Spectrochemical Studies on the Blue Copper Protein Azurin from *Alcaligenes denitrificans*†

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ABSTRACT: Spectroscopic and electrochemical studies, incorporating electronic spectra, electron paramagnetic resonance (EPR) spectra, resonance Raman (RR) spectra, and measurements of the redox potential, have been carried out on the blue copper protein azurin, from *Alcaligenes denitrificans*. These data are correlated with the refined crystal structure of this azurin and with corresponding data for other blue copper proteins. The electronic spectrum, characterized by an intense (ϵ = 5100 M^{-1} cm^{-1}) charge-transfer band at 619 nm, the EPR spectral parameters ($g_\perp$ = 2.059, $g_\parallel$ = 2.255, $A_\parallel$ = 60 × 10^{-4} cm^{-1}), and the resonance Raman spectrum are similar to those obtained from other azurins and from plastocyanins. Both the electronic spectrum and the EPR spectrum are unchanged over the pH range 4–10.5, but major changes occur above pH 12 and below pH 3.5. A small reversible change occurs at pH ~11.4. In the RR spectrum the Cu–S stretching mode is shown to contribute to all of the five principal RR peaks. Deuterium substitution produces shifts in at least seven of the peaks; these shifts may be attributable, at least in part, to the NH···S hydrogen bond to the copper-ligated Cys-112. Measurements of the redox potential, using spectroelectrochemical methods, over the temperature range 4.8–40.0 °C, give values for $\Delta H°'$ and $\Delta S°'$ of −55.6 kJ mol^{-1} and −97.0 J K^{-1} mol^{-1}, respectively. The redox potential of *A. denitrificans* azurin at pH 7.0, $E°'$, is 276 mV. These data are interpreted in terms of a copper site, in azurin, comprising three strong bonds, in an approximately trigonal plane, from Cys-112, His-46, and His-117 and much longer axial approaches from Met-121 and the peptide carbonyl oxygen of Gly-45. Spectral differences within the azurin family and between azurin and plastocyanin are attributed to differences in the strengths of these axial interactions. Likewise, the distinctly lower $E°$ values for azurins, as compared with plastocyanins, are related to the more copper(II)-like site in azurin [with a weaker Cu–S(Met) interaction and a Cu–O interaction not found in plastocyanin]. On the other hand, the relative constancy of the EPR parameters between azurin and plastocyanin suggests they are not strongly influenced by weakly interacting axial groups.

The "blue" copper proteins have been the subject of intensive spectroscopic and structural studies over a number of years because of their distinctive spectrochemical properties [for recent reviews, see Fee (1975), Lappin (1981), Gray and Solomon (1981), and Adman (1985)]. Particularly noteworthy are their very intense (ϵ = 3500–6000 M^{-1} cm^{-1}) absorption bands in the visible part of the spectrum (λ = 600–625 nm), an axial EPR spectrum with a very low hyperfine splitting constant ($A_\parallel$ = 0.006 cm^{-1}), and a relatively high redox potential.

The spectroscopic data are rich and varied, from methods such as absorption and circular dichroism (CD) spectroscopy (McMillin et al., 1974a,b; Solomon et al., 1976a,b), electron paramagnetic resonance (EPR) spectroscopy (Vanngard, 1972), nuclear magnetic resonance (NMR) spectroscopy (Markley et al., 1975; Hill et al., 1976; Ugurbil et al., 1977), resonance Raman spectroscopy (Miskowski et al., 1975; Siiman et al., 1974, 1976; Thamann et al., 1982; Maret et al., 1983; Nestor et al., 1984; Woodruff et al., 1984; Blair et al., 1985), electron nuclear double resonance (ENDOR) spectroscopy (Roberts et al., 1984), and X-ray absorption spectroscopy (Tullius et al., 1978; Peisach et al., 1982). In most cases, however, proper interpretation has been hampered by the paucity of small-molecule models with similar properties and by the relative lack of structural information on the proteins themselves.

The only blue copper proteins whose three-dimensional structures have been determined by X-ray crystallography are the two single-copper electron-transfer proteins plastocyanin and azurin. In the case of plastocyanin, the initial 2.7-Å analysis (Colman et al., 1978) has been extended to 1.6 Å and the structure refined (Guss & Freeman, 1983), allowing a detailed description of the whole structure and, in particular, of the geometry of the copper site. This has stimulated further spectroscopic studies on plastocyanin, with more detailed interpretations being possible (Penfield et al., 1981, 1985; Gray & Solomon, 1981; Scott et al., 1982). For azurin, medium-resolution crystallographic analyses (2.7 and 2.5 Å, respectively) have been reported for the azurins of two bacterial species, *Pseudomonas aeruginosa* (Adman et al., 1978; Adman & Jensen, 1981) and *Alcaligenes denitrificans* NCTC 8582 (Norris et al., 1983). Most of the spectroscopic properties that have been carried out on azurin have used the *P. aeruginosa* protein, and very little data are available on that from *A.*

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denitrificans. There are, however, significant amino acid sequence differences between the two azurins, with 38% of the amino acid residues being different (Ambler, 1971). Differences are found in the resonance Raman spectra of different azurins, and there is also evidence for subtle functional differences. For example, a conformational transition proposed for *P. aeruginosa* azurin apparently does not occur for *A. faecalis* azurin (Rosen et al., 1981), and sequence comparisons suggest that it may also not occur in *A. denitrificans* azurin (Adman, 1985).

Detailed interpretation of spectroscopic results requires knowledge of the spectral changes that accompany structural changes. We have therefore undertaken a series of spectrochemical investigations of *A. denitrificans* azurin, in order to be able to compare the results with those obtained for plastocyanin and for *P. aeruginosa* azurin, in the light of the known three-dimensional structures of these three blue copper proteins. The comparison is made more significant by the fact that the crystal structure of *A. denitrificans* azurin has now been refined at high resolution (1.8 Å) (Norris et al., 1986), so that structural details are now available for azurin that are comparable in accuracy with those derived for plastocyanin. This paper summarizes the results from electronic, electron paramagnetic resonance (EPR), and resonance Raman (RR) spectroscopies, measurements of the redox potential over a range of temperatures, and some details of the refined three-dimensional structure relevant to these results.

Materials and Methods

Preparation of Azurin. The bacterial strain used for preparation of our azurin was *Alcaligenes denitrificans* NCTC 8582. (Note that this organism is also listed as a subspecies of *Alcaligenes faecalis*, but it should not be confused with the type strain NCIB 8156 or ATCC 8750 of *A. faecalis* on whose azurin other studies have been reported.) Azurin was isolated as previously described (Norris et al., 1979), following slight modifications of earlier procedures (Ambler, 1963; Ambler & Brown, 1967; Ambler & Wynn, 1973). The spectral extinction ratio E^{625}/E^{280} was 0.30. The protein was stored at 4 °C as a crystalline suspension in 0.1 M phosphate buffer, pH 6.0, which was 70% saturated with ammonium sulfate. Prior to use, samples were dissolved in 0.1 M phosphate solution and brought to the appropriate pH and ionic strength by dialysis or with an Amicon ultrafiltrator.

Crystallography. Further refinement of the 2.5-Å resolution structure previously reported (Norris et al., 1983) was based on X-ray diffraction data to 1.8-Å resolution ($2\theta = 50.7°$). Measurements were made on a computer-controlled X-ray diffractometer. All X-ray data between 10 and 1.8 Å were used in the refinement, the only reflections omitted being those measured as having negative net intensities. Restrained least-squares procedures were employed, with bond lengths and angles within the protein molecules being restrained close to standard values. Initially the program PROLSQ, due to Hendrickson and Konnert (1980), was used; later a similar program, TNT, due to L. Ten Eyck and D. Tronrud, University of Oregon, was also used. No restraints were, however, imposed on any of the distances or angles involving the copper atom, and the two azurin molecules in the crystal asymmetric unit were allowed to refine quite independently. Full details of the refinement will be reported elsewhere (E. N. Baker, B. F. Anderson, and G. E. Norris, unpublished results).

Electronic Absorption Spectroscopy. Spectra were recorded over the range 400–1100 nm, with a protein concentration of 2×10^{-4} M. In the range 400–900 nm, a Cary 219 spectrophotometer was used, while a Shimadzu MPS 5000 spectrophotometer was used for the range 900–1100 nm. For the pH titrations the protein was dialyzed against 0.02 M bicarbonate buffer to bring it to pH 9.6. Higher pH values were obtained by incremental addition of 0.1 M NaOH. Low-pH values were obtained by utilization of a 0.05 M citrate/phosphate buffer at pH 3.8, followed by subsequent addition of 0.1 M citric acid.

