

# CONFORMATIONAL HETEROGENEITY OF THE COPPER BINDING SITE IN AZURIN

## A Time-resolved Fluorescence Study

A. G. SZABO, T. M. STEPANIK, D. M. WAYNER, AND N. M. YOUNG

*Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A 0R6*

**ABSTRACT** Comparison of the fluorescence spectra and the effect of temperature on the quantum yields of fluorescence of Azurin (from *Pseudomonas fluorescens* ATCC-13525-2) and 3-methylindole (in methylcyclohexane solution) provides substantive evidence that the tryptophan residue in azurin is completely inaccessible to solvent molecules. The quantum yields of azurin (CuII), azurin (CuI), and apoazurin ( $\lambda_{\text{ex}} = 291$  nm) were 0.052, 0.054, and 0.31, respectively. Other evidence indicates that there is no energy transfer from tyrosine to tryptophan in any of these proteins. The fluorescence decay behavior of each of the azurin samples was found to be invariant with emission wavelength. The fluorescences of azurin (CuII) and azurin (CuI) decay with dual exponential kinetics ( $\tau_1 = 4.80$  ns,  $\tau_2 = 0.18$  ns) while that of apoazurin obeys single exponential decay kinetics ( $\tau = 4.90$ ). The ratio of pre-exponentials of azurin (CuII),  $\alpha_1/\alpha_2$ , is found to be 0.25, and this ratio increases to 0.36 on reduction to azurin (CuI). The results are interpreted as originating from different interactions of the tryptophan with two conformers of the copper-ligand complex in azurin.

### INTRODUCTION

Studies on the structure and function of the blue copper protein, azurin, have been stimulated by its probable role as an electron carrier analogous to plastocyanin (1–3) and its unique spectral properties (4–10). Azurins isolated from different bacterial sources contain a single copper atom per molecule, have similar molecular weights (MW 14,000, 128 residues), and possess a high degree of homology (11–13). A variety of biophysical studies have been directed at elucidating the interactions in the vicinity of the copper binding site, the identity of the copper ligands and the geometry of this complex (14–18).

With the elucidation of the crystal structure of the azurin from *Pseudomonas aeruginosa* the four copper ligands were identified as His 46, His 117, Cys 112, and Met 121 (19). It has also been shown that homologous residues are the ligands to the single copper atom in plastocyanin (20).

The absorption spectrum of the oxidized form of azurin [hereafter designated azurin (CuII)] is characterized in the visible region by an intense band centered at 620 nm. This band is absent in the spectra of both the reduced form of azurin [azurin (CuI)] and apoazurin. In the ultraviolet spectral region there is a sharp maximum at 291 nm for those azurins containing a tryptophan residue, as well as the usual maximum at 278 nm. Excitation at 291 nm gives rise to an emission maximum at 308 nm in the fluorescence spectrum (5, 9). It has been suggested that this unusual spectral behavior indicates that the single tryptophan

residue is located in an hydrophobic environment in the protein. The x-ray crystallographic studies (19) supported this assignment, placing the tryptophan residue in the interior of the molecule within a "barrel" of peptide chains.

Apoazurin has a dramatically higher intensity of fluorescence when compared with azurin (CuI) and azurin (CuII). The fluorescence efficiencies of these species had not been quantified previously, and it was not clear from the literature whether energy transfer from tyrosine to tryptophan contributes to the fluorescence spectral intensities (14, 21).

Two groups have investigated the fluorescence decay parameters of azurin, and, although the lifetime values and pre-exponential terms differ, both groups report that the tryptophan fluorescence decays with double exponential kinetics (21, 22). For apoazurin however, one group (21) reported that its fluorescence decay obeys single exponential kinetics while the other group (22) reported dual exponential kinetics.

Because of the detailed knowledge of their structure and their unique spectroscopic properties, the azurins offer a convenient model for studies of the intramolecular interactions and properties of the copper binding site for other cuproproteins. In this report we present our ultraviolet absorption, steady-state fluorescence, and fluorescence decay experiments on the azurin from *Pseudomonas fluorescens*, which were undertaken to clarify the relationship between these properties and the structural features which give rise to them. From fluorescence decay measurements

at several emission wavelengths we have characterized the fluorescence heterogeneity of azurin and have determined the relative populations of at least two protein conformations in the vicinity of the tryptophan residue.

## MATERIALS AND METHODS

Azurin was isolated from the acetone powder of *P. fluorescens* (ATCC 13525-2) by the procedures of Ambler (23, and personal communication from R. P. Ambler) except for the following modifications. The step involving ion-exchange chromatography on DEAE-cellulose was omitted and the final purification step was by gel filtration on a Sephadex G-75 Superfine column ( $2.2 \times 100$  cm, flow rate 8.0 ml/h) using 10 mM cacodylate buffer, pH 5.1 as the eluant. Fractions from this column, with  $A_{620}/A_{280}$  ratio  $\geq 0.47$ , were combined. This stock solution was stored frozen at  $-20^\circ\text{C}$ . Protein concentrations were determined using  $A_{280}^{1\%} = 6.0$  (11).

In addition to examination of its spectral features, the purity of the preparation was checked by both disk gel electrophoresis (method 14 in reference 24) and slab gel electrophoresis in the presence of sodium dodecyl sulfate and 8 M urea (25). Under these conditions only one component, with an apparent molecular weight of 14,000, was evident in both gel systems.

Apoazurin was prepared from azurin (CuII) by dialysis (Spectra/Por dialysis tubing [Fisher Scientific Ltd., Ottawa, Ont.], MW cutoff 6,000–8,000) at  $4^\circ\text{C}$  against 0.1 M phosphate buffer, pH 7.6, containing 0.05 M potassium cyanide. The apoazurin was then equilibrated with 10 mM cacodylate buffer, pH 5.1, by gel filtration through Sephadex G-25. Azurin (CuI) was prepared by the addition of an excess of sodium hydrosulfite (dithionite), the reaction being monitored by the disappearance of the absorption band at 620 nm.

The ultraviolet difference spectrum between azurin (CuII) and azurin (CuI) was obtained in the following manner. Equal volumes (10  $\mu\text{l}$ ) of a fresh sodium hydrosulfite solution (0.1 M) in a 0.01 M cacodylate buffer, pH 5.3 or buffer solution only were added to equal volumes (2 ml) of azurin (CuII) ( $7 \times 10^{-5}$  M) contained in separate cuvettes. A blank containing the same concentration of dithionite as present in one of the above solutions was also prepared. A tenfold excess of fresh reducing agent was sufficient to cause complete loss of the 620 nm band in the spectrum of azurin (CuII). It was found that the spectrum of the sample containing dithionite alone included a broad band centered at 315 nm (minimum at 275 nm  $A_{315}/A_{275} = 3.5$ ). After a period of 2 h the dithionite was judged to have decomposed completely, by the loss of absorbance above 250 nm in its spectrum (compared with a buffer blank). Then the spectrum of each azurin sample was measured against its corresponding blank solution. Samples for the determination of the quantum yields of azurin (CuII) and azurin (CuI) were similarly prepared except that the absorbance at the excitation wavelength was between 0.05 and 0.07.

For some experiments azurin (CuII) and apoazurin were equilibrated with cacodylate buffer solutions of deuterium oxide (Merck Sharp & Dohme Canada Ltd, Montreal, Quebec, 99.7%  $\text{D}_2\text{O}$ ) as solvent or with aqueous cacodylate buffer solutions containing 6 M guanidinium chloride (Heico Inc., Delaware Water Gap, PA, extreme purity grade) by extensive dialysis of the protein stock solution against the appropriate buffer.

## Fluorescence Decay Techniques

The fluorescence decay experiments were performed using the technique of time-correlated single photon counting (26, 27). The instrumentation and data measurement methods and analysis used in our laboratory have been described in detail (28, 29).

The excitation time intensity profile and the sample fluorescence decay profile (hereafter referred to as the excitation profile and sample profile respectively) were each collected in a minimum of 512 channels in a multichannel analyser (MCA). The channel width was usually 0.043

ns/channel. To optimize the signal-to-noise ratio, a minimum of 10,000 counts were usually collected in the peak of the sample profile. The photomultiplier time response variation with wavelength was accounted for by correction techniques described earlier (28, 29).

The fluorescence decay parameters that describe the physical model of the excited state deactivation function,  $G_{em}(t)$ , are obtained by nonlinear least-squares fitting procedures. In these procedures a physical model for the sample decay is assumed, and the kinetic expression that describes this model is convolved with the excitation profile. The best fit parameter values are those that minimize the weighted sum of squares of residuals (WSSR) (30). The adequacy of the decay model and the parameter values are judged from statistical criteria such as WSSR and by an inspection of the weighted residual plot. If the residuals are not randomly distributed then the physical model and kinetic parameters do not properly describe the fluorescence decay behavior of the sample and the proposal of another physical model is required.

