaxed triplet wavefunction will be admixed with other excited electronic wavefunctions of the same multiplicity. There will occur a change in D resulting from a change in the average of r_{12}. It can be anticipated that, since the more highly excited admixed states generally are more diffuse, the average of r_{12} will increase, and D will decrease with increased solvent–chromophore interaction (i.e., with increased solvent-induced red shift).

E is complicated, depending not only on the interelectronic distance but also upon the symmetry both of the chromophore and its perturbing environment. As such, a correlation between E and phosphorescence red shift may not exist. Nevertheless,

TABLE III: Tyrosine Triplet State Data at 1.2 K.

Sample[a]	Frequency in GHz[b]			Zero-Field Splitting[c]	
	$D - E$ Transition	$2E$ Transition	$D + E$ Transition	$\|D\|/hc$ (cm^{-1})	$\|E\|/hc$ (cm^{-1})
Apoazurin A	2.19	3.40	5.59	0.1296 (4)	0.0567 (3)
Native azurin A[d]	2.23	3.41	5.62	0.1313 (4)	0.0569 (3)
Apoazurin B	2.11	3.51	5.63	0.1289 (5)	0.0585 (5)
Native azurin B[d]	2.12	3.54	5.65	0.1298 (5)	0.0590 (5)
L-Tyrosine[e]	2.20		5.59	0.130	0.056

[a] Azurin samples were $\sim 10^{-3}$ M in EG–ammonium acetate buffer, pH 6.5, 0.1 M (1:1). L-Tyrosine was in EG–H_2O (1:1). Azurin samples were excited at 280 nm; the emission was monitored at 383 and 395 nm for azurin A and B, respectively. Tyrosine sample was in EG–H_2O (1:1) (Zuclich et al., 1973). [b] The line widths (fwhm) of the three transitions for azurin A and B were ~150, 110, and 90 MHz for the $D - E$, $2E$, and $D + E$ transitions, respectively. [c] Uncertainty in the last figure is given in the parentheses. [d] The oxidized and the reduced forms of native azurin give the same results. [e] From Zuclich et al. (1973). The $2E$ transition was not detected.

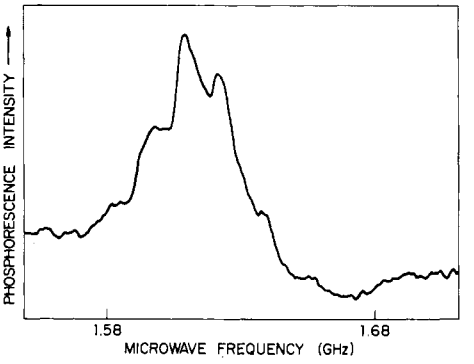

FIGURE 4: Slow-passage $D - E$ ODMR signal observed from azurin B tryptophan while monitoring phosphorescence at 411 nm.

the ZFS data suggest that larger E values are in general associated with buried residues. In the absence of rearrangement by the solvent molecules at low temperatures, the phosphorescence red shift, as pointed out earlier by Purkey and Galley (1970), and the decrease in D associated with exposed chromophores will be determined by the polarizability and not the polarity of the solvent.

The azurin tryptophan can be considered the least perturbed of any buried tryptophan in a protein observed thus far. The comparatively small ODMR line widths (Table II) indicate that, among the proteins studied, the relative homogeneity of the tryptophan environment is greatest in azurin. The hydrophobicity of the tryptophan site in azurin B is suggested by the extreme blue shift and the structure present in its fluorescence at ambient temperatures (Grinvald et al., 1975) and at 77 K (Figure 2). Moreover, its phosphorescence shows a relatively small red shift and its ZFS parameters are large. Therefore we conclude that the medium which surrounds the tryptophan in azurin B must be nonpolar and have a low polarizability, i.e., it must be hydrocarbon like. This conclusion is subtly supported by the fact that the tryptophan residue in azurin B is replaced by leucine in the "class A" azurin from *Pseudomonas fluorescens* ATCC 17467 (Ambler, 1971).

An interesting feature of the azurin tryptophan $D - E$ ODMR transition is the presence of fine structure in the signal at high microwave powers (~50 mW) (Figure 4). A similar structure was not detected for the $2E$ transition. When the microwave power is reduced to very low levels (~100 μW), the structure in the $D - E$ signal disappears. At high microwave power levels, small changes were detected in the signal shape when the line was swept twice as fast. Although the structure in tryptophan ODMR transitions is not unprecedented [similar structure was also evident in the slow passage ODMR transitions of α-chymotrypsin (Maki and Co, 1976)], its cause at present remains unexplained.