EPR Spectroscopy. Solutions with a protein concentration of about 5×10^{-4} M were made up in distilled water. For the pH study the acidity was changed as described above for the electronic spectra. Spectra were recorded from frozen solutions (at ~100K) on a Varian E-104A spectrometer equipped with a Varian E-257 variable-temperature accessory. Spectral g values were calibrated with a (diphenylpicryl)hydrazyl standard.

Resonance Raman Spectroscopy. For resonance Raman experiments, ~1 mM samples of azurin were made up in 0.05 M phosphate (Na^+) buffer at pH 6.5 (H_2O solution) or pD 6.5 (D_2O solution). Spectra were collected in a backscattering geometry with an automated Jarrell-Ash 25-300 scanning spectrophotometer described previously (Loehr et al., 1979). Laser light at 647.1 nm from a Spectra-Physics 164-01 Kr ion laser was filtered through a Pellin-Broca prism monochromator to reduce the intensity of plasma emission; only a weak laser line at 234 cm^{-1} was observed in some of the spectra. An RCA C31034A photomultiplier cooled to ~245 K served as the detector. Samples in glass melting point capillaries were placed in a Dewar fitted with a copper cold finger and cooled to 90 K (Sjöberg et al., 1982). Sample integrity was verified spectrophotometrically after laser exposure.

Spectroelectrochemical Measurements. The azurin solution was made up immediately before use, in 0.1 M ionic strength phosphate buffer, pH 7.0, in an Amicon ultrafiltrator. Pentaammine(pyridine)ruthenium(III) perchlorate ($[Ru(NH_3)_5py](ClO_4)_3$) (Cummins & Gray, 1977) was added to the protein solution, in an equimolar ratio, as a redox mediator. Measurements of the azurin redox potential were made with an optically transparent thin-layer electrochemical (OTTLE) cell, in a nonisothermal configuration (Taniguchi et al., 1982). Potentials were applied across the thin-layer cells with a PAR 174A polarographic analyzer and were measured with a Keithley 177 microvolt digital multimeter. The cell temperature was maintained with a Forma Scientific Model 2095 circulating water bath and monitored (±0.2 °C) with an Omega Engineering Inc. precision microthermocouple and a Fluke 2175A Digital Thermometer. The ratios $[Az^{ox}]/[Az^{red}]$ were obtained by monitoring the change in absorbance at 625 nm on a Cary 219 spectrophotometer.

Results and Discussion

Copper Site in Azurin, after Crystallographic Refinement. The final refined model of 1956 protein atoms (two molecules of azurin) and 218 water molecules gives a crystallographic R factor[1] of 0.164 for all 21779 reflections between 10- and 1.8-Å resolution. For bond lengths and angles that were restrained during refinement (i.e., all except those involving copper), the root mean square (rms) deviations from standard values were 0.016 Å and 3.0°. The *upper limit* for the error in atomic positions, derived from the variation of R with resolution, is 0.15 Å, but for well-ordered parts of the structure (such as the copper site), with low thermal parameters, the error is much less. Taking this into account and the high level of agreement between the two independent molecules in the asymmetric unit, the standard deviations for bond lengths and

[1] $R = \sum||F_o| - |F_c||/\sum|F_o|$.

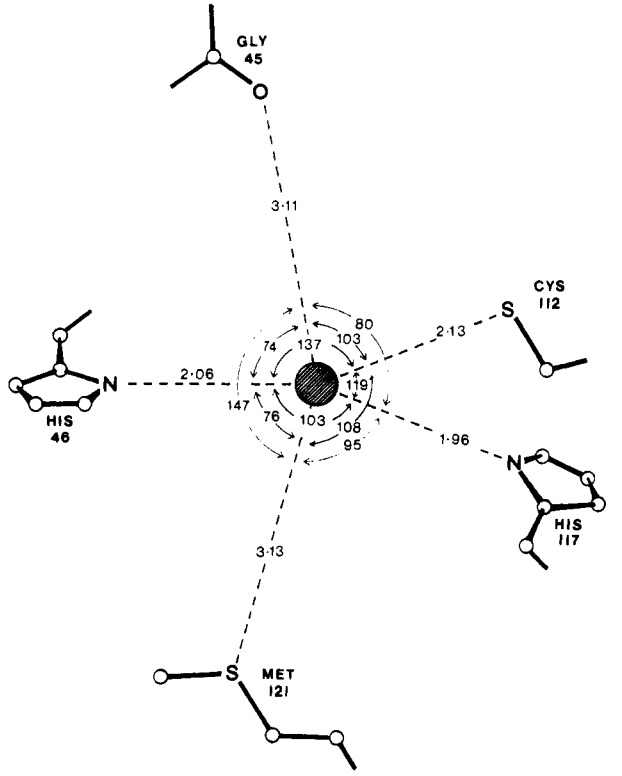

FIGURE 1: Schematic diagram showing distances and angles around the copper atom in *A. denitrificans* azurin. The values shown are the mean values for the two independent molecules in the crystallographic asymmetric unit.

angles involving copper are estimated to be about 0.05 Å and 3.0°, respectively.

A full description of the refined structure will be given elsewhere (E. N. Baker, B. F. Anderson, and G. E. Norris, unpublished results). Details of the copper geometry are as published previously (Norris et al., 1986) but will be summarized here as they are relevant to the following spectrochemical results. Bond lengths and angles around the copper atom are shown in Figure 1, which gives a simplified representation of the copper coordination. Figure 2 is a stereo view of the full copper site.

The copper site in *A. denitrificans* azurin is best described in terms of a basic three-coordinate, distorted trigonal-planar geometry, with two additional, much more weakly interacting groups in axial positions completing an axially elongated trigonal bipyramid. The three in-plane, strongly bound ligands are the thiolate sulfur of Cys-112 (mean Cu–S bond length 2.13 Å in the two independent molecules) and the imidazole nitrogens of His-46 and His-117 (mean Cu–N bond length 2.06 and 1.96 Å). The bond distances agree closely with those derived for *P. aeruginosa* azurin by X-ray absorption spectroscopy, viz., 2.10 Å for Cu–S and 1.97 Å for Cu–N (Tullius et al., 1978). The low symmetry of the site is emphasized by the in-plane ligand–copper–ligand angles of 103°, 119°, and 137°.

Much longer approaches to the copper atom are made by the thioether sulfur of Met-121 on one side of the trigonal plane (Cu–S, 3.13 Å) and to the main chain carbonyl oxygen of Gly-45 on the other side (Cu–O, 3.11 Å). At best, these can only be described as very weak interactions. Similar long axial Cu–S bonds, of 2.8–3.2 Å, have been observed in some low molecular weight Cu(II) complexes [e.g., see Guss and Freeman (1983) and references cited therein; Prochaska et al., 1981; Van Rijn et al., 1984]. The Cu–O distance is probably marginal for a weak Cu–O bond, but a Cu–O(carbonyl) interaction has been detected by NMR spectroscopy on *P. aeruginosa* azurin (Ugurbil et al., 1977). The copper atom lies very close to the N_2S trigonal plane, its deviation being 0.13 Å (toward Met-121) in molecule 1 and 0.08 Å in molecule 2.

The copper site in *A. denitrificans* azurin differs from that determined crystallographically for poplar plastocyanin (Guss & Freeman, 1983) in that although the three strong bonds (to the Cys and two His ligands) are essentially the same, the axial interactions are different. The Cu–S(Met) distance is 0.2 Å longer in azurin, and plastocyanin has no corresponding Cu–O interaction (the equivalent Cu···O distance in plastocyanin is 3.8 Å). The *P. aeruginosa* azurin structure has not yet been fully refined, but present coordinates (Adman et al., 1980) are not inconsistent with the *A. denitrificans* results reported above.

One other aspect of the copper site is that the copper ligands appear quite tightly constrained by the surrounding protein structure. The thiolate sulfur of Cys-112 is hydrogen-bonded to the peptide NH of residue 47 (S···N, 3.51 Å), and the imidazole ring of His-46 hydrogen bonds (through $N^{\epsilon 2}$) to the peptide C=O of residue 10, while that of His-117 is sandwiched between the side chains of Phe-114 and Met-13 and the side chain of Met-121 packs between the copper atom and the aromatic ring of Tyr-15. Similar constraints on the copper ligands have been noted in plastocyanin (Guss & Freeman, 1983), for which it has also been shown that removal of copper hardly affects the positions of the ligating residues (Garrett et al., 1984).

