Confirmation That Multiexponential Fluorescence Decay Behavior of Holoazurin Originates from Conformational Heterogeneity[†]

Cindy M. Hutnik[‡] and Arthur G. Szabo*

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

Received July 14, 1988; Revised Manuscript Received November 8, 1988

ABSTRACT: Homologous azurins from *Pseudomonas fluorescens* (ATCC 13525) and *Pseudomonas aeruginosa* (ATCC 10145) were examined by a number of electrophoretic techniques, and their copper to protein stoichiometry was determined by atomic absorption and amino acid analysis. Provided that the spectral ratio (A_{620}/A_{280} or A_{625}/A_{280}) was 0.53 and there was no evidence of a Soret band in the absorption spectrum, then these criteria can be used to judge the homogeneity of the azurin sample. If the spectral ratio was less than 0.50, evidence suggested a nonreconstitutable, non-trypsin-digestible apoazurin was present. The fluorescence decay of these homogeneous holoazurins included three components, not two as previously reported [Szabo, A. G., et al. (1983) *Biophys. J. 41*, 233-244]. Whereas the decay times were nearly the same for the azurins from the different sources, the fractional fluorescence of each component varied with the azurin measured. The fluorescence of the corresponding apoazurins, prepared by a refined procedure, obeyed monoexponential decay kinetics. The temperature and pH effects on the fluorescence behavior of these homologous azurins are presented with the pH study suggesting an influence by a group which titrates between pH 5 and pH 7. When taken together, these results confirm that the multiexponential decay behavior originates from conformational heterogeneity and not from contamination by an apo form [Petrich, J. W., et al. (1987) *Biochemistry 26*, 2711-2722].

The azurins comprise a homologous class (Ambler & Brown, 1967; Ryden & Lundgren, 1979) of blue copper-containing, low molecular weight (MW ≃ 14000) proteins which function as redox partners in bacterial electron transfer (Brunori et al., 1974, 1975; Wilson et al., 1975). Their study has been pursued not only to better understand the molecular basis of biological electron transfer (Wherland & Pecht, 1978; Brill, 1978) but also to determine what aspects of their structure give rise to their unique spectroscopic properties (McMillin & Tennent, 1979; Szabo et al., 1983; Tennent & McMillin, 1979; Ugurbil et al., 1977; Tullius et al., 1978). More recent spectroscopic studies have suggested the possible role of individual amino acids in the electron-transfer process (Adman et al., 1982; Mitra & Bersohn, 1982).

The azurins from several bacterial strains have been sequenced (Ambler, 1973), and the X-ray crystallographic structures of two azurins from *Pseudomonas aeruginosa* and *Alcaligenes denitrificans* have been determined (Adman & Jensen, 1981; Norris et al., 1986). The majority of the azurins contain 128 amino acid residues and only a single tryptophan residue at position 48 which, in all cases, is fully conserved.

The presence of this single intrinsic fluorescent amino acid has resulted in the use of azurins in a number of fluorescence spectroscopic studies (Grinvald et al., 1975; Mallinson et al., 1981; Munro et al., 1979). Upon excitation at 291 nm, the fluorescence emission maximum of the azurins is located at 308 nm (Burstein et al., 1977; Finazzi-Argo et al., 1973). As such, the fluorescence of Trp[1] in azurin has the most blue-shifted emission maximum of any tryptophan-containing protein reported to date. Previously, it had been shown that this fluorescence behavior closely paralleled that of 3-methylindole in methylcyclohexane solution, and this similarity was attributed to the complete inaccessibility of Trp-48 in azurin to solvent molecules (Szabo et al., 1983). This was consistent with the crystallographic data which revealed that the Trp was located in the highly hydrophobic interior of an eight-stranded, β-barrel structure constituting the bulk of the azurin molecule (Adman & Jensen, 1981; Adman et al., 1978).

Time-resolved fluorescence studies have revealed double-exponential decay behavior for the Trp residue in holoazurin (Grinvald et al., 1975; Munro et al., 1979; Szabo et al., 1983) with the long decay time being quite similar to the single decay time reported for the corresponding copper-less apoazurin. In agreement with Grinvald et al. (1975), this multiexponential decay behavior of holoazurin was interpreted to arise from conformational heterogeneity (Szabo et al., 1983). While our work was in progress, Petrich et al. (1987) reported their work on different azurin samples. They suggested that the long decay time component in holoazurin was due to an apoazurin contaminant. Because of the critical nature of this point in the interpretation of the fluorescence and other spectral data, it was necessary to establish definite biochemical criteria by which the homogeneity of azurin could be assessed. Once confidence in the purity of the samples was achieved; both steady-state and time-resolved fluorescence measurements on the homogeneous protein would confirm or refute the original interpretations (Szabo et al., 1983).

In this paper we present several lines of evidence which confirm the homogeneity of holoazurin samples isolated from *Pseudomonas fluorescens* (*Pfl*) and *Pseudomonas aeruginosa* (*Pae*). We also present the pH and temperature dependence of both the steady-state and time-resolved fluorescence be-

---

[†] This is NRCC Publication No. 29811.

[*] To whom correspondence should be addressed.

[‡] C.M.H. is a 1967 NSERC scholar and a predoctoral student in the Department of Biochemistry, University of Ottawa, Ottawa, Canada, working under the supervision of A.G.S. at the NRC.

[1] Abbreviations: *Pae*, *Pseudomonas aeruginosa*; *Pfl*, *Pseudomonas fluorescens*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; apo, apoazurin; holo, holoazurin; CN, cyanide; ASC, ascorbate; Trp, tryptophan; OD, optical density.

0006-2960/89/0428-3923$01.50/0 Published 1989 by the American Chemical Society

haviors of these two homologous azurins. In addition, we present information which characterizes the azurin isolated from *Pfl* (ATCC 13525), including its amino acid composition. We show how fluorescence results can discern differences in the conformational heterogeneity of homologous proteins. Finally, we present a refined procedure for the preparation of apoazurin and also present data to suggest that under certain conditions a nonreconstitutable, non-trypsin-digestible apoazurin may exist.

EXPERIMENTAL PROCEDURES

Materials. The bacterial cultures used were *P. aeruginosa* (ATCC 10145) and *P. fluorescens* (ATCC 13525). In addition, samples of *Pae* azurin were purchased from Sigma Chemical Co., St. Louis, MO. Buffalyte 3-10, used in isoelectric focusing, was purchased from Pierce Chemical Co., Rockford, IL. Isoelectric focusing p*I* markers were obtained from Pharmacia, Quebec, Canada. Potassium cyanide and cupric chloride were purchased from BDH Chemicals Ltd., Toronto, Canada, cupric nitrate was from Fisher Scientific Co., Fair Lawn, NJ, and cupric sulfate was from Anachemia, Montreal, Canada. Spectrapor membrane tubing (MW cutoff 3500) was purchased from Spectrum Medical Industries, Los Angeles, CA. Bovine pancreatic trypsin was obtained from Sigma Chemical Co., St. Louis, MO. All buffers were prepared from reagent-grade chemicals and double glass distilled, deionized water and, in some cases, were passed through Chelex-100 (Bio-Rad Laboratories, Richmond, CA) prior to use.

Methods. Azurin was purified from the acetone-dried cells of *P. fluorescens* (ATCC 13525) and *P. aeruginosa* (ATCC 10145) as described by Ambler and Wynn (1973) with the following modifications. The step involving ion-exchange chromatography on DEAE-cellulose was omitted, and instead, the chromatography on CM-cellulose was immediately followed by ammonium sulfate precipitation in 50 mM ammonium acetate buffer, pH 5.5. To remove the contaminating cytochrome, ammonium sulfate was gradually added at room temperature until the solution became turbid. After an approximately 2–5-min wait, the solution was centrifuged for 10 min at 9500 rpm. Both the supernatant and the pellet were monitored spectrophotometrically in order to locate the cytochrome. This procedure was repeated until the absorption spectrum of the supernatant showed no sign of a Soret band at 410 nm. The solution was then saturated with ammonium sulfate and left at 4 °C overnight. The precipitate was collected by centrifugation, redissolved in 1–2 mL of 50 mM ammonium acetate buffer, pH 5.0, and further purified by gel filtration on a Sephadex G-75SF column (2.2 × 100 cm, flow rate 8.0 mL/h) eluted with the same buffer. All blue-colored fractions were collected, and those having a spectral ratio (A_{620}/A_{280} for *Pfl*; A_{625}/A_{280} for *Pae*) greater than 0.50 were combined, concentrated on CM-52, and stored at −20 °C.

Several criteria were applied to assess the purity of the azurin samples. Molecular weight homogeneity was established by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10–15% gradient using the Pharmacia PhastSystem. Charge homogeneity was established by both cationic disk polyacrylamide gel electrophoresis and isoelectric focusing in polyacrylamide gels (Righetti & Drysdale, 1971). The latter gels were run in a pH gradient of 3–10. Estimates of the isoelectric points were made by comparison of the mobility of each sample with the mobility of p*I* marker proteins. Another test for homogeneity involved the stoichiometric determination of copper to protein by atomic absorption spectroscopy and amino acid analysis.

Apoazurin was prepared from holoazurin according to two general procedures. The first method was a modified version of the KCN dialysis procedure that was initially described by Yamanaka et al. (1963). The holo samples were initially dialyzed for 2 h against 2.0 L of 100 mM phosphate buffer, pH 7, at room temperature. The samples were then dialyzed at room temperature against 1.0 L of 0.5 M KCN in 100 mM phosphate buffer, the pH being adjusted down to pH 8.5 with concentrated HCl after cyanide addition, in the fume hood. Judging by the lack of blue color, the dialysis was considered complete after 20 min. The sample was then exhaustively dialyzed at 4 °C against the desired storage buffer for the apoazurin samples. The second method used in the preparation of apoazurin involved reduction of the Cu(II) azurin to Cu(I) azurin by addition of ascorbate followed by dialysis against thiourea as described by Blaszak et al. (1983).

