A Time-Resolved Fluorescence Study of Azurin and Metalloazurin Derivatives†

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ABSTRACT: Nickel and cobalt derivatives of *Pseudomonas fluorescens* (ATCC 13525) azurin were prepared and their steady-state fluorescence and time-resolved fluorescence monitored. Like the copper-containing native protein, the fluorescence decay of both metallo derivatives was best fit to a sum of three exponentials, whereas the apoazurin from which they were prepared obeyed single-exponential decay kinetics. However, comparison of the lifetimes and fractional fluorescence of each of the components in these derivatives to those in the oxidized and reduced native proteins revealed significant differences. These results suggest that the presence of a metal center in azurin imparts a conformational heterogeneity which is strongly dependent on the nature of the metal center. Further, the results are used to comment on current ideas concerning the geometry of the active site in this redox protein.

The azurins are bacterial blue copper proteins which have been the subject of numerous biophysical studies (Adman et al. 1982; Ainscough et al., 1987; Blair et al., 1985; Burstein et al., 1977; Finazzi-Agro et al., 1970; Grinvald et al., 1975; Groeneveld & Canters, 1985) attempting to relate their structure to their function as electron transferases (Farver & Pecht, 1984; Adman, 1985). The geometry and nature of the ligands at the active site have been deemed responsible for the protein's unique spectroscopic properties, as well as influencing the redox reactivity important for their function [this is summarized by Engeseth and McMillin (1986)]. There is additional evidence to suggest that the structure around the copper site, which includes closely connected residues such as His-35, exhibits "fluxionality". It has been suggested that this dynamic behavior may offer an explanation for the electron-transfer function of azurin (Adman et al., 1982, 1983; Canters et al., 1984).

The azurins from a number of bacterial species possess only a single tryptophan residue, which is fully conserved in position 48 (Ambler, 1971). Previous time-resolved fluorescence studies by ourselves as well as others revealed multicomponent decay kinetics in the azurin molecules that was not evidenced when the metal was removed (Szabo et al., 1983; Grinvald et al., 1975). In the preceeding paper (Hutnik & Szabo, 1989) we have shown that these decay kinetics have their origin in distinct protein conformations as opposed to possible contamination by an apo form. These studies included the pH and temperature dependence of each of the observed components.

It was shown that the conformational distribution of the holoazurin varied with pH, having more of the long lifetime component at pH 8 compared to pH 4. Also in two homologous azurins, *Pseudomonas fluorescens* (*Pfl*)[1] and *Pseudomonas aeruginosa* (*Pae*), the three fluorescence decay times observed for each protein were similar, but the fractional fluorescence and, hence, their conformational distributions were different by significant factors (Hutnik & Szabo, 1989).

Earlier it had been shown that cobalt and nickel bind to apoazurin to form a metalloazurin complex (Ferris et al., 1979; Tennent & McMillin, 1979; Blaszak et al., 1982; Engeseth et al., 1984; Engeseth & McMillin, 1986) having the same amino acid residues as ligands as the native cupro azurin. Furthermore, it had been shown that in the case of Co(II) and Ni(II) azurin, the metal–ligand cluster has a pseudotetrahedral structure not unlike the holoazurin. This implies that the protein structure restricts the metal–ligand complex from assuming its preferred planar geometry. It has been suggested that the nonplanar metallo ligand complex confers a more positive reduction potential on the metal, leading to a more favorable electron-transfer process (Gray & Solomon, 1981; Gray & Malmstrom, 1983). As different metals possess different sizes and different geometrical preferences (Ambler, 1971), they may serve to provide useful information on the active site and the factors controlling the electron-transfer process. Hence in order to obtain new information on the role the active site geometry has on the structure, dynamics, and function of azurin, the Co(II) and Ni(II) derivatives have been prepared and studied with fluorescence techniques. In this paper the time-resolved fluorescence and steady-state fluorescence measurements of these metalloazurins are presented and discussed in terms of the conformational heterogeneity of the protein.

Materials and Methods

Azurin was purified from the acetone-dried cells of *Pseudomonas fluorescens* (ATCC 13525) as previously described to a final spectral ratio of ≥0.56 and was judged homogeneous by newly established criteria. The apoazurin was prepared by the modified KCN dialysis method as described elsewhere (Hutnik & Szabo, 1989).