Electronic Absorption Spectroscopy. The electronic spectrum of the azurin was recorded in aqueous solution (pH 6.5) over the range 400–1100 nm (Figure 3 and Table I). As is typical for this type of protein, an intense absorption occurs at 619 nm, and this is flanked by weaker bands at 460 and 780 nm (a broad shoulder). The latter absorption does not peak as definitely as for plastocyanin or stellacyanin and in this respect differs from them. Also a long absorption tail runs into the near infrared from this 780-nm shoulder.

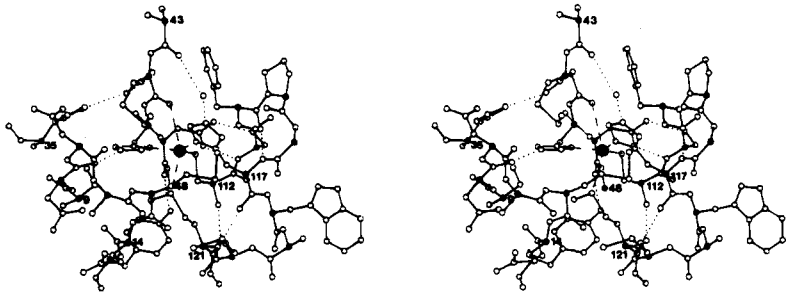

FIGURE 2: Stereo view of the copper site in *A. denitrificans* azurin, looking down on the nonpolar surface patch that surrounds the edge of the imidazole ring of His-117. α-Carbon atoms are shown as filled circles along the polypeptide chain. Some strong hydrogen bonds are indicated with dotted lines.

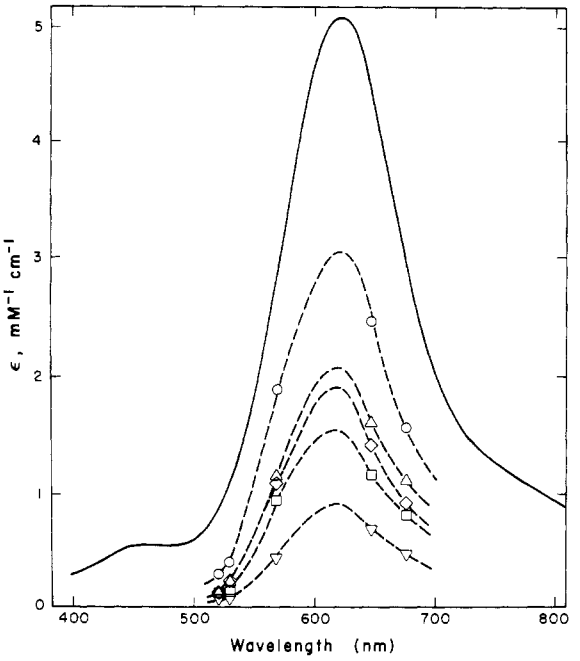

FIGURE 3: Electronic absorption spectrum (—) of *A. denitrificans* azurin, recorded in aqueous solution at pH 6.5. Also shown are the resonance Raman enhancement profiles of principal vibrational modes (--). Raman spectra were obtained at five excitation wavelengths on a single protein sample (0.5 mM) in 0.4 M $(NH_4)_2SO_4$ at 5 °C. Enhancement for vibrations at 375 (◇), 398 (△), 411 (○), 429 (□), and 459 (▽) cm^{-1} was measured as the height of the Raman peak relative to the height of the 980-cm^{-1} sulfate peak.

Table I: Electronic Spectral Data for Azurin (pH 6.5)

λ_{max} (nm) [ϵ (M^{-1} cm^{-1})]		
A. denitrificans azurin	*P. aeruginosa* azurin[b]	assignment
900–1000	~1100	d–d
780 (sh) [1040]	779 [686]	S(Cys)$\pi \rightarrow Cu(d_{x^2-y^2})$
619 [5100][a]	631 [3798]	S(Cys)$\sigma \rightarrow Cu(d_{x^2-y^2})$
	567 [504]	S(Cys)$\sigma \rightarrow Cu(d_{x^2-y^2})$
460 [580]	481 [198]	N(His)$\pi \rightarrow Cu(d_{x^2-y^2})$

[a] Estimated error ±8%. [b] Data from Solomon et al. (1976, 1980).

The assignments (Table I) are based on those of Penfield et al. (1981) for plastocyanin and stellacyanin. The absorptions at 619 and 780 nm are considered to arise from ligand to metal charge-transfer transitions between the cysteine (Cys) sulfur and copper(II) atoms, i.e., S(Cys)$\sigma \rightarrow Cu(d_{x^2-y^2})$ and S(Cys)$\pi \rightarrow Cu(d_{x^2-y^2})$, respectively. A Gaussian analysis of the latter band has not been performed, but by analogy with the other blue copper proteins, a component may be expected at about 560 nm. Its assignment has been the subject of some controversy. Penfield et al. (1981) favored another S(Cys)$\sigma \rightarrow Cu(d_{x^2-y^2})$ charge-transfer transition, whereas McMillin and Morris (1981) preferred a S(Met)$\sigma \rightarrow$ Cu assignment. The former assignment is preferable, however, because of the long Cu–S(Met) bond and the poor orientation of the $Cu(d_{x^2-y^2})$ orbital with respect to this bond. Moreover, while comparisons with small-molecule compounds should be treated with caution, we note that compounds with long apical Cu(II)–S(thioether) bonds (2.82–3.28 Å) have S$\sigma \rightarrow$ Cu charge-transfer absorptions in the 300–400-nm region if detected (Prochaska et al., 1981; Sakurai et al., 1981). The highest energy band is assigned to a N(His)$\pi \rightarrow Cu(d_{x^2-y^2})$ charge transfer. A d–d absorption is estimated to occur in the 900–1000-nm region, but is not resolved. For plastocyanin and stellacyanin, near-infrared CD and MCD spectra were employed to resolve the d–d bands (Solomon et al., 1980).

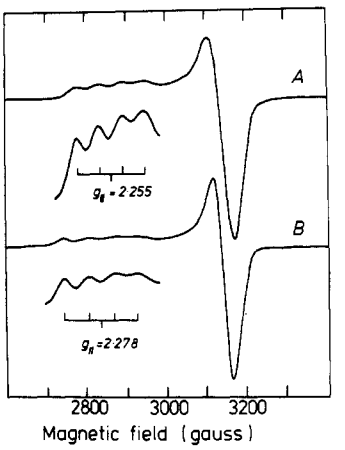

FIGURE 4: Frozen solution X-band EPR spectra for *A. denitrificans* azurin. Spectrum A was obtained in water at pH 6.5 and spectrum B in 0.02 M bicarbonate buffer titrated to pH 11.4 with 0.1 M NaOH. In each case the hyperfine splitting is shown below, on an expanded scale.

Table II: EPR Parameters for Azurins and Plastocyanin

protein	$g_{\parallel}$	$A_{\parallel}$ ($\times 10^{-4}$ cm^{-1})	$g_{\perp}$
azurin (*A. denitrificans*)			
pH 6.5	2.255	60	2.059
pH 11.4	2.278	69	2.058
azurin (*P. aeruginosa*)[a]	2.260	60	2.052
azurin (*P. denitrificans*)[b]	2.260	60	2.055
azurin (*B. bronchiseptica*)[b]	2.273	60	2.049
plastocyanin (spinach)[e]	2.226	63	2.059,[c] 2.042[d]

[a] From Brill et al. (1968). [b] From Lappin (1981). [c] g_y. [d] g_x. [e] From Penfield et al. (1985). Rhombic splitting of $g_{\perp}$ from Q-band spectrum.

Spectral features for *P. aeruginosa* azurin are also included in Table I, for comparison. The spectral profiles of the two azurins are more similar to each other than to either plastocyanin or stellacyanin, which are themselves not identical (Solomon et al., 1980). It is not clear what structural features may give rise to the small differences in the electronic absorption spectra of these various proteins. Comparing azurins and plastocyanins in general, the intense S(Cys)$\sigma \rightarrow$ Cu charge-transfer band is at distinctly higher energy in plastocyanins (λ_{max} ~600 nm compared with ~620 nm in azurins) [see also Lappin (1981)], implying some difference in the electron density on the copper atom. Both the crystallographic and X-ray absorption spectral results, however, suggest an almost identical Cu–S(Cys) bond length. A probable explanation is that the weaker axial interactions in azurin result in a lesser buildup of charge on the copper ion, an event that would be expected to decrease the energy of the charge-transfer band (Lever, 1984).