Cu(II)-reconstituted azurin samples were prepared in two ways. The first method involved dialysis of apoazurin samples ($\simeq 5 \times 10^{-5}$ M) against a 10-fold molar excess of either cupric nitrate, cupric sulfate, on cupric chloride in 50 mM ammonium acetate buffer at various pHs and for various times at 4 °C. In some cases nitrogen was bubbled into the copper solutions during dialysis. Cu(II)-reconstituted samples were also prepared by the addition of various aliquots of metal ion to the apoazurin samples ($\simeq 5 \times 10^{-5}$ M) at different metal:protein ratios. These solutions were then equilibrated at room temperature and monitored by absorption and fluorescence spectroscopy for reconstitution after various times.

Holoazurin samples having spectral ratios below 0.5 were treated with trypsin under the conditions of Ambler (1963). Samples were then applied to a CM-52 column (1 × 18 cm, 50 mM ammonium acetate buffer, pH 4.2) and eluted with the same buffer, pH 6.5. The blue fractions were pooled and then applied to a Sephadex G-25 column (1 × 30 cm) equilibrated and eluted in 50 mM ammonium acetate buffer, pH 5.0.

Holoazurin samples with spectral ratios below 0.5 were also subjected to chromatofocusing using a Pharmacia FPLC system. Blue fractions were collected and their spectral ratios determined.

Azurin samples were reduced according to the method of Corin et al. (1983).

Instrumentation. The fluorescence decay experiments were performed according to the time-correlated single photon counting technique with instrumentation described in detail elsewhere (Zuker et al., 1985). Essentially it included a Spectra Physics sync-pumped argon ion dye laser and cavity dumper system as the excitation source, operating at 825 kHz and with a pulse width of 15 ps. The excitation at 292 nm was vertically polarized. The emission was detected after passing through a polarizer set at 55° to the vertical and a JY H10 monochromator, with a 4-nm band-pass, on a Hamamatsu 1564U-01 microchannel plate photomultiplier. The channel width typically was 21.6 ps/channel, and fluorescence decay curves were collected in 1024 channels of a multichannel analyzer (MCA). To optimize the signal-to-noise ratio, a minimum of 25 000 counts was usually collected in the peak of the sample profile. The ratio of laser pulses to single photon events was 100:1. A buffer blank was also measured under identical conditions as the corresponding sample. The instrument response function was determined by measuring the scattered light from a glycogen sample having an optical density of 0.2 at the excitation wavelength. The buffer blank signal was subtracted from the sample decay curve, appropriate consideration being given to the weighting of the data points

Table I: Stoichiometry Determination of Copper to Protein by Atomic Absorption and Amino Acid Analysis[a]

	copper concn			
sample	µg/L	M	protein concn (M)	% copper
blank	<0.33	$<5.2 \times 10^{-9}$		
Pfl apo/CN	80 ± 0.7	$1.3 \times 10^{-6} \pm 0.01 \times 10^{-6}$	$5.74 \times 10^{-5} \pm 0.02 \times 10^{-5}$	2.3 ± 0.01
Pfl apo/ascorbate	220 ± 0.0	$3.5 \times 10^{-6} \pm 0.0$	$3.5 \times 10^{-5} \pm 0.05 \times 10^{-5}$	10.0 ± 0.1
Pfl holo, $R = 0.54$	5950 ± 106	$9.4 \times 10^{-5} \pm 0.17 \times 10^{-5}$	$9.2 \times 10^{-5} \pm 0.03 \times 10^{-5}$	102 ± 1.5
Pae holo, $R = 0.52$	3500 ± 0	$5.5 \times 10^{-5} \pm 0.0$	$5.5 \times 10^{-5} \pm 0.03 \times 10^{-5}$	100 ± 0.5
Pae holo, $R = 0.39$	4950 ± 35	$7.8 \times 10^{-5} \pm 0.06 \times 10^{-5}$	$11.5 \times 10^{-5} \pm 0.10 \times 10^{-5}$	68 ± 0.07
Pfl CN[b] + Cu(II) (reconstituted)	1748 ± 23	$2.8 \times 10^{-5} \pm 0.03 \times 10^{-5}$	$2.7 \times 10^{-5} \pm 0.08 \times 10^{-5}$	102 ± 1.9
Pfl ASC[c] + Cu(II) (reconstituted)	1810 ± 40	$2.9 \times 10^{-5} \pm 0.06 \times 10^{-5}$	$3.4 \times 10^{-5} \pm 0.07 \times 10^{-5}$	85 ± 0.02

[a] The samples were analyzed quantitatively by either graphite furnace atomic absorption spectrometry or inductively coupled plasma atomic emission spectrometry. Norleucine was added to the protein samples prior to amino acid analysis for concentration determination. Each value represents the mean of at least two individual samples, each done in duplicate, and the standard error of the mean is calculated. [b] The symbol CN denotes apoazurin preparation by the method of KCN dialysis as described under Methods. [c] The symbol ASC denotes apoazurin preparation by the ascorbate/thiourea method as described by Blaszak et al. (1983).

(Zuker et al., 1985). The data were analyzed with the nonlinear least-squares iterative convolution method based on the Marquardt algorithm. Adequacy of the exponential decay fitting was judged by the inspection of the plots of weighted residuals and other statistical parameters (MacKinnon et al., 1977). The standard error reported for the decay times in the tables indicates precision of an individual decay experiment. The errors shown in Figure 9 reflect the standard error between several measurements on different samples.

Steady-state fluorescence measurements were made on an SLM 8000C spectrofluorometer equipped with a Neslab Endocal refrigerated circulating bath for temperature control. Absorption spectra were measured on a Cary 219 spectrophotometer. Fluorescence quantum yields at 20 °C were measured with apoazurin ($\phi = 0.31$; Szabo et al., 1983) as a standard. The excitation wavelength was 292 nm, and the excitation and emission band-passes were both 2 nm. The OD of the samples was typically <0.05. The OD of every sample was measured prior to recording the fluorescence; for example, in the pH study of the OD for each pH at λ_{ex} was measured. Fluorescence quantum yields were corrected for absorbance at the excitation wavelength, and blank signals were subtracted from the spectra prior to integration. The reported quantum yields were the mean of at least three determinations, the accuracy of which is reported as the standard error of the mean.

RESULTS

Homogeneity of Protein Samples. The absorption spectrum of holoazurin from *P. fluorescens* illustrated in Figure 1 was virtually identical with spectra obtained from *Pae* holoazurin, although the 620-nm peak was generally slightly broadened in the latter. Removal of all of the cytochrome by ammonium sulfate precipitation was shown by the lack of any significant absorption near 410 nm.

Holoazurin samples of various spectral ratios were purified from *Pfl* and *Pae* and were compared by spectrophotometry and electrophoresis to commercially obtained *Pae* holoazurin samples. The absorption spectra of the *Pfl* and the two *Pae* azurins were identical in the UV region, and in fact, the only difference in the spectra was the ratio of the 620-nm (or 625-nm) band to the 280-nm band. Samples of each of these proteins were subjected to SDS–PAGE, and the results are illustrated in Figure 2. In all cases single bands of identical mobility were observed, indicating no proteins of molecular weight different from that of azurin were present in the samples.

When the various protein samples were analyzed for their copper content by atomic absorption and also for their protein concentration by amino acid analysis, a 1:1 copper to protein stoichiometry was obtained when the spectral ratios were above 0.50 (Table I). However, in the samples having spectral ratios less than 0.50, 1:1 stoichiometry was not obtained. Amino acid analysis results consistently revealed the same residue compositions, regardless of the spectral ratio. Table II gives the amino acid compositions of the two homologous azurins. The

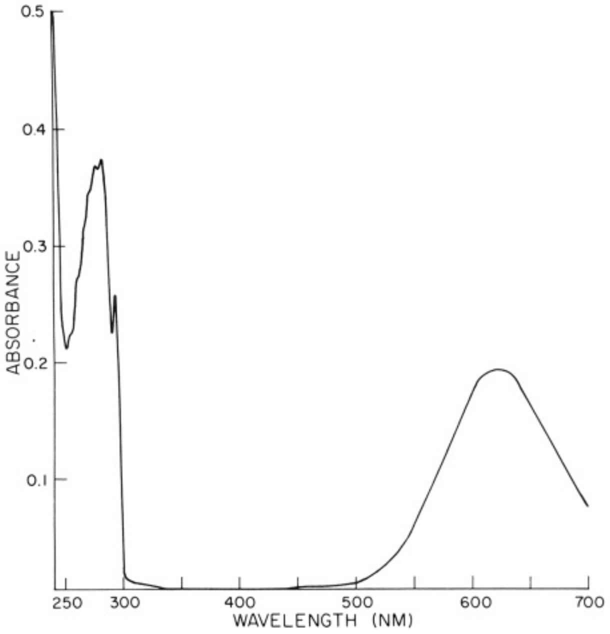

FIGURE 1: Absorption spectrum of *Pfl* holoazurin in 50 mM NH_4OAc buffer, pH 5; spectral ratio = 0.52.

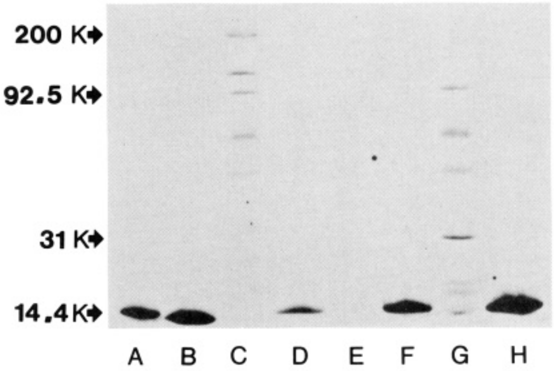

FIGURE 2: SDS–PAGE pattern of the following azurin samples: (lane A) *Pae* holo after G-75SF, spectral ratio = 0.30; (lane B) *Pfl* holo, spectral ratio = 0.55; (lane C) high molecular weight standards; (lane D) *Pae* holo before G-75SF, spectral ratio = 0.30; (lane E) sample buffer—control; (lane F) *Pae* holo, spectral ratio = 0.52; (lane G) low molecular weight standards; (lane H) duplicate sample as in lane A at double the concentration.

