Tyrosine Emission in the Tryptophanless Azurin from *Pseudomonas fluorescens*†

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ABSTRACT: A strain of *Pseudomonas fluorescens* contains an azurin with no tryptophan and two tyrosines. This protein is interesting because it allows one to study both the structure of azurin and the emission of tyrosines in proteins. Comprehensive measurements were carried out including spectrophotometric and fluorimetric titration, fluorescence quantum yield, fluorescence polarization, and I^- quenching. In the copper-containing protein, almost independent of the copper ion oxidation, the fluorescence quantum yield is ~60% of that of the apoprotein. The latter has the remarkable property that its quantum yield is even greater than free tyrosine. The two tyrosines in the metalloprotein have different pK_a's, 10.75 and 12.78, but there is only one average pK_a, 10.9, in the apoprotein. The polarization of the fluorescence at 310 nm (290-nm excitation) is 0.32 for the metalloproteins and 0.34 for the apoprotein. I^- hardly quenches the fluorescence. The conclusion is that the two tyrosines are inaccessible to the solvent, located in nonpolar environments, $\gtrsim$20 Å apart, and not adjacent to the disulfide bridge.

Azurins are low-molecular-weight (~14 000) bacterial proteins which contain one copper ion per molecule (Sutherland and Wilkinson, 1963; Sutherland, 1965; Ambler, 1963; Ambler and Brown, 1967). The copper coordination in azurin is classified as type 1 ("blue"); this type is characterized by a unique EPR[1] spectrum and an unusually strong absorption around 625 nm (Brill et al., 1968; Malkin, 1973).

Azurins from several different organisms have been sequenced (Ambler, 1971). Many but not all of these azurins contain a single tryptophan residue which therefore cannot have an irreplaceable functional role in the protein. All sequenced azurins, on the other hand, invariably possess tyrosines at positions 72 and 108. The positions of the additional tyrosines, 110 in *Alcaligenes faecalis* and 15 and 110 in *Alcaligenes denitrificans*, are occupied by phenylalanine residues in other azurins.

Little is known about the environments of the tyrosine residues and their function within azurin. Avigliano et al. (1970) and Finazzi-Agrò et al. (1970) reported that the two tyrosines in *Pseudomonas fluorescens*[2] azurin have an abnormally high pK_a (~12.4); in the latter work, the presence of a tyrosine contribution to the total fluorescence and a tyrosine to tryptophan energy transfer were also ruled out. Grinvald et al. (1975) have concluded that energy transfer from the tyrosine residues to the tryptophan in *P. aeruginosa* apoazurin is either totally absent or very fast and that tyrosine contribution to the emission of native azurin could not be significant. Recently, using natural abundance ^{13}C NMR spectroscopy, we have shown that in *P. aeruginosa* azurin the tyrosines have different pK_a's, estimated to be 11.4 and 12.5 (31 °C). The lower pK_a tyrosine is located near the Cu^{2+} with its hydroxyl group pointing away from the metal ion. The high pK_a tyrosine was found to be well removed from the copper and consequently from the low pK_a tyrosine (Ugurbil et al., 1977b).

In this paper, the results of an investigation on the fluorescence properties of the two tyrosine residues in *P. fluorescens* (American Type Culture Collection (ATCC) 13430) azurin are reported. This azurin does not have a tryptophan residue. Consequently, it provides an excellent system for the study of the tyrosine residues in azurins.

Experimental Section

Materials. CM- and DEAE-cellulose were obtained from Whatman, Inc., Clifton, N.J., as CM-52 and DEAE-52 wet powders. Sephadex G-25 (fine) was from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

A culture of *P. fluorescens* (ATCC 13430) was obtained from the American Type Culture Collection. The bacteria were grown in large-scale by the Grain Processing Corp., Muscatin, Iowa, according to conditions described by Ambler (1963). Azurin was extracted and purified according to the procedure described by Ambler (1963) with the following changes: (1) Rivanol treatment was altogether omitted. (2) After the acidification of the extract and the centrifugation to remove the precipitate, the clear supernatant was dialyzed with agitation against 20 volumes of 0.05 M ammonium acetate buffer, pH 3.9, at 4 °C for 14 h. The dialysis medium was changed once during the 14-h period. This process allowed us to collect the azurin and cytochrome *c* components of the supernatant as a narrow band on only one CM-cellulose column and increased our yield. (3) Precipitation with ammonium sulfate was also omitted; instead chromatography on DEAE-cellulose was performed twice. DEAE-cellulose was equilibrated with a pH 9.2, 0.02 M ammonium acetate buffer. Azurin was retarded by the column but did not adhere to it, whereas the contaminating respiratory enzyme components were absorbed at the top 1 or 2 cm. The column was washed with 0.02 M (pH 9.2) ammonium acetate buffer till the azurin band was eluted.