Metal-reconstituted azurin samples were prepared in two ways. The first method involved dialysis of apoazurin samples ($\sim 5 \times 10^{-5}$ M) against a 10-fold molar excess of either cupric chloride (BDH Chemicals Ltd., Toronto, Canada), cobaltous chloride (Fisher Scientific Co., Fair Lawn, NJ), or nickelous sulfate (Anachemia, Montreal, Canada) in 50 mM ammonium acetate buffer, pH 6.5 at 4 °C. In some cases the buffer was passed through Chelex-100 (Bio-Rad Laboratories, Richmond, CA) prior to addition of the metal salt, and in some cases nitrogen was bubbled into the metal solutions during the dialysis. M(II) azurins were also prepared by the addition of various aliquots of metal ion solution to the apoazurin

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[1] Abbreviations: *Pae*, *Pseudomonas aeruginosa*; *Pfl*, *Pseudomonas fluorescens*; Trp, tryptophan; OD, optical density.

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Table I: Fluorescence Decay Parameters of Apoazurin, Holoazurin, and M(II) Azurins[a]

sample	λ_{em} (nm)	τ_1	τ_2	τ_3	F_1	F_2	F_3	ϕ^b
holoazurin, pH 5	310	4.91	0.52	0.105	0.78	0.06	0.16	0.056
Cu(I) azurin	310	4.98	0.37	0.063	0.87	0.03	0.10	
apoazurin	310	5.10						0.330
Cu(II) azurin, reconstituted	310	4.94	0.52	0.097	0.83	0.04	0.13	0.055
Co(II) azurin	310	4.88	2.08	0.136	0.91	0.07	0.02	0.160
Ni(II) azurin	310	4.90	1.01	0.094	0.97	0.01	0.02	0.150

[a] Time-resolved measurements were performed in the presence of a 10-fold molar excess of each metal in 50 mM ammonium acetate buffer, pH 6.5 at 20 °C. All samples were prepared from protein purified from *P. fluorescens* having an original $A_{620}/A_{280} \geq 0.56$. The values presented are the mean of at least six determinants with standard errors as follows: for τ_1, ±0.005; for τ_2, ±0.01; for τ_3, ±0.001. [b] Fluorescence quantum yields of the various azurin samples were measured in 50 mM ammonium acetate buffer, pH 6.5 at 20 °C.

samples (~5×10^{-5} M) to yield various metal:protein ratios. These solutions were then equilibrated at room temperature and monitored for incorporation after various times.

Absorption, steady-state, and time-resolved fluorescence measurements were performed according to methods and instrumentation as previously described (Hutnik & Szabo, 1989).

Results

The UV–vis absorption spectrum of *Pfl* apoazurin in 50 mM ammonium acetate buffer, pH 6.5, featured a maximum at 280 nm and a sharp subsidiary maximum at 292 nm and lacked the 620-nm absorption. The protein was considered to be homogeneous on the basis of the original spectral ratio of at least 0.56 for the holoazurin and a lack of absorption in the UV–vis spectrum near 410 nm for both the holo and apo forms. This has been shown to be sufficient criteria by which to judge homogeneity. In addition, as judged by this spectrum of apoazurin, as well as atomic absorption analysis, less than 2% of the original copper remained in the apoazurin preparations.

The incorporation of the metal ions into apoazurin has been accomplished in two ways. In the first case solutions of apoazurin were dialyzed against solutions containing a 10-fold molar excess of each respective metal ion. The ultraviolet absorption spectra of the three metal derivatives were indistinguishable, while in the visible region each derivative had its characteristic absorption bands centered at 620 nm for Cu(II) azurin, 360 and 445 nm for Ni(II) azurin, and 340 and 380 nm for Co(II) azurin. The absorption spectrum of the Co(II) azurin derivative is shown in Figure 1. The fluorescence quantum yields obtained after dialysis for 24 h at 4 °C are shown in Table I. The same results were obtained regardless of whether nitrogen gas was bubbled into the solutions during dialysis or whether the buffer, prior to metal addition, was passed through Chelex-100 to remove adventitious metal ions. Addition of Cu(II) resulted in the greatest reduction of the quantum yield of fluorescence (6-fold), followed by Ni(II) (2.2-fold) and Co(II) (2.1-fold). The steady-state fluorescence emission spectra, when normalized at 308 nm, were superimposable for all of the M(II) azurins.