Table II: Amino Acid Composition of Holoazurin from *P. aeruginosa* and *P. fluorescens*

amino acid	*Pae* (ATCC 10145)	*Pfl* (ATC 13525)
Ala	7	13
Arg	1	1
Asx	18	15
Cys	3	3
Glx	10	6
Gly	11	11
His	4	4
Ile	4	7
Leu	10	5
Lys	11	12
Met	6	6
Phe	6	6
Pro	4	3
Ser	10	11
Thr	10	12
Trp	1	1
Tyr	2	2
Val	10	10
total	128	128

composition of *Pae* azurin was identical with that previously reported (Ambler, 1973). There have been no reports to confirm the composition of the *Pfl* azurin (ATCC 13525), but analyses from different preparations were reproducible.

The combination of the amino acid analyses, copper stoichiometry, and SDS-PAGE results strongly indicated that a protein contaminant having a composition similar to that of holoazurin was present when the spectral ratios were less than 0.50. In preparation for separation by chromatofocusing techniques, the protein preparations were subjected to isoelectric focusing to determine their isoelectric points. The isoelectric focusing results are shown in Figure 3. Several points were noted. The first was that the p*I* of *Pfl* azurin was higher than that of *Pae* azurin and that the former protein, having a spectral ratio greater than 0.50, migrated as only a single band. The corresponding *Pfl* apoazurin, which was fully reconstitutable upon addition of Cu(II), also migrated as a single band but with a more acidic p*I* compared to that of the holo form. In contrast, samples of *Pae* azurin displayed additional bands with only one of the bands (marked "B" in Figure 3) appearing blue prior to staining. In *Pae* holoazurin samples having spectral ratios less than 0.50, the bands which appeared second from the top of each gel upon staining varied in intensity depending upon the spectral ratio. The intensity of this band progressively *decreased* as the spectral ratio *increased*, and the band could not be detected when the spectral ratio was 0.53. This band was found to comigrate with *Pae* apo samples. A band appeared virtually at the top of the gel (p*I* < 3) in *Pae* azurin samples. The intensity of this band did not depend on the spectral ratio and was present in all *Pae* samples isolated by us, as well as in samples obtained from a commercial source. However, this additional band was barely detectable in the corresponding *Pae* apo samples. No such bands were ever detectable in the *Pfl* azurin samples.

Earlier it had been reported (Ambler & Brown, 1967) that holoazurin was markedly resistant to trypsin digestion. Hence, it was thought that the non-holoazurin protein contaminant might be removed when samples were subjected to tryptic digestion, leaving the holoazurin intact. After treatment with trypsin the impure azurin samples were purified by ion-exchange and gel chromatography. Only a small improvement of the spectral ratio from 0.30 to 0.35 was achieved.

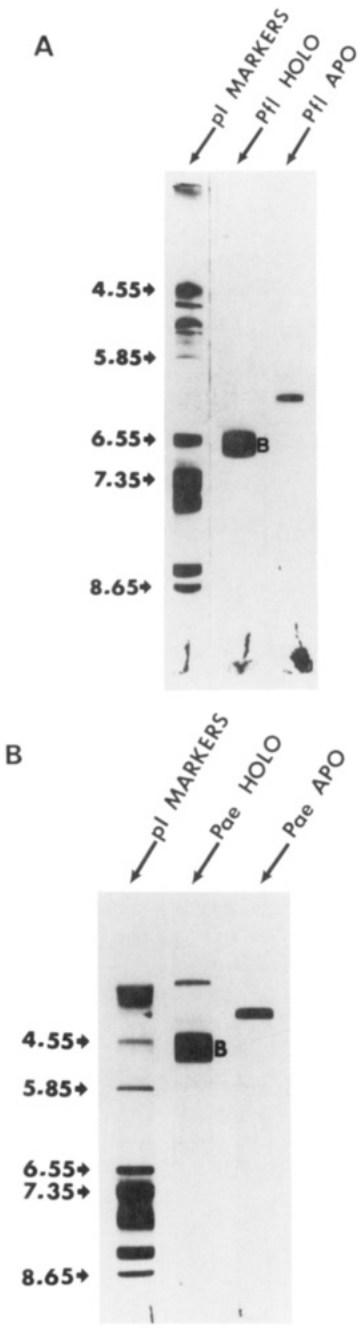

FIGURE 3: Polyacrylamide rod gels obtained upon isoelectric focusing in a gradient of pH 3–10 from top to bottom at a constant voltage of 250 V. The bands with a B located on the immediate right were blue prior to Coomassie staining and hence were assigned to blue copper-containing azurin. (A) p*I* markers and holoazurin and apoazurin from *P. fluorescens*; (B) p*I* markers and holoazurin and apoazurin from *P. aeruginosa*.

The same holoazurin samples were subjected to chromatofocusing, and an elution profile was obtained in which two closely eluting bands could be observed (data not shown). Upon monitoring of the spectral ratios, a distribution of ratios from 0.45 to 0.25 was obtained across the elution profile.

Preparation and Reconstitution of Apoazurin. Earlier reports (Blaszak et al., 1983) and the recent one of Petrich et al. (1987) suggested that it was difficult to produce an apoazurin preparation which could be fully reconstituted with copper. We reinvestigated this problem by treating holoazurin samples with different concentrations of KCN at different pH values ranging from 8.5 to 11. The critical criterion in choosing the best conditions in our investigations was the ability to fully reconstitute the holoazurin with its original 620/280 spectral ratio. The second criterion was the maximal removal of copper as judged by atomic absorption analysis.

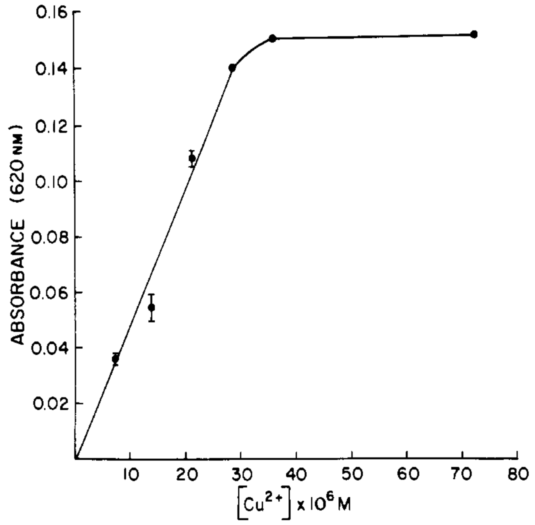

FIGURE 4: Plot of absorbance at 620 nm as a function of $CuCl_2$ concentration during the Cu(II) reconstitution of 3.6×10^{-5} M *Pfl* apoazurin. All solutions were in 50 mM NH_4OAc buffer, pH 6.5, and the values shown are the average values recorded after 6, 24, and 48 h of incubation at room temperature with duplicate samples. At the plateau a spectral ratio of 0.52 was obtained, and an extinction coefficient at 620 nm of 5100 M^{-1} cm^{-1} was calculated for this *Pfl* holoazurin. Errors are reported as the standard error of the mean.

In the apoazurin samples obtained, ≤2% copper was detected (Table I). Figure 4 shows the titration of a *Pfl* apoazurin sample with aqueous Cu(II) from which an extinction coefficient for *Pfl* azurin of 5100 $M^{-1}{\cdot}cm^{-1}$ at 620 nm was calculated.

It was found that dialysis against lower concentrations of cyanide greatly increased the length of time required to remove the copper, from 20 min at 0.5 M KCN to over 2 days at 0.05 M KCN at pH 8.5. In addition, more than 10% of the copper remained bound to holoazurin under these conditions. If the pH of the solution was not adjusted down to pH 8.5 after 0.5 M KCN addition, the dialysis was found to take at least 2 h and sometimes resulted in protein denaturation as witnessed by a loss of the characteristic azurin sharp peak at 292 nm in the absorption spectrum. The azurin spectrum could not be regenerated by addition of Cu(II) (aqueous) to these samples.

Upon preparation of the apoazurin by the method of Blaszak et al. (1983) using ascorbate and thiourea, it was found that no more than 90% of the original copper could be removed and full reconstitution could not be achieved (≈85% at best). In addition there was evidence of absorbance at 310 nm which indicated that trace amounts of ascorbate, or an ascorbate decomposition product, remained, even after exhaustive dialysis against ascorbate-free buffer.

The Cu(II) reconstitution of apoazurin was performed at a variety of pH values and in the presence of three different counterions, chloride, nitrate, and sulfate. In some cases nitrogen was bubbled through the copper solutions during dialysis, and the buffers were Chelex-treated prior to addition of copper. These latter two precautions, however, did not affect the final results. The nature of the counterion appeared to have no effect on the reconstitution as judged by the final spectral ratio. Neither did the pH of the solution have any effect on the final spectral ratio between pH 5 and pH 7. However, at pH > 8, a complete restoration of the spectral ratio could not be achieved. All further reconstitution experiments were conducted at pH 6.5.