In many cases the fluorescence of a molecule obeys decay kinetics described by a sum of exponentials:

$$G_{em}(t) = \sum_{i=1}^n \alpha_i \exp(-t/\tau_i) \quad (1)$$

where  $\alpha_i$  is the pre-exponential term and  $\tau_i$  is the singlet lifetime of the  $i$ th component of the fluorescence. The  $\alpha_i$  terms, in simple models, may be related to the fluorescence quantum efficiency and the  $\tau_i$  are the reciprocal of the sum of the excited singlet state deactivation rate constants.

The wavelength of the excitation monochromator was 290 nm unless otherwise noted and the excitation and emission monochromator bandwidths were 4 and 8 nm, respectively.

In experiments that checked for the effect of fluorescence depolarization of emission on the decay parameters, ultraviolet polarizer filters (Polacoat) (Polaroid Corp., Cambridge MA) were used. The excitation polarizer was set for vertical polarization and the emission polarizer was set at an angle of  $55^\circ$  from the vertical.

Corrected emission spectra were measured on a Perkin-Elmer MPF-44A spectrofluorimeter equipped with a DCSU-2 microprocessor correction unit. The analog signal was passed into an analog-to-digital converter that was interfaced to a 32K Commodore PET minicomputer having a Commodore 2040 dual disk drive for data storage (Commodore Business Machines, Santa Clara, CA). This configuration allowed the storage and manipulation of individual spectra. Solvent blanks could be subtracted and the spectra integrated using a trapezoidal approximation for quantum yield measurements. Absorption spectra were measured on a Cary 219 or 118 spectrophotometer (Cary Instrumans, Monrovia, CA).

All solutions except those for temperature studies were in 0.01 M cacodylate buffer (pH 5.3). For the temperature studies the solutions were 20% ethylene glycol (Matheson Coleman and Bell [MCB Reagents, Cincinnati, OH], chromatography grade) – 0.01 M cacodylate. The optical densities of the solutions at 292 nm were between 0.05 and 0.07 for quantum yield measurements. A degassed solution of freshly sublimed 3-methylindole (Aldrich Chemical Co., Inc., Milwaukee, WI) in methylcyclohexane (Caledon Laboratories, Georgetown, Ontario, distilled in glass grade, distilled from  $\text{P}_2\text{O}_5$ ) was used as a quantum yield standard. The quantum yields were corrected for the difference in refractive index between the reference solvent and buffer where required.

## RESULTS

### Absorption Spectra of Azurins and the Effect of Dithionite Reduction

The absorption spectra of azurin (CuII), reduced azurin (CuI), apoazurin, and denatured azurin (6 M guanidinium chloride solution) are similar to those reported earlier (4, 5, 9). The absorption spectra of azurin (CuII), azurin (CuI)

and apoazurin all have a sharp transition near 291 nm. In the absorption spectrum of azurin (CuII) in 6 M guanidinium chloride solution, the band at 291 nm is lost but a shoulder at 287 nm is found on the main ultraviolet band ( $\lambda_{\text{max}} = 285 \text{ nm}$ ).

When azurin (CuII) is reduced by dithionite to azurin (CuI), ultraviolet absorbance changes were observed. The spectra (Fig. 1) have similar band shapes at wavelengths  $>270 \text{ nm}$  except for the loss of the 625 nm absorbance. On reduction of azurin (CuII) there is an increase in absorbance of between 5 to 15% over the measured spectral range. There is also a small reproducible shift in the position of the sharp maximum from 291 nm in the spectrum of azurin (CuII) to 292 nm in the spectrum of azurin (CuI). These changes are evident in the difference spectrum shown in Fig. 1b.

This difference spectrum is very similar to that reported by Pecht et al. (31) for the azurin from *P. aeruginosa*. The small shift of the 291 nm band is also reflected in their difference spectrum. In contrast Ugurbil and Bersohn (32), in their work on the azurin from *P. fluorescens* (ATCC 13430) which lacks tryptophan, showed an increase in absorbance of  $>100\%$  in the ultraviolet spectral region, upon dithionite reduction. The general shape of their difference spectrum was similar to that we observed, but is greatly different in intensity, and lacks the feature arising from the 291 nm band. Yamanaka et al. (4) reported an increase of 45–60% in the absorbance of the

ultraviolet spectrum of *P. aeruginosa* azurin after reduction.

### Fluorescence Spectra and Fluorescence Efficiencies of Azurin

The fluorescence spectra [ $\lambda_{\text{ex}} = 291 \text{ nm}$ ] of azurin (CuII), azurin (CuI), and apoazurin have similar band shapes with a maximum at 308 nm (Fig. 2). The fluorescence intensity of the apoazurin spectrum was nearly six times that of azurin (CuII). There was negligible fluorescence intensity difference between the spectra of azurin (CuII) and azurin (CuI) solutions, which contained equal concentrations of protein. Denaturation of azurin (CuII) with 6 M guanidinium chloride shifted the maximum in the fluorescence spectrum to 351 nm (Fig. 3).

The excitation spectra [ $\lambda_{\text{em}} = 310 \text{ nm}$ ] of samples of azurin (CuII) and azurin (CuI) containing equal concentrations of azurin were compared to determine whether the increase in absorbance of the latter sample was related to the fluorescence transitions. Fig. 4 clearly shows that the absorption spectral differences were not reflected in their fluorescence spectra. Furthermore, when the excitation spectra, normalized at their maxima, (excitation spectrum  $\lambda_{\text{max}} = 278 \text{ nm}$ ) of azurin (CuII), azurin (CuI), and apoazurin were compared, they were all found to be nearly superimposable.

We found that 3-methylindole in methylcyclohexane solution is a useful model for the tryptophan residue in azurin. The fluorescence spectrum of 3-methylindole in

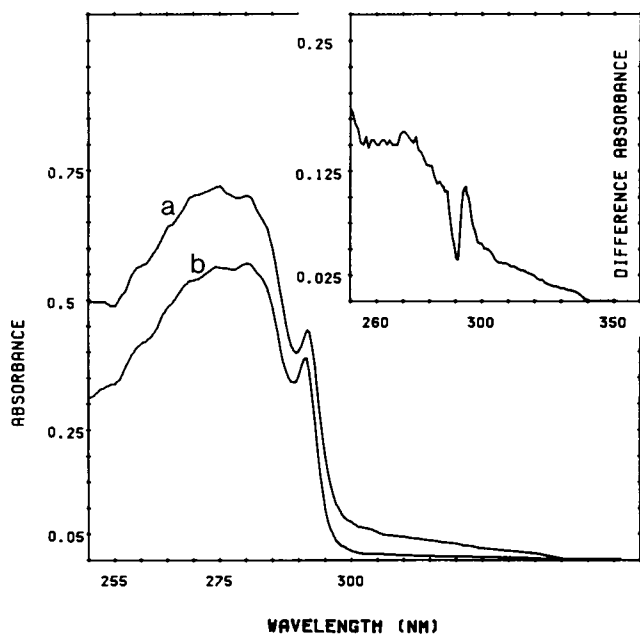


FIGURE 1 Absorption spectra of (a) azurin (CuII) vs. buffer blank; (b) azurin (CuI) vs. blank containing buffer and same amount of sodium hydrosulfite required to reduce azurin (CuII). The azurin (CuI) spectrum was recorded 2.5 h after the addition of sodium hydrosulfite to azurin (CuII) and buffer blank. Concentrations were  $7 \times 10^{-5} \text{ M}$  in azurin, pH 5.3. *Inset* is the difference between the above absorption spectra.

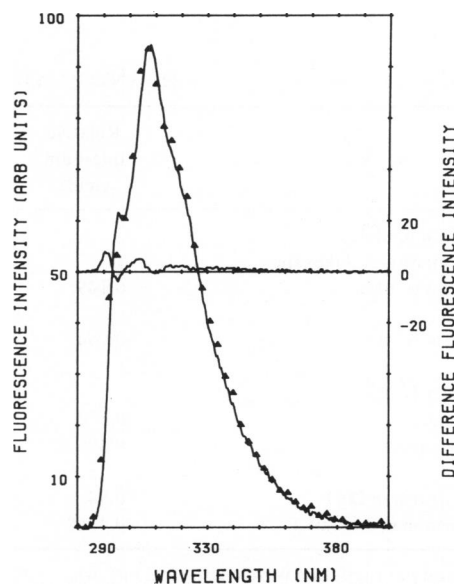


FIGURE 2 Corrected fluorescence spectra of apoazurin (0.01 M cacodylate buffer, pH 5.3) (solid line) and 3-methylindole in methylcyclohexane solution (triangles) normalized to have the same fluorescence intensity at their maxima. The respective blanks have been subtracted. The center line is the difference between these spectra.  $\lambda_{\text{ex}} = 292 \text{ nm}$ ; excitation and emission bandwidths were 3 nm.