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[1] Abbreviations used: EPR, electron paramagnetic resonance; CM, carboxymethyl; DEAE, diethylaminoethyl.

[2] A strain of *Pseudomonas aeruginosa* was misnamed *Pseudomonas fluorescens* in the past (Ambler and Wynn, 1973). Consequently as pointed out earlier by Grinvald et al. (1975), it is unclear which strain Finazzi-Agrò et al. (1970) had used.

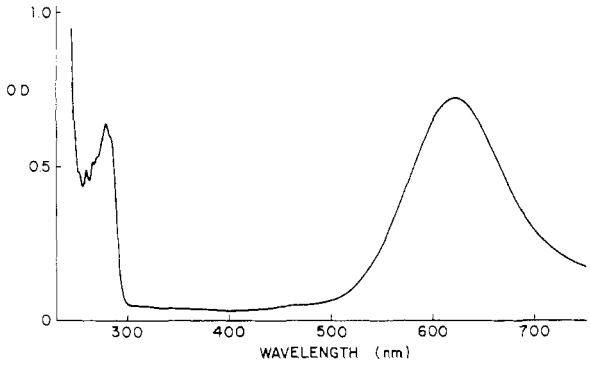

FIGURE 1: Absorption spectrum of native *P. fluorescens* (ATCC 13430) azurin; 0.05 M ammonium acetate buffer, pH 6.0, 1.63×10^{-4} M.

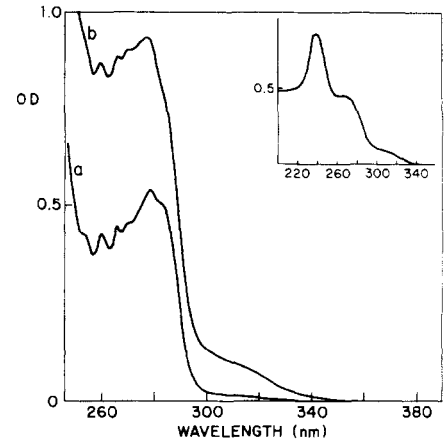

FIGURE 2: Effect of copper ion oxidation state on the ultraviolet absorption of *P. fluorescens* (ATCC 13430) azurin. UV absorption spectrum of oxidized azurin, 1.56×10^{-4} M, pH 6.0 (a); reduced azurin, 1.56×10^{-4} M, pH 6.0 (b). Insert: difference of b from a.

The purity of the preparation was monitored by the $A_{622}{}^{ox}/A_{280}$ ratio, where the $A_{622}{}^{ox}$ is the absorption of a ferricyanide oxidized sample and the A_{280} is the absorption of the untreated native preparation. The purest preparation had an $A_{622}{}^{ox}/A_{280}$ of 1.27 at pH 6.0. Azurin was stored as a pH 3.9, $\sim 10^{-3}$ M solution at 1 °C.

Azurin yields from this organism were very low. From 1 kg of frozen wet paste of bacteria, approximately 60 mg of pure azurin was obtained. With the identical extraction procedure, we were able to obtain yields of 200 mg of azurin per kg of frozen wet paste of *P. aeruginosa*. The large difference in the azurin yields of two closely related species is not understood.

Methods. Reduced azurin was prepared by the addition of a slight excess of dithionite, followed by chromatography on a Sephadex G-25 (fine) column. Reduced azurin samples were used immediately after their preparation; although azurin is classified as nonautooxidizable, a small amount of autooxidation does occur after prolonged exposure to air. Apoazurin was prepared by dialysis at 4 °C against 0.5 M KCN in 0.1 M ammonium acetate buffer; the pH of this solution was 10.

Absorption spectroscopy was performed on a Cary-17 instrument at room temperature. Fluorescence spectra and polarizations were measured on a Schoeffel RRS-1000 fluorimeter. Resolution of the emission and excitation monochromators were set at 3.3 and 3.2 nm, respectively. The fluorimeter was equipped with a quantum counter to which a small fraction of the excitation light is diverted at all times. Excitation spectra were corrected for the wavelength dependent variations in lamp intensity by dividing the signal from the sample with the quantum counter output. Relative quantum yields were calculated from the equation, $Q_1/Q_2 = (A_1/A_2)(OD_2/OD_1)$, where Q is the quantum yield, A is the area of the corrected emission spectrum, and OD is the optical density at the excitation wavelength; the unknowns and the reference (L-tyrosine in H_2O, pH 6.0) were excited at 280 nm. All samples had optical densities ≤ 0.05 at 280 nm and all spectra used in quantum yield calculations were obtained at 25 °C.