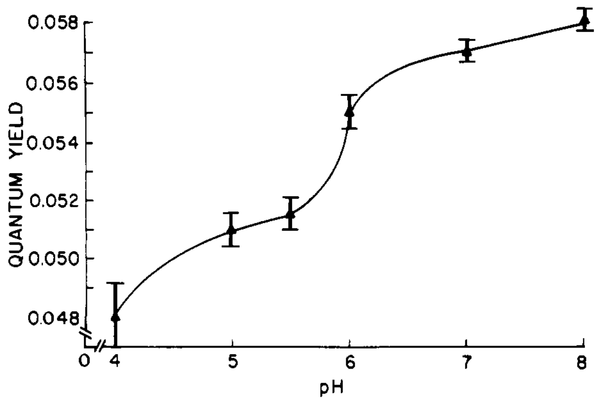

FIGURE 5: Effect of pH on the quantum yield of fluorescence of *Pfl* holoazurin in 50 mM NH_4OAc buffer. The sample OD at the excitation wavelength was always less than 0.1, and the quantum yields were calculated relative to previously determined values at 20 °C (Szabo et al., 1983). Errors are reported as the standard error of the mean.

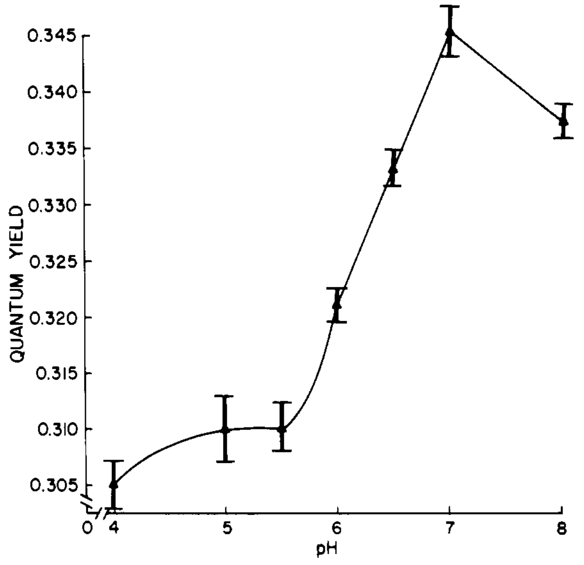

FIGURE 6: Effect of pH on the quantum yield of fluorescence of *Pfl* apoazurin prepared by the modified cyanide dialysis method in 50 mM NH_4OAc buffer. The sample OD at the excitation wavelength was always less than 0.1, and the quantum yields were calculated relative to previously determined values at 20 °C (Szabo et al., 1983). Errors are reported as the standard error of the mean.

Steady-State and Time-Resolved Fluorescence. The relative fluorescence quantum yields measured in this work were 0.051 for *Pfl* holoazurin and 0.018 for *Pae* holoazurin. The effect of pH on the quantum yield of fluorescence for both holo- and apoazurin obtained from *Pfl* is shown in Figures 5 and 6. In both cases a sigmoidal curve was obtained displaying an inflection near pH 5.5–6. However, whereas the quantum yield remained relatively high at pH 8 for the holoazurin, it appeared to fall off at pH 8 for the corresponding apoprotein. This trend was confirmed by continued titration which at pH 9 resulted in a relative fluorescence quantum yield equal to 42% of the pH 7 value for the apoprotein.

Upon the addition of Cu(II) ion to a solution of apoazurin, a 6-fold quenching of the Trp fluorescence was observed. The resulting quantum yield was the same in the presence of either a 2-fold or a 10-fold molar excess of Cu(II). This amount of quenching corresponded to a 1:1 copper to protein sample.

The fluorescence decay results from the various holoazurin, reduced azurin, apoazurin, and Cu(II)-reconstituted azurin samples are given in Table III, a typical decay curve of holoazurin is shown in Figure 7, and weighted residual plots are shown in Figure 8. On the basis of the randomness of residuals, as well as other statistical criteria [see Zuker et al.

Table III: Fluorescence Decay Parameters[a]

sample[b]	λ_{em} (nm)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	F_1	F_2	F_3	α_1	α_2	α_3
Pfl apo[c]	310	5.10								
	320	5.20								
	330	5.04								
Pae apo[d]	310	5.11								
	330	5.14								
Pae apo[e]	310	5.08								
Pfl holo, pH 5.0	310	4.91	0.52	0.105	0.78	0.06	0.16	0.09	0.06	0.85
	330	4.95	0.50	0.106	0.79	0.04	0.17	0.09	0.04	0.87
Pfl Cu(II) azurin	310	4.94	0.52	0.097	0.83	0.04	0.13	0.11	0.05	0.85
	330	4.85	0.48	0.100	0.80	0.05	0.15	0.09	0.06	0.85
Pfl Cu(I)	310	4.98	0.37	0.063	0.87	0.03	0.10	0.09	0.04	0.86
	330	4.96	0.39	0.068	0.86	0.02	0.12	0.09	0.03	0.89
Pae holo, pH 5.0	310	4.89	0.36	0.098	0.49	0.08	0.44	0.02	0.05	0.93
	330	4.95	0.35	0.100	0.49	0.08	0.44	0.02	0.05	0.93
Pae Cu(II) azurin	310	4.80	0.36	0.097	0.51	0.09	0.41	0.02	0.05	0.92
Pae Cu(I)	310	4.76	0.47	0.061	0.46	0.09	0.46	0.01	0.02	0.96
	330	4.74	0.46	0.063	0.46	0.10	0.45	0.01	0.03	0.96
Pae holo, *R* = 0.40	310	5.01	0.12		0.96	0.04		0.37	0.63	
	330	5.03	0.15		0.96	0.04		0.42	0.58	

[a] 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. The precision of the data per exponential decay was typically as follows: τ_1, ±0.01; τ_2, ±0.01; τ_3, ±0.001. Standard errors were typically as follows: τ_1, ±0.03; τ_2, ±0.03; τ_3, ±0.002. The lifetime value of 5.20 ns for *Pfl* apoazurin at 320 nm is slightly less accurate due to interference at this λ by the Raman scattering of water. τ_i is the symbol used for lifetime, F_i represents the fractional fluorescence for each respective component, and α_i represent the normalized preexponential terms. [b] All samples were in 50 mM NH_4OAc buffer, pH 6.5, unless otherwise stated. All samples were measured at 20 °C. All holoazurin and Cu(II)-reconstituted azurin samples had spectral ratios greater than 0.50 unless otherwise stated. λ_{ex} = 292 nm. [c] This apoazurin was prepared from *Pfl* holo having an original spectral ratio = 0.55. [d] This apoazurin was prepared from *Pae* holo having an original spectral ratio = 0.33. [e] This apoazurin was prepared from *Pae* holo having an original spectral ratio = 0.51.

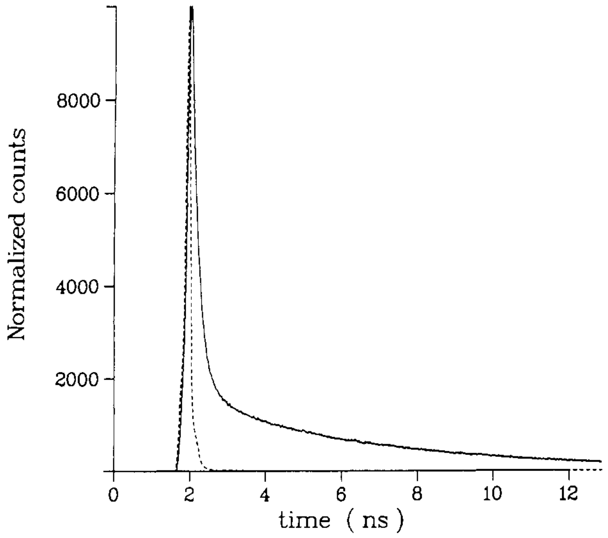

FIGURE 7: Typical normalized fluorescence time-intensity profiles. The lamp intensity profile (dotted line) and holoazurin decay profile were recorded with a channel width = 21.6 ps/channel; λ_{ex} = 292 nm; λ_{em} = 310 nm; emission bandwidth = 4 nm.

(1985)], all apoazurin samples were found to adequately fit single-exponential decay kinetics with a decay time near 5 ns, whereas the best fit for reduced Cu(I) azurin, holoazurin, and Cu(II)-reconstituted holoazurin samples having spectral ratios above 0.50 was found for a triple-exponential decay function. All the copper-containing azurin samples had one long decay time component with a value near 5 ns. The fractional fluorescence of this component varied depending on the source of the holoazurin sample. In the case of *Pfl* holoazurin, this was 0.78 while for *Pae* holoazurin F_1 was 0.49, at 310 nm. The fractional fluorescence of this long decay time component in the reconstituted Cu(II) samples was virtually the same as that of the originally isolated protein. In the case of the reduced Cu(I) azurins, the fractional fluorescence of the long decay time components was 0.87 and 0.46 for *Pfl* Cu(I) azurin and *Pae* Cu(I) azurin, respectively. The shortest decay time component observed in all copper-containing samples was ca. 100 ps. Consistent with the observed variation in fractional fluorescence of the long lifetime component, the fractional fluorescence of this short decay time component significantly changed depending on the source of the azurin. Contrary to earlier reports (Szabo et al., 1983; Munro et al., 1979; Grinvald et al., 1975) and that recently published by Petrich et al. (1987), a third decay time component was required in order to obtain a satisfactory fit to the decay data. The value of this middle decay time component varied depending on the source of the azurin, being 0.52 ns in *Pfl* azurin and 0.36 ns in *Pae* azurin. In all cases the fractional fluorescence corresponding to this component was relatively small (0.04–0.09).

Reduction of the Cu(II) azurins to Cu(I) azurins resulted in a decrease in the value of τ_3 for both *Pae* and *Pfl* azurins to a value of approximately 0.063 ns. In *Pfl* azurin τ_1 increased slightly while the opposite was true in *Pae* azurin. A corresponding increase in F_1 was noted upon reduction of *Pae* azurin. τ_2 followed opposite trends: τ_2 decreased upon reduction of *Pfl* azurin but increased upon reduction of *Pae* azurin with only slight changes in the F_2 values.

In samples which had a spectral ratio of 0.4 the best-fit fluorescence decay kinetics was obtained with a double-exponential model. The decay times were similar to those reported earlier, with the long decay time being again near 5 ns while the short decay component had a value of 0.12 ns at 310 nm which was slightly longer than the shortest component found in the azurin samples with a spectral ratio of >0.50.