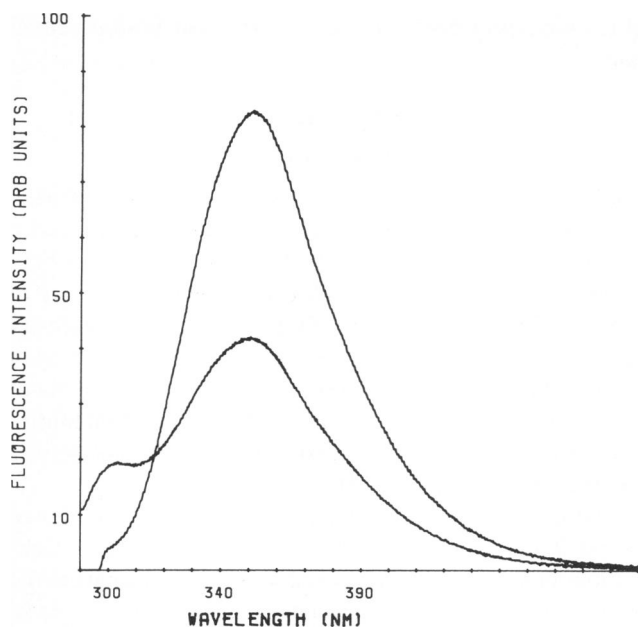


FIGURE 3 Corrected fluorescence spectra of azurin (CuII) ( $\sim 8 \times 10^{-6}$  M) in 6 M GdmCl, pH 5.3. Excitation and emission bandwidths were 3 nm. (a)  $\lambda_{ex} = 275$  nm; (b)  $\lambda_{ex} = 292$  nm.

methylcyclohexane solution (Fig. 2) had a band shape that was very similar to that of the azurins except that the maximum was at 305 nm. In Fig. 2 the difference between the fluorescence spectra of apoazurin and 3-methylindole is shown. As suggested by the preliminary spectral examination there were no significant differences between these spectra.

Table I documents the quantum yields at 20°C of azurin

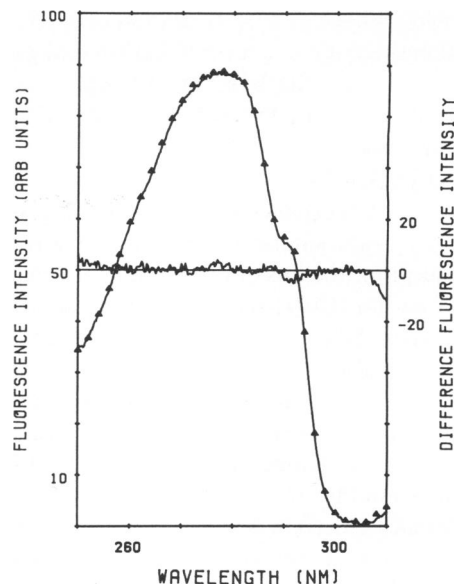


FIGURE 4 True fluorescence excitation spectra of azurin (CuII) (solid line) and azurin (CuI) (triangles) containing equal concentrations of azurin ( $7 \times 10^{-6}$  M) in 0.01 M cacodylate buffer, pH 5.3.  $\lambda_{em} = 310$  nm. Excitation and emission bandwidths were 3 nm. The buffer blank spectrum has been subtracted from each spectrum. The center line is the difference between the two excitation spectra multiplied by a factor of 5.

and apoazurin samples when excited at 291 nm and the conflicting values from two previous studies. 3-Methylindole in methylcyclohexane solution was used as a quantum yield standard ( $\phi = 0.35$  [Skalski, Krajcarski, and Szabo, unpublished manuscript]) for all samples except azurin in guanidinium chloride solution. The refractive index differ-

TABLE I  
QUANTUM YIELDS OF AZURIN AND APOAZURIN\*

Sample	Relative quantum yield‡	Quantum yield	Literature value§	Reference
3-Methylindole in methylcyclohexane	1	0.35	0.35	47
Azurin (CuII)	0.149	0.052	0.031–0.037 0.04	21 5
Azurin (CuI)	0.154	0.054   0.048		
Azurin (CuII) in D <sub>2</sub> O	0.177	0.062		
Apoazurin	0.89	0.31	0.20 0.10	21 5
Apoazurin in D <sub>2</sub> O	0.94	0.33	0.23	21
Azurin in 6M GdmCl	0.486‡	0.068		

\*Optical density at excitation wavelength (292 nm) was  $<0.06$ ; excitation and emission bandwidth, 3 nm.

‡Relative quantum yield (corrected for refractive index difference) is that compared with the integrated fluorescence intensity of a standard, which was 3-methylindole in methylcyclohexane solution in all cases except for azurin in 6M GdmCl where it was *N*-acetyltryptophanamide in aqueous buffer at pH 7.

§The literature values of Grinvald et al. (21) used *N*-acetyltryptophanamide as a standard that had a quantum yield of 0.13. The standard used by Finazzi-Agro (5) was not given.

||Two values for the quantum yield are given for azurin (CuI). The larger value (0.054) is that obtained when the absorbance is taken as that of an equivalent concentration of azurin (CuII). The lower value (0.048) is that which takes into account the increase in absorbance on reduction.

ence of the reference and sample solutions was taken into account. For the latter sample, *N*-acetyltryptophanamide (NATA) in aqueous buffer, pH 7 was used as a quantum yield standard ( $\phi = 0.14$  [26]). Two values are given for azurin (CuI). The larger value ( $\phi = 0.054$ ) is that value based on the absorbance at 291 nm of the azurin (CuII) sample from which it was prepared. The smaller value ( $\phi = 0.048$ ) was obtained when the increase in absorbance at the excitation wavelength is taken into account.

There was a sixfold increase in the fluorescence efficiency when the copper atom is removed from azurin to

give apoazurin. This agrees with the ratio found for *P. aeruginosa* azurin as reported by Steinberg and co-workers (21), while Finazzi-Agro et al. (5) reported a 2.5-fold increase in the fluorescence efficiency of apoazurin compared with azurin. The similarity between the quantum yields for 3-methylindole in the nonpolar solvent, methylcyclohexane, and of apoazurin is noteworthy.

The effect of temperature on the quantum efficiency of azurin (CuII), apoazurin and 3-methylindole (methylcyclohexane solution) was studied. As described by Kirby and Steiner (33) if the radiative rate constant,  $k_f$  is independent of temperature, and there is only a single significant excited state deactivation process, then the following expression describing the temperature effect of fluorescence quantum yield may be written:

$$1/\phi - 1 = (f/k_f) \exp(-E/RT). \quad (2)$$

Hence a plot of  $\log(1/\phi - 1)$  vs.  $1/T$  would be linear with slope equal to  $-E/(\ln 10)R$  and an intercept of  $\log(f/k_f)$  where  $f$  is the frequency factor of the temperature dependent deactivation process.

The fluorescence efficiency as a function of temperature of these samples is shown in Fig. 5a. It is evident that the fluorescence decreased uniformly with temperature from 5° to 40°C with a change of 15% between these two temperatures. The small increase in the fluorescence of azurin and apoazurin between -5° and 5°C was reproducible.

A plot of  $\log(1/\phi - 1)$  against  $1/T$  for each sample is shown in Fig. 5b and the slopes, intercepts, and activation energies for the deactivation process are given in Table II. These plots were all linear and the activation energies for the temperature-dependent deactivation process for each protein was of the same order as that for 3-methylindole in methylcyclohexane. It is interesting that the activation energy for azurin (CuII) was the lowest measured and the entropic frequency factor term,  $f$ , was the highest. Similar activation energies for excited state deactivation processes of indoles in nonpolar solvents have been reported (34). In the few cases where the temperature dependence of the tryptophan fluorescence of proteins has been studied activation energies between 2.5 and 4 kcal/mol have been measured (35).