The second method of introducing the metals into apoazurin involved addition of aliquots of stock metal solutions to test tubes containing apoazurin, yielding a variety of metal:protein ratios. Figure 2 shows the quantum yield of each of the derivatives as a function of metal:protein ratio; the data shown are the mean of three trials recorded after 48 h. The quantum yields recorded after dialysis against a 10-fold molar excess of each metal are also shown in this figure. The 1:1 complex plateau values corresponded to relative fluorescence quantum yields of 0.15 for Ni(II) azurin, 0.16 for Co(II) azurin, and 0.055 for Cu(II) azurin.

It is important to note that a plateau quantum yield was achieved when either Cu(II), Co(II), or Ni(II) was added to

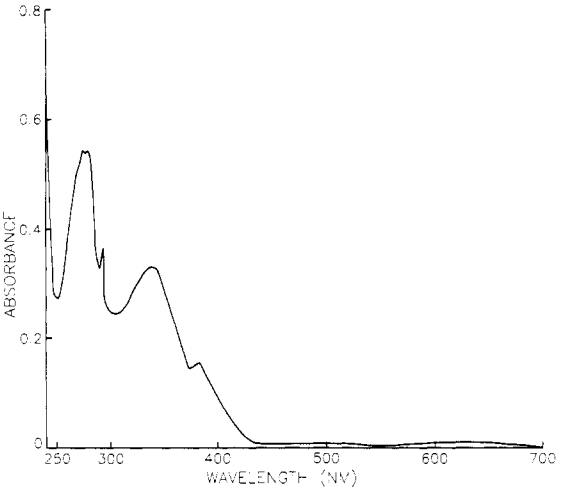

FIGURE 1: UV–vis absorption spectrum of *Pfl* Co(II) azurin in 50 mM ammonium buffer, pH 6.5.

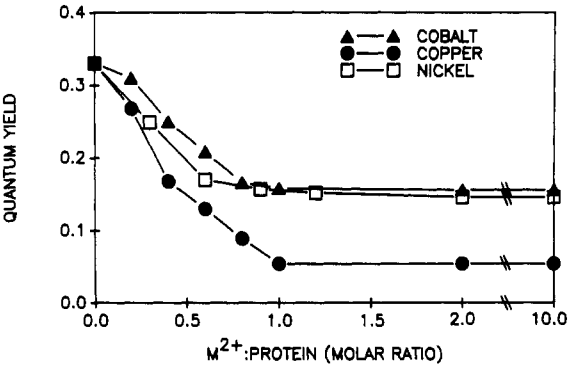

FIGURE 2: Effect of M(II) titration on the quantum yield of fluorescence of azurin derivatives in 50 mM ammonium acetate buffer, pH 6.5, 20 °C. Note that in the case of the Co(II) derivative the optical density at 292 nm of apoazurin [instead of the Co(II) azurin] was used as an approximation in the quantum yield calculation. This avoids interference caused by the visible absorbance of the Co(II)–chelate complex. All values shown are the mean of three determinations whose standard errors are within the size of the symbols.

apoazurin. When the copper solutions were also monitored by absorption spectroscopy, full restoration of the original spectral ratio was achieved, which suggested that this preparation of apoazurin was completely reconstitutable. Only small changes in the absorbances at 295 nm were observed. Atomic absorption and amino acid analysis revealed a 1:1 copper–protein complex had been formed (prior to these analyses the samples were either dialyzed against copper-free buffer or desalted by G-25 gel filtration). All quantum yields were calculated with the previously reported quantum yield of apoazurin as a standard (Szabo et al., 1983), and as can be seen in Table I and Figure 2, the quantum yields for the Ni(II) and Co(II) derivatives were similar and different from that of Cu(II) azurin.

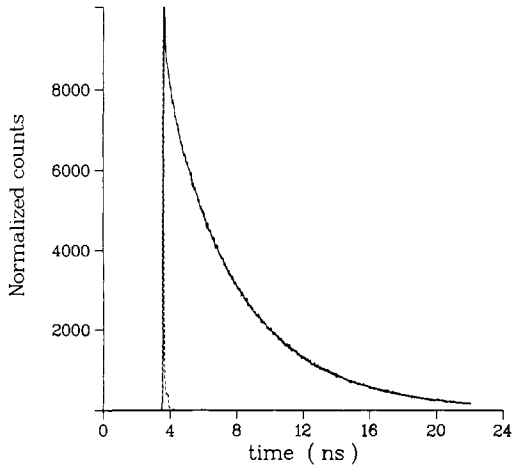

FIGURE 3: Typical normalized fluorescence time–intensity profiles. The lamp intensity profile (dotted line) and Co(II) azurin decay profile were recorded with channel width = 21.6 ps/channel, λ_{ex} = 292 nm, λ_{em} = 310 nm, and emission band-pass = 4 nm.