The pH dependence of the fluorescence decay parameters is highlighted in Table IV and Figure 9 for *Pfl* holoazurin. The long and middle decay components, τ_1 and τ_2, were titratable between pH 5 and pH 7 whereas the shortest decay component, τ_3, was not affected by pH. The fractional fluorescence of the decay components hardly varied with pH.

The temperature dependence of the fluorescence decay parameters of *Pfl* holoazurin was determined, and the data are given in Table V. The long decay component, τ_1, gradually decreased with increasing temperature whereas the middle decay component, τ_2, followed the opposite trend. The shortest decay component, τ_3, was relatively unaffected by temperature changes. Again, there was negligible change in

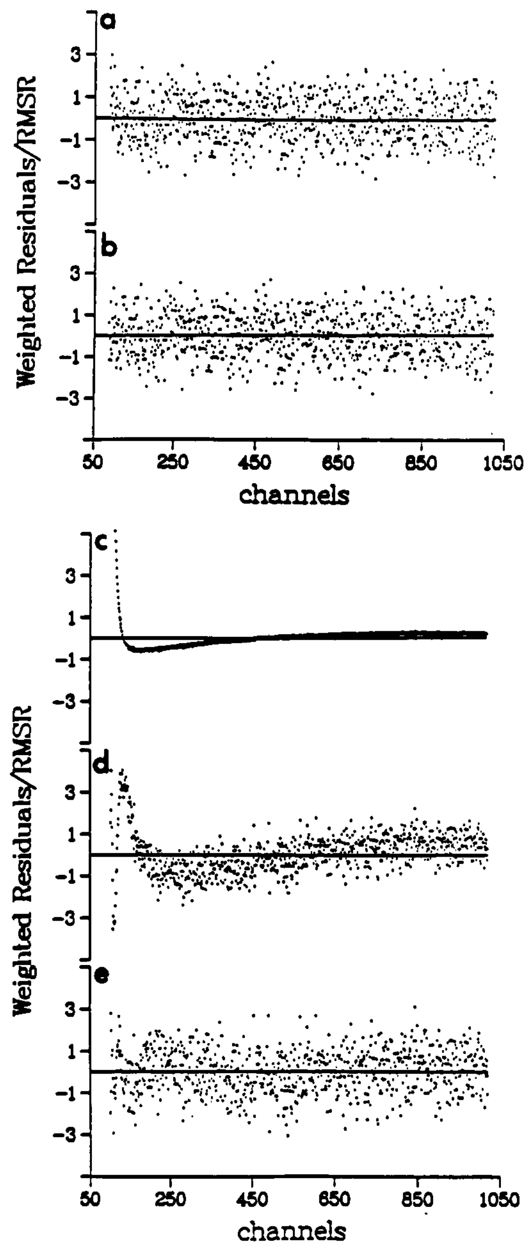

FIGURE 8: Weighted residual plots for the calculated best-fit emission decay profile after deconvolution of the emission decay profile. Panels a and b correspond to those plots for *Pfl* apoazurin fitted to single- and double-exponential decay components, respectively, and panels c–e correspond to those plots for *Pfl* holoazurin fitted to a single-, double-, and triple- exponential decay components, respectively.

Table IV: Influence of pH on Fluorescence Decay Parameters[a] of *P. fluorescens* Holoazurin[b] in 50 mM NH_4OAc Buffer, 20 °C

pH	λ_{em} (nm)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	F_1	F_2	F_3
4	310	4.95	0.48	0.085	0.77	0.07	0.16
	330	4.97	0.50	0.107	0.77	0.04	0.19
5	310	4.91	0.52	0.105	0.78	0.06	0.16
	330	4.95	0.50	0.106	0.79	0.04	0.17
6	310	4.79	0.54	0.105	0.77	0.05	0.18
	330	4.82	0.53	0.102	0.78	0.04	0.18
7	310	4.71	0.55	0.101	0.77	0.05	0.18
	330	4.71	0.55	0.101	0.78	0.04	0.18
8	310	4.69	0.57	0.100	0.77	0.04	0.19
	330	4.70	0.60	0.102	0.78	0.03	0.19

[a] Each value is the mean of at least three determinations, and typical standard errors are as follows: τ_1, ±0.03; τ_2, ±0.03; τ_3, ±0.002. τ_i is the symbol used for lifetime, and F_i represents the fractional fluorescence for each respective component and is determined according to $F_i = \alpha_i\tau_i/\sum\alpha_i\tau_i$, where α_i is the preexponential of the corresponding ith exponential term. [b] Each holoazurin sample used had a spectral ratio greater than 0.52.

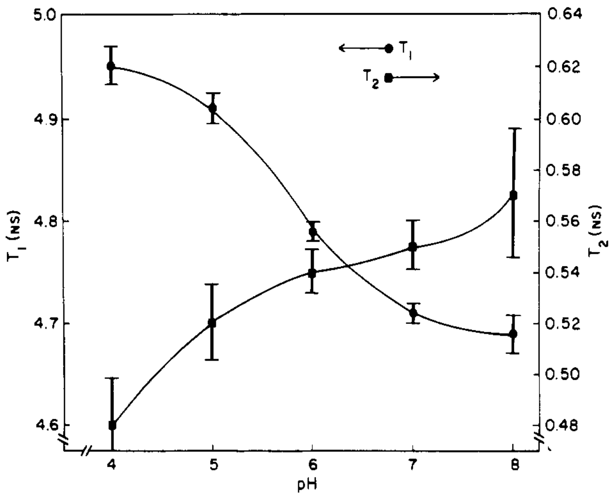

FIGURE 9: Plot of the variation of the long decay component, τ_1 (●), and the middle decay component, τ_2 (■), with pH. The left-hand ordinate is that for τ_1, and the right-hand ordinate is that for τ_2. The samples were excited at 292 nm, and the emission was monitored at 310 nm. Errors are reported as the standard error of the mean.

Table V: Influence of Temperature on Fluorescence Decay Parameters[a] of *P. fluorescens* Holoazurin[b] in 50 mM NH_4OAc Buffer, pH 5.0

temp (°C)	λ_{em} (nm)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	F_1	F_2	F_3
5	310	5.11	0.44	0.100	0.79	0.04	0.17
	330	5.14	0.45	0.101	0.80	0.03	0.17
10	310	5.01	0.46	0.099	0.79	0.06	0.15
	330	5.04	0.47	0.091	0.80	0.05	0.15
16	310	4.96	0.48	0.104	0.78	0.04	0.18
	330	4.98	0.48	0.097	0.79	0.04	0.17
20	310	4.91	0.52	0.105	0.78	0.06	0.16
	330	4.95	0.50	0.106	0.79	0.04	0.17
29	310	4.61	0.71	0.111	0.78	0.04	0.19

[a] Each value is the mean of at least three determinations, and typical standards error are the same as those in Table IV. [b] Each holoazurin sample used had a spectral ratio greater than 0.52.

the fractional contribution of each decay component to the total fluorescence with temperature.

DISCUSSION

Homogeneity of Protein Samples. In 1967, Ambler reported that azurin samples having a spectral ratio ranging from 0.48 to 0.60 may be regarded as homogeneous on the basis of results from starch–gel electrophoresis, although some difficulty in removing all of the cytochrome which copurified with the azurin was reported (Ambler & Brown, 1967). Since then, most workers cite either this criterion alone, no criteria, or the spectral ratio plus SDS–PAGE when discussing azurin homogeneity (Blaszak et al., 1983; Canters et al., 1984; Blair et al., 1985; Szabo et al., 1983; Finazzi-Agro et al., 1970; Engeseth & McMillin, 1986; Petrich et al., 1987). In some cases, azurins with spectral ratios below 0.48 have been routinely used, and reports using azurin with spectral ratios as low as 0.4 have been published. Grinvald et al. (1975) used the rationale that if the apoazurin prepared from the corresponding holoazurin gave monoexponential decay kinetics, then the holoazurin used originally must be pure. In agreement with these workers it was stated (Szabo et al., 1983) that holoazurin samples could not be initially contaminated with apoazurin since the addition of excess Cu(II) ion did not result in either an increase in absorbance of the 620-nm band or a decrease in the fluorescence intensity. However, no effort was made at that time toward correlating this spectral evidence with biochemical techniques capable of detecting protein heterogeneity.

This paper includes biochemical evidence which establishes definite criteria by which homogeneity can be monitored. It is shown that homogeneous azurin samples free from apoazurin or other protein contaminants can be obtained for spectroscopic studies. The various electrophoretic results presented herein and the stoichiometry determination using amino acid and atomic absorption analyses show that a single UV–vis absorption spectrum may provide satisfactory evidence as to whether an azurin sample was homogeneous.

When the spectral ratio was below 0.50, neither SDS–PAGE nor the shape of the UV–vis absorption spectrum revealed the obvious presence of any protein contaminant other than a diminished absorbance at 620 nm relative to that of 280 nm. Addition of either Cu(II) ion in a 25-fold molar excess or an oxidizing agent such as 20 μM $K_3Fe(CN)_6$ had no effect upon either the absorption spectrum or the steady-state fluorescence intensity. These observations provided evidence that the protein samples did not contain either a colorless, reconstitutable apoazurin or a colorless reduced Cu(I) azurin. The combined amino acid analyses and atomic absorption stoichiometry determination of holoazurins having spectral ratios of <0.50 suggested contamination by a copper-less protein. The reproducibility of the amino acid composition results, which in the case of *Pae* azurin matched the previously published sequence data (Ambler, 1973), as well as the detection of only single bands in SDS–PAGE suggested that any proteins present had compositions similar to holoazurin. This would be consistent with holoazurin being contaminated with a non-copper-reconstitutable apoazurin when the spectral ratio was less than 0.50. The nature of this "nonreconstitutable" apoazurin is unknown at the present time. The most likely possibility is that the ligands responsible for Cu binding have been altered either during the workup or during biosynthesis.

