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Internal Motion and Electron Transfer in Proteins: A Picosecond Fluorescence Study of Three Homologous Azurins†

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ABSTRACT: We have carried out a picosecond fluorescence study of holo- and apoazurins of *Pseudomonas aeruginosa* (azurin Pae), *Alcaligenes faecilis* (azurin Afe), and *Alcaligenes denitrificans* (azurin Ade). Azurin Pae contains a single, buried tryptophyl residue; azurin Afe, a single surface tryptophyl residue; and azurin Ade, tryptophyl residues in both environments. From anisotropy measurements we conclude that the interiors of azurins Pae and Ade are not mobile enough to enable motion of the indole ring on a nanosecond time scale. The exposed tryptophans in azurins Afe and Ade show considerable mobility on a few hundred picosecond time scale. The quenching of tryptophan fluorescence observed in the holoproteins is interpreted in terms of electron transfer from excited-state tryptophan to Cu(II). The observed rates are near the maximum predicted by Marcus theory for the separation of donor and acceptor. The involvement of protein matrix and donor mobility for electron transfer is discussed. The two single-tryptophan-containing proteins enable the more complex fluorescence behavior of the two tryptophans of azurin Ade to be understood. The single-exponential fluorescence decay observed for azurin Pae and the nonexponential fluorescence decay observed for azurin Afe are discussed in terms of current models for tryptophan photophysics.

Proteins are dynamic structures, continuously exchanging thermal energy with their surroundings; their constituent atoms are in a state of continuous motion (Cooper, 1984). The goal of relating protein structure and dynamics, and the possible relation of motion to function, has attracted the efforts of both experimentalists (Ringe & Petsko, 1985) and theoreticians (Karplus & McCammon, 1983; McCammon, 1984).

The naturally occurring aromatic amino acid tryptophan has proved to be a useful fluorescence probe of protein structure (Longworth, 1971, 1983; Beecham & Brand, 1985). In particular, the decay of tryptophyl fluorescence anisotropy has indicated the existence of a local mobility in proteins (Hochstrasser & Negus, 1984; Lakowicz et al., 1983; Munro et al., 1979; Scarlata et al., 1984; van Hoek et al., 1983).

In this paper, we present a study of the time-resolved fluorescence spectroscopy of blue-copper bacterial electron-

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transfer proteins, azurins, and their apoproteins. Azurins are redox enzymes isolated from several denitrifying bacteria. The enzyme contains a single copper atom as the active site in an amino acid chain of 128 or 129 amino acids and has a molecular mass of 14 kDa. The Cu(II) atom has an intense blue coloration, arising from a charge transfer from a cysteine thiolate anion ligand to the Cu(II) cation (Gray & Malmstrom, 1983). The copper atom exists in a flattened tetrahedral coordination with two histidines and a methionyl thioether sulfur in addition to the cysteinyl thiolate. The enzyme is found in the cytoplasm of several facultatively anaerobic denitrifying water and soil bacteria; nitrate or nitrite and oxygen can function as the final electron acceptor for respiration. A definite role for azurin has yet to be established (Henry & Bessiers, 1984). Azurins will react rapidly with cytochrome *c*-551 and with cytochrome *cd*, the soluble dimeric terminal oxidase (Corin et al., 1983; Mitra & Bersohn, 1980; Silvestrini et al., 1981; Takano et al., 1979; Ugurbil et al., 1985). We have chosen to study holo- and apoazurins of *Pseudomonas aeruginosa* (azurin Pae),[1] *Alcaligenes faecalis* (azurin Afe), and *Alcaligenes denitrificans* (azurin Ade). The amino acid sequences of nine azurins isolated from different bacterial species were established by Ambler (1973), and sequence homologies suggest a common evolutionary origin (Ryden & Lundgren, 1976; Schwartz & Dayhoff, 1978). The amino acid sequence of azurin Pae is 64% homologous with that of azurin Afe (Dayhoff, 1978). Azurin Pae and azurin Ade are 63% sequence homologous; azurin Afe is 67% homologous with azurin Ade. X-ray crystallographic analyses have established molecular structures for azurin Pae (Adman & Jensen, 1981) and azurin Ade (Norris et al., 1983). Visual inspection of the two structures with a graphics system using the X-ray coordinates obtained from the Brookhaven data bank (Bernstein et al., 1977) shows marked topological homology (Norris et al., 1983). Thus, a large topological homology is expected among all members of the azurin family, a feature observed with several families of proteins (Richardson, 1981). Azurins show a marked sequence homology with plastocyanins (Dayhoff, 1978), and plastocyanin has a remarkably similar molecular structure to that of azurin (Coleman et al., 1978; Guss et al., 1983; Norris et al., 1983). Garrett et al. (1984) have found that apoplastocyanin has a crystal structure homologous with that of plastocyanin. Crystal structures of the apoazurins are not yet available.

The crucial difference in sequence between azurin Pae and azurin Afe for this study lies in the location of the single tryptophyl residue contained by each enzyme. Azurin Pae has W-48 located in the core of a β-barrel structure, entirely surrounded by hydrophobic side chains. This residue is exceptional in that its indole moiety is not engaged in any H-bonding interactions (Adman et al., 1978; Chothia & Lesk, 1982). Azurin Afe also contains a single tryptophyl residue, W-118. Comparison with the homologous W-118 of azurin Ade (Ambler, 1973; Norris et al., 1983) indicates that this tryptophyl residue is located at the surface of the protein. This residue is probably engaged in H-bonding interactions with the solvent and is largely exposed (Norris et al., 1983). Azurin Ade contains both the buried and exposed tryptophyl residues. Thus, azurins Pae and Afe provide interesting systems to study the influence of location in the protein and accessibility to solvent on the mobility of tryptophyl residues. Azurin Ade provides an excellent model system for an attempt to decompose the fluorescence of a two-tryptophan protein into its constituents since the relevant spectral and dynamic information can be transferred from azurin Pae and azurin Afe. The fluorescence behavior in azurin Ade is, however, considerably complicated by energy transfer from the interior tryptophan (W-48) to the exposed tryptophan (W-118). We will take up this topic in more detail in a forthcoming paper (J. W. Petrich, J. W. Longworth, and G. R. Fleming, unpublished research).

The azurin from *Pseudomonas fluorescens* (azurin Pfl) contains a single tryptophan, W-48, which is 65% homologous in sequence with azurin Pae. Its fluorescence maximum is located at 308 nm at 298 K and at 298 nm at 77 K (Finazzi-Agro et al., 1970, 1973). To our knowledge, no proteins other than azurins containing W-48 have a fluorescence maximum at such a short wavelength [see Longworth (1971)]. A structured fluorescence was observed from azurin Pae at 298 K (Grinvald et al., 1975). Subsequently, this structure was also observed in azurin Pfl at both 298 K (Szabo et al., 1983) and 77 K (Burstein et al., 1977). All these studies noted a dramatic increase in fluorescence quantum yield and lifetime accompanying demetallization, though there were no changes in spectral distribution and location.

Szabo et al. (1983) drew attention to the remarkable similarity in fluorescence behavior of skatole (3-methylindole) in methylcyclohexane to that of azurin Pfl and Pae at 298 K. This similarity also extends to the absorption spectrum of skatole in hydrocarbon solvents (Martinaud & Kadiri, 1978) and is noted as well in the phosphorescence and optical magnetic double resonance behavior of azurin Pae (Hershberger et al., 1980).

The fluorescence decay of azurin and apoazurin Pae and Pfl has been studied by several groups (Grinvald et al., 1975; Munro et al., 1979; Szabo et al., 1983). Though there is little agreement in detail, double-exponential decay kinetics were noted for the holoprotein. In marked contrast, monoexponential decay kinetics are found for apoazurin (Grinvald et al., 1975; Szabo et al., 1983). Another indication of the ability of copper to influence W-48 is found in the marked increase of triplet yield upon reduction to Cu(I) (Ugurbil et al., 1977) measured at 77 K.

Munro et al. (1979) measured the decay of the fluorescence anisotropy of apoazurin Pae and found the presence of a subnanosecond depolarization in addition to the depolarization from the overall protein tumbling; they also, however, report a double-exponential decay for the fluorescence of apoazurin Pae, unlike other reports of a monoexponential decay.

