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Environment of Copper in *Pseudomonas fluorescens* Azurin: Fluorometric Approach*

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ABSTRACT: The relationships between tertiary structure and copper binding in *Pseudomonas fluorescens* azurin have been studied by fluorescence, absorption, and electron paramagnetic resonance spectra. The fluorescence spectrum at neutral pH shows an emission maximum at 308 nm when excited at 275 nm. Below pH 2 or in the presence of 6 M guanidine hydrochloride at neutral pH the fluorescence maximum shifts toward longer wavelengths. The addition of sodium dodecyl sulfate at low pH restores the original wavelength of emission. The loss of the blue color at low pH is not due to the reduction of copper, whereas in the presence of guanidine hydrochloride it is concomitant with copper reduction. Above pH 9 copper is reduced without any apparent change in protein structure. The recombination of apoprotein with copper causes a quenching of protein fluorescence, without any increase of absorbance at the wavelengths of the emission spectrum of the protein. Mercuric ion resembles copper in its quenching effect. *p*-Mercuribenzoate reacts only with the copper-free protein but does not influence the fluorescence.

The results suggest the interaction of copper with tryptophan and sulfhydryl in a strongly hydrophobic site. Copper is reduced by SH when the site becomes exposed to the solvent (*i.e.*, in guanidine hydrochloride at neutral pH) or when the pH is sufficiently high to favor the oxidation of the sulfhydryl in the native site.

The nature of the amino acid residues which form the copper binding site in copper proteins has been only tentatively established in the proteins studied so far, on the basis of mostly indirect evidence or of a questionable use of protein reagents, since more direct and reliable methods did little to solve the question. Electron paramagnetic resonance spectroscopy—in the absence of ligand hyperfine structure, as in native copper proteins—can give only suggestions and working hypotheses; X-ray crystallography is in its initial stages in this field, because of the difficulties involved in crystallizing copper proteins. A great deal of the theory on the copper binding sites in proteins rests on information derived from spectroscopy (optical spectroscopy, optical rotatory dispersion, and electron paramagnetic resonance); this, however, mostly concerns the symmetry of the sites

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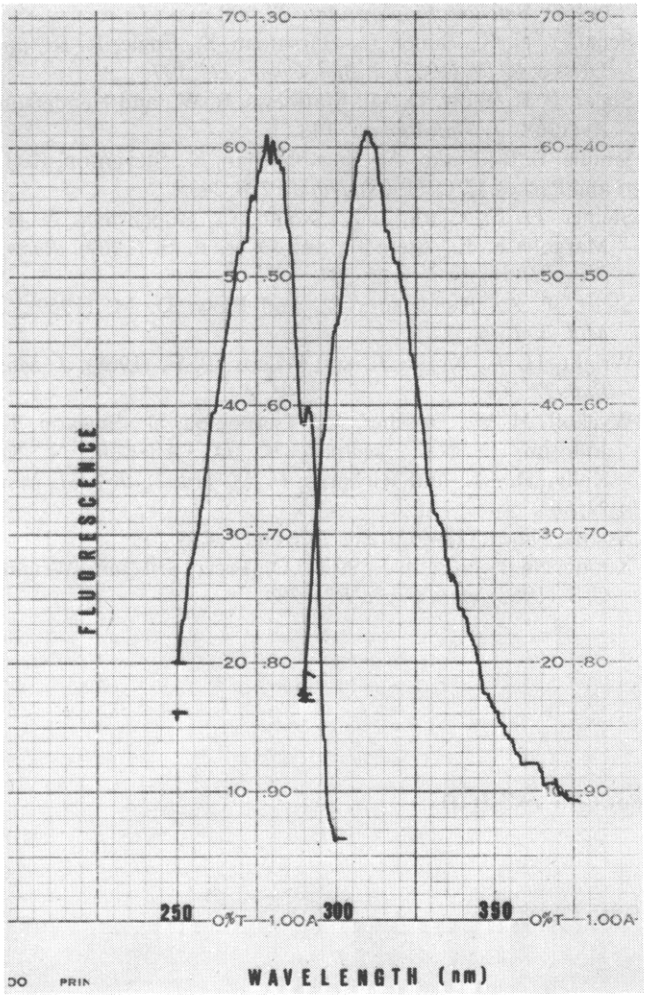

FIGURE 1: Excitation and emission spectra of azurin. Excitation maximum 280 nm. Emission maximum 308 nm.