The isoelectric focusing results strongly supported this suggestion since apoproteins could be distinguished from holoazurins as they migrated at more acidic p*I*s. In the samples whose spectral ratios were below 0.50, the intensity of the extra bands attributed to apoazurin increased as the spectral ratio decreased. Two preliminary attempts have been made to remove this putative, nonreconstitutable apoazurin from the holo samples by both trypsin digestion and chromatofocusing. Trypsin digestion improved the spectral ratio slightly (0.30–0.35) due to the removal of cytochrome but could not digest the nonreconstitutable apoazurin contaminant.

Chromatofocusing was also attempted and revealed that two closely eluting proteins were present in holo samples having spectral ratios below 0.50. However, due to the poor resolution of the two peaks, appreciable amounts of the separate components were not obtained by this method.

In the isoelectric focusing of the *Pae* holo samples (spectral ratio 0.53), an additional band, beside that ascribed to holoazurins, was observed. This band was noted in all samples of *Pae* azurin, independent of spectral ratio and independent of protein source (purified or commercially obtained). The band migrated to an extremely acidic position (p*I* less than 3.00). It is suggested that it may be due to artifacts arising from interactions with the ampholytes or represent non-protein particles which were also stained (Illingworth, 1972). Additional arguments which support this view follow. Samples with a spectral ratio of 0.53 containing this band were found to possess a 1:1 copper to protein stoichiometry on the basis of amino acid and atomic absorption analyses. Amino acid analysis revealed a composition indistinguishable from that which has already been published (Ambler, 1973). Apo samples prepared from these holo samples were fully Cu(II) reconstitutable, and it was noted that the presence of this extra band diminished significantly upon copper removal. All of these observations, including single bands observed upon SDS–PAGE, support the idea that no other non-azurin protein could be present in the samples and it is due to a complex between ampholyte and *Pae* azurin. The results also argue against some other form of an azurin sample accounting for these bands. The corresponding apo samples were found to obey monoexponential fluorescence decay kinetics. At the level of resolution obtainable with the instrumentation employed, no other protein has been reported which displays such behavior. It is difficult to imagine that a protein (azurin derivative or oxidized apo) with such a markedly different p*I* would have an amino acid composition essentially identical with that of the azurin of interest and have identical fluorescence properties. Finally, it was interesting to note that this additional band was not observed when the *Pfl* holoazurin was run under identical conditions. This suggests that this latter protein does not form a similar complex with ampholytes.

Provided that the spectral ratio was above 0.53 or greater and no absorbance at 410 nm (attributed to cytochrome) was observed, neither the biochemical tests nor the spectral results could detect any spurious protein contamination. Such criteria would also rule out contamination by an "apoazurin". Since it was possible to obtain spectral ratios above 0.50 even when a Soret band was detectable, it is important that an entire UV–vis absorption spectrum, as illustrated in Figure 1, be reported when purity is being attested.

Preparation of a Reconstitutable Apoazurin. The method first reported for the preparation of apoazurin involved dialysis against potassium cyanide (Yamanaka et al., 1963). More recently, Blaszak et al. (1983) stated that the use of this method yielded an apoprotein which gave variable kinetics for Cu(II) uptake, depending upon the number of times the protein was exposed to cyanide solution. They suggested that it was likely that Yamanaka et al. failed to reduce the pH after addition of 0.5 M KCN (pH 10.5). Our initial apo preparation results support this view, and in addition, when the solution was dialyzed overnight at this pH, changes in the absorption spectrum at 280–300 nm suggested protein denaturation. By careful control of the pH after cyanide addition, a fully Cu(II)-reconstitutable apoazurin retaining less than 2% of its original copper can be obtained.

We also attempted the preparation of apoazurin according to the method of Blaszak et al. (1983). These samples did not show full Cu(II) reconstitutability, and significant amounts of copper remained in the original samples. Frequently, spectral interference was caused by the presence of ascorbate or an ascorbate decomposition product. Recently, Petrich et al. (1987) also reported that traces of ascorbate or a yellow impurity remained in the apoazurin samples even after dialysis when the protein was prepared by this method.

As judged by the final spectral ratio and recovery of the fluorescence decay parameters, the full reconstitution of apoazurin could be achieved in the range of pH 5–7. However, in contrast to the work of Tennent and McMillin (1979), we could not achieve full reconstitution at pHs above 7.5. The examination of the pH dependence of the quantum yield of apoazurin (Figure 6) showed a marked decrease in the quantum yield at pHs above 7 which was not seen in the holoform. These results may suggest the apoazurin was less stable at these high pH values.

Fluorescence Behavior of Homogeneous Cu(II) Azurin. On the basis of the evidence presented in this paper, the holoazurin samples used in the fluorescence study were considered homogeneous. An analysis of the decay data obtained for those holo samples having a spectral ratio above 0.50 was best fit by a sum of three exponential decay components, instead of a sum of two exponentials as previously reported (Szabo et al., 1983; Grinvald et al., 1975; Munro et al., 1979; Petrich et al., 1987). Double-exponential decay was observed when the spectral ratios were below 0.50. In these latter cases the fraction of the fluorescence of the long-lived component (~5 ns) was considerably greater, consistent with presence of a nonreconstitutable apoazurin. In all apoazurin samples, single-exponential decay behavior with a decay time near 5 ns was observed at all emission wavelengths. This confirmed earlier published values. The lack of reconstitutability was not reflected in a heterogeneity of Trp fluorescence in the apoprotein preparations. This suggests that if there were any conformational changes associated with the inability to take up Cu, they were not sensed by the Trp residue. The results also suggest that in the absence of copper the Trp in the two homologous azurins existed in comparable molecular environments.

Contrary to earlier work from this laboratory and that recently reported by Petrich et al. (1987), the fluorescence decay of the holoazurin or copper-reconstituted samples obeyed triple-exponential decay kinetics. The fact that the fluorescence decay parameters could be recovered on copper reconstitution of the apoprotein strongly implies that the component with the intermediate decay time was not due to any experimental artifact. The observation of three exponential decay components was highly reproducible. We are unable to account for the difference between our results and those recently reported by Petrich et al. (1987), who found only double-exponential decay behavior for holoazurin. In their work they proposed that the long decay component found in holoazurin was due to an apoazurin impurity. However, the long decay time value reported by Petrich et al. for *Pae* apoazurin (5.16 ± 0.19 ns) was significantly different from that found in the corresponding holoazurin (4.15 ± 0.53 ns). For this long decay component of *Pae* holoazurin the fractional fluorescence amounted to 55% of the total. A value similar to that is reported herein. The large error on a decay component comprising 55% of the total fluorescence is surprising. In the case of the middle decay time component reported in this work, simulation studies in our laboratory have shown that the ability to resolve a fluorescent component which makes only a small contribution (<5%) to the total fluorescence may depend on the total number of counts in a fluorescence decay curve. In our experiments we typically collected more than 20 000 counts in the maximum channel of the fluorescence decay curve, which has been shown to be sufficient to recover decay components such as we report in this work.

In comparing the decay parameters obtained from the two homologous azurins, it can be seen that the largest difference was observed in the fractional contribution of the longest and shortest lifetime components to the total observed fluorescence. As the residues in the vicinity of the active copper sites in azurin are highly conserved (Ambler, 1973), this suggests that the less highly conserved amino acids have an influence upon the conformational distribution of these two proteins. Recently, the secondary structure of *Pfl* holoazurin has been examined by resonance-enhanced infrared spectroscopy (Surewicz et al., 1987) and was shown to be quite similar at that level to the structure of *Pae* azurin, on the basis of the known X-ray data of the latter (Adman & Jensen, 1981; Adman et al., 1978). This requires that the Trp fluorescence was sensitive to subtle structural or dynamic differences in the two proteins.

The lack of exact agreement in detail of the Trp fluorescence decay behavior in holoazurin between different laboratories may be due to one of several factors. One may be the homogeneity of the various azurin preparations. When the spectral ratios were below 0.50, only two decay components were observed. As apoazurin was on average 6 times more fluorescent than the corresponding holoform, even a small fraction could potentially "mask" a weak component if it were present.

Another factor may be the sensitivity and resolution capabilities of the time-resolved instrumentation. Improvements in the instrumental stability and lamp intensity profile would logically permit a more accurate analysis of the sample decay curve. In earlier work from this laboratory, the instrument response function was >2 ns wide and had a long tail. The data were collected at a resolution of 0.043 ns/channel, and the technique used for correction of the photomultiplier "color effect" may have masked a small amount of a third component.

Another source of variability may be the confusion associated with the taxonomic numbering of the various *Pseudomonas* species and strains. Initial reports of the amino acid sequence of *Pfl* azurin were later corrected by the realization that the protein was that of *Pae* rather than that of *Pfl* (Ambler & Brown, 1967; Ambler & Wynn, 1973). To complicate matters further, seven biotypes of *Pfl* exist (Stanier et al., 1966) while only a few have been sequenced. Petrich et al. (1987) reported using a bacterial strain whose ATCC number 13525 corresponding to *P. fluorescens* biotype A, not *P. aeruginosa* as they indicate.

Conformational Heterogeneity versus an "Apo-like" Contaminant. It is clearly shown in this paper from the electrophoretic results and the determination of the copper to protein stoichiometry by atomic absorption and amino acid analysis that the holoazurin samples did not contain any apoazurin contamination as long as the spectral ratio was 0.53 or greater. When the gels were heavily loaded with azurin samples used for the fluorescence studies, no trace of any apoazurin could be detected. We believe that the combination of the electrophoretic experiments and the results obtained suggest a conformational heterogeneity of the holoazurin molecule as proposed earlier (Szabo et al., 1983).