In this paper we present evidence obtained from time-resolved fluorescence anisotropy measurements for internal motion in apoazurin Afe and its absence in apoazurin Pae. In addition, we present the different fluorescence spectra and decay kinetics for apoazurin Pae and Afe and their holo counterparts, which are evidence of the extensive quenching that occurs upon metallization of the apoproteins. These observations will be interpreted with the molecular models for azurin Pae and Ade and their sequence homologies.

Materials and Methods

Sample Preparation. Holoazurin Pae (ATCC 13525) and Afe (ATCC 8750) were isolated (Parr et al., 1976) and supplied by Dr. R. Timkovich, Department of Chemistry, University of Alabama. Holoazurin Ade (NCTC 8582) was prepared by the method of Norris et al. (1979) and supplied by Dr. G. E. Norris, Department of Chemistry, Massey University, Palmerston North, New Zealand. The holoazurins from *P. aeruginosa* and *A. denitrificans* did not require further

[1] To identify the origin of a protein, we have adopted the naming convention used for DNA restriction endonucleases; a protein isolated from a given *Genus species* is identified as Gsp.

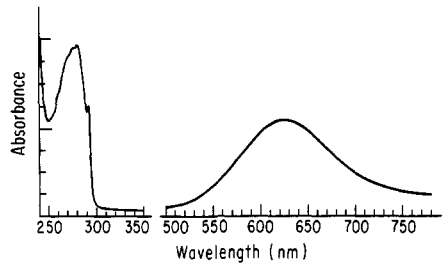

FIGURE 1: Absorption spectrum of holo Pae azurin: 50 mM acetate, pH 5.0, and $A_{625}/A_{280} = 0.56$. The absorption spectra of the Afe and Ade azurins differ from that of Pae azurin only in that the shoulder at 292 nm is less well defined.

purification. The ratio of the absorbance at 625 nm to that at 280 nm, roughly the maxima of the absorption in the visible and the UV, is customarily taken as the criterion of purity for the azurins; the larger the A_{625}/A_{280}, the more blue-copper-containing protein is present. For the holoazurins from Pae and Ade, the A_{625}/A_{280} ratios were 0.56 and 0.25, respectively. The UV/visible absorption spectrum of holoazurin Pae is shown in Figure 1.

The holoazurin Afe initially had $A_{625}/A_{280} = 0.39$, and we ran it once through a column suggested by Dr. Timkovich to purify it further. A 1.5 cm × 30 cm column of CM-52 (carboxymethyl)cellulose (Whatman) was equilibrated with 50 mM acetate at pH 3.9. The holoazurin Afe was dialyzed into the same buffer and loaded onto the column where it bound at the top as a tight blue band ~1 cm wide. The column was run in a cold box at 4 °C and eluted with a flow rate of 20 mL/h with 50 mM acetate buffer, whose pH was increased in 0.2 increments. The azurin came off the column at pH 4.9; 2.5–3.0-mL fractions were collected and were checked for their A_{625}/A_{280}. Those fractions with the highest purity ratio were pooled together, yielding $A_{625}/A_{280} = 0.55$, and were used for the experiments discussed below.

Apo derivatives of the azurins were prepared by the method of Blaszak et al. (1983). The Cu(II) azurin was first reduced to Cu(I) azurin by the addition of a concentrated solution of ascorbate buffered at pH 5 with acetate. The ascorbate solution was added until the absorbance at 625 nm disappeared. The Cu(I) was then removed from the azurins by dialyzing for a total of 12 h against a solution of 0.1 M thiourea, 0.25 M NaCl, 0.2 mM ascorbate, and 50 mM acetate adjusted to pH 4.5. Fresh dialyzate was provided approximately every 4 h, and nitrogen was bubbled through the solutions to prevent oxidation of any copper present. To remove the complexing agent and the copper complex, samples were dialyzed against 50 mM acetate at pH 5.0 for at least 4 h 3 times. All dialyses took place in a cold room at 4 °C. Traces of ascorbate remained in solution, even after dialysis, yielding a faint yellowish tint to the solutions of apoprotein. This did not seem to pose any problem, however, as can be seen from the fluorescence data below. Blaszak et al. (1983) found that the commonly used alternative method of preparing apoazurin by dialysis against 0.5 M KCN at pH 7 (Yamanaka et al., 1963) gave rise to variable kinetics for the uptake of Cu(II) depending on the number of times the protein was exposed to the cyanide solution. This implies that cyanide may induce some small changes in the azurin.

Fluorescence Measurements. Fluorescence lifetime and depolarization measurements were carried out with the time-resolved single-photon-counting apparatus described earlier (Chang et al., 1986). Depending on the experiment, fluorescence was collected through a polarizer oriented parallel, perpendicular, or 54.7° (the magic angle) to the excitation polarization. All fluorescence was collected with a monochromator placed against the photocathode of an ITT F4129 multichannel plate photodetector. We used a J-Y H10 (Instruments SA) monochromator ($f/3.5$) with an ion-etched holographic grating blazed at 250 nm. A 16-nm band-pass was used for all experiments. In all cases, excitation was at 292 nm. In order to check that our fluorescence measurements were not contaminated by scattered light, we periodically performed the photon-counting experiment with only the buffer solution for a length of time equivalent to that required to collect an actual fluorescence decay. Azurin samples were used with $0.5 \leq A_{280} \leq 2.4$ in a 1-cm cell depending on the nature of the experiment and the availability of the sample. Fluorescence decays and spectra did not vary over this range of concentration. The settings of our photon-counting apparatus were adjusted to provide a full scale of 6.02 ns (11.75 ps/channel) for the fluorescence decay measurements of the holoazurins and a full scale of 30.08 ns (58.75 ps/channel) for the fluorescence and anisotropy decay measurements of the apoazurins.

Fluorescence anisotropy decays were analyzed by methods of simultaneous fitting of the parallel and perpendicular fluorescence intensities (Cross & Fleming, 1984) and of fitting to the difference, $D(t)$, between the parallel and perpendicular fluorescence intensities (Barkley et al., 1981; Kinoshita et al., 1981). Cross and Fleming (1984) review and give a thorough discussion of methods of analysis for fluorescence anisotropy decays. Simultaneous and difference fitting gave nearly identical results, and the data in Table III and Figure 5 were obtained with the difference method. Acceptable fits had $0.9 \lesssim \chi^2 \lesssim 1.1$ and $|Z_{runs}| < 2$.

Samples were prepared with pH 5.0, 50 mM acetate buffer and were maintained at 20 °C with a Neslab RTE4 temperature bath. The samples were studied at pH 5.0 because the pK_a of the H-35 of oxidized azurin Pae is ~7 (Rosen et al., 1981). Adman et al. (1982) have noted that deprotonation of H-35 in azurin Pae was accompanied by increased motion in the copper ligand M-35. Since the state of protonation of the H-35 may directly or indirectly influence the motion of the W-48, we postponed any investigation of the pH effects on the azurin photophysics and dynamics and carried out this study well below the H-35 pK_a. The H-35 is not titratable in azurin Afe (Mitra & Bersohn, 1982; Ugurbil et al., 1985). When experiments were not being performed, samples were either refrigerated or quick-frozen in liquid nitrogen and stored in a freezer. Sample quality was frequently monitored by checking the A_{625}/A_{280} and by checking the steady-state fluorescence spectra with a Perkin-Elmer MPF4 fluorometer.

The maxima of the azurin Pae UV absorption spectra (Figure 1) are at approximately 292 and 280 nm and can be attributed to the ^1L_a and ^1L_b absorption bands (Martinaud & Kadiri, 1978; Strickland & Billups, 1973; Valeur & Weber, 1977; Yamamoto & Tanaka 1972). We attempted to effect a better resolution between these maxima by cooling samples of holoazurin Pae in $MeOH/H_2O$ mixtures to −60 °C in order to prepare a more well-defined initial population of molecules in the ^1L_a and ^1L_b states for studies of the effects of level kinetics on the fluorescence anisotropy (Cross et al., 1983). The absorption measurements carried out with the apparatus of Dr. M. Makinen (Makinen et al., 1982) of the Department of Biochemistry and Molecular Biology at the University of Chicago indicated, however, that low temperatures did not significantly resolve the two maxima in the spectrum of azurin Pae.