EPR Spectroscopy. The X-band EPR spectrum for a frozen solution (pH 6.5) of *A. denitrificans* azurin is shown in Figure 4, and the parameters, both for this azurin and for other azurins and plastocyanin, are listed in Table II. A striking feature of the spectrum is the narrow hyperfine splitting by copper of 60×10^{-4} cm^{-1} in the $g_{\parallel}$ region. This, of course, is now a recognized trademark of blue copper proteins. As in other azurins (Table II), an axial-type spectrum, with $g_{\parallel} > g_{\perp} > 2$, consistent with a $d_{x^2-y^2}$ ground state, is observed. Essentially the same EPR parameters are also found for plastocyanin.

Interpretation of this common EPR spectrum in terms of structure demands a careful examination of the azurin and plastocyanin copper sites. It is clear that these two proteins

share the same three strongly bound copper ligands (one Cys, two His) in an approximately trigonal-planar arrangement, with very similar bond distances and angles. As already noted, however, they differ significantly in the nature and extent of the weaker axial interactions with the copper atom. This suggests that attention should be focused primarily on the distorted trigonal-planar $CuSN_2$ moiety and that the weak axial interactions of the methionine sulfur and (in the case of azurin) peptide oxygen atoms with copper have little influence on the EPR parameters.

Early attempts to rationalize the low values of $A_\parallel$ attributed them to a distorted tetrahedral site, and some Cu(II) complexes with tetrahedral or flattened tetrahedral geometries have indeed been shown to give low $A_\parallel$ values (Gould & Ehrenberg, 1968; Forster & Weiss, 1968; Solomon et al., 1980). The mixing of 4p character into 3d levels (allowed in near-tetrahedral symmetries) has been cited as a contributor to low values of $A_\parallel$ (Bates et al., 1962; Bates, 1964; Sharnoff, 1965).

The structural arguments above suggest that analysis of the EPR spectrum in terms of tetrahedral, or near-tetrahedral, symmetry, approximating to C_{3v}, is inappropriate. A recent theoretical analysis by Penfield et al. (1985) in terms of lower symmetry C_s or C_1 models does, however, give very good agreement with observed EPR spectra. Models were based on the geometry of the copper site in plastocyanin, as determined crystallographically, and the results showed that similar EPR parameters and wave functions are obtained whether or not an axial ligand equivalent to S(Met) is included in the model. The low value of $A_\parallel$ is explained in terms of the high degree of delocalization, in this geometry, of the unpaired electron density in the copper $3d_{x^2-y^2}$ orbital on to the p_π orbital of the thiolate sulfur. (Interestingly their results also show that mixing the copper $4p_z$ orbital into the $3d_{x^2-y^2}$ orbital does not produce low $A_\parallel$ values.) The calculations also suggest a small rhombic splitting in the g values ($g_y - g_x = 0.017$), and this was in fact observed for spinach plastocyanin when the high-resolution Q-band EPR spectrum was measured (see Table II). We have not resolved any rhombic splitting in our lower resolution X-band EPR spectrum for *A. denitrificans* azurin.

Model studies have had little success in reproducing the EPR parameters of blue copper centers in low molecular weight compounds. Several studies have shown that thiolate coordination to copper(II) is not in itself sufficient to produce low $A_\parallel$ values [e.g., Bharadwaj et al. (1986)]. Most synthetic work has, however, been directed toward four-coordinate, tetrahedral or distorted tetrahedral compounds. One example is the thermally unstable complex $[Cu^{II}(SR)N_3]$ [where N_3 = hydrotris(3,5-dimethyl-1-pyrazolyl)borate and SR = *p*-nitrobenzenethiolate or *O*-ethylcysteinate) (Thompson et al., 1977, 1979). While the g parameters are similar to those of blue copper centers, the $A_\parallel$ parameter is not. It was proposed that the complex had an elongated C_{3v} symmetry, but it is now clear that this geometry alone does not give rise to a low $A_\parallel$ value. A second example involves the cluster $[Cu^{I}{}_{10}Cu^{II}{}_2L_{12}(MeCN)_4](BPh_4)_2$ (L = the anion of 1-methyl-2-mercaptoimidazole) (Agnus et al., 1980), which was reported to display a low $A_\parallel$ value [the copper(II) centers have a distorted square-pyramidal geometry with a N_3S_2 donor set]. However, on reexamination of the spectrum we find there are five unevenly spaced hyperfine lines in the $g_\parallel$ region, suggesting the initial interpretation was too simplified and either weak dipolar interactions occur between the copper(II) centers (Addison et al., 1984) or two species are present, both with low $A_\parallel$ values. Our results indicate that pertinent small models should be based on a trigonal-planar geometry with approximate C_{2v} symmetry. No such systems have yet been prepared, and coordination number three is as yet unknown in small molecule copper(II) chemistry. Five-coordinated models with long axial bonds may be useful alternatives.

Effects of pH on Electronic and EPR Spectra. Over the pH range 4–10.5, the electronic and EPR spectral properties of *A. denitrificans* azurin remain unchanged. When the pH is increased above 10.5, to 11.4, the main visible band at 619 nm moves to higher energy (to 610 nm) without a marked change in intensity, and the shoulder at 460 nm moves to 452 nm. These spectral changes are reversible. Concomitantly, the EPR spectrum is modified, with $g_\parallel$ moving to lower field and $A_\parallel$ increasing slightly (see Figure 4 and Table II). The fact that the spectral properties of azurin at high pH are only slightly modified indicates that the essential features of the copper coordination site remain intact. However, since the visible absorption bands are assigned to ligand to metal charge-transfer transitions, e.g., $S(Cys)\sigma \rightarrow Cu(d_{x^2-y^2})$ and $N(His)\pi \rightarrow Cu(d_{x^2-y^2})$, their increase in energy points to a buildup of electron density on the copper (Ainscough et al., 1981) arising perhaps from some small change in metal–ligand bond distances or angles. As the pH is increased above 12 the intense blue color of the protein starts to disappear, and an EPR spectrum typical of a copper–biuret complex (Finazzi-Agro et al., 1970; Malmström et al., 1970) is observed, indicating that the copper coordination sphere has been dramatically altered and the copper is nonspecifically bound. These results may be compared with those obtained for other blue copper proteins. For azurin from *Pseudomonas fluorescens* up to a pH of 10 only a decrease in intensity of the EPR signal was reported, with no significant changes in its shape. Above pH 10 the signal modified gradually, with the appearance of a biuret-type spectrum (Finazzi-Agro et al., 1970). On the other hand, stellacyanin, from the lacquer tree *Rhus vernicifera*, exhibits a change in its EPR spectrum above pH 9, with a new spectrum being fully developed at pH 11. This new spectrum is also typical of a blue copper center, but the modification is greater than we observe for *A. denitrificans* azurin, in that $A_\parallel$ decreases from 35×10^{-4} to less than 17×10^{-4} cm^{-1}. Paralleling this change, the main visible absorption band also shows a larger increase in energy, going from 604 to 588 nm (Peisach et al., 1967; Malmström et al., 1970). This movement of the "600-nm" absorption to shorter wavelengths at higher pH appears to be a general feature of type 1 copper proteins, having also been observed for *R. vernicifera* laccase (Malmström et al., 1970), mavicyanin (from green squash fruit) (Marchesini et al., 1970), and umecyanin (from horseradish root) (Stigbrand & Sjöholm, 1972).

As the pH of *A. denitrificans* azurin is decreased, the blue color fades, and below pH 3.5 the EPR spectrum gradually changes. At pH 2.9 a spectrum ($g_\perp = 2.072$, $g_\parallel = 2.352$, $A_\parallel = 159 \times 10^{-4}$ cm^{-1}) almost identical with that observed for copper(II) bound nonspecifically to human lactoferrin (Ainscough et al., 1980) is observed. The parameters point to oxygen-donor ligands (Peisach & Blumberg, 1974), such as protein carboxylate residues binding to Cu(II). At still lower pH, a more complex spectrum is seen, similar to that reported for *P. fluorescens* azurin at pH 1.75 (Malmström et al., 1970).