### Fluorescence Decay Measurements

The fluorescence decays of the various azurin samples were measured at several emission wavelengths using the time-correlated single-photon counting technique. Typical excitation and sample fluorescence time-intensity profiles are shown in Fig. 6. It was found that the fluorescence decay data of azurin (CuII) and azurin (CuI) could not be adequately fitted to a single exponential decay model. The plot of weighted residues shown in Fig. 7a is clearly not random. The residual plot, Fig. 7b, and other statistical criteria show that the fluorescence decay is best repre-

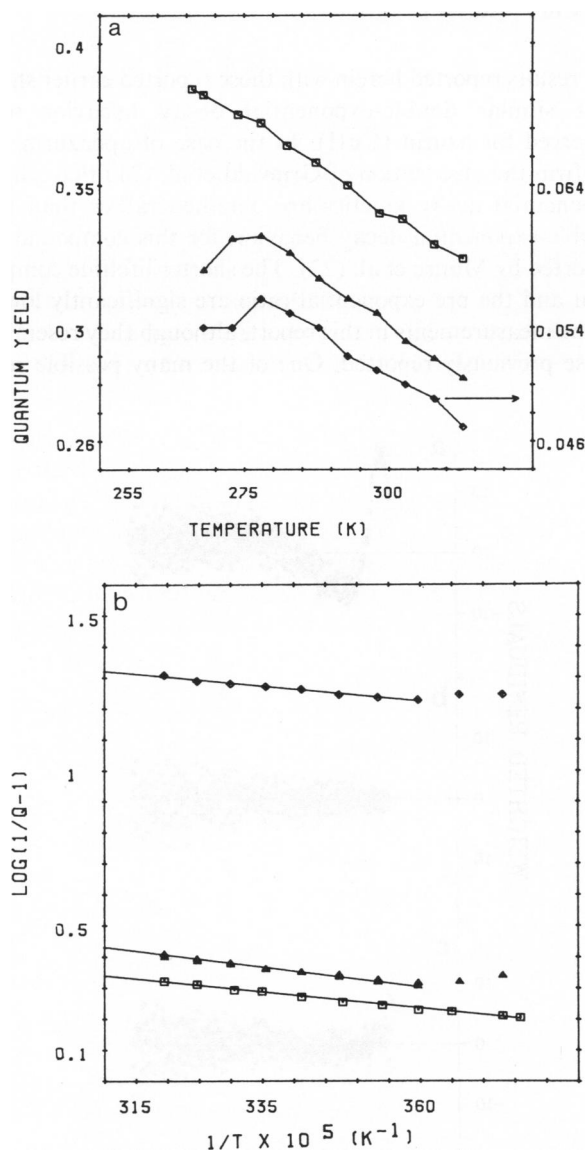


FIGURE 5 (a) Plot of temperature effect on fluorescence quantum yield of 3-methylindole (methylcyclohexane solution),  $\square$ ; apoazurin,  $\Delta$ ; and azurin (CuII),  $\diamond$ . The left hand ordinate is that for 3-methylindole and apoazurin samples and the right hand ordinate is for azurin (CuII). The azurin samples were in 0.01 M cacodylate buffer, pH 5.3, 20% ethylene glycol.  $\lambda_{ex} = 292$  nm;  $\lambda_{em} = 310$  nm. (b) Plot of  $\log(1/\phi - 1)$  vs.  $1/T$  for 3-methylindole (methylcyclohexane solution),  $\square$ ; apoazurin,  $\Delta$ ; and azurin (CuII),  $\diamond$ ; of data presented in Fig. 5a.

TABLE II  
PARAMETERS OF THE TEMPERATURE EFFECT ON FLUORESCENCE\*

Sample	Slope‡	Intercept§	Frequency factor	Activation energy
	( $10^3 K^{-1}$ )		( $10^8 s^{-1}$ )	( $kcal \cdot mol^{-1}$ )
3-Methylindole in methylcyclohexane	$0.21 \pm 0.01$	$0.98 \pm 0.02$	6.6	$0.97 \pm 0.07$
Azurin (CuII)	$0.17 \pm 0.02$	$1.8 \pm 0.07$	43	$0.78 \pm 0.09$
Apoazurin	$0.23 \pm 0.01$	$1.14 \pm 0.02$	9.5	$1.06 \pm 0.07$

\*The slope and intercepts were obtained by a linear regression analysis of the combined data from two separate experiments.

‡The slope is obtained from the plots in Fig. 5b and equals  $-E/(\ln 10)R$ .

§The intercept from the same plots equal  $\log(f/k_f)$ .

||The frequency factor,  $f$ , was estimated from the intercept and a value of  $k_f = 6.9 \times 10^7 s^{-1}$  (see text).

sented by a double exponential decay function with lifetimes for azurin (CuII) [ $\lambda_{ex} = 290$  nm,  $\lambda_{em} = 310$  nm] of 4.78 and 0.17 ns with a ratio of pre-exponential terms,  $\alpha_1/\alpha_2$ , of 0.25. Similar singlet lifetime values were obtained for azurin (CuI) but the ratio of pre-exponentials,  $\alpha_1/\alpha_2 = 0.36$ , was higher in this case.

For apoazurin the fluorescence decay obeyed single exponential kinetics, as evidenced by an inspection of the residual plot, Fig. 7c, giving a single lifetime of 4.86 ns.

The fluorescence decay of azurin samples measured at several emission wavelengths showed no significant differences in the decay parameters obtained. The fluorescence decay of azurin or apoazurin in 6 M guanidinium chloride also obeyed double exponential decay kinetics but with markedly different values from the native forms ( $\tau_1 = 2.92$  ns,  $\tau_2 = 0.72$  ns,  $\alpha_1/\alpha_2 = 0.66$  [330 nm]). These values and other fluorescence decay results are summarized in Table III, along with the values reported by Grinvald et al. (21), who used sampling techniques, and Munro et al. (22) who used time-correlated single-photon counting, but with synchrotron-generated excitation pulses. A comparison of

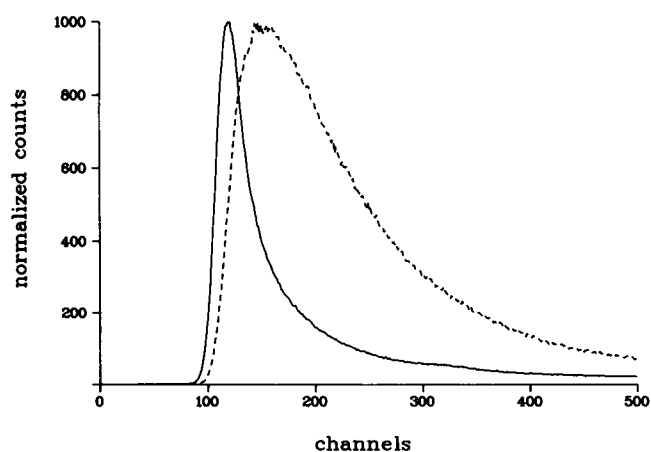


FIGURE 6 Typical normalized fluorescence time-intensity profiles for decay measurements reported here. The lamp intensity profile (solid line) and azurin fluorescence decay profile were recorded with a channel width = 0.043 ns.  $\lambda_{ex} = 290$  nm;  $\lambda_{em} = 310$  nm; excitation bandwidth = 4 nm; emission bandwidth = 8 nm.

the results reported herein with those reported earlier show that similar double-exponential decay behavior was observed for azurin (CuII). In the case of apoazurin we confirm the observation of Grinvald et al. (21) that single exponential decay kinetics are obtained rather than the double-exponential decay behavior, for this compound as reported by Munro et al. (22). The shorter lifetime component and the pre-exponential ratio are significantly lower in the measurements in this report, although they resemble those previously reported. One of the many possible rea-

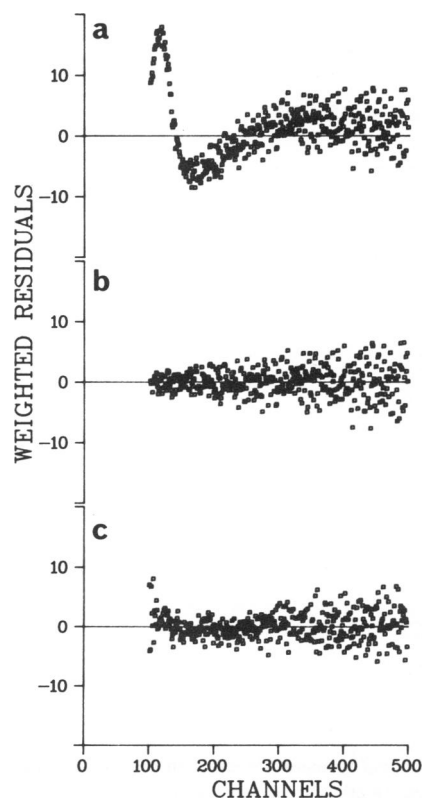


FIGURE 7 Weighted residual plots for calculated best fit emission decay profile after deconvolution of the corrected emission decay profile: (a) for azurin Cu(II) fitted to a single exponential decay component; (b) for azurin (CuII) fitted to two exponential decay components; (c) apoazurin fitted to one exponential decay component.