Table I: Fluorescence Lifetimes of Holoazurins

azurin[a]	A_{625}/A_{280}	Δt_i (ps)[b]	A_1[c]	τ_1 (ps)	τ_2 (ps)	$k_{NR} \times 10^{-9}$ (s^{-1})[d]	λ_{em} (nm)	ref
Pae	0.56	70–100	0.97 ± 0.01	102 ± 8	4150 ± 530	9.61 ± 0.78	325	*e*
			0.57	278	4320		*g*	Gratton et al., 1985
	0.50	650	0.50	750	4150	1.12	*h*	Munro et al., 1979
	0.48	760	0.44	800	4500	1.04	>310	Grinvald et al., 1975
Pfl	0.47[f]	1500	0.80	170 ± 40	4780 ± 30	5.62 ± 1.38	310	Szabo et al., 1983
	0.47	1500	0.79	190 ± 40	4860 ± 30	5.06 ± 1.11	325	Szabo et al., 1983
	0.47	1500	0.79	180 ± 40	4750 ± 30	5.35 ± 1.23	345	Szabo et al., 1983
Pfl Cu(I)		1500	0.74	150 ± 40	4890 ± 30	6.46 ± 1.78	310	Szabo et al., 1983
		1500	0.76	160 ± 40	4980 ± 30	6.05 ± 1.56	325	Szabo et al., 1983
Afe	0.55	70–100	0.92 ± 0.01	212 ± 13	2800 ± 300	4.23 ± 0.29	340	*e*
Ade	0.25	70–100	0.95 ± 0.01	89 ± 8	1776 ± 12[i]	10.46 ± 0.95	310	*e*
		70–100	0.92 ± 0.01	167 ± 10	3210 ± 100[i]	5.75 ± 0.03	355	*e*

[a] The Cu(II) form of the azurin unless otherwise indicated. [b] Full width at half-maximum of the instrument response function used in the time-resolved measurement. [c] $A_1 + A_2 = 1.00$. [d] The nonradiative rate associated with the electron transfer of excited-state tryptophan to copper was calculated by subtracting the inverse of the fluorescence lifetime (or average lifetime where appropriate) of the apoprotein from the inverse of the fluorescence lifetime of the corresponding holoprotein. [e] λ_{ex} = 292 nm; pH 5.0, 50 mM acetate, 20 °C. This work. [f] This is A_{620}/A_{280} rather than A_{625}/A_{280}. If the UV/vis spectra of holoazurins Pae and Pfl are the same, then $A_{625}/A_{280} = 0.55$. [g] Emission was collected with a 0-52 Corning filter. [h] Emission was collected through 1.0 M $CuSO_4$ (1-cm path length). [i] Assuming our assignment of the long-lived component in the fluorescence decay of the holoazurins to an "apo-like" impurity is correct (see text), then the long-lived decay components of holoazurin Ade represent a weighted average of the lifetime components observed in its apo counterpart (Table II). No attempt, however, was made to decompose the long-lived components observed in the apoprotein because the fluorescence decays of the holoazurin were collected with a full-scale time base of 6.02 ns (see text) in order to attain better resolution of the short-lived component. Such a time base is too small to justify decomposition of fluorescence decay times of the order of several nanoseconds.

Table II: Fluorescence Lifetimes of Apoazurins

azurin	A_1[a]	τ_1 (ns)	τ_2 (ns)	λ_{em} (nm)	ref
Pae			5.16 ± 0.19	325	*c*
	0.40	0.88	4.79	*b*	Munro et al., 1979
			4.7	320–380	Grinvald et al., 1975
Pfl			4.94 ± 0.03	325	Szabo et al., 1983
Afe	0.43 ± 0.03	1.06 ± 0.06	2.79 ± 0.03	340[d]	*c*
Ade	0.78 ± 0.04	1.28 ± 0.05	3.19 ± 0.17	310	*c*
	0.54 ± 0.02	1.28 ± 0.06	3.55 ± 0.06	325	*c*
	−0.16 ± 0.01	0.91 ± 0.13	3.82 ± 0.13	355	*c*

[a] $A_1 + A_2 = 1$. The absence of a value for A_1 implies the fluorescence decay was best fit to a single exponential. [b] Emission was collected through 1.0 M $CuSO_4$ (1-cm path length). [c] λ_{ex} = 292 nm; pH 5.0, 50 mM acetate, 20 °C. This work. [d] The decay parameters at 330 and 360 nm were the same as those at 340 nm.

Molecular Graphics. Visual inspection of the structures of azurin Pae and Ade (Adman & Jensen, 1981; Norris et al., 1983) was made possible by displaying the X-ray coordinates obtained from the Brookhaven data bank (Bernstein et al., 1977) on an Evans and Sutherland PS300/VAX11-750 graphics system. Figure 2 is a close-up of the structure of holoazurin Ade and reveals the relationship among W-48, W-118, and Cu(II).

Results

Fluorescence Lifetimes and Purity of the Holoazurins. Table I presents a compilation of the fluorescence lifetimes obtained for holoazurins Pae, Afe, and Ade along with their A_{625}/A_{280} and literature values for these quantities. In all three of our holoazurins we found a small amount (3–8%) of a long-lifetime component, which agrees very well with the corresponding lifetime components of the apoazurins (Table II; Figure 3). Several investigators (Munro et al., 1979; Szabo et al., 1983; Grinvald et al., 1975; E. Gratton, R. Alcala, G. Marriott, and F. Prendergast, unpublished data) have previously observed a similar long-lifetime component in the fluorescence decay of holoazurin Pae and of azurin Pfl (Table II), which also has only a W-48. In all cases, however, they have observed a larger amplitude for this component.

The long-decay component of each holoprotein is very similar to that of its apo counterpart (Tables I and II). In azurins Pae and Pfl the long-lifetime component is unusually blue for tryptophyl fluorescence, and the weight of the long component in azurin Pfl (Szabo et al., 1983) does not change with emission wavelength. These findings suggest that the long-lived component comes from a small amount of contamination by protein with photophysical properties very similar to those of the apoprotein.

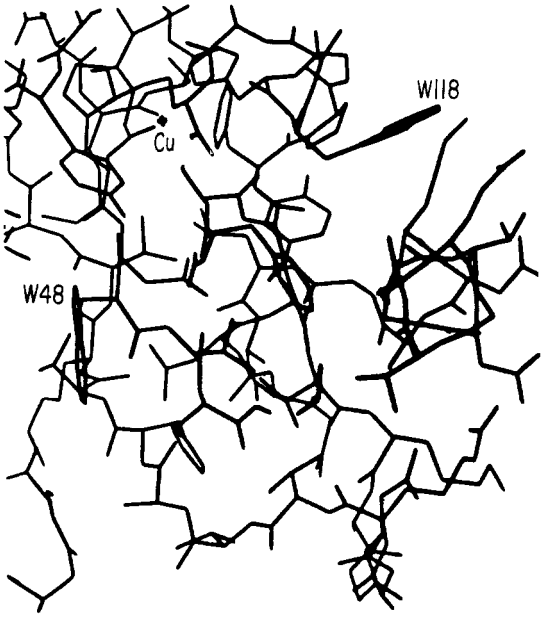

FIGURE 2: Section of the azurin Ade structure showing the position of W-48 and W-118. W-118 is located in the upper right-hand corner of the figure, and the plane of the indole ring of W-48 is on the middle of the left-hand side of the figure. The copper atom appears as a cross in the upper left-hand corner and is roughly equidistant from W-48 and W-118.

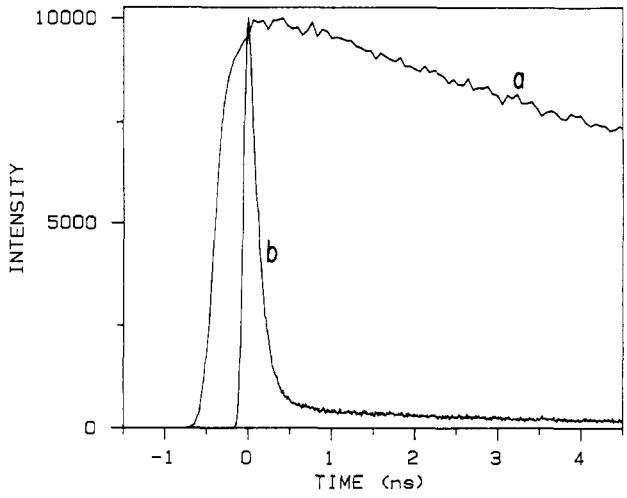

FIGURE 3: Fluorescence decays of apo- (a) and holo- (b) azurin Pae: pH 5.0, 50 mM acetate, and 20 °C. See Table I and II for the lifetimes of these species. The apparent noisiness of the apoazurin decay reflects the fact that only a few of the channels comprising it are displayed. The instrument function for both decays is ~100 ps FWHM. Zero time has been defined arbitrarily.