Perpendicular and parallel components of the emission at 310 nm were monitored simultaneously through two different monochromators at 10-nm resolution. The excitation source polarization was first set so that the excitation polarization axis and the axes of polarization of the two emission polarizers were all mutually perpendicular. The difference between the readings under these conditions was adjusted to zero through small changes in the two photomultiplier voltages. The excitation polarization was then rotated 90° to obtain the polarization of emission. Readings were signal averaged by sampling the signal approximately every 0.2 s and recording it in the 300 addresses of a Tektronix 31 calculator. All measurements were carried out at 4 °C and pH 6.0. Ethylene glycol–0.05 M ammonium acetate buffer (50:50) was used as the solvent.

I^- quenching measurements were carried out at 22 °C and pH 6.0, using 280-nm excitation. Solutions containing increasing amounts of KI were prepared by diluting stock solutions of KI, KCl, azurin, L-tyrosine, and 0.05 M ammonium acetate buffer into volumetric flasks. KCl was used to keep the ionic strength constant. The iodide solution contained 10^{-4} M $S_2O_3{}^{2-}$ to prevent the formation of $I_3{}^-$. The final protein concentrations were 3×10^{-5} M.

pH was measured with a Corning Model 12 pH meter equipped with a Sargent microelectrode. The pH meter was calibrated at pH 4, 7, 8, and 10. pH titrations were performed by adding microliter quantities of concentrated HCl or NaOH. All solutions used for spectrophotometry were routinely filtered through Millipore filters before they were used.

Results

Absorption Spectra. The oxidized azurin absorption spectrum (Figure 1) has a visible maximum at 622 nm. As expected, the spectrum in the UV lacks the 292-nm peak which is characteristic of the tryptophan residue. When the protein is reduced, the UV spectrum changes drastically (Figure 2). The insert (Figure 2) shows the difference between the reduced and oxidized azurin absorption spectra in the UV. Similar increases in the UV absorption upon reduction of azurin have been reported for tryptophan-containing *P. aeruginosa* and *P. fluorescens* azurins (Yamanaka et al., 1962; Avigliano et al., 1970). The absorption spectrum of the apoazurin (Figure 3a) shows changes both in the UV and in the visible upon addition of Cu^{2+} (Figure 3). From the apoazurin titration with Cu^{2+}, as monitored with absorption spectroscopy, a molar extinction coefficient of 4413 M^{-1} cm^{-1} was obtained for the oxidized azurin maximum in the visible (622 nm); the apo-, oxidized, and reduced azurin molar extinction coefficients for the maximum in the UV (277.5 nm) were calculated to be 3500, 3610 (from Figure 3), and 5950 M^{-1} cm^{-1} (using Figure 2), respectively. The reconstituted azurin had exactly the same $A_{620}{}^{ox}/A_{280}$ value and EPR spectrum as the native oxidized azurin stock solution from which the apoprotein was prepared. The 622-nm extinction coefficient is larger than the 3500 M^{-1} cm^{-1} reported by Brill et al. (1968) for *P. aeruginosa* azurin;

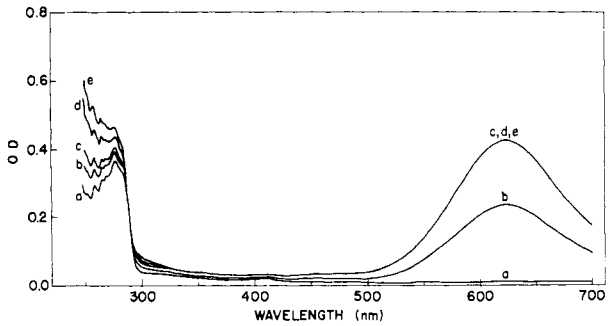

FIGURE 3: Effect of addition of increasing amounts of Cu^{2+} to the absorption spectrum of *P. fluorescens* (ATCC 13430) apoazurin in 0.05 M ammonium acetate buffer, pH 6.0, 9.6×10^{-5} M. Apoazurin (a) plus 0.55 equiv of $CuCl_2$ (b); 1.1 equiv of $CuCl_2$ (c); 1.4 equiv of $CuCl_2$ (d); 1.6 equiv of $CuCl_2$ (e).

however, Rosen and Pecht (1976) have measured an extinction coefficient of 5700 M^{-1} cm^{-1} at 625 nm for *P. aeruginosa* azurin.