Resonance Raman Spectroscopy. The resonance Raman (RR) spectrum of *A. denitrificans* azurin obtained in frozen H_2O solution in the region from 200 to 500 cm^{-1} is shown in Figure 5. It is characterized by the presence of four strong

Table III: Resonance Raman Vibrational Frequencies for Azurins[a]

	P. aeruginosa azurin[b]		*A. faecalis* azurin[c]		*A. denitrificans* azurin[d]	
peak	freq	shift in D_2O	freq	shift in D_2O	freq	shift in D_2O
A	116 (w)				112 (w)	
B	138 (vvw, sh)					
C	165 (vvw, sh)					
D	188 (w)				184 (w)	
E	199 (vw, sh)					
F	222 (w)		225 (vw)			
G	266.1 (w)		251 (vw)		255 (w, br)[f]	0
H	286.7 (w)	−5	272 (vw)		282 (w, br)[f]	0
I	308 (vw)		307 (vw)		[g]	
J	333 (vw)				336 (vw)	−1
					350 (w)	−2
K	348.2 (w)	−2	357 (w, sh)		363 (w)	−3
L	372.6 (m)	−0.9	372 (m)	−0.7	375 (s)	−0.4
M	400.5 (m, sh)		394 (m)		397.9 (s)	0
N	408.6 (s)	−1.0	412 (vs)	−0.4	411.2 (vs)	−1.0
O	427.9 (m)	−1.2	426 (s)	−1.5	429.4 (s)	−0.4
P	441 (w, sh)	−1.5			444.2 (m)	0
Q	454.6 (w)	0[e]	456 (w)	−1.3	459 (m)	−1.4
R	474 (w)	−1.0	471 (w)	−2.1		
S	492 (w)	+1.3	497 (w)			
T	569 (vvw)	−2			565 (vw)	
U	657.1 (w)				[h]	
V	678 (vw)					
W	753.2 (m)				756 (m, br)	
X	932 (vvw)				937 (vw)	
Y	975 (vvw)					

[a] Frequencies and isotope shifts in cm^{-1}; qualitative intensity descriptions abbreviated as follows: w, weak; m, medium; s, strong; br, broad; sh, shoulder; v, very. Negative numbers indicate that the frequencies in D_2O solution are lower than in H_2O solution of the respective azurin. [b] Frequencies (12 K) and isotope shifts (25 K) from Blair et al. (1985). [c] Frequencies and isotope shifts (300 K) from Blair et al. (1985). [d] This work. [e] A shift of −0.6 cm^{-1} is reported for data obtained at 300 K. [f] In some spectra, an indication of resolution into two components at ~252 and 258 cm^{-1} and 280 and 285 cm^{-1}, respectively, has been observed. [g] Frozen solutions exhibit an ice mode at 308 cm^{-1}, which shifts 11 cm^{-1} in D_2O. [h] We have observed a band at 656 cm^{-1} in our azurin spectra but also note the presence of an acetate band at this frequency.

and well-resolved bands, at 375, 398, 411, and 429 cm^{-1}, flanked by many weaker features whose frequencies are listed in Table III. Broad features at ~255 and 282 cm^{-1} appear to be made up of two or more components, resolution being apparent in some of the RR spectra. The peak at 234 cm^{-1} is a strong plasma emission that is incompletely removed by the Pellin–Broca prism monochromator, but otherwise useful as a frequency marker. The peak at 228 cm^{-1} and a broad feature at 308 cm^{-1} are due to ice from the frozen sample; all other spectral features arise from the blue copper protein site.

The intensities of the five principal RR peaks in the *A. denitrificans* azurin spectrum were studied as a function of excitation wavelength. All of these vibrational modes show similar resonance enhancement profiles that are plotted in Figure 3 under the electronic absorption band. Although the exact shape of these profiles could not be established from the limited number of excitation wavelengths, the data indicate that the resonance enhancement of the entire RR spectrum faithfully tracks the $S(Cys)\sigma \rightarrow Cu(d_{x^2-y^2})$ transition. The congruence of all of the scattering profiles with the absorption band suggests that each of these resonant vibrations has a Cu–S stretching component in its normal mode. The very strong enhancement of this blue copper chromophore is demonstrated by comparing the area of the 411-cm^{-1} peak to that of the 980-cm^{-1} peak of sulfate ion and normalizing each to a molar concentration. A resultant scattering factor of ~2000 is calculated for spectra obtained with 647.1-nm excitation; this number would be significantly elevated by excitation at λ_{max}. For comparison, the strongly enhanced tyrosine peak at 1280 cm^{-1} in purple acid phosphatase has a molar scattering factor of ~1000 relative to sulfate when excited near the λ_{max} for the Tyr → Fe(III) LMCT transition (Averill et al., 1987).

To establish the influence of deuterium-sensitive vibrational modes of the azurin, the RR spectrum of a sample dissolved in D_2O was compared with that of a sample in H_2O. These data are shown in Figure 6. Frequency shifts for the six peaks in the 375–459-cm^{-1} range were quantitated both by curve resolution using 30% Gaussian–70% Lorentzian product functions with peak widths of 8.5 cm^{-1} and by difference spectral analysis techniques (Kiefer, 1977; Laane & Kiefer, 1980). Both methods yielded similar results, with a precision of 0.2 cm^{-1}. Isotope shifts for the peaks at 336–363 cm^{-1} were discerned by visual analysis using a graphics cursor. Of the 11 spectral features between 255 and 459 cm^{-1} in *A. deni-*

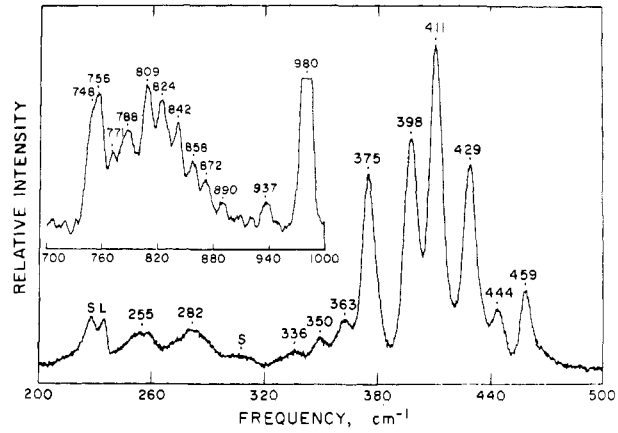

FIGURE 5: Resonance Raman spectrum of *A. denitrificans* azurin at 90 K. Concentration was ~2 mM in 0.05 M phosphate buffer, pH 6.5. Instrumental conditions: 647.1-nm excitation (160 mW at the sample Dewar); resolution, 4 cm^{-1}; scan rate, 0.5 cm^{-1}/s; 25 accumulations; no smoothing applied. The bands marked "S" and "L" are from solvent (ice) and plasma emission, respectively. The inset shows the higher frequency region of a similar sample in 0.05 M acetate buffer excited with 568.2-nm radiation; other conditions are also similar except for a 25-point smoothing of the spectral data. The peak at 980 cm^{-1} is $\nu_1(SO_4^{2-})$ added as an internal standard.

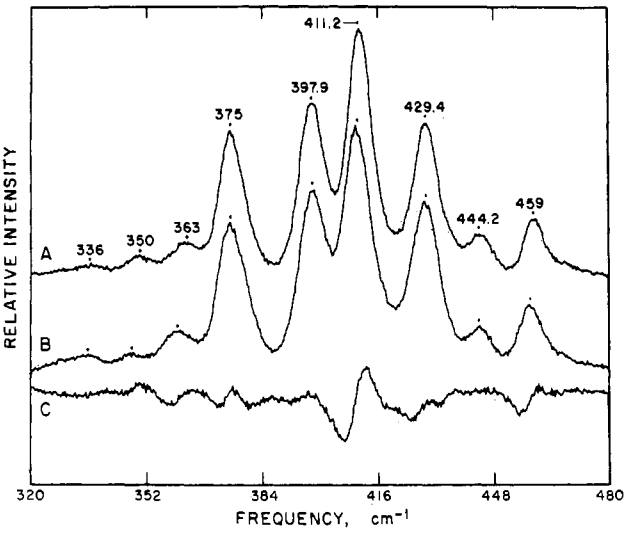

FIGURE 6: Resonance Raman spectra of *A. denitrificans* azurin at 90 K in H_2O (trace A) and D_2O (trace B). Sample and instrumental conditions are as in Figure 5. The data in trace A are identical with those shown in Figure 5 except for abscissa expansion. The difference spectrum (A − B) is shown in trace C.

Table IV: Overtone and Combination Bands (in cm^{-1}) in Resonance Raman Spectra of *A. denitrificans* Azurin

fundamentals	overtones and combinations		
	assignment	predicted	observed
L (375)	2L	750	748
M (398)	L + M	773	771
N (411)	L + N	786	788 (br)
O (429)	2M	796	
P (444)	L + O	804	
Q (459)	M + N	809	809
	L + P	819	
	2N	822	
	M + O	827	824
	L + Q	834	
	N + O	841	842
	M + P	842	
	N + P	855	
	M + Q	857	858
	2O	859	
	N + Q	870	872
	O + P	874	
	2P	888	890
	O + Q	888	
	P + Q	903	
	2Q	918	

trificans azurin, seven exhibit a deuterium isotope effect (Table III).