TABLE III  
FLUORESCENCE DECAY PARAMETERS\*

Sample‡	$\lambda$ (em)	$\tau_1$	$\tau_2$	Ratio of preexponentials§
	(nm)	(ns)	(ns)	
3-Methylindole in methylcyclohexane	310	4.20	—	—
Azurin (CuII)	310	4.78	0.17	0.25
	325	4.86	0.19	0.27
	345	4.75	0.18	0.26
Literature value (21)	>310	4.5	0.8	0.54
Literature value (22)	>300	4.15	0.75	1
Azurin (CuII) in D <sub>2</sub> O	310	5.04	0.20	0.27
	325	5.05	0.19	0.28
Azurin (CuI)	310	4.89	0.15	0.36
	325	4.98	0.16	0.31
Literature value (21)	>310	4.8	0.4	0.7
Azurin (CuI) in D <sub>2</sub> O	310	5.03	0.14	0.35
	325	5.13	0.16	0.37
	345	5.0	0.16	0.36
Apoazurin	310	4.86	—	—
	325	4.94	—	—
Literature value (21)	>320	4.7	—	—
Literature value (22)	>300	4.79	0.88	0.67
Apoazurin in D <sub>2</sub> O	310	5.30	—	—
	325	5.25	—	—
Literature value (21)	>310	5.1	—	—
Azurin   in (6M GdmCl)	330	2.99	0.76	0.66
	330	2.87	0.61	0.64
$\lambda$ (em) = 280 nm				
Apoazurin   in 6M GdmCl	330	2.92	0.72	0.66
	360	3.12	0.85	0.66
Literature value (21)	>320	3.4	1.2	0.6

\*The fluorescence decay parameters are the best fit values, which are obtained from the exponential decay model. Each value is the mean of at least two determinations. Typical standard errors are for  $\tau_1$ ,  $\pm 0.03$ ; for  $\tau_2$ ,  $\pm 0.04$ ; for  $\alpha_1/\alpha_2$ ,  $\pm 0.04$ .

‡All samples were in aqueous buffer, 0.01 M cacodylate, pH 5.3 unless otherwise stated. The samples in D<sub>2</sub>O were similarly buffered.

§The ratio of pre-exponentials is  $\alpha_1/\alpha_2$ .

||The samples were in 6M guanidinium chloride (GdmCl) with the excitation polarized vertically and the emission polarizer set at 55° to the vertical.

sons for these discrepancies is that these measurements are for the fluorescence decay of azurin from *P. fluorescens* while the other reports are for the azurin from *P. aeruginosa*. Also, the earlier reports did not indicate whether corrections for the variation in instrument response with wavelength were made, nor was the channel width reported.

## DISCUSSION

### 3-Methylindole as a Model for Azurin Fluorescence

The spectral and quantum yield measurements detailed in this report provide convincing evidence that the single tryptophan residue in azurin and apoazurin is in a very hydrophobic, nonpolar environment and is completely

inaccessible to aqueous solvent. Further, the results show that the spectral properties of 3-methylindole in methylcyclohexane solution serve as a convenient model for those of the tryptophan in azurin and apoazurin.

The sharp peak at 291 nm in the absorption spectra of the intact azurin samples corresponds to the distinct 289 nm transition in the spectrum of 3-methylindole in nonpolar solvents. The absorption spectrum of 3-methylindole in aqueous solution has lost this feature and instead a broad shoulder at 287 nm is observed, similar to that found in the spectrum of azurin in the denaturing solvent system of 6 M guanidinium hydrochloride.

This absorption band correspondence between azurin and 3-methylindole, together with their similar fluorescence spectra, as shown by the difference fluorescence spectrum (Fig. 2), and the effect of temperature on the fluorescence quantum yield of azurin and 3-methylindole (methylcyclohexane), confirms that the emission spectrum [ $\lambda(ex) = 291$  nm] for azurin originates from the tryptophan residue in azurin.

It has been shown that the fluorescence maxima of many proteins containing "buried" tryptophan residues occurs between 325 and 335 nm (36), while the maxima for proteins containing "exposed" tryptophan residues are shifted to lower energy. In all cases their fluorescence band shapes are broad. Similarly it has been adequately demonstrated that the fluorescence spectral maxima of indoles are shifted to lower energy in polar solvents (37). Recently Skalski et al. (38) have shown that this spectral shift can be rationalized in terms of ground state complex formation between the indole and the polar solvent. The similarity not only in the emission maxima but also in the band shape in the fluorescence spectra of 3-methylindole in methylcyclohexane and the azurins indicate that water is excluded from the interior of the protein in the region of the tryptophan residue at ambient temperatures. This has been suggested by the crystallographic model of azurin (CuII) (*P. aeruginosa*) (19) and the low temperature (1.2 °K) ODMR (optical detection of magnetic resonance) measurements (14, 39). The lack of quenching of azurin fluorescence by iodide ion (up to 0.5 M) (40) and more recently (41) evidence using acrylamide quenching confirm the inaccessibility of the tryptophan to solvent.

The similarity in the fluorescence spectral band shapes of the intact azurin samples show that reduction to azurin (CuI) or removal of the copper does not significantly affect the environment of the tryptophan within the protein. Moreover the identical excitation spectra [ $\lambda_{em} = 310$  nm] of azurin (CuII), azurin (CuI), and apoazurin requires that there are no differences in the tyrosine environments in these three samples, nor in the tyrosine-tryptophan interactions. In the case of azurin (CuII) and azurin (CuI) where an increase in the absorption is observed for the reduced form, the excitation spectral evidence indicates that the increased absorbance cannot be due to changes in the interactions or environments of the aromatic amino

acids, tyrosine or tryptophan. Rather the implication is that the increase in absorbance originates from a contribution to the total absorbance by the reduced copper atom.

### Energy Transfer in Azurins

The contribution of tyrosine fluorescence and tyrosine-to-tryptophan energy transfer in azurin has not been heretofore clearly resolved. The establishment of 3-methylindole in methylcyclohexane as a good model for the tryptophan emission in azurin or apoazurin allows us to address this issue.

The ratio of the fluorescence intensities of azurin (CuII) at 308 and 320 nm depends on the excitation wavelength (Table IV) being 1.51 for excitation at 275 nm, and 1.41 for excitation at 291 nm. The inequality of these ratios indicates that there must be a tyrosine fluorescence contribution to the fluorescence spectrum obtained with 275 nm excitation. The extent of this contribution and whether there is any energy transfer from tyrosine to tryptophan as well can be deduced from the following arguments.

The absorbance of 3-methylindole at 275 and 289 nm are nearly equivalent. Because it has been shown that 3-methylindole in methylcyclohexane solution is a good model for the spectral properties of the tryptophan in azurin, the assumption is made that the absorbance of this tryptophan at 275 nm is nearly equivalent to the absorbance of the band at 291 nm which corresponds to the 289 nm band in the 3-methylindole spectrum. It has previously been concluded (see above) that there cannot be any contact between the aqueous solution and the azurin tryptophan, and therefore standard spectral data (42) cannot be taken as estimates of the absorbance data of the tryptophan in this protein. We therefore suggest that the fluorescence resulting from 291 nm excitation may be taken as a good estimate of the tryptophan fluorescence resulting from the direct excitation at 275 nm of the tryptophan residue. In the absence of fluorescence energy transfer from tyrosine to tryptophan, the tyrosine fluorescence contribution at 308 nm may be calculated

$${}_{275}F_{308}(\text{Tyr}) = {}_{275}F_{308} - {}_{291}F_{308} \quad (3)$$

where the first subscript represents the excitation wave-

TABLE IV  
EFFECT OF EXCITATION WAVELENGTH ON  
FLUORESCENCE INTENSITIES OF AZURIN (CuII)

Excitation wavelength	Emission wavelength	Fluorescence intensity	Ratio of intensities*
(nm)	(nm)		
275	308	50.5	1.51
275	320	33.5	
291	308	26.6	1.41
291	320	18.8	

\*The ratio of fluorescence intensities at 308 and 320 nm, at the excitation wavelength indicated.

length and the second represents the emission wavelength. Then  ${}_{275}F_{308}(\text{Tyr}) = 50.5 - 26.6 = 23.9$ . The next step is to estimate the tyrosine contribution to the total fluorescence at 320 nm,  ${}_{275}F_{320} = 33.5$  (Table IV). Fluorescence spectra have been obtained from several peptide toxins isolated from snake venoms which contain tyrosine as the only fluorescent amino acid and have no unusual tyrosine fluorescence features (Stepanik and Szabo, submitted for publication.) The ratio ( $R$ ) of fluorescence at 320 nm to that at 308 nm was found to be 0.59 in these toxins. The contribution of the tyrosine fluorescence at 320 nm in azurin may then be estimated

$$\begin{aligned} {}_{275}F_{320}(\text{Tyr}) &= {}_{275}F_{308}(\text{Tyr}) \times R \\ &= 23.9 \times 0.59 = 14.1. \end{aligned} \quad (4)$$

The balance of the fluorescence at 320 nm with 275 nm excitation is then assigned to that due to tryptophan

$$\begin{aligned} {}_{275}F_{320}(\text{Trp}) &= {}_{275}F_{320} - {}_{275}F_{320}(\text{Tyr}) \\ &= 19.4. \end{aligned} \quad (5)$$

This value is very close to that actually observed at 320 nm with 291 nm excitation,  ${}_{291}F_{320} = 18.8$ . Hence, it is implied that there is no energy transfer from the tyrosine to the tryptophan in azurin (CuII). The validity of this conclusion is dependent on the assumptions made above but the agreement between the calculated tryptophan contribution to the fluorescence at 320 nm and that observed is striking.