The possible existence of a number of conformations of the azurin molecule and the pH dependence of their interconversion has been proposed earlier. NMR measurements showed that in *Pae* azurin His-35 slowly exchanged between its protonated and nonprotonated forms (Hill & Smith, 1979; Ugurbil & Bersohn, 1977a; Ugurbil et al., 1977b). It was also found that two forms of His-83 were in fast exchange, and the X-ray structure of *Pae* azurin crystals grown at pH 5.5–5.8 clearly showed that the imidazole group of His-83 juts out into the solution whereas that of His-35 lies in a crevice near the surface (Adman & Jensen, 1981; Adman et al., 1978). Structural and kinetic data collectively suggested that the fast reaction of imidazole protonation could be coupled to a slower conformational change involving His-35 (Corin et al., 1983).

In the earlier work from this laboratory it was proposed that the two fluorescence decay components observed in holoazurin resulted from two different conformational states of the copper–ligand complex. The long decay time component (5 ns) was assigned to a conformer with the copper–ligand complex in a more tetrahedral structure and at some distance from Trp-48 (Szabo et al., 1983). In this case fluorescence quenching by interaction with the copper–ligand complex would not be important. The short decay time component was assigned to a conformer in which the copper–ligand complex had a flattened tetrahedral structure bringing potential quenching groups closer to the Trp residue. Obviously, the observation of a third decay time component in the holoazurin samples requires a revision of this former assignment.

From the fluorescence results, the longest lifetime was essentially the same for the two homologous holoazurins. In addition, it was similar to the single decay time observed for apoazurin (4.9 ns vs 5.1 ns). This decay time component of the holoazurins was titratable between pH 5 and pH 7, decreasing with increasing pH (pH 4, 4.95 ns; pH 8, 4.70 ns). As was proposed earlier, this long decay component is assigned to a protein conformation in which the interactions of the Trp with the Cu binding site are not important. The pH behavior of this decay time component suggests that it may be attributed to a conformer whose structure was influenced by a titratable histidine residue. Both Canters et al. (1984) and Corin et al. (1983) have suggested the protonation–deprotonation of His-35 (pK = 5.9 ± 0.4), a nonliganded residue lying adjacent to the ligand His-46, leads to conformational changes of the protein. The decrease in the value of the long decay time component with increasing pH rules out any close interaction of the surface histidine residues 35 and 83 with the Trp. If there were such an interaction, it would be expected that the fluorescence decay time should increase as the histidine becomes increasingly deprotonated since a proton-transfer quenching process from histidine to tryptophan would be eliminated (Masotti et al., 1988). The results then are most consistent with alteration of the protein conformation with protonation or deprotonation of His-35, which alters the interaction of the Trp with other parts of the protein structure leading to a reduction of the fluorescence decay time. The increase in quantum yield (20% between pH 4 and pH 8) (Figure 5) is difficult to rationalize in terms of the fluorescence decay parameters (Table IV). The fractional fluorescence of the long decay component remained constant with increasing pH. There are two possible explanations to account for this behavior. Either there is a nonfluorescent conformer which becomes fluorescent at higher pH, or the radiative lifetime of the Trp residue in the holoprotein changes with pH. A rationalization which favors this latter alternative is presented below.

The quantum yield of the apoazurin also was titratable between pH 5 and pH 7, although the inflection appeared at a slightly higher pH (Figure 6). This suggests that the same protonation–deprotonation of a His residue may be influencing the Trp fluorescence even in the absence of the copper center and supports the view that comparable conformational changes in the holoazurin may occur as the pH is varied. In contrast to that of the holoazurin, the fluorescence decay time of the apoazurin remained constant with pH, lending support to the proposal that the radiative lifetime changed with pH.

The remaining two lifetimes observed in the holoazurin must be the result of an influence by the copper–ligand complex on the conformers, since they are absent in the apoprotein. The decay time of the shortest component, τ_3, remained essentially constant, not being influenced by temperature, pH, or even the different amino acid composition in the proteins. The major difference was its contribution to the total fluorescence observed in the two homologous azurins ($\simeq$16% for *Pfl* vs $\simeq$44% for *Pae*). On the other hand, the middle lifetime component, τ_2, was affected by pH, temperature, and amino acid differences, but its contribution to the total fluorescence was not that much different in the two homologous azurins.

We propose that the two shorter decay components represent conformations wherein the copper–ligand complex is closer to the tryptophan residue. It is suggested that the two shorter decay components may be due to local conformational differences of the tryptophan residue with respect to a proximate ligand complex. The greater effect of pH on the intermediate decay time suggests that the protein conformational changes resulting from protonation of His-35 influence the local interactions of the tryptophan with the copper–ligand complex.

In our discussion we clearly put considerable emphasis on the effect of protonation of the histidine residues on the conformational states of the protein. However, the photophysical origin of the quenching of the tryptophan excited state leading to the short decay times remains speculative in our opinion. When the copper is reduced from Cu(II) to Cu(I), changes in the decay times and fractional fluorescence values are observed. The shortest decay time becomes even shorter on reduction, changing from 0.10 ns in the Cu(II) form to 0.06 ns in the Cu(I) form. It may be that the short decay time results from an electron-transfer mechanism from the excited state of the tryptophan to the copper center. However, we cannot distinguish such an excited state deactivation process from other possibilities. For example, the quenching may result from an interaction between the charge-transfer excited state of tryptophan (Meech & Phillips, 1983) and the electronic structure of the copper–ligand complex. Another possible quenching mechanism may involve either the ligand histidines or ligand cysteine or methionine in a special interaction with Trp-48. It may be that upon reduction of the copper the proximity of the ligand complex to the tryptophan is altered, leading to a more efficient deactivation process. It is interesting that the changes observed upon reduction of *Pae* azurin are different from those observed in *Pfl* azurin (Table III). We are unable to offer any rationalization of these differences.

The assignment of the three decay components to three different conformational states of the protein leads to the question of estimating the relative concentrations of these components. It can readily be shown according to equations presented by Donzel et al. (1974) that the relative ground-state concentrations can be estimated from the preexponential terms. For example

$$\frac{c_j}{c_k} = \frac{\alpha_j \tau_j}{\alpha_k \tau_k} \frac{\phi_k}{\phi_j} \qquad (1)$$

where c_j and c_k are the relative concentrations of the jth and the kth decay components, respectively, and α, τ, and ϕ are the normalized preexponential term, decay time, and quantum yield of the corresponding components. Since $\phi_i = \tau_i/\tau_r$, where τ_r is the reciprocal radiative rate constant, and if τ_r is the same for each component, then eq 1 reduces to

$$c_j/c_k = \alpha_j/\alpha_k \qquad (2)$$

A priori one would consider that τ_r would be the same for each component. Then according to eq 2 the normalized preexponential terms (Table III) represent the relative concentration of each component, where $c_1 + c_2 + c_3 = 1$. Their values clearly show that the concentration of the long decay time conformer changed from 9% to 2% for *Pfl* and *Pae* azurins, respectively; the concentration of the short decay time conformer changed from 85% to 93% while the concentration of the intermediate decay time conformer was 6% and 5%, respectively, for the two proteins.

Using these relative concentration values and the value of the radiative lifetime determined earlier (15.5 ns; Szabo et al., 1983), it is possible to calculate an expected steady-state quantum yield for each of the proteins. The calculations were made with

$$\phi = c_1\frac{\tau_1}{\tau_r} + c_2\frac{\tau_2}{\tau_r} + c_3\frac{\tau_3}{\tau_r} \qquad (3)$$

The calculated quantum yields using the decay parameters were 0.036 and 0.014 for *Pfl* and *Pae* holoazurins, respectively. These calculated quantum yields are approximately 75% of the measured values. It is expected that there should be a closer correspondence between the measured quantum yield and that calculated (note that the quantum yield accuracy was better than 5%); hence, one of the assumptions must be incorrect (Donzel et al., 1974). The most likely possibility was that τ_r for the holoazurin was different from that of the apoazurin, owing perhaps to an electronic perturbation of the Trp-48 excited singlet state by the copper–ligand complex. With the measured quantum yield values of 0.051 and 0.018 for *Pfl* and *Pae* holoazurins, respectively, a value of τ_r for each protein can be calculated from eq 3. This calculated value of τ_r was 11.0 ns and 11.7 ns for *Pfl* and *Pae* azurins, respectively. These values are significantly different from that calculated for apoazurin. They are not unreasonable since radiative lifetimes similar to this have been reported for several indole derivatives in nonpolar environments (Meech & Phillips, 1983). This difference between the estimated radiative lifetimes for the apo- and holoazurins suggests the interesting speculation of whether τ_r for the 5-ns component was different from that of the other two components.

If we assume that τ_r for the two short decay components are similar and the τ_r for the long decay component is the value obtained from apoazurin (15.5 ns), then it is possible to estimate the former value. If this were the case, the value of the relative concentrations are not represented by eq 2 but are related according to

$$\frac{c_1}{c_2} = \frac{\alpha_1}{\alpha_2}\frac{15.5}{\tau_r'} \qquad \frac{c_1}{c_3} = \frac{\alpha_1}{\alpha_3}\frac{15.5}{\tau_r'} \qquad (4)$$

where 15.5 ns is the radiative lifetime of the long decay time component and τ_r' is the radiative lifetime of the two short decay components. With $c_1 + c_2 + c_3 = 1$ and eq 3 and 4, the values of τ_r' equal 10.2 ns for *Pfl* and 11.6 ns for *Pae* holoazurins, respectively, are calculated. Following these calculations, then the relative concentrations require recalculation according to eq 4. When this was done, the relative concentrations for *Pfl* holoazurin were $c_1 = 0.13$, $c_2 = 0.07$, and $c_3 = 0.81$, while for *Pae* holoazurin they were $c_1 = 0.02$, $c_2 = 0.05$, and $c_3 = 0.93$. This suggestion that the different conformers have different radiative lifetimes would be consistent with the conformer model which we have proposed. In the conformers with the short decay times the electronic interaction of the copper–ligand complex with Trp-48 might lead to a shorter radiative lifetime.