Although the absorption spectrum in the visible remains unaltered when Cu^{2+} in excess of 1 equiv is added, the absorption in the UV continues to change (Figure 3). A similar change in the UV absorption is observed when Cu^{2+} is added to the native (oxidized) azurin. This is in contrast to what has been reported for a tryptophan-containing *P. fluorescens* azurin (Finazzi-Agrò, 1970). A second binding site, other than the "blue" coordination moiety, appears to exist for this protein. The maximum for the increase in absorption caused by the excess Cu^{2+} is at approximately 250 nm. An external histidine residue could be the site of binding since a 4:1 imidazole-cupric complex exhibits a maximum in the absorption curve at 278 nm with the maximum shifting to about 250 nm in complexes with fewer imidazole groups (Brill et al., 1964).

Fluorescence Spectra. The fluorescence and the corrected excitation spectra (monitored at 310 nm) for equimolar reduced and oxidized azurin solutions are shown in Figure 4. For both the oxidized and reduced azurins and for apoazurin (not shown), the excitation and emission spectral shapes are clearly that of tyrosine. The difference in intensity between the reduced and oxidized azurin excitation spectra reflects the difference in the intensity of emission at 310 nm. The peak of emission for the oxidized and reduced forms and for apoazurin was at 308 nm (uncorrected spectrum). The tyrosine monomer in H_2O, pH 6.0, however, also showed a 308-nm emission maximum on the same instrument. Although the shapes of the excitation and emission spectra were totally independent of the copper ion oxidation state and the presence or absence of the metal ion, quantum yields showed variations. At 25 °C and in 0.05 M ammonium acetate buffer, pH 6.0, the quantum yields of apo-, oxidized, and reduced azurins were 0.162, 0.097, and 0.087, respectively. The apoazurin and oxidized (native) azurin quantum yields were measured relative to the L-tyrosine monomer whose yield was taken as 0.14 (Chen, 1967). The quantum yield of reduced azurin was measured relative to the oxidized protein. The absorptivity of azurin in the UV increases upon reduction of the azurin bound Cu^{2+} and is larger for the oxidized (native) azurin than for the apoprotein. Consequently, the calculated quantum yields depend on whether these increases in the absorptivity are ascribed to the copper or to the tyrosines themselves. Despite the large changes in the UV absorption spectra, the spectral shape of the fluorescence excitation remains the same for apo-, reduced, and oxidized azurins. Since, as discussed further on, both of the tyrosines

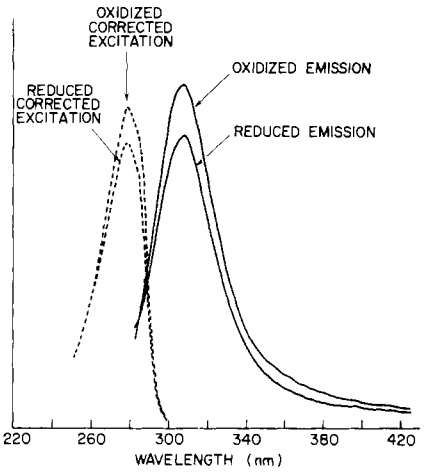

FIGURE 4: Fluorescence emission and corrected excitation spectrum of oxidized and reduced *P. fluorescens* (ATCC 13430) azurin, pH 6.0, 25 °C. Emission monitored at 310 nm for the excitation spectrum. 278-nm excitation was used for the emission spectrum.

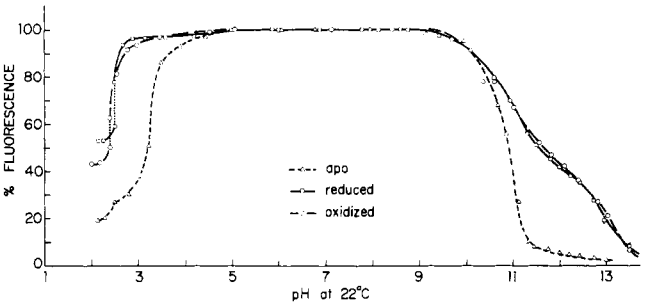

FIGURE 5: Fluorimetric titration of apo, reduced, and oxidized *P. fluorescens* (ATCC 13430) azurin, 22 °C. Excitation λ = 284 nm, emission λ = 294 nm.

appear to be fluorescing with comparable yields, the insensitivity of the fluorescence excitation spectral shape to the metal ion presence or its oxidation state excludes the tyrosines from being the source of the differences in the UV absorptivity. Therefore, in the quantum yield calculations, tyrosine absorptivity was assumed unchanged in the apo-, oxidized, and reduced species; the 280-nm absorption of the reduced and oxidized samples was corrected for the copper center contribution using Figures 2 and 3.