The present results may be compared with the studies reported for a variety of azurins and other blue copper proteins (Blair et al., 1985; Nestor et al., 1984). Table III illustrates the many similarities in peak positions and deuterium isotope shifts among the azurins from *P. aeruginosa*, *A. faecalis*, and *A. denitrificans*. For example, in each case there are four strong bands, L, M, N and O, three of which (with the exception of M) exhibit a 0.4–1.5-cm^{-1} downshift in frequency in D_2O solution. The presence of three further bands, at 336, 350, and 363 cm^{-1}, in the *A. denitrificans* azurin RR spectrum, each showing a deuterium isotope effect, appears to be an unusual feature, since other bacterial azurins show only one to two bands in this region, peaks J and/or K (Blair et al., 1985). However, the blue copper site of *R. vernicifera* laccase also has three bands here, at nearly identical frequencies (Nestor et al., 1984; Blair et al., 1985). Another comparison of interest is with the RR spectra of the azurins of *Bordetella bronchiseptica* and *Bordetella pertussis* (Blair et al., 1985). Although no deuterium isotope effects have been reported for the latter two, their spectra appear to be highly similar to that of *A. denitrificans* azurin, in both peak positions and intensities. Finally, a notable difference between the *A. denitrificans* azurin spectrum and those of other azurins is the absence of resolved peaks between 460 and 500 cm^{-1} (i.e., peaks R and S).

A characteristic signature of the RR spectra of blue copper proteins is an abundance of overtone and combination bands arising from the high-intensity fundamentals in the 350–450-cm^{-1} region. The azurin spectrum in this study is no exception and is shown as an inset to Figure 5. A strong feature at 750 cm^{-1}, generally assigned to C–S stretching modes of ligated cysteinates, is here observed at 756 cm^{-1}. The large number of features resolved in this region in this work are given in Table IV, with proposed assignments, where it was assumed that any bands within 10 cm^{-1} of each other would not be resolved.

Detailed assignment of the unusually rich RR spectra of blue copper proteins has been of interest to many laboratories. Various experimental and theoretical approaches have been brought to bear on this issue including comparisons with model systems (Miskowski et al., 1975; Siiman et al., 1976), metal ion substitution (Ferris et al., 1979), ligand atom substitution in the protein by growth of an auxotrophic bacterial strain (Thamann et al., 1982), isotopic substitution of copper and hydrogen–deuterium exchange (Nestor et al., 1984; Blair et al., 1985), spectral enhancement through signal averaging or cryogenic temperatures (Woodruff et al., 1983; Maret et al., 1983; Nestor et al., 1984; Blair et al., 1985), and normal coordinate calculations (Thamann et al., 1982; Nestor et al., 1984). As previously pointed out (Thamann et al., 1982), the RR spectra are best explained as coupled vibrations of the fundamental Cu–S stretching modes with those of other ligands. Subsequent studies have expanded on the nature of such coupling interactions and have emphasized the importance of the internal modes of the Cys and His ligands (Nestor et al., 1984; Blair et al., 1985). This coupling is evident from the large number of vibrational frequencies exhibiting small (<2 cm^{-1}) deuterium isotope shifts (especially among the strong, high-frequency fundamentals). Deuterium substitution, however, has no significant effect on the intensities of any of the RR bands.

For *A. denitrificans* azurin, we observe at least seven D-sensitive modes, consistent with similar numbers and magnitudes of shifts in other blue copper proteins. These shifts have been attributed to proton exchanges at the C2 and N3 positions of imidazole (Nestor et al., 1984), in which case the large number could be due to the two imidazole ligands making separate contributions to the coupled modes involving the vibrations of the single cysteinate ligand. This contrasts with the situation for Cu(II)-substituted liver alcohol dehydrogenase, which has blue copper optical and EPR properties and a qualitatively similar RR spectrum (Maret et al., 1983, 1986). However, this protein has only one histidine ligand, and yet in the coenzyme-bound form, which is the closest spectroscopically to the blue copper proteins, six of the eight peaks in the 320–450-cm^{-1} region have significantly altered frequencies and/or intensities following deuteration. These findings imply that other factors besides imidazole coupling must be contributing to the unusual behavior of these proteins in D_2O.

Another possible source of deuterium isotope effects is from the NH···S hydrogen bond linking the cysteinate sulfur ligand in *A. denitrificans* azurin to an amide of the polypeptide

backbone (residue 47, see above). A similar hydrogen bond apparently occurs in *P. aeruginosa* azurin (Adman et al., 1980) and certainly does in plastocyanin (Guss & Freeman, 1983). Furthermore, in the native zinc form of liver alcohol dehydrogenase one of the active site metal ligands (Cys-46) is also hydrogen bonded to a main chain NH (48) (H. Eklund, personal communication). Deuterium substitution into a hydrogen-bonded M–L system can lead to either decreases or increases in ν(M–L), depending on whether the particular hydrogen bond is stronger or weaker with deuterium. Such changes in frequency have been well documented for deuterium isotope effects in the hydrogen-bonded active site of the binuclear iron protein hemerythrin (Shiemke et al., 1986). Hence, a hydrogen-bonding proposal for the deuterium sensitivity of the blue copper protein Raman spectra seems quite plausible.

The most marked deuterium effect in other blue copper proteins is generally seen in a small spectral feature in the 250–290-cm^{-1} range. Blair and co-workers (Blair et al., 1985) reported a −5-cm^{-1} shift of band H at 286.7 cm^{-1} in *P. aeruginosa* azurin, and Nestor et al. (1984) observed a −2-cm^{-1} shift at 273 cm^{-1} for stellacyanin. In Cu(II)-substituted liver alcohol dehydrogenase, a similar 2-cm^{-1} decrease for a peak at 254 cm^{-1} occurs on changing the solvent from H_2O to D_2O (Maret et al., 1986). These observations have led to the logical assignment of bands in this region as Cu–N(Im) stretches. In *A. denitrificans* azurin, however, neither of the two bands at 255 and 282 cm^{-1} exhibits a deuterium isotope effect. Similarly, in laccase the 265-cm^{-1} band also fails to shift in D_2O (Nestor et al., 1984). Thus in these two proteins, and perhaps the others, assignment as a Cu–S–C_β deformation of copper–cysteinate may be more appropriate [as is borne out by frequency calculations (Thamann et al., 1982; Nestor et al., 1984)].

In spite of the large amount of spectral data now available, it is still difficult to relate resonance Raman effects to the three-dimensional structure of the proteins with confidence. Our results, and those of Blair et al. (1985), show that the RR spectra of the blue copper proteins are very similar, implying a very similar structure for the copper chromophore, yet subtle differences exist, in peak positions and intensities, even among different azurins. In this respect, a close comparison of the *A. denitrificans* and *P. aeruginosa* azurin structures, when the latter has been fully refined, may be of considerable value. Since most of the bands are influenced strongly by the copper–cysteinate interaction, its geometry may be of most importance. The latter appears to be so similar in *A. denitrificans* azurin and poplar plastocyanin, however [Cu–S distance 2.12 and 2.13 Å, Cu–S–C_β angle 106° and 110°, dihedral angles Cu–S–C_β (S–C_β–C_α) 169° and 170°, with errors of the order of 0.05 Å and 3.0° respectively for distances and angles) that differences may be too small to be detected crystallographically, unless perhaps a number of structures are compared. (Note also that the S–C_β–C_α angle was in each case restrained close to a standard value during refinement.) The one feature that can be compared with confidence is the extent of axial interactions with the copper. Woodruff et al. (1984) have suggested that differences between the azurin and plastocyanin RR spectra may be attributable to a weaker apical Cu–S(Met) interaction in plastocyanin, whereas the crystal structures now show that the reverse is the case [with the Cu–S(Met) distance 0.2 Å shorter in plastocyanin]. The apparently more constrained Cu site in azurin, as deduced from RR spectra, could instead be due to weak interaction with the carbonyl oxygen of Gly-45 on the other side of the trigonal plane. Suggestions

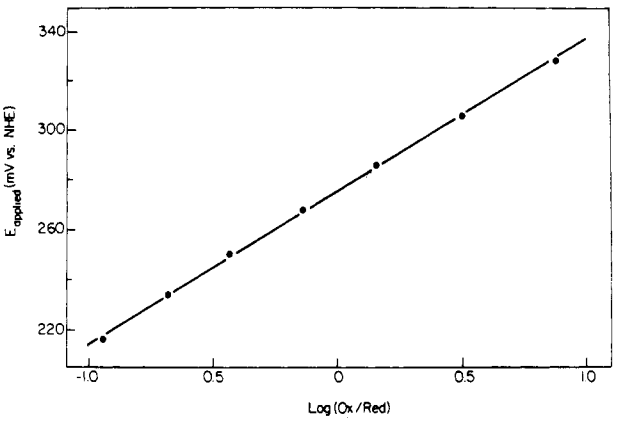

FIGURE 7: Nernst plot (E_{app} vs. log $[Az^{ox}]/[Az^{red}]$) for *A. denitrificans* azurin at 25 °C, in 0.1 M ionic strength phosphate buffer, pH 7.0. Measurements were made in an OTTLE cell with $[Ru(NH_3)_5py]^{3+}$ as redox mediator. The value of $E°'$ is 276 mV.