The identity of the fluorescence excitation spectra [ $\lambda_{em} = 310$  nm] of azurin (CuII), azurin (CuI), and apoazurin suggests that the contribution of the tyrosine fluorescence to the total fluorescence is the same in each protein. It then follows that the intramolecular relationship of the tyrosine and tryptophan residues is not altered on reduction or removal of the copper atom.

### Fluorescence Decay Kinetics

The fluorescence decay data reported in this work shows that the fluorescence decay of azurin (CuII) and azurin (CuI) from *P. fluorescens* is heterogeneous but that of the apoazurin is homogeneous. Similar observations were reported by Grinvald et al. (21) on the azurins from *P. aeruginosa*. We are confident that the observation of dual exponential decay behavior is not due to an instrumental or sample artifact. If the short lifetime component ( $\tau_2 = 0.18$  ns) was due to a scattered light contribution to the measured decay profiles one would expect to have observed similar behavior for apoazurin. But an excellent fit is obtained to the fluorescence decay data of the latter material with single exponential kinetics (Fig. 7c) ( $\tau = 4.90$  ns). Using a scatterer solution, such as glycogen, with the excitation monochromator set at 290 nm (4 nm bandwidth) and the emission monochromator set at 310 nm (7 nm bandwidth), the ratio of the number of lamp pulses



to single photon counts was >10,000:1. The ratio for the sample fluorescence under similar conditions was typically 40:1. It has also been verified that the dual exponential decay behavior was not due to a polarization effect. When the sample was excited with vertically polarized light and the emission observed through a polarizer set at 55° to the vertical ("magic angle" [46]) similar results to those measured without using polarizers were obtained.

Because the decay time of the long-lived component ( $\tau_1 = 4.80$  ns) is similar to the single decay time of apoazurin ( $\tau = 4.90$  ns) one may suggest that this was due to a significant fraction of apoazurin in our azurin sample. However, sequential addition of small volumes of  $\text{CuCl}_2$  to an azurin sample until there was a twenty-five-fold excess of copper to the amount of azurin in the sample, did not result in any increase in the absorbance at 620 nm, the maximum of the blue copper-ligand transition, nor did it result in any decrease in the fluorescence intensity. If there had been any apoazurin in the azurin samples an increase in the absorbance of the 620 nm band and a loss of fluorescence intensity should have been observed.

If the dual exponential decay behavior of azurin fluorescence was due to the presence of denatured azurin in the sample, the decay parameters at the longer wavelengths, 325 and 345 nm, should be altered because the denatured azurin would make a greater contribution to the fluorescence spectrum at these wavelengths. In fact the fluorescence decay parameters  $\tau_1$ ,  $\tau_2$ , and the ratio of pre-exponentials,  $\alpha_1/\alpha_2$ , were constant over the entire spectrum. Also, the measured decay parameters of denatured azurin ( $\tau_1 = 3.0$ ,  $\tau_2 = 0.76$ ,  $\alpha_1/\alpha_2 = 0.66$ ) are significantly different from those of azurin.

### The Two-Conformer Model For Azurin

We feel that the dual exponential decay behavior of the tryptophan fluorescence of azurin can best be rationalized as resulting from at least two protein conformers of the copper binding site which interact differently with the tryptophan. Then each decay time can be assigned to one of these two conformers and the fluorescence from each conformer has a similar spectral distribution. This suggestion was originally made by Grinvald et al. (21), but questioned by Ugurbil et al. (14) in their interpretation of the fluorescence decay results of *P. aeruginosa* azurin. The presence of two copper site conformations is supported by the observations and interpretations of the results of other studies on azurin structure and mode of action (44–48).

Very recently Wisenfeld et al. (52), using subpicosecond laser pulse techniques, measured the ground-state repopulation kinetics of the 620 nm charge transfer absorption band of azurin. They observed two-step relaxation kinetics, and suggest that the fast rate (<0.5 ps) corresponds to a relaxation between two excited states ( $\sigma S \rightarrow \text{Cu } d_{x^2-y^2}$  to  $\pi S \rightarrow \text{Cu } d_{x^2-y^2}$ ), and the slower rate (1.6 ps) to the reverse-charge transfer relaxation to the ground state. Alternatively one might consider whether their data may

not be interpreted in terms of different electronic relaxations in two protein conformers.

We therefore consider that it is reasonable to assign the two decay times as the excited singlet state lifetimes of the tryptophan in two conformers of the protein. The similarity of the decay times of the long lifetime component in azurin and the single decay time of apoazurin allows us to correlate the 4.8 ns component with a protein conformation in which the interactions of the tryptophan with the copper binding site are unimportant. The short lifetime component (0.18 ns) is assigned to a conformer in which the tryptophan interacts with the copper ligand, quenching the excited singlet state and hence the tryptophan fluorescence.

Using the relationship  $\phi \tau_r = \tau_s$ , where  $\tau_r$  is the excited singlet state radiative lifetime, and  $\tau_s$  is the measured excited singlet decay time, the value of  $\tau_r$  may be calculated for apoazurin. These values are tabulated in Table V. There is a close correspondence between the radiative lifetimes of apoazurin ( $\tau_r = 15.5$  ns) and 3-methylindole (methylcyclohexane solution) ( $\tau_r = 12.0$  ns). Whereas the radiative lifetimes of NATA and 3-methylindole in aqueous solution ( $\tau_r = 21.4$  ns, and  $\tau_r = 25.3$  ns, respectively) are significantly different from that of apoazurin.

These results again show that the polarity and solvent interactions of the indole nucleus in azurin are very similar to those of 3-methylindole in nonpolar hydrocarbons. They add conclusive evidence that the tryptophan residue is inaccessible to solvent and in a very nonpolar hydrophobic environment. The solution properties are therefore consistent with the structure determined by x-ray crystallographic analysis (19) in which the tryptophan is located in the hydrophobic interior of a  $\beta$ -barrel structure and is not hydrogen bonded to any other residue.

Because there is no difference in the absorption spectra or the fluorescence spectra band shapes for azurin (CuII) and apoazurin we can assume that the radiative lifetimes of the two components in azurin are equal to the radiative

TABLE V  
SUMMARY OF FLUORESCENCE PARAMETERS\*

Sample	Quantum yield	$\tau_s$	$\tau_r$	$k_{nr}$
		ns	ns	( $10^8 \text{s}^{-1}$ )
3-Methylindole in methylcyclohexane	0.35	4.20	12.0	1.5
Apoazurin	0.31	4.90	15.5	1.4
Azurin	0.052	$\tau_1 = 4.80$ $\tau_2 = 0.18$	—	1.4 54.9
3-Methylindole in buffer (pH 5.3)	0.37	9.37	25.3	0.67
N-Acetyltryptophanamide in buffer (pH 5.3)	0.14	3.00	21.4	2.9

\* $\tau_s$  and  $\tau_r$  are the measured singlet state and calculated radiative lifetimes respectively.  $k_{nr}$  is the sum of the nonradiative rate constants calculated from the relationship  $1/(k_r + k_{nr}) = \tau_s$ .

lifetime of apoazurin ( $\tau_r = 15.5$  ns). Then the sum of the nonradiative rate constants,  $k_{nr}$ , for both components can be calculated. For the 4.8 ns component we obtain a value of  $k_{nr} = 1.4 \times 10^8$  s<sup>-1</sup>, and for the 0.18 ns component a value of  $k_{nr} = 5.49 \times 10^9$  s<sup>-1</sup>. The rate constant for the extra quenching process in the short lifetime conformer is then the difference which is  $k_q = 5.35 \times 10^9$  s<sup>-1</sup>. This quenching process, which is absent when the copper atom is removed, must result from a close interaction between the indole nucleus and one or more of the copper ligands. From C-13 NMR data, Ugurbil et al. (47) have suggested that the C- $\gamma$  and C- $\delta_2$  carbons of the indole are as close as 8.4 and 9.8 Å to the copper atom, respectively. It is reasonable to expect then that the indole residue is even closer to one of the ligands.

There are two quenching mechanisms that may be operative in the 0.18 ns component. One is a proton transfer from His 46 or His 117 to the excited indole ring, while the other is an electron transfer involving either Cys 112 or Met 121. If the histidine protons could be exchanged with deuterons, then it is expected that a proton transfer quenching process could be operative. However, no change in the decay times of the short lifetime component of samples of azurin (CuII) which were equilibrated with D<sub>2</sub>O was found. Then, either proton transfer is not the quenching mechanism, or the N—H protons of the liganded imidazole ring may not have exchanged under the equilibrated conditions. NMR experiments (15, 16) indicate that the hydrogen-deuterium exchange of the imidazole N—H may not be facile. Further experiments are required to distinguish which quenching mechanism is operative.