The pH dependence of the fluorescence intensity at 294 nm was studied at 22 °C, using excitation at the isosbestic point (284 nm) (Figure 5). In the alkaline pH range, consistent with the ionization of the tyrosine residues, the fluorescence intensity at 294 nm decreases with increasing pH. Both the biphasic shape of the titration curve and the pH range (~4.5 units) over which the titration occurs indicate that the two tyrosines are both fluorescing, they have different pK_a's, and that tyrosine to tyrosinate energy transfer is negligible. A least-squares fit of the emission intensity (normalized to neutral pH value) vs. pH data for pH ≥8 to the equation

$$f = \frac{f_1}{1 + 10^{(pH - pK_{a1})}} + \frac{f_2}{1 + 10^{(pH - pK_{a2})}} \qquad (1)$$

gave $pK_{a1} = 12.75 \pm 0.04$, $pK_{a2} = 10.78 \pm 0.03$, $f_1 = 0.46 \pm 0.02$, $f_2 = 0.54 \pm 0.02$ for the reduced and $pK_{a1} = 12.80 \pm 0.04$, $pK_{a2} = 10.75 \pm 0.02$, $f_1 = 0.49 \pm 0.02$, $f_2 = 0.51 \pm 0.02$ for the oxidized forms. In this equation $f = F/F_0$ where F_0 is the total fluorescence intensity at neutral pH, and f_i (i = 1, 2) is the fractional contribution to the fluorescence at neutral pH

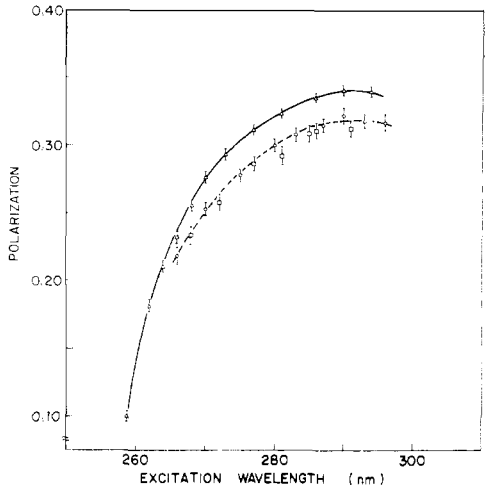

FIGURE 6: Fluorescence emission polarization of *P. fluorescens* (ATCC 13430) apoazurin, 5×10^{-5} M (△), oxidized azurin, 8×10^{-5} M (○), and reduced azurin, 8×10^{-5} M (□). Emission $\lambda = 310$ nm (10-nm resolution). Excitation monochrometer resolution, 3.2 nm. 50:50 ethylene glycol:0.05 M ammonium acetate buffer, pH 6.0, 4 °C.

of the tyrosine which titrates with the ionization constant K_{at}. The titration was reversible from a pH of ~12.5; however, prolonged exposure at this alkalinity prevented the recovery of the original neutral pH yields. In contrast, apoazurin fluorescence decays over approximately 2 pH units and does not exhibit the well-resolved biphasic shape observed for the native protein in its oxidized and reduced forms. The pH at which the apoprotein fluorescence decreases to 50% of its neutral pH value is 10.9 ± 0.1.

The deprotonation of the tyrosines was also followed by monitoring the optical density changes at 295 nm. These optical density changes were converted to changes in the 295-nm extinction coefficient on the basis of azurin concentrations which were calculated from the neutral pH optical density readings and the azurin extinction coefficient at 277.5 nm. A biphasic titration was obtained for the oxidized and reduced azurin; each phase of the titration corresponded to a total change of 2470 in the 295 nm extinction coefficient. From the extinction coefficient of tyrosinate which is ~2300 at 293 nm (Weinryb and Steiner, 1971), it can be seen that each phase of the titration curve indeed corresponds to the deprotonation of one tyrosine. The dissociation constants obtained from these data were also in excellent agreement with those derived from the fluorimetric titration curve. Apoprotein exhibited a monophasic titration of the two tyrosines identical with what was observed by monitoring the fluorescence.

The reduced and oxidized protein fluorescence behave very similarly in the acidic pH range; a time-dependent decrease in the fluorescence intensity (indicated by the dotted lines in Figure 5) was observed at pH 2.5 for the reduced and pH 2.4 for the oxidized protein. This process took place within approximately half an hour, after which no further time dependence in the fluorescence was observed. Concomitant with the decrease in the fluorescence intensity with time at pH 2.4, the characteristic blue color of the oxidized protein also disappeared. Prior to the onset of the time-dependent transition, both the reduced and oxidized azurin fluorescence intensity titrated back along the same curve, initially obtained as a function of decreasing pH. Once the time dependent transition had taken place, neither the original fluorescence intensities nor the blue color of the oxidized protein were recoverable when the pH was raised. A similar time dependence of the fluorescence was not