Over the temperature range studied (5–29 °C) there were only small changes in the decay parameters obtained. Importantly, the fractional fluorescence values remained constant. Using these parameters and the radiative decay times calculated for long decay and short decay components, it is possible to calculate the relative concentrations of each component at each of the measured temperatures according to eq 4. It was found that there was hardly any change in the relative concentrations over the temperature range studied in this paper. The decrease in value of the long decay time is considerably less than that seen for other tryptophan proteins or peptides. This is in keeping with the relatively stable structure of azurin.

Table VI: Summary of pH Effect on Radiative Rate Constants of the Short Decay Time Components, Relative Concentration of the Long Decay Component, and Fractional Quantum Yield of This Component in *Pfl* Holoazurin

pH	τ_r' (ns)[a]	c_1[b]	ϕ_1[b]
4	9.9	0.12	0.037
5	10.2	0.13	0.041
6	8.7	0.14	0.043
7	8.1	0.14	0.044
8	7.4	0.15	0.045

[a] Radiative decay time of the short decay time components calculated according to the text. [b] The relative concentration, c_1, and fractional quantum yield, ϕ_1, of the long decay time component.

As indicated above, the fluorescence quantum yield of holoazurin (*Pfl*) increased with pH, yet the fractional fluorescence values remained constant, and the decay time of the major fluorescent component, the long decay time component, decreased with increasing pH. If we assume that this component has a radiative lifetime equivalent to that of apoazurin, 15.5 ns, then again the radiative lifetimes of the short decay components (assuming they are equal) can be calculated. These values are tabulated in Table VI. Using the various parameters, it is possible to calculate the relative concentrations of each component, and the fractional quantum yield at each pH value. These values for the long decay component are tabulated as well in Table VI. The relative concentration of the long decay component increases from a value of 0.115 at pH 4 to 0.148 at pH 8. The fractional quantum yield increases from 0.037 to 0.045 between the same pH range, easily accounting for the change in total quantum yield observed. This result is significant in another respect since it shows that the relative concentrations of the various decay components or conformers are affected by pH. A rationalization of the decrease in radiative lifetime of the short decay components may be that, on an increase in the pH, the histidine residues become neutral, leading to changes in the conformational distribution and a more effective electronic perturbation of the tryptophan excited state by the copper–ligand complex.

In summary, we report evidence which strongly indicates that holoazurin samples free of any apoazurin contaminant can be obtained. Further, we show that absorption spectral ratios of >0.50 can be used as a convenient measure of the homogeneity of holoazurin samples. The fluorescence decay behavior of the holoazurins from two different sources was best fit by three components whose values corresponded closely in the two proteins. One decay time was found at 5 ns, another between 0.5 and 0.4 ns, and a third at 0.10 ns. These three decay components are suggested to originate from three different protein conformational states. It is possible to estimate the relative concentrations of these three conformers, and it was shown that they were different for the two proteins. In addition, we suggest that the radiative rate constant of tryptophan, when it is proximate to the copper–ligand complex, is affected by the pH of the system owing to structural changes of the protein with pH. Furthermore, we suggest that the distribution of conformational states also changes with pH. In the accompanying paper (Hutnik & Szabo, 1989), we present a study of other metallo derivatives of azurin which also demonstrate conformational heterogeneity which varies depending on the nature of the metal–ligand complex.

ACKNOWLEDGMENTS

We thank P. Fleming and D. Griffith for the growth and harvesting of the *P. fluorescens* and *P. aeruginosa*, respectively. We also thank Dr. M. Yaguchi for his assistance in obtaining the amino acid composition of the azurins. We also thank Dr. D. Williamson and S. Moulton for their guidance and the use of their equipment for chromatofocusing. The technical assistance of D. T. Krajcarski is gratefully acknowledged.

Registry No. Cu, 7440-50-8.

REFERENCES

Adman, E. T., & Jensen, L. H. (1981) *Isr. J. Chem. 21*, 8.

Adman, E. T., Stenkamp, R. E., Sieker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol. 123*, 35.

Adman, E. T., Canters, G. W., Hill, H. A. O., & Kitchen, N. A. (1982) *FEBS Lett. 143*, 287.

Ambler, R. P. (1963) *Biochem. J. 89*, 349.

Ambler, R. P. (1973) in *Recent Developments in the Chemical Study of Protein Structures* (Previero, A., Pechere, J. F., & Coletti-Previero, M. A., Eds.) pp 289–305, INSERM Press, Paris.

Ambler, R. P., & Brown, L. H. (1967) *Biochem. J. 104*, 784.

Ambler, R. P., & Wynn, M. (1973) *Biochem. J. 131*, 485.

Blair, D. F., Campbell, G. W., Schoonover, J. R., Chan, S. I., Gray, H. B., Malmstrom, B. G., Pecht, I., Swanson, B. I., Woodruff, W. H., Cho, W. K., English, A. M., Fry, H. A., Lum, V., & Norton, K. A. (1985) *J. Am. Chem. Soc. 107*, 5755.

Blaszak, J. A., McMillin, D. R., Thornton, A. T., & Tennent, D. L. (1983) *J. Biol. Chem. 258*, 9886.

Brill, A. S. (1978) *Biophys. J. 22*, 139.

Brunori, M., Greenwood, C., & Wilson, M. T. (1974) *Biochem. J. 137*, 113.

Brunori, M., Parr, S. R., Greenwood, C., & Wilson, M. T. (1975) *Biochem. J. 151*, 185.

Burstein, E. A., Permyakov, E. A., Yashin, V. A., Burkhanov, S. A., & Finazzi-Agro, A. (1977) *Biochim. Biophys. Acta 491*, 155.

Canters, G. W., Hill, H. A. O., Kitchen, N. A., & Adman, E. T. (1984) *Eur. J. Biochem. 138*, 141.

Corin, A. F., Bersohn, R., & Cole, P. E. (1983) *Biochemistry 22*, 2032.

Donzel, B., Gauduchon, P., & Wahl, Ph. (1974) *J. Am. Chem. Soc. 96*, 801.

Engeseth, H. R., & McMillin, D. R. (1986) *Biochemistry 25*, 2448.

Finazzi-Agro, A., Rotilio, G., Avigliano, L., Guerrieri, P., Boffi, V., & Mondovi, B. (1970) *Biochemistry 9*, 2009.

Finazzi-Agro, A., Giovagnoli, C., Avigliano, L., Rotilio, G., & Mondovi, B. (1973) *Eur. J. Biochem. 34*, 20.

Grinvald, A., Schlessinger, J., Pecht, I., & Steinberg, I. Z. (1975) *Biochemistry 14*, 1921.

Hill, H. A. O., & Smith, B. E. (1979) *J. Inorg. Biochem. 11*, 79.

Hutnik, C. M., & Szabo, A. G. (1989) *Biochemistry* (following paper in this issue).

Illingworth, J. A. (1972) *Biochem. J. 129*, 1125.

MacKinnon, A. E., Szabo, A. G., & Miller, D. M. (1977) *J. Phys. Chem. 81*, 1564.

Mallinson, R., Carter, R., & Ghiron, C. A. (1981) *Biochem. Biophys. Acta 671*, 117.

Masotti, L., Cavatorta, P., Szabo, A. G., Farruggia, G., & Sartor, G. (1988) in *Fluorescent Biomolecules* (Jameson, D., & Gratton, E., Eds.) Plenum Press, New York (in press).

McMillin, D. R., & Tennent, D. L. (1979) in *ESR and NMR of Paramagnetic Species in Biological and Related Systems* (Bertini, I., & Drago, R. S., Eds.) pp 369–379, D. Reidel, Dordrecht, The Netherlands.

Meech, S. R., Phillips, D., & Lee, A. G. (1983) *Chem. Phys. 80*, 317.

Mitra, S., & Bersohn, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6807.

Munro, I., Pecht, I., & Stryer, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 56.

Norris, G. E., Anderson, B. F., & Baker, E. N. (1986) *J. Am. Chem. Soc. 108*, 2784.

Parr, S. R., Barber, D., & Greenwood, C. (1976) *Biochem. J. 157*, 423.

Petrich, J. W., Longworth, J. W., & Fleming, G. R. (1987) *Biochemistry 26*, 2711.

Righetti, M., & Drysdale, J. W. (1971) *Biochim. Biophys. Acta 236*, 7.

Ryden, L., & Lundgren, J. O. (1979) *Nature 261*, 344.

Stanier, R. Y., Palleroni, N. J., & Doudoroff, M. (1966) *J. Gen. Microbiol. 43*, 159.

Surewicz, W. T., Szabo, A. G., & Mantsch, H. H. (1987) *Eur. J. Biochem. 167*, 519.

Szabo, A. G., Stepanik, T. M., Wayner, D. M., & Young, N. M. (1983) *Biophys. J. 41*, 233.

Tennent, D. L., & McMillin, D. R. (1979) *J. Am. Chem. Soc. 101*, 2307.

Tullius, T. D., Frank, P., & Hodgson, K. O. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4069.

Ugurbil, K., & Bersohn, R. (1977) *Biochemistry 16*, 3016.

Ugurbil, K., Maki, A. H., & Bersohn, R. (1977a) *Biochemistry 16*, 901.

Ugurbil, K., Norton, R. S., Allerhand, A., & Bersohn, R. (1977b) *Biochemistry 16*, 886.

Wherland, S., & Pecht, I. (1978) *Biochemistry 17*, 2585.

Wilson, M. T., Greenwood, C., Brunori, M., & Antonini, E. (1975) *Biochem. J. 145*, 449.

Yamanaka, T., Kijimoto, S., & Okunuki, K. (1963) *J. Biochem. 53*, 256.

Zuker, M., Szabo, A. G., Bramall, L., Krajcarski, D. T., & Selinger, G. (1985) *Rev. Sci. Instrum. 56*, 14.