Azurin Tyrosines

The ZFS parameters of tyrosine itself have been studied by ODMR (Zuclich et al., 1973); however, an ODMR study of a tyrosine residue in a protein has not previously been reported. The results are summarized in Table III.

For both proteins, the tyrosine ZFS parameters are independent of the copper ion oxidation state but show a decrease of ~0.001 cm^{-1} in the $|D|$ values upon removal of the copper ion. The difference indicates that the tyrosines in both azurins have slightly different environments in the absence of the copper ion. This is not surprising since the copper ion which is located at a hydrophobic internal site in native azurin (Koenig and Brown, 1973) is expected to bring about some degree of structural reorganization upon binding to the apoprotein. In fact, at least in the case of azurin A, the pK_a of one of the two tyrosines decreases from 12.80 to ~10.9 upon removal of the copper ion (Ugurbil and Bersohn, 1977). Whether a similar change occurs in azurin B is not known.

The tyrosine ZFS parameters are not the same for the two proteins; in azurin B, where the phosphorescence is red shifted by 4 nm with respect to that of azurin A, the $|E|$ is larger by 0.002 cm^{-1} and $|D|$ is smaller by 0.001 cm^{-1}. The direction of the change in $|D|$ and the phosphorescence wavelength are consistent with the correlation earlier suggested (for tryptophan) between these two parameters. Fluorescence studies have shown that both of the tyrosines in azurin A occupy hydrophobic sites (Ugurbil and Bersohn, 1977). That the azurin B tyrosines are not solvent exposed is indicated by their anomalous pK_a's (Ugurbil et al., 1977). Therefore, the differences in the ZFS parameters and phosphorescence wavelength between the two proteins cannot be ascribed to radically different environments, such as being solvent exposed in one azurin and buried in the other. Since the phosphorescence red shifts are thought to originate from dipole-induced-dipole interaction with a polarizable medium (Purkey and Galley, 1970), the red shift of azurin B phosphorescence alone suggests a higher degree of polarizability in the environment of one or both of the tyrosines in azurin B.

Besides the ZFS parameters, the slow passage ODMR of azurin tyrosines showed three important results. (1) Tyrosine in EG–H_2O gives only two slow passage ODMR signals; the $2E$ transition is undetectable by this method (Zuclich et al.,

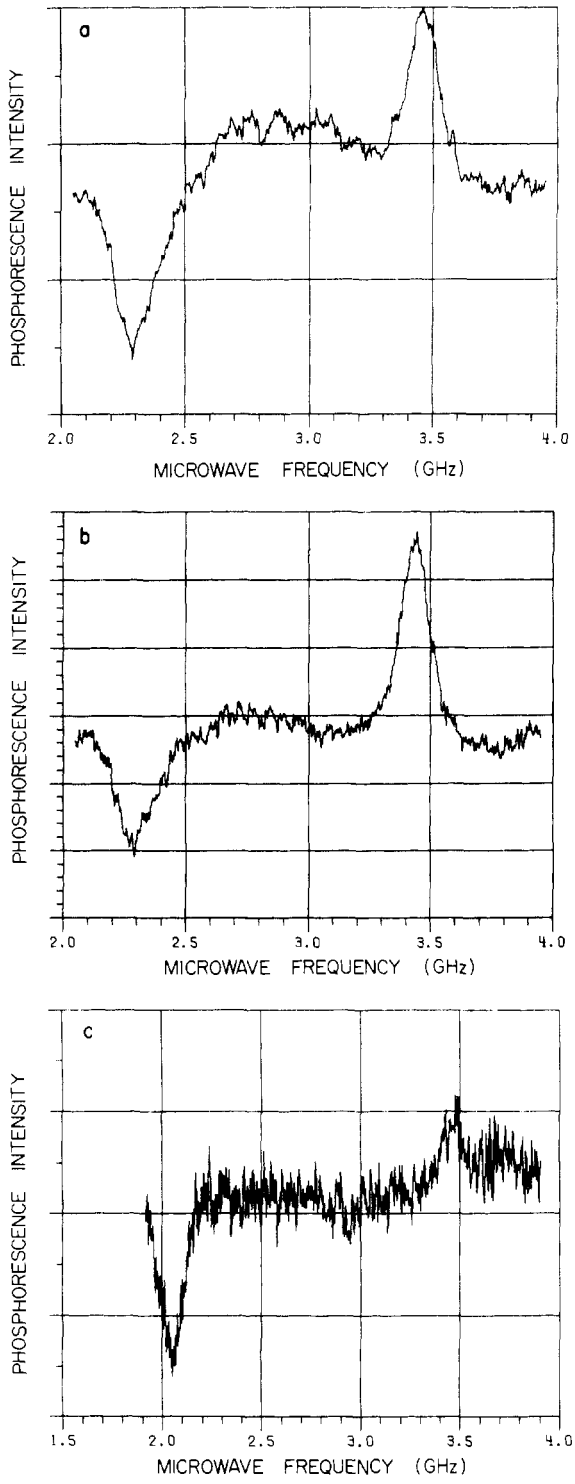

FIGURE 5: $D - E$ and $2E$ slow-passage ODMR signals observed for the tyrosines. (a) Oxidized azurin A; (b) reduced azurin A; (c) reduced azurin B.