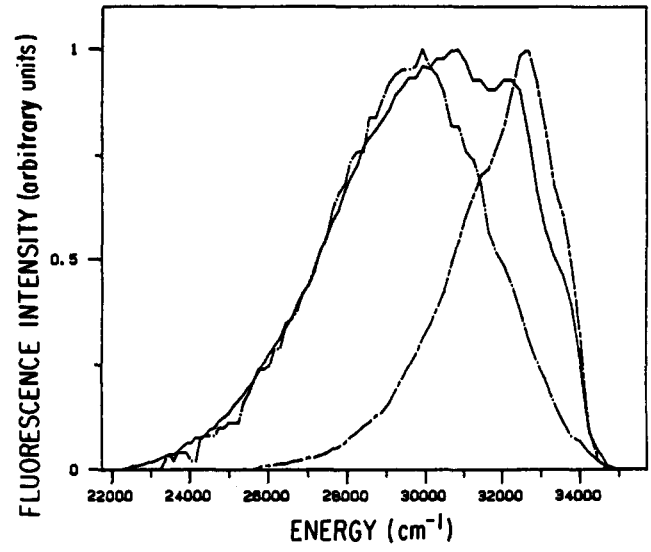

FIGURE 4: Fluorescence spectra of apo Afe azurin (-·-), apo Ade azurin (—), apo Pae azurin (- - -). The fluorescence spectra of the holoproteins only differ from that of the apoproteins in that they have a reduced intensity.

In their study of holoazurin Pfl, Szabo et al. (1983) initially suggested that the long-lived component may be due to contamination by the apoazurin; but they dismissed this possibility because they observed no change in the absorbance or fluorescence spectrum of the protein upon addition of a 25-fold excess of $CuCl_2$. They then invoked a two-conformer model to explain the azurin double-exponential decay on the basis of the biphasic ground-state recovery measurements of Wiesenfeld et al. (1980) of the charge-transfer band of azurin Pae. Considering the high affinity of apoazurin for various metal ligands (Blaszak et al., 1983; Engeseth & McMillin, 1986; D. R. McMillin, personal communication), a 25-fold excess of $CuCl_2$ ought to metallate any residual apoazurin. If, however, there is azurin present that contains a metal other than Cu or if one or several of the metal ligands are damaged so that metal uptake is impossible, addition of excess Cu(II) would have no effect on the azurin photophysics. For example, Engeseth (1983) has shown that AzCd(II) and AzZn(II) are nearly as fluorescent as apoazurin Pae, and Tennent and McMillin (1979) have observed that when *p*-(hydroxymercuri)benzoic acid is bound to apoazurin (presumably at C-112, one of the metal ligands), the characteristic blue color of the holoazurin does not appear with the addition of Cu(II). We therefore propose that the long component arises from one or more of these "apo-like" azurins.

If this assignment is correct, we should ideally be able to determine quantitatively the amount of the holoprotein from the A_{625}/A_{280} and from the weight of the short-lifetime component, A_1, in the fluorescence decay. If, however, the A_{625}/A_{280} and the A_1 values from two different laboratories are compared (Table I), one notices an apparent inconsistency: the change in A_{625}/A_{280} from laboratory to laboratory for holoazurins Pae and Pfl is smaller than the change in A_1.

That the change in A_1 is larger than the change in A_{625}/A_{280} can be rationalized by noting that the value of A_1 (and τ_1) can be strongly influenced by the time resolution (the instrument function) of the photon-counting system. If the instrument function is too broad, rapidly decaying lifetime components will be broadened and will contribute less to the overall fluorescence decay. Table I also correlates the weight and the lifetime of the short component of the holoazurin Pae and Pfl decay with the instrument function width. This suggests that one need not attribute the *entire* change in A_1 with respect to A_{625}/A_{280} to the presence of a long-lived species. Our fluorescence measurements for holoazurin Pae indicate that the long-lived component makes an upper limit contribution of 3% to the fluorescence decay.

It is interesting in this context to note the analogous situation that occurs in the fluorescence quenching of tryptophyl residues by energy transfer in human hemoglobin (Szabo et al., 1984), sperm whale myoglobin (Hochstrasser & Negus, 1984), and *Aplysia* myoglobin (Janes et al., 1986). In all cases, a long component of several nanoseconds was found. Because the presence of the long component (i) was insensitive to the heme protein investigated, (ii) could not be attributed to any conformational changes produced in orientational energy-transfer calculations or molecular dynamics simulations, and (iii) decreased as a function of purification by isoelectric focusing in *Aplysia* myoglobin, Janes et al. (1986) attributed the long component in sperm whale and *Aplysia* myoglobin to an impurity. The impurity, however, could not be assigned to apoprotein since an excess of hemin failed to reduce the presence of the long component in both sperm whale and *Aplysia* myoglobin.

Fluorescence Lifetimes of the Apoazurins. Table II presents our results for the fluorescence lifetimes of the apoazurins along with the literature values for apoazurins Pfl and Pae. The fluorescence decays of azurin Pae and azurin Pfl are single-exponential, whereas the decays of azurins Afe and Ade are fit well to a sum of two exponential components.

(*i*) *P. aeruginosa Azurin.* The single-exponential fluorescence decay of the W-48 of azurin Pae (5.16 ns) and of azurin Pfl is not completely unexpected given the very blue fluorescence maximum of 308 nm (Figure 4), the bluest protein tryptophyl fluorescence maximum of which we are aware. Szabo et al. (1983) have shown that the fluorescence spectrum of 3-methylindole in methylcyclohexane is a good model for the apoazurin Pfl fluorescence and there is a very well documented relationship between the Stokes shift of indole fluorescence and the polarity of the indole environment (Meech et al., 1983). Mallinson et al. (1981) have shown that the W-48 of azurin Pae is inaccessible to acrylamide and the tightly wrapped β-barrel structure of azurin (Adman & Jensen, 1981; Norris et al., 1983), and the results of accessibility calculations (Chothia & Lesk, 1982) indicate that the azurin W-48 is inaccessible to water. In polar solvents, or in the

Table III: Fluorescence Anisotropy Decay Parameters of Apoazurins

azurin[a]	$r_1(0)$[b]	τ_1 (ns)	$r_2(0)$[c]	τ_2 (ns)[c]	λ_{em} (nm)
Pae			0.260 ± 0.011	4.94 ± 0.23	325
Pae	0.139 ± 0.002	0.49 ± 0.01	0.092 ± 0.0006	6.84 ± 0.12	*d*
Afe	0.082 ± 0.022	0.16 ± 0.06	0.221 ± 0.009	6.88 ± 0.45	340
Ade			0.252 ± 0.004	3.55 ± 0.38	325
Ade	0.125	0.69	0.126	4.94	355

[a] Unless otherwise specified, results are from this laboratory: pH 5.0, 50 mM acetate, 20 °C, λ_{ex} = 292 nm, and emission band-pass 16 nm. [b] The weight of the short component of the anisotropy decay extrapolated to time zero. The absence of a value for $r_1(0)$ implies that the anisotropy decay was best fit to a single exponential. [c] The value of $r(0)$ obtained by extrapolating the long-term behavior of the anistropy to zero time. $r_2(0) = r(0^+)$, see text. τ_2 is the long-time behavior of the anisotropy associated with τ_r in the text. [d] Emission was collected through 1.0 M $CuSO_4$ (1-cm path length). Munro et al., 1979.

presence of a strong electrophile such as Cu(II), the major process responsible for quenching tryptophan fluorescence is believed to be electron transfer (Petrich et al., 1983). In the absence of copper and water molecules the charge-transfer rate is expected to be negligible, and although there may be multiple conformations of the tryptophyl residue, these have no influence on the radiative and intersystem crossing rates. Thus a single-exponential decay is expected, as is observed in anionic tryptophan, deprotonated tryptamine, or indole-3-propronic acid (Petrich et al., 1983).