When monitored after 6 h of equilibration at room temperature, both Cu(II) azurin and Co(II) azurin had quantum yields which remained constant over the next 48 h; however, the quantum yields observed for Ni(II) azurin after 6 h were higher than those recorded after 24 and 48 h which suggested that more than 6 h was required to achieve full Ni(II) incorporation.

The fluorescence decay parameters of these various M(II) azurin samples were measured in the presence of excess metal to ensure that no dissociation of the metal–protein complexes had take place. These results are summarized in Table I. In the absence of any metal, single-exponential decay kinetics existed, but in the presence of any of the metals, the data were best fit by a sum of three exponentials. Figure 3 gives a typical decay curve for a M(II) azurin [Co(II) azurin in this case] while the plots of weighted residuals corresponding to this decay curve are shown in Figure 4. The longest decay component was essentially identical in all M(II) azurins but possessed different contributions to the total fluorescence. The fractional fluorescence of the shorter decay components constituted only 3% of the total fluorescence in Ni(II) azurin and 9% in Co(II) azurin. However, the plots of weighted residuals show satisfactory fits to the data could only be obtained when the data were fit with three exponential decay components. The shortest decay component was identical in Cu(II) and Ni(II) forms while for Co(II) azurin it was about 30% longer (0.13 ns). The fractional contribution of τ_3 to the total observed fluorescence was much less in the Co(II) and Ni(II) derivatives when compared to that in Cu(II) azurin.

Discussion

Preparation of Metal Derivatives of Azurin. It has already been demonstrated that different metals, including Ni(II) and Co(II), all coordinate at the copper binding site in azurin (Blaszak et al., 1982; Engeseth et al., 1984; Engeseth & McMillin, 1986) and that once bound these derivatives display negligible affinities for either Cu(II) or *p*-(hydroxymercuri)benzoate (Tennent & McMillin, 1979). In addition, by use of a variety of spectrophotometric assays, it has been shown that even after exhaustive dialysis or gel filtration the metal derivatives retained "significant quantities of metal ions" (Tennent & McMillin, 1979).

Consistent with these findings, we observed a plateau quantum yield when the metal to apoazurin stoichiometry was 1:1. No further change in fluorescence was seen when there

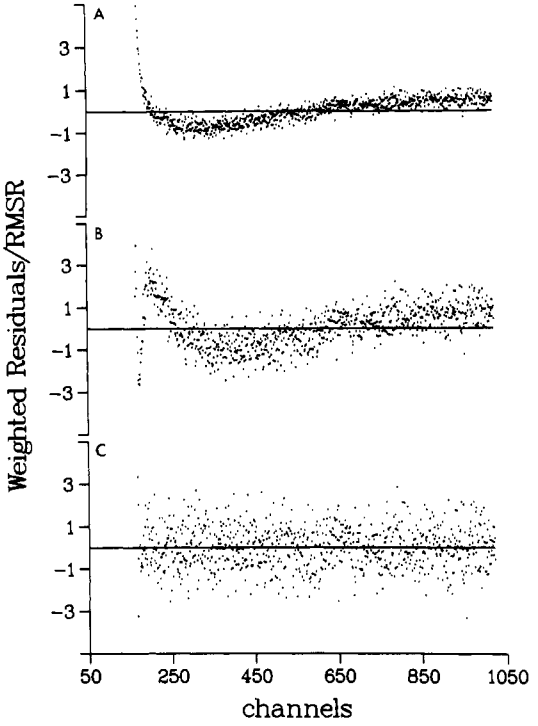

FIGURE 4: Weighted residual plots for the calculated best-fit emission decay profile after deconvolution of the emission decay profile (in Figure 4). Panels A–C correspond to the plots for Co(II) azurin fitted to single-, double-, and triple-exponential decay components, respectively.

was either a 2- or 10-fold molar excess of metal ion. This same apoazurin could be fully Cu(II) reconstituted as judged by the A_{620}/A_{280} spectral ratio and the 1:1 copper to protein stoichiometry established by amino acid and atomic absorption analysis. These results indicated that the various samples had become fully metal incorporated with a 1:1 stoichiometry and that no free apoazurin was present. Excess metal did not alter the quantum yields or lifetimes observed for any of the native, reconstituted, or metal-incorporated azurins, allowing measurements to be made in the presence or absence of excess cations.