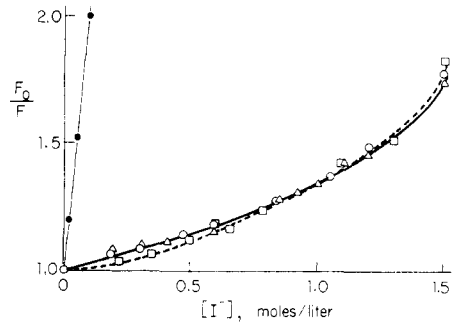

FIGURE 7: Quenching of *P. fluorescens* (ATCC 13430) azurin fluorescence by iodide ion, 22 °C, pH 6.0. Oxidized azurin (○); reduced azurin (△); apoazurin (□); L-tyrosine (●).

detected in the case of the apoprotein. Although the fluorescence intensity decreased with decreasing pH, the onset of the decrease is shifted to higher pH values. In general, the tyrosine fluorescence is quenched by negatively charged moieties such as carboxylic groups (Longworth, 1971). In the acidic pH region where such groups are neutralized upon protonation, an increase in the fluorescence yield of tyrosine containing proteins is seen (Longworth, 1971). This is in marked contrast to what is observed for this protein.

When excess Cu^{2+} was added to a native (oxidized) azurin solution, the fluorescence quenching was minimal; at a level of 2.5 equiv of excess Cu^{2+}, the quenching was only 7%. This indicates that the second binding site for excess Cu^{2+} detected with absorption spectroscopy is not located very near the tyrosines.

Fluorescence Polarization. Protein-bound tyrosine residues could participate in singlet–singlet energy transfer among each other with a consequent decrease in the emission polarization (Longworth, 1971). The results of the fluorescence polarization measurements as a function of the excitation wavelength are shown in Figure 6. The reduced and oxidized azurin polarizations are similar within experimental error. The emission of the apoprotein is polarized to a higher degree than that of the native azurin in the oxidized and reduced forms and is not much different from values observed for *N*-acetyl-L-tyrosine amide (Knopp et al., 1969).

Fluorescence Quenching by Iodide. In order to further investigate the environments of the two tyrosines within azurin, the quenching of the fluorescence by I^- was studied. The results are shown in Figure 7. Although I^- can react with aqueous Cu^{2+} compounds to give Cu^+, the analogous reaction was not observed for oxidized azurin. The quenching profiles of oxidized and reduced azurin were the same within experimental error. Apoprotein quenching exhibited a small but repeatable difference at low I^- concentrations. Azurin, as oxidized, reduced, and apoprotein, does not behave according to the Stern–Volmer law over the range of iodide concentrations used in this work. However, at iodide concentrations lower than 0.6 M, the quenching of the oxidized and reduced azurin fluorescence is linear in the iodide concentration. The Stern–Volmer constant obtained from the linear region is 0.27 M^{-1}. A Stern–Volmer plot for the quenching of the L-tyrosine fluorescence by I^- under identical conditions, on the other hand, is a straight line (Figure 7) and gives a Stern–Volmer constant of 11.4 M^{-1}.

The apoazurin data can be fitted satisfactorily to the equation

$$F_0/F = 1 + 0.132(I^-) + 0.244(I^-)^2 \quad (2)$$

The haloprotein (oxidized and reduced) deviates from this

TABLE I: pK_a's of Tyrosines in Azurins.

Organism	pK's	Source
P. fluorescens (ATCC 13430)		
Oxidized	10.75 ± 0.03, 12.80 ± 0.04	Fluorimetric titration
Reduced	10.78 ± 0.04, 12.75 ± 0.04	
Apo	10.9	
P. fluorescens (containing Trp)		
Oxidized	~12.4	Absorption spectroscopy (Avigliano et al., 1970)
Apo	~12.4	
P. aeruginosa		
Reduced	11.4, 12.5	^{13}C NMR (Ugurbil et al., 1976b)
Reduced	~11.9	absorption spectroscopy

equation for $(I^-) < 0.6$ M. A possible explanation for the quadratic term of eq 2 is that the protein structure is altered at high iodide ion concentrations, allowing more efficient quenching of the tyrosines.

Discussion

The fluorimetric titration data of oxidized and reduced native azurin indicate that the two tyrosines have different pK_a's, both contribute to the total protein fluorescence, and that tyrosine to tyrosinate energy transfer from an un-ionized high pK_a tyrosine to an already ionized lower pK_a tyrosine is negligible. This interpretation is confirmed by the observation that the titration of the tyrosines followed by monitoring the changes in the azurin extinction coefficient at 295 nm yield results in excellent agreement with the fluorimetric data.