(*ii*) *A. faecalis Azurin.* In contrast to azurin Pae, the double-exponential fluorescence decay of the W-118 of apoazurin Afe and its emission maximum of 334 nm are as expected for surface-exposed tryptophan residues (Longworth, 1971, 1983). The fluorescence decay of apoazurin Afe is very similar to that of polypeptide hormones such as glucagon (Cockle & Szabo 1981; Beddard et al., 1980) and ACTH (L. X.-Q. Chen, J. W. Petrich, and G. R. Fleming, unpublished results), where the tryptophan is expected to be fully exposed to the solvent. As we discuss in detail elsewhere (L. X.-Q. Chen, J. W. Petrich, and G. R. Fleming, unpublished results), if a tryptophan occupies a position in the middle of a polypeptide sequence of length greater than four amino acids, then the fluorescence lifetime of the polypeptide is almost always double exponential with lifetimes of approximately 1 and 3 ns with nearly equal weights.

(*iii*) *A. denitrificans Azurin.* As can be seen from Table II, the fluorescence properties of apoazurin Ade are much more complicated than those of apoazurin Pae or Afe. The wavelength-dependent decay is a result of the large shift (~26 nm or ~2500 cm^{-1}) between the fluorescence maxima of the interior W-48 and the exterior W-118 (Figure 4). When the very blue (λ_{em} = 310 nm) fluorescence from W-48 is observed, the fluorescence decay can be fit to a double exponential with a fast-decay component of 1 ns; the redder fluorescence (λ_{em} = 355 nm) from the exterior W-118 has a rise time of 1 ns. The overlap between the absorption spectrum of W-118 and the fluorescence spectrum of W-48 suggests that the 1-ns decay of the blue azurin Ade emission and the 1-ns rise of the red azurin Ade emission very likely result from energy transfer between these two tryptophans. The relatively slow rate of energy transfer results from the unfavorable overlap between the donor and acceptor transition dipole moments (J. W. Petrich, J. W. Longworth, and G. R. Fleming, unpublished research).

The 3-ns lifetime observed for apoazurin Ade can be attributed to energy transfer. First, to perform our lifetime measurement properly, it is necessary to collect the fluorescence through a polarizer placed at 54.7° to the vertical polarization of our excitation beam in order to discriminate against fluorescence depolarization that is occurring on the same time scale as the decay of the excited-state species. Since, however, there is energy transfer from W-48 to W-118, a portion of the W-118 residues are indirectly excited with depolarized light. The analyzer polarizer set as 54.7° to the vertical still screens out the fluorescence depolarization as it does in the former case. Second, energy transfer itself takes place on a time scale comparable to the fluorescence decay and thus can complicate the observed decay constants. The energy-transfer dynamics will be taken up in more detail in a future publication.

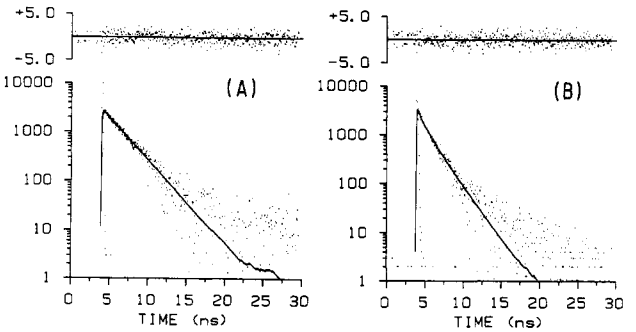

FIGURE 5: (A) $D(t)$ for apo Pae azurin. $D(t) = r(t)K(t)$. The fixed $K(t)$ parameters were obtained from Table II. The fit of $D(t)$ corresponds to an anisotropy decay of 4.81 ns with an $r(0)$ = 0.27. χ^2 = 0.995, and Z_{runs} = −0.064. The residuals for the fit are displayed at the top of the figure. (B) $D(t)$ for apo Afe azurin. The fixed $K(t)$ parameters were obtained from Table II. The fit of $D(t)$ corresponds to an anisotropy decay with $r_1(0)$ = 0.10, τ_1 = 0.15 ns and $r_2(0)$ = 0.22, τ_2 = 7.22 ns. See Table III. χ^2 = 1.075, and Z_{runs} = 1.19.

Anisotropy Decays of the Apoazurins. (*i*) *Overall Protein Reorientation Times.* Adman et al. (1978) have described the shape of azurin Pae as a "flattened pear of approximate dimensions 25 Å × 30 Å × 45 Å with the Cu atom toward the small end of the molecule". If we assume for the sake of simplicity that azurin is a prolate ellipsoid of dimensions 45 × 27.5 × 27.5 Å and that the emission and absorption dipoles of the tryptophans are aligned with the principle rotation axes of the protein, three overall rotation times, τ_r, are predicted (although it is unlikely that all three will be resolved in a given experiment): 4.17, 5.06, and 5.45 ns. As can be seen from Table III, azurin Pae yields a rotation time of 4.94 ns and azurin Afe one of 6.88 ns. On the basis of the homology with azurin Ade, we expect the planes of W-48 in azurin Pae and W-118 in azurin Afe to lie almost exactly perpendicular to each other. The indole plane of W-48 in azurin Pae lies nearly perpendicular to the long axis of the ellipsoid, and the anisotropy would thus be expected to decay more rapidly for W-48 of azurin Pae, as is observed. Whether the entire decrease in reorientation time can be ascribed to anisotropic motion or whether some contribution comes from structural differences, or solvent attachment differences, cannot be stated with certainty. It seems likely, however, that the present data do provide a direct observation of anisotropic reorientation of a macromolecule. The anisotropy decays of apoazurins Pae and Afe are shown in Figure 5.

The anisotropy decay of apoazurin Ade is more complicated. On the basis of the similarity of the azurin Ade W-48 with the azurin Pae W-48 and of the azurin Ade W-118 with the azurin Afe W-118, one would expect to observe anisotropy decays of 4.94 and 6.88 ns for the W-48 and W-118 of apoazurin Ade. Instead, we observe 3.55 and 4.94 ns for the reorientation times of the W-48 and the W-118, respectively. Again, these differences may result from slight differences among the structures of the three proteins. The presence of energy transfer on a time scale comparable to the overall reorientation times provides an additional complication in the interpretation of the azurin Ade data.

(*ii*) *Internal Motion in the Azurins.* The fluorescence anisotropy decays were fit to

$$r(t) = r_1(0)e^{-t/\tau_1} + r_2(0)e^{-t/\tau_2} \quad (1)$$

The anisotropy decay of apoazurin Pae fit well to a single-exponential decay component, implying the absence of detectable internal motion. The anisotropy decay of apoazurin Afe was nonexponential but fit well to a double exponential with a short component of ~160 ps. Figure 5 gives a comparison of the anisotropy decays. A short component was also observed in the anisotropy decay of apoazurin Ade detected at 355 nm (W-118 emission); however, when emission at 325 nm (W-48) was detected, a single-exponential decay was observed. These findings are consistent with the buried W-48 in azurin Pae and Ade being rigidly held, whereas the exposed W-118 in azurins Afe and Ade has substantial flexibility.

To quantify the degree of flexibility, we use the order parameter S^2, which is a quantitative and model-independent measure of the extent to which restricted motion can occur (Lipari & Szabo, 1982; Chang et al., 1983a). S^2 is defined as

$$S^2 = \frac{r(t)}{r(0)}e^{t/\tau_r} = \frac{r(0^+)}{r_{eff}(0)} \quad (2)$$

τ_r and $r(0^+)$ are defined by the fit of the long time behavior (overall protein reorientation) of the anisotropy to a single exponential and are equivalent to τ_2 and $r_2(0)$ in eq 1. $r_{eff}(0)$ is the initial value of the anisotropy (Chang et al., 1983a) less those nonmotional factors contributing to the anisotropy decay (Cross et al., 1983). In the discussion below, $r_{eff}(0) = r_1(0) + r_2(0)$. S^2 gives an indication of the magnitude of the depolarizing motions that are present in addition to the overall protein reorientation. A value of $S^2 < 1$ implies motion with respect to the body of the protein. The order parameter can be related to a hypothetical cone semiangle, θ_0, that the transition dipole can diffuse within:

$$S = \frac{1}{2}\cos\theta_0\,(1 + \cos\theta_0) \quad (3)$$

Applying eq 2 and 3 to the anisotropy decay of W-118 in Afe azurin, we obtain a cone semiangle of $\theta_0 = 38°$. For W-118 in azurin Ade we find $\theta_0 = 26°$. From Figure 2, this seems to be a reasonable range for the excursion of the W-118. Note in Table III that the maximum value of $r(0)$ [$r_1(0) + r_2(0)$] obtained is 0.30 with 292-nm excitation. Thus, as is typical with tryptophyl anisotropies, $r(0)$ is never equal to the limiting value of 0.4.