For reasons similar to those given above for equating the radiative lifetimes of both components in azurin it is reasonable to assume that their extinction coefficients are also equal. Then the total quantum yield,  $\phi_T$ , of azurin is the sum of the products of the quantum yield and fraction of each component,

$$\phi_T = \phi_{4.8}(X) + \phi_{0.18}(1 - X) \quad (6)$$

where  $\phi_{4.8}$  and  $\phi_{0.18}$  are the quantum yields of the 4.8 and 0.18 ns components and  $X$  is the fraction of the 4.8 ns component in azurin. Because  $\phi = \tau_s/\tau_r$ , the quantum yields of each component may be estimated from their respective singlet state decay times and the radiative lifetime. In this way the fraction of the 4.8 ns component in azurin (CuII) is calculated to be 0.14. The relative amount of the 4.8 ns conformer to the 0.18 ns conformer,  $C_{4.8}/C_{0.18}$ , is 0.16. Similarly it can be shown that the relative concentrations of the components in azurin (CuI) increase to 0.20 (a value of 0.054 for the  $\phi$  of tryptophan in azurin (CuI) is used).

The ratio of the concentrations of the 4.8 and the 0.18 ns components may also be estimated using the fluorescence decay data only. When the fluorescence consists of two components each of which have similar spectral distribu-

tions it can be shown that the relative concentration of the two components,  $C_1/C_2$ , is simply equal to the ratio of the pre-exponential terms,  $R = \alpha_1/\alpha_2$ , obtained from the fluorescence decay data analysis (26, 50). This requires the same assumptions that were used above of equal extinction coefficients and radiative lifetimes of the two components. The ratio  $C_{4.8}/C_{0.18}$  for azurin (CuII) and azurin (CuI) were found to be 0.26 and 0.35, respectively, from the decay data.

These latter values are higher than those calculated from the quantum yield data. Both calculations are subject to measurement errors, especially the measurement of  $R$  from the fluorescence decay data. Regardless of the method of calculation the results show that the fraction of the long-lived component increases on reduction of azurin (CuII) to azurin (CuI). This implies that the change in relative concentration of the two components is related to the structure of the copper-ligand complex.

Spectroscopic studies on azurin and other blue copper proteins have convincingly concluded that the structure of the copper II ligand complex is that of a distorted or flattened tetrahedron (1, 10, 54, 55). This geometry is not too different from the preferred tetrahedral geometry for copper I ligand complexes. Brill (48) has suggested that there is only a small displacement between the configurations of the cuprous and cupric complexes in azurin. The X-band EPR spectra (48) indicate there are fluctuations of the copper binding site geometry. NMR spectra are interpreted in terms of two azurin conformers that have very small local structural differences (16). Kinetic experiments have shown that the rate constant for interconversion between two azurin conformers is small, with values of 5 to 40 s<sup>-1</sup> (44, 46).

We propose that the two components that are indicated by the fluorescence decay results correspond to two interconverting conformers that have different copper-ligand geometries. On reduction, the fraction of the more tetrahedral structure would increase. But the continued observation after reduction of the copper of two components with similar decay times indicates that the copper-ligand complex must be restrained by the protein structure and hence slow interconversion between the two forms continues to occur. The constant decay times of the tryptophan residue are further evidence that the two geometries after reduction are not too different from those of the oxidized azurin, otherwise changes in intramolecular interactions should be reflected in the fluorescence decay times.

Based on the above proposed rationalization of the dual exponential decay behaviour of azurin, we assign the 4.8 ns component to a conformer that is close to the usual tetrahedral geometry of Cu(I) ligand complexes. The 0.18 ns component is assigned to the flattened tetrahedral conformer which is the predominant conformation in azurin.

It was suggested by Grinvald et al. (21) that there was a significant fraction of azurin that was nonfluorescent. This

conclusion derived from the assumption that  $\tau_r$  for apoazurin was the same as that for NATA in aqueous buffer. In this work it has been shown that this assumption is not valid. Nevertheless, one can calculate a nonfluorescent fraction in azurin using the relationship derived by Steinberg (see reference 21), assuming (reasonably) that the  $\tau_r$  for azurin is the same as that for apoazurin. Then from the data herein it would appear that ~30% of the azurin is nonfluorescent. However, it has also been reported (18, 21) that the ultraviolet absorbance of azurin was 15 to 20% greater than apoazurin. This increase may likely be due to the absorbance of the bound copper atom. Hence the quantum yield of azurin should be increased by this factor, and it would account for a significant part of the nonfluorescent fraction.

Errors in the measured parameters could make up the balance. Finally, while it is a reasonable assumption that both fluorescent components in azurin have similar radiative lifetimes, this cannot be verified. Although at present we cannot definitively eliminate the possibility of a third nonfluorescent protein conformation, we suggest that the calculated nonfluorescent fraction may be accounted for by the above considerations. Certainly, if such a component were present it would complicate the resolution of the conformational heterogeneity of the tryptophan site in azurin.

The fluorescence decay results have been discussed above in terms of two protein conformers whose relative concentrations were not altered in the excited singlet state. However, changes in equilibrium constants in the excited state are well known (27, 50). Rapid reaction technique experiments have estimated the equilibrium constant between the two conformers and found  $K = 1$  (44). Our results show that the relative concentrations are between 0.15 and 0.26, or the inverse 3.85 to 3.67, depending on how the equilibrium equation is written. The fluorescence decay data were analyzed in a manner similar to that discussed in other work on glucagon conformers (53) and diketopiperazine conformers (50). We found that the data were completely inconsistent with the existence of changes in the equilibrium constant in the excited state. This is not surprising since the rate constant for conformational inter-change in the ground state is between 5 and 40 s<sup>-1</sup> (44, 45), significantly lower than the singlet decay rate constants.

It has been suggested from NMR experiments that the two conformers result from the acidity properties of a histidine residue that has a pK near 7. At the pH of these experiments (pH 5.3) it is expected that only the conjugate acid form of this histidine is present. This does not preclude the possibility that another histidine acid-based equilibrium might be involved. Only two of the four histidines can be titrated in azurin and they have pKs above 7 (15).

The interpretation that azurin exists as a mixture of at least two distinct components that correspond to two different conformations of the copper-ligand complex has important implications for the analysis of the absorption

and circular dichroism spectra of azurins. It is beyond the scope of this work to attempt an analysis of the data available in the literature on this subject.

The authors thank Professor R. P. Ambler for providing details and advice on the isolation and purification of the azurin samples. They also thank Mr. D. T. Krajcarski for his expert technical assistance, Mrs. L. Bramall for computational assistance, and Dr. S. Martin for growing the bacteria used in this study. NRCC #20885.

Received for publication 17 September 1981 and in final form 4 August 1982.

## REFERENCES

- Holwerda, R. A., S. Wherland, and H. B. Gray. 1976. Electron transfer reactions of copper proteins. *Annu. Rev. Biophys. Bioeng.* 5:363-396.
- Rosen, P., and I. Pecht. 1976. Conformational equilibria accompanying the electron transfer between cytochrome C (P551) and azurin from *Pseudomonas Aeruginosa*. *Biochemistry.* 15:775-786.
- Muir Wood, P., and D. S. Bendall. 1975. The kinetics and specificity of electron transfer from cytochromes and copper proteins to P700. *Biochim. Biophys. Acta.* 387:115-128.
- Yamanaka, T., S. Kijimoto, and K. Okunuki. 1963. Some properties of *Pseudomonas* blue protein and its apoprotein. *J. Biochem. (Tokyo).* 53:256-259.
- Finazzi-Agro, A., G. Rotilio, L. Avigliano, P. Guerrieri, V. Boffi, and B. Mondovi. 1970. Environment of copper in *Pseudomonas fluorescens* Azurin: fluorometric approach. *Biochemistry.* 9:2009-2014.
- Finazzi-Agro, A., C. Giovagnoli, L. Avigliano, G. Rotilio, and B. Mondovi. 1973. Luminescence quenching in azurin. *Eur. J. Biochem.* 34:20-24.
- Brill, A. S., G. F. Bryce, and H. J. Maria. 1968. Optical and magnetic properties of *Pseudomonas* azurins. *Biochim. Biophys. Acta.* 154:342-351.
- Solomon, E. I., J. W. Hare, D. M. Dooley, J. H. Dawson, P. J. Stephens, and H. Gray. 1980. Spectroscopic studies of stellacyanin, plastocyanin, and azurin. Electronic structure of the blue copper sites. *J. Am. Chem. Soc.* 102:168-178.
- Burstein, E. A., E. A. Permyakov, V. A. Yashin, S. A. Burkhanov, and A. Finazzi-Argo. 1977. The fine structure of luminescence spectra of azurin. *Biochim. Biophys. Acta.* 491:155-159.
- Brill, A. S. 1977. Copper. In *Transition Metals in Biochemistry*, Springer Verlag KG, Berlin, W. Germany. 26:47-77.
- Ambler, R. P., and L. H. Brown. 1967. The amino acid sequence of *Pseudomonas Fluorescens* azurin. *Biochem. J.* 104:784-825.
- Ryden, L., and J.-O. Lundgren. 1979. On the evolution of blue proteins. *Biochimie (Paris).* 61:781-790.
- Ryden, L., and J.-O. Lundgren. 1976. Homology relationships among the small blue proteins. *Nature (Lond.).* 261:344-346.
- Ugurbil, K., A. H. Maki, and R. Bersohn. 1977. Study of the triplet state properties of tyrosines and tryptophan in azurin using optically detected magnetic resonance. *Biochemistry.* 16:901-907.
- Ugurbil, K., and R. Bersohn. 1977. Nuclear magnetic resonance study of exchangeable and non exchangeable protons in azurin from *Pseudomonas aeruginosa*. *Biochemistry.* 16:3016-302.
- Hill, H. A. O., and B. E. Smith. 1979. Characteristics of azurin from *Pseudomonas aeruginosa* via 270 MHz <sup>1</sup>H nuclear magnetic resonance spectroscopy. *J. Inorg. Biochem.* 11:79-93.
- Tullius, T. D., P. Frank, and K. O. Hodgson. 1978. Characterization