The fluorimetric monophasic titration curve of the apoprotein could occur because: (a) the high pK_a emission is quenched by energy transfer to an already ionized lower pK_a tyrosine; (b) only the lower pK_a tyrosine is emissive; or (c) both pK_a's are very similar. The first two explanations are ruled out by the fact that the results obtained by monitoring the absorption or the fluorescence leads to the same single pK. Explanation b would also demand that the fluorescence yield of the emissive residue be 0.32, an unprecedented value. We conclude that the tyrosine of lower pK_a in the metalloprotein has about the same pK_a in the apoprotein but the tyrosine of higher pK_a undergoes a large change in pK_a. This implies that, at neutral pH, the high pK_a tyrosine of the native azurin is in a different environment in the apoprotein, or the apoazurin fails to maintain an intact structure at *alkaline* pH, or both.

The known pK_a's of tyrosines in azurin are listed in Table I. The occurrence of tyrosines with two different pK_a's may be a general phenomenon in azurins. Optical spectroscopy is not as effective as ^{13}C NMR in resolving these two pK_a's. ^{13}C NMR results showed that the lower pK_a tyrosine and the tryptophan are near to but not ligands of the Cu^{2+} in *P. aeruginosa* azurin (Ugurbil et al., 1977b).

The time-dependent decrease in the fluorescence intensity accompanied by the disappearance of the blue color in oxidized azurin indicates that, in the acid pH range, azurin structure about the tyrosines and the coordination of the copper ion are simultaneously perturbed. The shift to higher pH values of this titration process in the apoprotein suggests that the structural perturbation may stem from the protonation of one or more of the copper ligands. The titration of a nonligand residue located in the immediate hydrophobic environment of the metal ion cannot be ruled out. The quantum yields for apo-, oxidized, and reduced azurins at pH 2.1 are 0.03, 0.04, and 0.04 (assuming the tyrosine monomer yield to be 0.14), respectively. These yields are still very large for tyrosines bound to proteins (see below). The acid pH quenching of the fluorescence probably occurs because the unique hydrophobic environment of the two tyrosines (discussed further on) is destroyed at low pH.

The fluorescence from "class A" proteins (those containing phenylalanines and tyrosines but no tryptophan) is generally characterized by very small quantum yields (Longworth, 1971). Possible causes of this quenching are hydrogen bonding of the tyrosine phenoxyl groups to the oxygens of amide carbonyls or carboxylates, collisional quenching by carboxylate side chains, or close proximity to a disulfide bridge (Longworth, 1971). The quantum yield of the "class A" azurin used in this work is unprecedentedly high: 69 and 116% of the L-tyrosine monomer yield for native (oxidized) and apoazurin, respectively. The phosphorescence spectra of the apo and native azurins are both of the *N*-acetyltyrosine amide type (Ugurbil et al., 1977a) and different from the distinct phosphorescence spectra exhibited by tyrosines near disulfide bridges (Longworth, 1968). In the acid pH range, an increase in the fluorescence yield is expected if collisional quenching by carboxylate moieties is significant. Such an increase in the acid pH range does not occur for the native or apoazurin. Therefore, we conclude that *the two tyrosines in both apo- and native azurin are not adjacent to the disulfide bridge, are not surrounded by carboxylate side chains, and are not internally hydrogen bonded.*

At low iodide ion concentrations, where the quenching is linear in the iodide ion concentration, the Stern–Volmer quenching constant is only 2.4% of that for L-tyrosine under identical conditions. This could not be ascribed to short fluorescence lifetimes since the large quantum yields indicate that the fluorescence lifetimes of the azurin bound tyrosines should be comparable to that of free tyrosine. The inefficient quenching could, however, arise from inaccessibility of the tyrosines to the solvent and/or the presence of a large negative change density, i.e., carboxylate moieties, in the immediate vicinity of the tyrosines. The latter possibility can be disregarded on the basis of the quantum yields and fluorimetric titration data in the acid pH range, as has already been discussed.