It is possible that our experiment failed to resolve a short component in the anisotropy decay of azurin Pae due to insufficient data collection. In order to test this hypothesis, we convoluted trial double-exponential anisotropy decays that were similar to our experimental data with a real instrument response function similar to that in Figure 5A. The resulting curve was fit to single- and double-exponential anisotropy decays by the difference method to find what set of decay parameters (for a given peak height in the polarized fluorescence curves) would prevent us from observing a fast component in the anisotropy decay. An order parameter can then be calculated from the preexponential factors of the anisotropy decay with eq 1 and 2, and from this value of S^2 we can obtain a lower limit on rapid depolarizing motions that can be resolved with a given set of experimental conditions.

With anisotropy decay times similar to those of Afe azurin, the simulations show that, for the maximum number of counts used in our measurements, it would be difficult to detect nonexponentiality in $r(t)$ for $S^2 \gtrsim 0.8$. In other words, for $\tau_1 \sim 160$ ps, if $\theta_0 \lesssim 22°$ in Pae azurin, it is unlikely that we would detect a rapid component in the anisotropy decay. The W-48 of azurin Ade is surrounded by Y-110, V-31, V-95, I7, and M-20, all of which are between 3 and 4 Å away (these are *atom to atom* distances) from the indole ring of W-48. While the relatively large amplitude motion similar to that of W-118 is prohibited given the tight packing about W-48, smaller amplitude motions (with a similar decay constant) may be present that are undetectable with our current experimental conditions.

Discussion

Role of Copper in the Tryptophyl Photophysics of Azurins. The lifetimes of the holoazurins are much shorter than those of their apo counterparts (Tables I and II). The increase in quantum yield upon demetallization has been measured by steady-state methods as 2.5-fold (Finazzi-Agro et al., 1970) and as approximately 6-fold for both azurin Pae and Pfl (Grinvald et al., 1975; Szabo et al., 1983). But if the long component in the fluorescence decays of the holoproteins is due to an impurity, the contribution of the impurity to the steady-state quantum yield measurement will be large. This is so, even if the weight is small, because of the long lifetime of the impurity fluorescence. (The quantum yield is the area under the fluorescence decay curve.) If we instead calculate the increase in the quantum yield upon demetallization by assuming that the lifetimes of the holoproteins are given by the short components listed in Table I, we then find that for azurin Afe demetallization produces a 10-fold increase in the quantum yield and for azurin Pae a 51-fold increase in the quantum yield.

The large decrease in the steady-state quantum yield associated with the presence of copper is due to a nonradiative process with a rate ranging from 5×10^9 to 1×10^{10} s^{-1} (Table I). The two most likely possibilities for the nonradiative process in the holoazurins are electronic energy transfer and electron transfer from the tryptophan to the copper. We first consider the possibility of energy transfer. Aqueous Cu(II) [and Cu(I), see below] both have oscillator strength, albeit very small, in the region where azurin Pfl and Pae fluoresce. The similarity, however, of the ultraviolet absorption spectra of the holoproteins with that of their apo counterparts suggests that copper plays a negligible role in quenching by energy transfer.

On the other hand, it has been shown that Cu^{2+} is a powerful quencher of the fluorescence of *N*-acetyltryptophanamide (NATA) with $k_q = 3 \times 10^{10}$ s^{-1} M^{-1} (Anbar, 1965; Steiner & Kirby, 1969), and our earlier model that charge transfer from the indole ring to an electrophile is a major nonradiative process in tryptophyl compounds (Chang et al., 1983b; Petrich et al., 1983) strongly suggests that copper quenches the tryptophyl fluorescence in the azurins by an electron-transfer reaction.

(*i*) *Dependence of Rate on Thermodynamic Parameters.* Electron transfer over distances up to 15 Å has been observed in solid solution (Miller et al., 1982), in model compounds where the donor and acceptor are separated by a rigid hydrocarbon spacer (Calcaterra et al., 1983; Miller et al., 1984), and in proteins (Isied et al., 1982; Kostic et al., 1983; McGourty et al., 1983, Nocera et al., 1984; Winkler et al., 1982). Particularly intriguing in these experiments is the range of observed electron-transfer rates. For example, while Miller et al. (1984) observe rates of 2×10^9 s^{-1} from electronically excited states and suggest that electron transfer across a 10-Å insulating barrier can occur in ~100 ps if conditions are optimized, Gray and co-workers (Kostic et al., 1983; Margalit et al., 1984) observe an electron transfer rate of 1.9 ± 0.4 s^{-1} in the ground-state system pentammine–ruthenium–(histidine-83) azurin over a distance of 11.8 Å.

We have calculated the nonradiative rate constant for the quenching of fluorescence of W-48 and W-118 in holoazurins Pae and Afe, respectively, by subtracting the inverse of the fluorescence lifetimes of apoazurins Pae and Afe from the inverse of the fluorescence lifetimes of their holo counterparts. We obtain approximately 1×10^{10} and 5×10^9 s^{-1} for the rate of the nonradiative process quenching the fluorescence of W-48 and W-118, respectively (see Table I). We treat the fluorescence quenching reaction as an electron-transfer process. Theories of electron transfer in condensed media predict a Gaussian or a skewed Gaussian dependence of the electron-transfer rate on the reaction exothermicity (Dogonadze, 1971; Levich, 1966; Marcus, 1956; Ulstrup & Jortner, 1975), and we have used the Marcus theory to carry out a thermodynamic analysis of an electron transfer for the reaction

$$\text{Trp}^* + \text{AzCu(II)} \rightarrow \text{Trp}^{*\bullet+} + \text{AzCu(I)} \qquad (4)$$

(We have written the reaction as forming the excited-state tryptophyl radical cation for thermodynamic reasons that are discussed below.) In particular, we hoped to discover whether the electron-transfer rates we observe are in agreement with those predicted by theory and whether the approximately 2-fold difference in electron-transfer rate between azurin Pae and azurin Afe could be attributed to the different environments of the W-48 and the W-118.

The rate of electron transfer can be written as (Beitz et al., 1979; Hopfield, 1974; Mauk et al., 1980; Miller et al., 1982, 1984)

$$k_{ET}(r) = \nu \exp[-(r - R_0)/a] \qquad (5)$$

where in the simplest formulations of electron-transfer theory, the frequency factor, ν, may be expressed as

$$\nu = \nu_0 \exp[-(\Delta G^\circ_{ET} + \lambda)^2/(4\lambda kT)] \qquad (6)$$

λ is the reorganization energy, and ΔG°_{ET} is the standard free energy change (Rehm & Weller, 1970) for the electron transfer reaction:

$$\Delta G^\circ_{ET} = E(D/D^+) - E(A^-/A) - E_D^* + E_P^* - e^2/(\epsilon r) \qquad (7)$$

$E(D/D^+)$ is the oxidation potential of the donor, $E(A^-/A)$ is the reduction potential of the acceptor, and E_D^* is the energy of the first excited singlet state of the donor. E_P^* is the energy of an excited-state product. Inclusion of this term takes into account the fact that the product of the electron-transfer reaction may be an excited-state species. Equation 6 indicates that when $-\Delta G^\circ_{ET} > \lambda$, the electron-transfer rate is reduced. Thus, the formation of excited-state product reduces the reaction exothermicity, $-\Delta G^\circ_{ET}$, and allows the reaction to proceed with a much smaller rate reduction (Efrima & Bixon, 1974; Ulstrup & Jortner, 1975). The last term in eq 7 is a correction for the Coulomb energy changes associated with charge separation. Because, however, our electron-transfer reaction most likely involves not two point charges but rather the π-clouds of the tryptophyl and the histidyl ligands of the copper, we expect the last term in eq 7 to be small and omit it in our calculations (Miller et al., 1982).