- of the blue copper site in oxidized azurin by extended X-ray absorption fine structure: Determination of a short Cu-S distance. *Proc. Natl. Acad. Sci. USA.* 75:4069-4073;
18. Tennant, D. L., and D. R. McMillin. 1979. A detailed analysis of the charge-transfer bands of a blue copper protein. Studies of the nickel (II), manganese (II), and cobalt (II) derivatives of azurin. *J. Am. Chem. Soc.* 101:2307-2311.
  19. Adman, E. T., R. E. Stenkamp, L. C. Sieker, and L. H. Jensen. 1978. A crystallographic model for azurin at 3 Å resolution. *J. Mol. Biol.* 123:35-47.
  20. Colman, P. M., H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris, J. A. M. Ramshaw, and M. P. Venkatappa. 1978. X-ray crystal structure analysis of plastocyanin at 2.7 Å resolution. *Nature (Lond.)* 272:319-324.
  21. Grinvald, A., J. Schlessinger, I. Pecht, I. Z. Steinberg. 1975. Homogeneity and variability in the structure of azurin molecules studied by fluorescence decay and circular polarization. *Biochemistry.* 14:1921-1929.
  22. Munro, I., I. Pecht, and L. Stryer. 1979. Subnanosecond motions of tryptophan residues in proteins. *Proc. Natl. Acad. Sci. USA.* 76:56-60.
  23. Ambler, R. P., and M. Wynn. 1973. The amino acid sequences of cytochromes c-551 from three species of *Pseudomonas*. *Biochem. J.* 131:485-498.
  24. Gabriel, O. 1971. Analytical gel electrophoresis. *Methods Enzymol.* 22:565-578.
  25. Swank, R. T., and K. D. Munkres. 1971. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. *Anal. Biochem.* 39:462-477.
  26. Szabo, A. G., and D. M. Rayner. 1980. Fluorescence decay of tryptophan conformers in aqueous solution. *J. Am. Chem. Soc.* 102:554-563.
  27. Badea, M. G., and L. Brand. 1979. Time-resolved fluorescence measurements. *Methods Enzymol.* 61:378-425.
  28. Rayner, D. M., A. E. McKinnon, A. G. Szabo, and R. A. Hackett. 1976. Confidence in fluorescence lifetime determinations: a ratio correction for the photomultiplier time response variation with wavelength. *Can. J. Chem.* 54:3246-3259.
  29. Rayner, D. M., A. E. McKinnon, and A. G. Szabo. 1977. Correction of instrumental time response variation with wavelength in fluorescence determinations in the ultraviolet region. *Rev. Sci. Instrum.* 48:1050-1054.
  30. McKinnon, A. E., A. G. Szabo, and D. R. Miller. 1977. The deconvolution of photoluminescence data. *J. Phys. Chem.* 81:1564-1570.
  31. Pecht, I., O. Farver, and M. Goldberg. 1977. Electron transfer pathways in blue copper proteins. *Adv. Chem. Ser.* 162:179-206.
  32. Ugurbil, K., and R. Bersohn. 1977. Tyrosine emission in the tryptophanless azurin from *Pseudomonas fluorescens*. *Biochemistry.* 16:895-901.
  33. Kirby, E. P., and R. F. Steiner. 1970. The influence of solvent and temperature upon the fluorescence of indole derivatives. *J. Phys. Chem.* 74:4480-4490.
  34. Klein, R., and I. Tatischeff. 1977. Temperature fluorescence quenching of indole and water structure. *Chem. Phys. Lett.* 51:333-338.
  35. Weinryb, I., and R. F. Steiner. 1970. Luminescence of the tryptophan and tyrosine residues of papain in solution. *Biochemistry.* 9:135-146.
  36. Burnstein, E. A., N. S. Vedenkina, and M. N. Ivkova. 1973. Fluorescence and the location of tryptophan residues in proton molecules. *Photochem. Photobiol.* 18:263-279.
  37. Sun, M., and P. Song. 1977. Solvent effects on the fluorescent states of indole derivatives-dipole moments. *Photochem. Photobiol.* 25:3-9.
  38. Skalski, B., D. M. Rayner, and A. G. Szabo. 1980. Ground-state complexes between solvents and l-Methylindole: the origin of the Stokes shift in their fluorescence spectra. *Chem. Phys. Lett.* 70:587-590.
  39. Hershberger, M. V., A. H. Maki, and W. C. Galley. 1980. Phosphorescence and optically detected magnetic resonance studies of a class of anomalous tryptophan residues in globular proteins. *Biochemistry.* 19:2204-2209.
  40. Avigliano, L., A. Finazzi-Agro, and B. Mondovi. 1974. Perturbation studies on some blue proteins. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 38:205-208.
  41. Mallinson, R., R. Carter, and C. A. Ghiron. 1981. Acrylamide quenching studies with Azurin B. *Biochim. Biophys. Acta.* 671:
  42. Sober, H. A., editor. 1970. Handbook of Biochemistry. Chemical Rubber Co., Cleveland. Section B. 75.
  43. Spencer, R. D., and G. Weber. 1970. Influence of brownian rotations and energy transfer upon the measurements of fluorescence lifetime. *J. Chem. Phys.* 52:1654-1663.
  44. Wilson, M. T., C. Greenwood, M. Brunovi, and E. Antonini. 1975. Electron transfer between azurin and cytochrome c-551 from *Pseudomonas aeruginosa*. *Biochem. J.* 449-457.
  45. Parr, S. R., D. Barber, C. Greenwood, and M. Brunovi. 1977. The electron-transfer reaction between azurin and the cytochrome c oxidase from *Pseudomonas aeruginosa*. *Biochem. J.* 167:447-455.
  46. Werhland, S., and I. Pecht. 1978. Protein-protein electron transfer. A Marcus theory analysis of reactions between c type cytochromes and blue copper proteins. *Biochemistry.* 17:2585-2591.
  47. Ugurbil, K., R. S. Norton, A. Allerhand, and R. Bersohn. 1977. Studies of individual carbon sites of azurin from *Pseudomonas aeruginosa* by natural-abundance carbon-13 nuclear magnetic resonance spectroscopy. *Biochemistry.* 16:886-894.
  48. Brill, A. S. 1978. Activation of electron transfer reactions of the blue proteins. *Biophys. J.* 22:139-142.
  49. Wiesenfeld, J. M., E. P. Ippen, A. Covin, and R. Bersohn. 1980. Electron relaxation in azurin: picosecond reverse charge transfer. *J. Am. Chem. Soc.* 102:7256-7258.
  50. Donzel, B., P. Gauduchon, and P. Wahl. 1974. Study of the conformation in the excited state of two tryptophanyl diketopiperazines. *J. Am. Chem. Soc.* 96:801-808.
  51. Brill, A. S., and G. F. Bryce. 1968. Cupric ion in blue proteins. *J. Chem. Phys.* 48:4398-4404.
  52. Solomon, E. I., J. W. Have, and H. B. Gray. 1976. Spectroscopic studies and a structural model for blue copper centers in proteins. *Proc. Natl. Acad. Sci. USA.* 73:1389-1393.
  53. Cockle, S. A., and A. G. Szabo. 1981. Time resolved fluorescence spectra of tryptophan in monomeric glucagon. *Photochem. Photobiol.* 34:23-28.