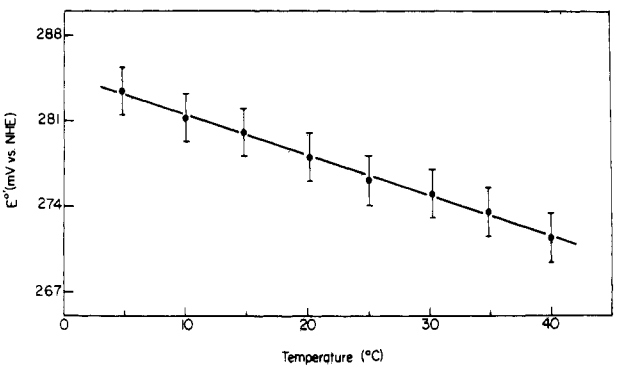

FIGURE 8: Plot of $E°'$ as a function of temperature. The line is fitted by least squares, and experimental conditions are as for Figure 7.

Table V: $E°'$ Values for *A. denitrificans* Azurin

temp (°C)	$E°$ (mV)[a]	slope[b]	r^2[c]
4.8	283.4	57.7	0.999
10.0	281.2	58.8	1.000
14.8	280.0	61.8	0.999
20.2	278.0	61.6	0.999
25.0	276.2	62.2	1.000
30.2	275.0	65.4	0.999
34.8	273.5	64.8	1.000
40.0	271.4	68.0	1.000

[a] Relative to NHE. [b] Experimental Nernst slope, $2.303RT/F$, at temperature T. [c] Linear correlation coefficient.

(Blair et al., 1985) that the temperature dependence and species variation of azurin RR spectra may arise from differences in the apical interactions can be tested when the crystal structure of another azurin, such as *P. aeruginosa* azurin, is fully refined.

Redox Potential. Values for the redox potential $E°$ were determined by plotting the applied potential E_{app} against the logarithm of the ratio of concentration of oxidized azurin to reduced azurin, following the Nernst equation:

$$E_{app} = E° + \frac{2.303RT}{nF} \log \frac{[Az^{ox}]}{[Az^{red}]}$$

At least seven points were included in each plot and lines fitted by linear least squares. A typical plot, that at 25 °C, is shown in Figure 7. This yields a value of $E°$ = 276 mV at 25 °C and pH 7.0, with an estimated error of less than 2 mV.

The temperature dependence of the redox potential was also investigated, by measuring $E°'$, in the same way, at eight different temperatures between 4.8 and 40.0 °C. These values

Table VI: Thermodynamic Parameters[a] for Azurins and Plastocyanin

protein	$\Delta H^{\circ\prime}$ (kJ mol^{-1})	$\Delta S^{\circ\prime}$ (J K^{-1} mol^{-1})	$\Delta S^{\circ\prime}_{rc}$ (J K^{-1} mol^{-1})	ref
azurin (*A. denitrificans*)	−55.6	−97.0	−31.8	this work
azurin (*P. aeruginosa*)	−69.4	−132.5	−67.3	Taniguchi et al., 1980
azurin (*A. faecalis*)	−62.7	−123.7	−58.5	W. R. Ellis, H. B. Gray, and I. Pecht, unpublished results
plastocyanin (*Phaseolus vulgaris*)	−57.3	−75.2	−10.0	Taniguchi et al., 1980

[a] All values obtained at 25 °C and pH 7.0.

are plotted in Figure 8 and listed in Table V. The values of the thermodynamic quantities[2] $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ for the complete cell reaction [i.e., referenced to the standard hydrogen electrode (NHE)] were calculated from the slope of this plot. The value for the "reaction entropy" $\Delta S^{\circ\prime}_{rc}$ was also calculated, as in Taniguchi et al. (1982). The values obtained, $\Delta H^{\circ\prime}$ = −55.6 kJ mol^{-1}, $\Delta S^{\circ\prime}$ = −97.0 J K^{-1} mol^{-1}, and $\Delta S^{\circ\prime}_{rc}$ = −31.8 J K^{-1} mol^{-1}, are compared with those obtained for other azurins and plastocyanin in Table VI. The large negative entropy change accompanying the reduction of azurin has been attributed to an increase in the ordering of water molecules in the vicinity of copper (Sailasuta et al., 1979). We note, however, that there are no internal water molecules in the azurin structure, and a more likely explanation might be an increased interaction of the Cu(I) atom with the thioether sulfur of Met-121, leading to a tightening up of the copper site structure (though this could conceivably also affect water molecules bound to the molecular exterior, more distant from the copper atom). The lower entropy for plastocyanin, where the interaction with the axial Met ligand is already stronger in the oxidized form, is consistent with this explanation. It is likely, however, that subtle variations in the packing of groups around the copper and its ligands also play a part, and this could explain the differences between different azurins. The large negative $\Delta H^{\circ\prime}$ values imply substantially stronger bonding in the reduced form of the proteins, and much of this, too, may derive from increased Cu–S(Met) interaction.

The redox potential of *A. denitrificans* azurin makes an interesting comparison with other single-copper blue proteins (Table VII). The azurins as a group have $E^{\circ\prime}$ values in the range 230–308 mV, with *A. denitrificans* azurin in the middle of the range. The azurin with the highest redox potential, that from *P. aeruginosa*, may be somewhat atypical in that it has been shown to undergo a pH-dependent conformational change (Rosen & Pecht, 1976; Wherland & Pecht, 1978; Silvestrini et al., 1981), which NMR studies suggest may be linked to the deprotonation of a histidine residue, His-35, with pK ~ 7 (Ugurbil & Bersohn, 1977; Ugurbil et al., 1977; Hill & Smith, 1979; Farver & Pecht, 1981). Adman et al. (1982) have further suggested that this may affect the Cu–S(Met) interaction, and this could in turn affect E°. The azurin from *A. faecalis*, however, does not show the same conformational change (Rosen et al., 1981), and sequence similarities suggest *A. denitrificans* azurin may not either (Adman, 1985). The $E^{\circ\prime}$ values of these two are very similar (266 and 276 mV), and they may be inferred to have very similar copper sites.

Comparison of *A. denitrificans* azurin with plastocyanin offers a valuable insight into the relationship between structure and redox potential. Although the redox potentials of both plastocyanins and azurins have been shown to vary with pH (Katoh et al., 1962; Pettigrew et al., 1983; W. R. Ellis, unpublished work), present data indicate that the E° values of azurins are in all cases lower than those of plastocyanins. At pH 7 the redox potential of *A. denitrificans* azurin is ~100 mV lower than that of plastocyanin (Table VII). At the same time the crystal structures show that in *A. denitrificans* azurin the axial Cu–S(Met) bond is 0.2 Å longer than in poplar plastocyanin, and there is a carbonyl oxygen 3.1 Å from the copper, compared with 3.8 Å in plastocyanin. Both these changes should produce a more Cu(II)-like copper site and a lower E° value (as observed). Thus, although possible influence by more remote groups cannot be excluded, the present structural and electrochemical results give firm experimental support for the view that it is variation in these axial groups that tunes the redox potential (Gray & Malmström, 1983), while the interaction with the copper atom with its three strongly bound ligands (one Cys and two His) remains essentially constant. Further support for this is given by measurements on a *P. aeruginosa* azurin in which selenomethionine is substituted for methionine (Frank et al., 1985). The redox potential of this selenoprotein is higher than that of the native protein (333 mV compared with 308 mV) as would be expected when the axial S(Met) ligand is substituted by the softer [Cu(I)-stabilizing] Se(Met). Moreover, in a series of pseudotetrahedral complexes, it has been shown that the redox potential increases in the order $CuS_2N_2 > CuN_4 > CuO_2N_2$ (Yokoi & Addison, 1977).