Table IV: Thermodynamic Data and Relative Electron-Transfer Rates

thermodynamic quantity	energy (V)
ionization potential (gas phase) of *N,N*-dimethylaniline (DMA)	7.13[a]
ionization potential (gas phase) of indole	7.87[b]
oxidation potential of DMA	1.02[c]
oxidation potential of indole	1.13[d]
reduction potential of Pae AzCu(II) (pH 5.0)	0.36[e]
excited-state energy of W-48 (azurin Pae)	4.03[f]
excited-state energy of W-118 (azurin Afe)	3.71[f]
excited-state energy of indole	2.19[g]
ΔG°_{ET}[W-48* + AzCu(II)][h]	−1.07
ΔG°_{ET}[W-118* + AzCu(II)][h]	−0.75

ratios of rates and prefactors of electron-transfer reactions	ratios of rates and prefactors (theor)	ratio of rates (exptl)
ν[W-118* + AzCu(II)]/ν[W-48* + AzCu(II)][i]	0.53	
k_{ET}[W-118* + AzCu(II)]/k_{ET}[W-48* + AzCu(II)][j]	10.56	0.44,[k] 0.55[l]
k_{ET}[W-48* + AzCu(II)]/k_{ET}[W-48* + AzCu(I)]		0.78[m]

[a] Larson et al., 1982. [b] Dolby et al., 1976. [c] The polarographic half-wave oxidation potential relative to the saturated calomel electrode (Rehm & Weller, 1970; Zweig et al., 1964) and scaled to the normal hydrogen electrode. [d] The oxidation potential of indole was obtained by scaling the oxidation potential of DMA relative to the normal hydrogen electrode to the ratio of the ionization potentials of indole and of DMA. This calculation assumes that the solvation energies of indole and of DMA are equivalent. The indole oxidation potential is likely to be sensitive to an aqueous or a hydrocarbon environment. Equation 7 addresses this problem by including the excited-state energies of the indoles, which are very sensitive to solvent environment. [e] This value has been determined for both azurin Pae and azurin Afe. The values for azurin Pae vary from 0.36 to 0.30 V depending on the pH and the method of determination. Silvestrini et al., 1981; Lappin et al., 1979; Myer et al., 1983. [f] Maximum of the appropriate fluorescence spectrum in Figure 5. [g] Maximum of the absorption spectrum of aqueous indole radical cation (Bent & Hayon, 1975). [h] Calculated from eq 7. See discussion in text. [i] Ratio of the prefactors for the electron-transfer rates (eq 6). [j] Ratio of the electron-transfer rates (eq 5) calculated with a value of 0.53 for the ratio of the prefactors (this table) and the distances between the closest aromatic atoms of W-48 and W-118 to aromatic atoms of the histidyl ligands of copper. See text. [k] Ratio of electron-transfer rates in azurins Afe and Pae. This work. See Table I. [l] Ratio of electron-transfer rates in azurin Ade. This work. See Table I. [m] Szabo et al., 1983. See Table I.

The thermodynamic data and relative rates for the electron-transfer reaction are compiled in Table IV. To the extent that the environment of the tryptophans in azurin Pae and Afe resemble a hydrocarbon such as methylcyclohexane (Szabo et al., 1983) or water, respectively, reasonable *estimates* for the reorganization energy are $\lambda_{W48} = 0.75$ eV and $\lambda_{W118} = 1.25$ eV, respectively (Miller et al., 1982, 1984; J. R. Miller, personal communication). Equation 6 indicates that the maximum rate occurs when $-\Delta G^\circ_{ET} = \lambda$. Assuming that except for solvent environment and excited-state energies everything is equivalent in the Pae and Afe systems, substitution of the appropriate values in Table IV into eq 6 and 7 indicates that the rate for the W-118 reaction lies on the rising edge of the

curve defined by eq 6 ($-\Delta G°_{ET}$(W-118) < λ_{W118}) and that the W-48 reaction lies on the maximum of the curve ($-\Delta G°_{ET}$(W-48) = λ). That both reactions are near the peak rate ($-\Delta G°_{ET} = \lambda$) is implied by the large observed electron-transfer rates, which attain the upper limit of 1×10^{10} s^{-1} suggested by Miller et al. (1982) for a 10-Å separation.

If we use eq 5 and 6 to predict the rates of the electron-transfer reaction of W-48* and W-118* with AzCu(II) assuming ν_0 and the distance, r, is the same for both reactions, we find that k_{ET}[W-118* + AzCu(II)]/k_{ET}(W-48* + AzCu(II)] = 0.53 (Table IV), which is in excellent agreement with the ratio of the rates obtained experimentally: 0.44 from azurins Pae and Afe and 0.55 from azurin Ade (Tables I and IV). We note, however, that this agreement may be fortuitous given the nature of the approximations required to obtain some of the parameters in Table IV. Furthermore, we have, in the above analysis, assumed equal distances between W-48 and W-118 and the Cu(II) and thus have not taken into account the effect of distance on the electron-transfer rates. We take up this point in the next section.

(*ii*) *Dependence of Rate on Distance.* In the previous section we assumed that the electron-transfer reactions in the Pae and Afe systems were identical in every respect except for donor excited-state energy and solvent environment. The extensive sequential homology among the azurins and the high structural similarity between azurins Pae and Ade imply that the W-118 of azurin Afe is very similar to the W-118 of azurin Ade. This contention is supported by the similarity between the 102-ps holoazurin Pae lifetime and the 89-ps lifetime of holoazurin Ade at 310 nm and the similarity between the 212-ps holoazurin Afe lifetime and the 167-ps lifetime of holoazurin Ade at 355 nm. Here we consider the distance dependence of the reaction rate using the coordinates from the holoazurin Ade structure.

Equation 5 shows that the rate of electron transfer decreases exponentially with distance. Since the electron transfer most likely occurs not between two point sources but between the π-clouds of the indole and the histidyl ligands of the copper, the determination of this distance is not trivial. We have estimated the distance over which the electron transfer occurs as the shortest distance between any aromatic atoms of the donor and the aromatic ligands of the copper. From the crystal structure of azurin Ade (Norris et al., 1983), these distances are W-48(C_γ)–H-46(C_γ) (9.558 Å) and W-118(C_{δ_1})–H-117-(C_γ) (7.314 Å). If we use 0.75 Å for the value of a (Miller et al., 1982, 1984), we obtain from eq 5 k_{ET}(W-118)/k_{ET}(W-48) = 10.56, assuming that ν(W-118)/ν(W-48) = 0.53 (see above and Table IV). If we have correctly assigned the nonradiative process to electron transfer and have accurately estimated the distance dependence of the electron-transfer rate, there must be something peculiar to the protein matrix between donor and acceptor that compensates for the factor ~20 between theory and experiment.

Calcaterra et al. (1983) and Miller et al. (1984) have shown for two isomers of the same model compound with identical donor and acceptor that the rate of electron transfer can be faster for the isomer with the greater donor–acceptor distance. Ohta et al. (1986) have investigated the effect of geometry and the role of the σ-orbitals connecting the donor and acceptor on the electron-transfer rate. Their calculations indicate that the σ-orbitals of the spacers can provide a rate enhancement of approximately 10^4 and that large differences in the rate of electron transfer can be found for donor–acceptor orientations where there is no change in the donor–acceptor separation. The possibility also exists that the π-orbitals of intervening aromatic amino acids in the protein matrix enhance the electron transfer rate. To this end, it is interesting to note that in azurin Ade and Pae the distances between W-48 and the copper ligand H-46 are bridged by the phenol ring of Y-15 and the phenyl ring of F-15, respectively, whereas no aromatic residues lie between W-118 and H-117.

Lastly, one might naively expect the oxidation state of the azurin copper to influence the rate of electron transfer. Szabo et al. (1983), however, measured the fluorescence decay of the Cu(I) form of azurin Pfl and found it to be the same as that of the Cu(II) azurin (Table I). This result suggests that it may be rash to correlate the electron affinity of an acceptor with its oxidation state. For example, Cu^{2+} and NO_3^- quench indole fluorescence at the diffusion-controlled rate $k_q = 3 \times 10^{10}$ s^{-1} M^{-1} (Steiner & Kirby, 1969; Anbar, 1965). Furthermore, since the coordination geometry of the azurin copper is midway between that of Cu(II) and Cu(I), one should not necessarily expect AzCu(II) to behave like either Cu(II) or Cu(I). If the major nonradiative process in the holoazurins is indeed electron transfer, it seems difficult to accept the suggestion of Szabo et al. (1983) for the long component in the holoprotein fluorescence decay. If the two decay components arise from two conformers, then using eq 5 we find that the donor–acceptor distance must increase by 3.1 Å in order to raise the lifetime from 100 ps to 5 ns.