1973). The tyrosines in both azurins, on the other hand, give signals for all three transitions. (2) In both azurins, the relative intensities of the $D - E$ and $2E$ transitions are very similar in the apo- and reduced protein; however, the relative intensity of the $2E$ signal decreases considerably in the oxidized protein (see Figures 5a and 5b). (3) The $2E$ transition is much weaker in azurin B than it is in azurin A (Figure 5). The differences in ODMR signal intensities between the apo-, oxidized, and reduced forms of azurin may stem from changes in the radiative decay rates, lifetimes or the relative populating rates of the individual sublevels. Nonvanishing spin-lattice relaxation rates at 1.2 K (Co et al., 1974) and complex decay kinetics (with microwave saturation of all three sublevels) exhibited by tyrosine prevent determination of these different rates. However, it can be concluded that the triplet state kinetic parameters of tyrosine are sensitive to its environment.

The oxidation state dependent changes seen in the metalloprotein slow passage ODMR signals may stem from the same process responsible for the oxidation state dependence of the overall phosphorescence intensity in both azurins (see above). If this is the case, most likely it is accomplished by perturbation of the overall and relative intersystem crossing rates to the individual sublevels of the triplet state.

Hole Burning

The line widths of the ODMR transitions are broad suggesting a distribution of values of D and E due to local inhomogeneities, in which case a hole can be burned in these lines (Leung and El Sayed, 1972).

The 40-MHz (fwhm) line width of the $D - E$ transition observed for tryptophan in azurin B is considerably narrower than the line widths previously seen in lysozyme and in HLAD (Table II); however, it is still larger than the 10-MHz homogeneous width of tryptophan (Zuclich et al., 1974). When continuous wave microwave power at the $D - E$ transition even at as low a level as 20 μW was applied to apo-, oxidized, and reduced azurin B, the tryptophan ODMR signal collapsed uniformly and no hole was burned. In previous experiments a hole was burned in HLAD but not in lysozyme or its inhibitor complex (von Schütz et al., 1974). Because in the absence of neighboring tryptophans energy exchange cannot occur, a fast exchange of local environments at 1.2 K is indicated. The rate of exchange must be faster than lifetimes of the individual sublevels but slower than the inverse of the line width so that motional narrowing does not occur. Zero-point vibrations and librations are too fast by this criterion. At this low temperature large amplitude nuclear displacements if any can only occur by proton tunnelling between potential minima differing in energy by only a few cm^{-1}. A possibility is the tunnelling of the protons of methyl groups of isopropyl radicals (valines, isoleucine) between minima which are equivalent for the isolated isopropyl group but exert slightly different potentials on the rigid indole side chain.

A hole could not be burned in the $2E$ transition of the azurin A tyrosines either. Because these tyrosines are too far apart to exchange energy (Ugurbil and Bersohn, 1977), the mechanism operative in case of the tryptophan may be operative here as well.

Conclusions

Our central conclusion is that the internal tryptophan of azurin B is located in a hydrocarbon-like site. This is indicated by the narrowness of the ODMR lines suggesting a narrow distribution of possible environments, by the large value of D, the small red shift of the phosphorescence, and the highly structured 77 K fluorescence emission. A definite tyrosine contribution to the emission of azurin B has been shown.

The outstanding problems are: (1) oxidation of copper reduces the phosphorescence quantum yield of both tryptophan and tyrosines sharply; yet the phosphorescence lifetimes do not change in any way. It is curious that the copper which is a neighbor but not a ligand of the tryptophan and a tyrosine and quite far from the other tyrosine should have about the same effect on all three groups. (2) Both tryptophan and the tyros-

ines exhibit effectively homogeneous line broadening of their ODMR lines at 1.2 K. (3) Tyrosine triplet state kinetics at 1.2 K are sensitive to the oxidation state of Cu^{2+}; specifically the 2*E* signal becomes much weaker when the protein is oxidized.

Acknowledgment

Thanks are due Dr. P. Aisen for help with a preliminary experiment, to Kathleen Ugurbil for aid in isolating the azurins, to S. Siegel, R. Anderson, and Tiao-te Co for help with the ODMR and phosphorescence decay measurements, and to Dr. K. P. Dinse for helpful discussions.

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