Quenching of Fluorescence by Metal Incorporation. In agreement with Tennent and McMillin (1979), we observed a reduction of the quantum yield of fluorescence upon the addition of metal ions to apoazurin in the order Cu(II) > Co(II) ≫ Ni(II) when the actual OD_{292} values of the samples were used. All the normalized emission spectra of the various metal derivatives were superimposable upon that of apoazurin. However, earlier Tennent and McMillin (1979) reported that Co(II) azurin had a σ S (Cys) → Co(II) electronic transition near 330 nm. The absorption band corresponding to this transition is clearly seen in Figure 1. The overlap of this band with the 292-nm band of apoazurin resulted in a significantly higher optical density measurement at λ_{ex} for the Co(II) derivative. In order to make the appropriate comparison with the quantum yields of the other metallo derivatives (where the quantum yield is essentially that of the Trp residue), the quantum yield of the Trp residue in the Co(II) derivative of azurin shown in Figure 2 was determined from the optical density of the original apoazurin solution. When this was done, the relative reduction in the quantum yields of fluorescence was different, occurring as Cu(II) > Co(II) = Ni(II).

Recently, Engeseth and McMillin (1986) have reported metal ion binding energies of azurin and various metal-substituted derivatives. Their results suggested that Ni(II) was much less strongly bound than the Cu(II) to azurin on the basis

of the comparison of enthalpies of denaturation. They suggested that Ni(II) suffered the greatest loss in ligand field stabilization energy when bound to the protein and thus a thermodynamic effect may dictate the lack of selectivity. The steady-state fluorescence measurements revealed that Ni(II) required more time than either Cu(II) or Co(II) in order for the protein to reach its final quantum yield. These data do not allow us to determine whether the affinity of Ni(II) for the binding site was less or whether all of the metals had comparable binding affinities but differed in their ability to induce protein conformational changes necessary for incorporation of the metal ions.

The mechanism by which the metal ion (especially Cu^{2+}) quenches the Trp fluorescence has been of some interest, and it has recently been suggested that the fluorescence quenching reaction is an electron-transfer process from the excited state of Trp to the liganded copper atom (Petrich et al., 1987). This quenching mechanism, if operative, would suggest a correlation between the quenching efficiency and the relative redox potentials of the metal ions. Both Co(II) and Ni(II) have negative reduction potentials (−0.283 V and −0.236 V, respectively), while the reduction potential for Cu(II) is positive (+0.153 V) (Glasstone, 1942; Atkins, 1978). This implies that both Co(II) and Ni(II) would not efficiently quench Trp fluorescence via an electron-transfer process from the excited Trp chromophore. Despite this point the fluorescence quantum yield of the azurin is considerably reduced when these metals bind to the protein. It appears therefore the quenching efficiency of the Trp is not primarily related to the reduction potential of the metal. This is reinforced by the comparatively similar quantum yield and lifetime behavior of Cu(II) and Cu(I) azurin where one expects significant differences owing to the differences in the reduction potential of these two oxidation states of copper (+0.153 V [Cu(II)] versus +0.521 V [Cu(I)]; Atkins, 1978). It cannot be ruled out however that in this latter case a limiting electron-transfer rate constant may have been realized at the reduction potential of Cu(II).

It is possible to calculate a fluorescence quantum yield of the metallo derivatives from the preexponential terms and the decay times (τ_i) of each decay component from

$$\phi_T = c_1\frac{\tau_1}{\tau_r} + c_2\frac{\tau_2}{\tau_r} + c_3\frac{\tau_3}{\tau_r} \quad (1)$$

where τ_r is the radiative lifetime and c_i are the fractional concentrations of each component. If τ_r is the same for each component, then c_i can be replaced by the normalized preexponential terms α_i ($\sum\alpha_i = 1$). In the previous paper it was found that the calculated quantum yield for Cu(II) azurin was *lower* than the measured value. It was suggested that the two short decay components in this case had a lower τ_r value (10.2 ns) than the long decay time (5 ns) component (τ_r = 15.5 ns). It was proposed that the shorter radiative lifetime arose because of an electronic perturbation of the Trp excited state by the copper–ligand complex.

When the quantum yield of the Ni(II) derivative was estimated from the decay parameters, a value of $\phi_T = 0.153$ was estimated when the radiative lifetime value of each component was considered to be the same, with a value of 15.5 ns. A value of $\phi_T = 0.156$ was calculated when the long decay time component had a τ_r = 15.5 ns and the other two decay components had τ_r = 10.2 ns. The measured quantum yield of 0.15 agreed quite well with either of these two calculated values.