Our assignment of the nonradiative process to electron transfer is tentative at this stage, and we cannot unambiguously rule out other explanations, such as energy transfer, for the quenching of the holoazurin fluorescence. Experiments are currently in progress to establish whether the nonradiative process is indeed electron transfer from excited-state tryptophan to copper.

Nonexponential Fluorescence Decay of W-118 and Protein Conformations. A conformer-based model for nonexponential fluorescence decay in tryptophan and its derivatives, in small peptides, and in proteins is based upon two major assumptions (Chang et al., 1983b; Petrich et al., 1983): (1) Charge transfer from the indole to a nearby electophile is the major nonradiative process in tryptophan and its derivatives. (2) Different lifetime components are obtained from conformers that do not interconvert during the excited-state lifetime and that have different distances or interactions between the indole and the nearby electrophile.

We have recently proposed a conformational model for tryptophan (Engh et al., 1986) which suggests that the nonexponential fluorescence observed for tryptophan itself arises from two groups of rapidly interconverting χ_1 conformers, distinguished by their χ_2 angle. In this section we explore the applicability of this idea to tryptophyl fluorescence in proteins.

We have already discussed the single-exponential fluorescence decay of apoazurin Pae and the more complicated fluorescence decay of apoazurin Ade. The protein whose fluorescence is most suitable for a discussion in terms of a conformer model is apoazurin Afe, which has a nonexponential fluorescence decay that is not complicated by energy transfer. The W-118 of azurin Ade in the crystal structure occupies the g^+ χ_1 conformer and the perpendicular χ_2 conformer. The anisotropy data imply substantial freedom of motion for W-118, and so in solution it is very likely that several other conformations are occupied. The crystal structure of azurin Ade indicates that the indole nitrogen of W-118 is 5.2 Å away from the carbonyl carbon of the G-116 peptide bond when W-118 occupies the perpendicular conformation. If the χ_2 angle is rotated by 180° so that W-118 occupies the anti-perpendicular conformation, the indole nitrogen is 6.0 Å away

from the carbonyl carbon of the peptide bond of G-116. If this carbon atom is an efficient electrophile, if the W-118 occupies both χ_2 conformers, and if ν in eq 5 does not change appreciably in the two χ_2 conformations, we calculate the ratio of the nonradiative rates in the perpendicular conformation to the antiperpendicular conformation to be 2.8. We can calculate the charge-transfer rates in apoazurin Afe by subtracting the values of the radiative and intersystem crossing rates (Petrich et al., 1983; Robbins et al., 1980) (5.0×10^7 and 3.3×10^7 s^{-1}, respectively) from the inverse of the two fluorescence lifetimes, 1.06 and 2.79 ns. The ratio of the charge-transfer rates giving rise to the short- and long-lifetime components is then 3.1. Similar results are obtained for an analogous calculation involving S-60. These results support our identification of G-116 and/or S-60 as the electrophile. We must, however, view this result skeptically since, as we have shown earlier, there is considerable uncertainty in determining the separation of the donor and acceptor sites in the electron-transfer reaction.

Mobility of the Tryptophyl Residues. The very different environments of W-48 and W-118 suggest that substantially different mobilities may be observed. The lack of mobility found for W-48 in azurin Pae differs from the finding of Munro et al. (1979) for azurin Pae where a short component of 0.51 ns was observed. In contrast to the suggestions of Beecham and Brand (1985), we conclude that the interior of azurin Pae is more crystalline than fluid on a nanosecond time scale. The molecular structure of azurin Pae shows that considerable protein rearrangement is necessary for substantial motion of W-48. W-118 in azurin Afe, on the other hand, from the spectral evidence and the molecular structure of the homologous azurin Ade, partially protrudes into solvent. Our inspection of the molecular model suggests W-118 could rotate about its χ_2 bonds without requiring motions of other residues.

The motions of the two tryptophyl residues provide an interesting challenge for molecular dynamics simulations (Karplus & McCammon, 1983, 1984). It is unfortunate that lack of knowledge about the limiting anisotropy of tryptophan makes it difficult to place precise limits on the lack of mobility of W-48. It is unlikely that rapid librational motion would be detected at the present level of precision, and we plan to carry out polarization spectroscopy (Waldeck et al., 1981) studies with single picosecond resolution to address this question. Simulation of the motion of W-118 should provide a good test of how well solvent–protein interactions are modeled in current calculations.

Conclusions

Our study of three homologous azurins in both holo and apo forms has led to the following conclusions.

(1) The interiors of azurins Pae and Ade (and most likely of azurin Afe) are not mobile on the nanosecond time scale. (This statement refers to a distance scale large enough to enable motion of an indole ring.)

(2) The external tryptophan in azurins Afe and Ade is exposed to the solvent and has substantial mobility. The cone model suggests an angular motion of about 30°.

(3) The nonradiative rate in the holoazurins is tentatively assigned to electron transfer from the excited-state tryptophyl residues to copper. This electron transfer occurs at near the maximum rate expected from Marcus theory for their separation. The rate is comparable to that observed in photosynthetic reaction centers [e.g., 200 ps from bacteriopheophytin to the primary quinone acceptor (Q_A) (Kaufman et al., 1975)] and very much faster than the rates observed by Kostic et al. (1983) and Margalit et al. (1984) for electron transfer from ruthenium to Cu(II) in azurin. At present it is not clear whether the tryptophyl environments, the intervening protein matrix, or the differing mobilities and orientations are responsible for the difference in electron-transfer rates observed between W-48 and W-118.

(4) Knowledge of the spectra and the lifetimes of the two single-tryptophan-containing proteins enables the fluorescence lifetime behavior of the azurin Ade containing two tryptophans to be understood.

(5) The fluorescence decay of the rigid interior W-48 is single-exponential, whereas the decay of the mobile solvent-exposed W-118 is nonexponential. The decay of W-118 can be interpreted in terms of χ_2 conformers. It should be noted, however, that the fluorescence behavior of W-118 is very similar to that in single-tryptophan hormones such as glucagon or ACTH. There thus seems rather little structural information to be obtained from the fluorescence lifetime data.

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Structure of Porcine Heart Cytoplasmic Malate Dehydrogenase: Combining X-ray Diffraction and Chemical Sequence Data in Structural Studies†

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ABSTRACT: The amino acid sequence of cytoplasmic malate dehydrogenase (sMDH) has been determined by a combination of X-ray crystallographic and chemical sequencing methods. The initial molecular model incorporated an "X-ray amino acid sequence" that was derived primarily from an evaluation of a multiple isomorphous replacement phased electron density map calculated at 2.5-Å resolution. Following restrained least-squares crystallographic refinement, difference electron density maps were calculated from model phases, and attempts were made to upgrade the X-ray amino acid sequence. The method used to find the positions of peptides in the X-ray structure was similar to those used for studying protein homology and was shown to be successful for large fragments. For sMDH, X-ray methods by themselves were insufficient to derive a complete amino acid sequence, even with partial chemical sequence data. However, for this relatively large molecule at medium resolution, the electron density maps were of considerable help in determining the linear position of peptide fragments. The N-acetylated polypeptide chain of sMDH has 331 amino acids and has been crystallographically refined to an R factor of 19% for 2.5-Å resolution diffraction data.

The extent to which the function of an enzyme, a protein, or in general any biomacromolecule can be understood depends to a large extent on available structural information. Single-crystal X-ray diffraction has been the most productive method for conformational analysis of proteins, but it requires knowledge of the amino acid sequence as additional input. In the absence of this information, it is still possible to interpret the electron density map of a protein but only in terms of an α-carbon model or, perhaps in somewhat more detail, a polyglycine or polyalanine model. Such models suffice to establish the overall folding patterns, as well as the presence of domain structures, subunit–subunit interactions, and structural relationships to other protein structures, but do not define the nature of the amino acids participating in active sites or other important locations.

Several attempts have been made to interpret electron

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