We conclude therefore that all the azurins probably have a similar copper site, with axial approaches by Met-121 and the carbonyl oxygen of Gly-45 (consistent with their uniformly lower E° values than those of plastocyanins), and that variation in the extent of interaction of Met-121 may be the best explanation of the pH dependence of the redox potential of *P. aeruginosa* azurin, as suggested by Adman et al. (1982). Further, these conclusions have a bearing on stellacyanin, whose redox potential (184 mV) is lower still (Reinhammar, 1972) and which contains no methionine (Bergman et al., 1977). There is a wide range of evidence that stellacyanin, too, has the same three basic ligands as azurin and plastocyanin (one Cys and two His) [e.g., Lappin (1981) and references cited therein; Hill & Lee, 1979; Roberts et al., 1980; McMillin & Morris, 1981; Peisach et al., 1982], and there are suggestions (Ferris et al., 1978; McMillin & Morris, 1981) that a

Table VII: Redox Potentials for Some Blue Copper Proteins

protein	E° (mV)	pH	ref
azurin (*A. denitrificans*)	276	7.0	this work
azurin (*P. aeruginosa*)	308	7.0	Taniguchi et al., 1980
azurin (*A. faecalis*)	266	7.0	Rosen et al., 1981
azurin (*A.* sp.)	230	6.8	Suzuki & Iwasaki, 1962
azurin (*Paracoccus denitrificans*)	230	7.0	Martinkus et al., 1980
azurin (*Achromobacter cycloclastes*)	245	7.0	Iwasaki & Matsubara, 1973
plastocyanin (spinach)	370	7.0	Katoh et al., 1962
plastocyanin (bean)	360	7.0	Taniguchi et al., 1980
stellacyanin (*R. vernicifera*)	184	7.1	Reinhammar, 1972
mavicyanin (zucchini)	285	7.0	Marchesini et al., 1979
umecyanin (horseradish)	283	7.0	Stigbrand, 1972
plantacyanin (cucumber seedlings)	317	7.0	Murata et al., 1982

[2] $E^{\circ\prime}$, $H^{\circ\prime}$, and $S^{\circ\prime}$ refer to values at pH 7.0.

disulfide sulfur may substitute for methionine. It is a logical extension of the azurin and plastocyanin structures and $E°$ values to suggest that stellacyanin, like azurin, may have a peptide carbonyl oxygen making a relatively close axial approach to copper and the lower $E°$ value of stellacyanin may arise from slightly stronger copper–oxygen interaction, and slightly weaker interaction of copper with the disulfide (if present) in the other axial position. This ultimately should be tested by crystallographic analysis, but we note that (i) NMR studies on Cd(II)-substituted proteins indicate that the azurin and stellacyanin spectra are very similar, but different from plastocyanin, in a way that suggests a higher coordination number in the former two (Engeseth et al., 1984) and (ii) the RR spectrum of stellacyanin contains a prominent feature at 1233 cm^{-1}, which suggests resonance enhancement of a peptide mode, consistent with backbone peptide carbonyl coordination (Blair et al., 1985).

Conclusions

The spectrochemical results reported here fit well into the patterns observed for other single-copper blue copper proteins. Certain features remain essentially constant, such as the intense (Cys)S → Cu charge-transfer transition at 600–625 nm and the ENDOR and EPR spectral parameters (although stellacyanin is rather different with respect to the latter), and most other spectral features are broadly similar. These similarities can probably be attributed to an essentially similar arrangement of the copper atom with three strongly bound, trigonally disposed ligands (one Cys, two His), including an unusually short Cu–S(thiolate) bond, in all the proteins. At the same time there are differences in detail, as in the resonance Raman spectra, and $E°$ values, which are probably derived from differences in the weaker axial interactions, viz., a weak Cu–S(Met) interaction in plastocyanin, even weaker Cu–S(Met) and Cu–O interactions in azurin, and (speculatively) Cu–S(disulfide) and Cu–O interactions in stellacyanin.

These effects are clearly seen in comparing azurin and plastocyanin, whose electronic absorption, EPR, and ENDOR parameters are very similar but where in going from a weak axial Cu–S(Met) interaction in plastocyanin to weaker axial Cu–S(Met) plus Cu–O interactions in azurin the $E°$ value is markedly lowered and details of the RR spectra are changed (as are the ^{113}Cd NMR spectra of the Cd-substituted proteins). Some of the detailed spectral differences within a family such as the azurins probably also derive from subtle differences in these weak axial interactions, caused ultimately by amino acid sequence differences. Further refined crystal structures, complementary to the many spectroscopic approaches, will be necessary to clarify these variations.

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Picosecond Resolution of Tyrosine Fluorescence and Anisotropy Decays by 2-GHz Frequency-Domain Fluorometry†

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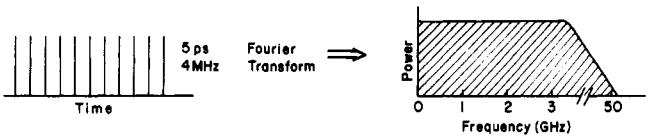
ABSTRACT: We extended the technique of frequency-domain fluorometry to an upper frequency limit of 2000 MHz. This was accomplished by using the harmonic content of a laser pulse train (3.76 MHz, 5 ps) from a synchronously pumped and cavity-dumped dye laser. We used a microchannel plate photomultiplier as the detector to obtain the 2-GHz bandwidth. This new instrument was used to examine tyrosine intensity and anisotropy decays from peptides and proteins. These initial data sets demonstrate that triply exponential tyrosine intensity decays are easily recoverable, even if the mean decay time is less than 1 ns. Importantly, the extended frequency range provides good resolution of rapid and/or multiexponential tyrosine anisotropy decays. Correlation times as short as 15 ps have been recovered for indole, with an uncertainty of ±3 ps. We recovered a doubly exponential anisotropy decay of oxytoxin (29 and 454 ps), which probably reflects torsional motions of the phenol ring and overall rotational diffusion, respectively. Also, a 40-ps component was found in the anisotropy decay of bovine pancreatic trypsin inhibitor, which may be due to rapid torsional motions of the tyrosine residues and/or energy transfer among these residues. The rapid component has an amplitude of 0.05, which is about 16% of the total anisotropy. The availability of 2-GHz frequency-domain data extends the measurable time scale for fluorescence to overlap with that of molecular dynamics calculations.

There is considerable interest in obtaining picosecond time resolution of the intrinsic fluorescence from proteins. The time-resolved intensity decay parameters may be correlated with structural features of the proteins. The anisotropy decay times may be compared with calculations of segmental motions or with hydrodynamic theories for global rotational diffusion. There are two alternative methods to obtaining the time-dependent data. These are direct measurements in the time domain (O'Connor & Phillips, 1985; Cundall & Dale, 1980; Brand et al., 1985) or measurements in the frequency domain (Lakowicz et al., 1984a,b; Lakowicz, 1985, 1986a,b). The time-domain measurements are dominant because of the availability of instrumentation, the increasing availability of picosecond pulsed laser sources, and the intuitive advantages of seeing the time-resolved data. Surprisingly, these instruments have yielded rather few direct measurements of the decays of protein fluorescence or anisotropy (Lee et al., 1985; Ludescher et al., 1985). We know of no tyrosine anisotropy decays obtained with a picosecond laser source, but intensity decays of tyrosine have been reported for histone H1 (Libertini & Small, 1985), and the intensity decays of a variety of phenol and tyrosine derivatives have been examined by using pulse synchrotron radiation with pulse widths near 500 ps (Laws et al., 1986). Picosecond tryptophan intensity decays have been observed for hemoglobin (Albuni et al., 1985), which illustrates the need for picosecond resolution of protein fluorescence.

Scheme I



During the past 3 years, this laboratory and others have developed instrumentation for variable-frequency phase-modulation fluorometry (Lakowicz & Maliwal, 1985; Lakowicz et al., 1986a; Gratton & Linkeman, 1983). To date, the upper frequency limit has been about 200 MHz. This limitation was the result of two factors. First, the available light modulators could only provide adequate modulation to about 250 MHz. Second, the time response of standard photomultipliers (1.5-ns full width at half-maximum) limited their bandwidth to about 220 MHz (3dB point; Wilson & Hawkes, 1983). We overcame the 200-MHz limit using two modifications of our original instrument (Lakowicz & Maliwal, 1985). First, we avoided the use of a light modulator by using the intrinsic high-frequency harmonic content of a 5-ps laser

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