In the case of the Co(II) derivative, the calculated quantum yield was 0.176 if all components have the same τ_r = 15.5 ns and was 0.184 when values of τ_r = 15.5 and 10.2 ns were used. These values are both higher than the measured quantum yield of 0.16. However, the measured value may be lower due to the fact that the quantum yield was calculated with the OD_{292} of the starting apoazurin solution as an estimate of the Trp absorbance in order to avoid interference by the 330-nm peak of the Co(II)–chelate complex.

As was the case for Cu(II) azurin, the long decay time components of approximately 4.9 ns in both the Co(II) and Ni(II) derivatives of azurin are assigned to a conformer where the tryptophan is distant from the metal–ligand complex. The other two components are assigned to conformational states in which the distance and/or orientation of the Trp residue relative to the ligand center are different. The significant differences in the decay parameters of these two shorter components in the various derivatives suggest that the geometrical constraints placed upon the metal center influence the Trp fluorescence to a considerable extent. Clearly careful temperature studies would be informative but to date have not been performed. Tennent and McMillin (1979) presented evidence that the Co(II) azurin–ligand complex existed in a distorted tetrahedral structure. The results reported herein suggest that the Co(II)–ligand structure is heterogeneous and the relationship of these structures to the single Trp residue is reflected in fluorescence behavior unique to this derivative.

In the case of Ni(II) azurin, the fluorescence decay components have similar values to those of the Cu(II) derivative, except for τ_2 and the preexponential terms. This suggests that in the Ni(II) and Cu(II) azurins the structure of the metal complex is similar, varying primarily in the relative concentration of the conformational components. The relative concentration of the two short components in the Ni(II) derivative is very low when compared to that of the long decay component and significantly less than the relative concentrations of these two components in Cu(II) azurin. It implies that the Ni(II) protein structure is most similar to the apoazurin structure. The work of Engeseth and McMillin (1986) is interesting in this respect since they reported that the calorimetric scans of Ni(II) azurin resembled that of apoazurin, having two well-separated thermal transitions. The work of Engeseth and McMillin is very relevant to these studies since they suggest that apoazurin also exists in two conformational states. It is rather surprising that if this were the case, the fluorescence decay behavior of tryptophan in apoazurin did not reflect that heterogeneity. In view of the results presented in the previous paper showing that some azurin samples contain nonmetal reconstitutable components, verification of the calorimetric scans on azurin samples known to be homogeneous would be appropriate.

In summary, the results on the different metal derivatives suggest that the different metallo derivatives have similar conformational heterogeneity. The structures of the Cu(II) and Ni(II) conformers may be comparable, but their proportions are different. The Co(II) derivative is the most different in terms of the interactions of these components with the tryptophan residue. Obviously, metal complexes in protein centers are varied and complex. This suggests that the approach of comparing metalloproteins with small model compounds requires care in interpretation. For example, it has been shown, on the basis of the hydrolysis of tetrabromide adducts, that the divalent transition metal ions have an increased stability in tetrahedral complexes as one progresses from Co(II) to Zn(II) (Bianchi & Paoletti, 1984). However, it was found that Cu(II) azurin was more stable than either Ni(II) or Zn(II) azurin, which was rationalized as being due to a particularly low-symmetry structure of the metal binding

site in azurin (Engeseth & McMillin, 1986). Because of a pseudo Jahn–Teller effect, Cu(II)/Cu(I) in the binding site may achieve stabilization by virtue of a distortion from idealized tetrahedral symmetry. It has also been demonstrated that the extinction coefficient of the band near 600 nm in the absorption spectrum of holoazurin is approximately 100 times larger than that, apparent in the same spectral region, of most small model Cu^{2+} complexes (Fee, 1975). This suggests the Cu^{2+}–protein combination possesses a structural arrangement which is entirely unique among Cu^{2+} coordination complexes.

Although this fluorescence study cannot determine the geometry of ligands in the coordination sphere, it very clearly reveals that the geometric constraints placed upon the center by various metals are quite different. The possible participation of a fifth ligand, contributed by Gly-45 (Norris et al., 1986), highlights the complexity that may be involved. The fluorescence results also indicate that the nature of the metal center can alter the conformational heterogeneity of the protein. It is this effect upon conformation by the redox-altered geometry at the active center which most probably mediates the electron-transfer process.

Acknowledgments

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