The high quantum yields and the results of the iodide ion quenching of the fluorescence together indicate that the two tyrosine residues are located in *nonpolar* environments and are *inaccessible* to the solvent, a conclusion consistent with the anomalous pK_a's of these residues. The absence of tyrosine-to-tyrosinate energy transfer at alkaline pH values where the lower pK_a tyrosine is ionized implies that the two tyrosines are well separated from each other. For RNase A, an R_0 (the distance at which energy transfer efficiency is 50%) of 14.4 Å was calculated for a donor quantum yield of 0.1, assuming κ^2 to be $\frac{2}{3}$ (Eisinger and Lamola, 1969). Since 0.1 is equal to the native azurin yield within experimental error, and the emission

spectral shape of azurin is similar to that of RNase A, R_0 of 14.4 Å is applicable to azurin. This implies that the separation between the two tyrosines has a lower limit of ~20 Å, in agreement with the ^{13}C NMR results which indicated that the two tyrosines are not very close to each other (Ugurbil et al., 1977b). Intertyrosine energy transfer is not expected to occur in azurin since the R_0 for this process is less than that for tyrosine-to-tyrosinate transfer (Eisinger and Lamola, 1969). Consequently, a high degree of polarization is expected for azurin fluorescence in the absence of rotational depolarization. For 290-nm polarized excitation, the apoazurin emission polarization is 0.340 ± 0.005, not very different from the reported values of 0.37 ± 0.01 (Knopp et al., 1969) and 0.36 ± 0.01 (corrected to polarized excitation condition from Helene et al., 1968) for *N*-acetyl-L-tyrosine amide and tyrosine, respectively, and much larger than 0.25 for RNase and 0.19 for insulin (corrected to polarized excitation condition from Weber, 1960). In native azurin, the polarization decreases slightly to 0.320 ± 0.007 for 290 nm excitation. Some energy transfer to the metal center which has nonvanishing absorption in the two oxidation states (Figures 2 and 3) could be the cause of this decrease.

^{13}C NMR experiments (Ugurbil et al., 1977a,b) have shown that the tryptophan and the lower pK_a tyrosine in *P. aeruginosa* azurin are both relatively near the copper ion and consequently not very far from each other; an upper limit of ~16 Å for the lower pK_a tyrosine C^γ–tryptophan C^γ separation has been calculated from the ^{13}C NMR data (Ugurbil and Bersohn, 1977). Considering the lower limit of ~20 Å obtained in this work for the tyrosine–tyrosine separation, efficient fluorescence quenching of both tyrosines by the tryptophan residue in the tryptophan-containing azurins appears unlikely. Therefore, contrary to the conclusions reached previously (Finazzi-Agrò et al., 1970; Grinvald et al., 1975), existence of a tyrosine contribution to the fluorescence and phosphorescence of tryptophan containing azurins is indicated. In agreement with this, a distinct tyrosine contribution to the phosphorescence is detectable in *P. aeruginosa* azurin (Ugurbil et al., 1977a).

The cause of the 40% decrease in the native (oxidized) azurin fluorescence relative to the apoprotein yield is not clear. It could not, however, be ascribed to metal ion coordination by either of the tyrosines since ^{13}C NMR results (Ugurbil et al., 1977b) and the presence of emission from both of the tyrosine residues in native azurin rule out this possibility. In the tryptophan containing *P. fluorescens* azurin, the tryptophan quantum yield has been reported to be independent of the metal ion oxidation state and equal to 40% of the apoprotein yield (Finazzi-Agrò et al., 1970). Grinvald et al. (1975) report a decrease in the *P. aeruginosa* apoazurin yield upon binding to Cu^{2+} and ascribe this to a heterogeneous tryptophan population where approximately 60% of the tryptophans are nonfluorescing. Probably, the same process by which the native azurin tryptophan fluorescence is quenched is also responsible for the decrease in the total tyrosine fluorescence in this "class A" native azurin. The nature of this process, however, remains unexplained.

The best characterized "class A" protein is probably RNase A. RNase A has six tyrosines, three of which are buried and hydrogen bonded to two amide and one carboxylate moieties, and three are exposed. The exposed residues titrate normally; however, the protein has to be irreversibly denatured for the deprotonation of the other three (Tanford et al., 1955). Iodide ion quenches 88% of the RNase A emission efficiently (Stern–Volmer constant of 23.2 M^{-1} (Lehrer, 1971)). Therefore it has been concluded that the small ambient yield (~7% of tyrosine yield (Teale, 1960; Cowgill, 1964)) predominantly stems from the exposed residues whose average yields were calculated to be 11 to 14% of the tyrosine yield (Burstein, 1968). The two strongly emitting tyrosines in azurin are internal as evidenced by the inefficient iodide ion quenching (Stern–Volmer constant of 0.27 M^{-1}) and higher than normal pK_a's. The protein need not be denatured for the titration of the tyrosines and is recoverable from a pH of ~12.5. The tyrosines, though internal, are not complexed to amides or carboxylate moieties. Primarily on the basis of RNase A protein-bound tyrosines have been divided into two general classes: (1) fluorescent surface tyrosines, and (2) weakly fluorescent tyrosines which are buried and hydrogen bonded. Tyrosines in azurin, however, with their unique properties fit into a third class: tyrosines buried in nonpolar regions which are very strong emitters of fluorescence.

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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.