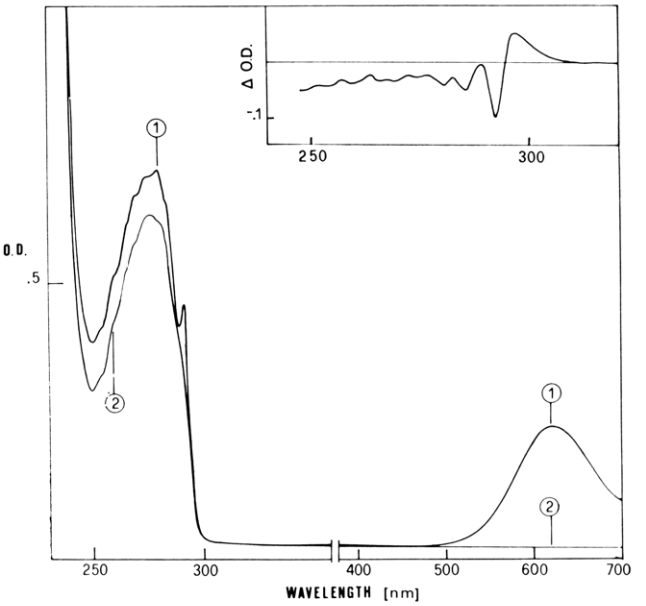

FIGURE 2: Effect of low pH on absorption spectrum of azurin. Azurin, 6.5×10^{-5} M: pH 6.5 (1) and pH 1.5 (2). Insert: ultraviolet difference spectrum of azurin at pH 1.5 against azurin at pH 6.5. Concentration of the protein: 5×10^{-5} M.

rather than the individual ligands, even though the energy of optical transitions could be related to the nature of the ligand atoms (Brill *et al.*, 1964).

The object of this investigation is the *Pseudomonas fluorescens* azurin. Azurins are a well-known class of bacterial proteins (Sutherland and Wilkinson, 1963). Common features of these proteins are the low molecular weight (15,000), the presence of only one copper atom per molecule and the high optical absorption around 600 nm. This last property, together with the singular electron paramagnetic resonance parameters, is common to other copper proteins (*i.e.*, laccase, ceruloplasmine, plastocyanine, etc.) (Gould and Ehrenberg, 1968).

The relatively simple structure and the well-defined spectral properties make this protein fairly suitable for an investigation of the nature of its copper binding site. In this paper we shall describe the relationships between changes in environment of aromatic chromophores, as monitored by fluorescence, and changes in environment of copper as indicated by optical and electron paramagnetic resonance spectra. The results obtained have lead to a tentative identification of some amino acid residues involved in the copper binding site.

Materials and Methods

All chemicals used were of reagent grade. Gd·HCl[1] was obtained from Mann Research Laboratory, New York. Azurin from *Pseudomonas fluorescens* was purified according to Ambler (1963) except that bacterial walls were disrupted by sonication and that the DNase and rivanol treatments were omitted. The purity of the protein was tested by starch gel electrophoresis according to Poulik (1957). The copper-free azurin was prepared according to Yamanaka *et al.* (1963).

Optical spectra were carried out with a Beckman DK2A ratio recording spectrophotometer. Fluorescence spectra were recorded with a G. K. Turner Model 210 spectrofluorometer; all experiments were performed at 20°, using excitation band widths of 25 or 100 Å. Quantum yields were calculated from

$$\varphi_u = \varphi_s \frac{A_u F_s \lambda_{ex_s} E_s C_s}{A_s F_u \lambda_{ex_u} E_u C_u}$$

where the subscript "u" indicates the unknown and the subscript "s" indicates the standard of known quantum yield. φ is the quantum yield. F is the amplification factor used. A is the area below the emission peak, after subtracting the blank. λ_{ex} is the excitation wavelength. E is the extinction coefficient. C is the concentration of the fluorescent species.

Tryptophan determination was performed by the method of Edelhoch (1967). Determination of SH groups was carried out according to Boyer (1954). The copper content of samples was measured by atomic absorption spectroscopy using a Hilger and Watts Atomspek Model H1170.

[1] Abbreviations used are: Gd·HCl, guanidine hydrochloride; PMB, *p*-mercuribenzoate.

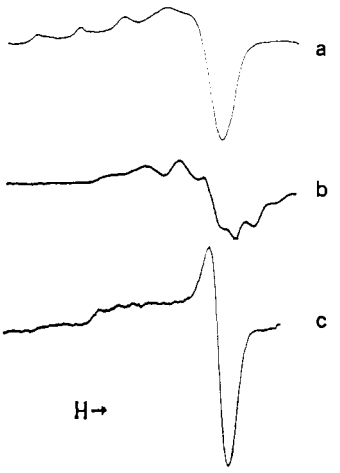

FIGURE 3: Electron paramagnetic resonance spectra of azurin. (a) 10^{-3} M $CuCl_2$ at pH 1.75. (b) 10^{-3} M azurin at pH 1.75. (c) The same sample as in part b, brought to pH 6.5.

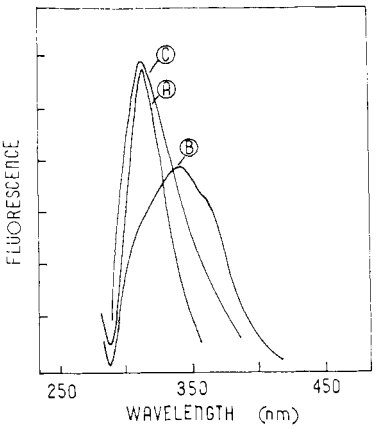

FIGURE 4: Effect of low pH and sodium dodecyl sulfate on fluorescence of azurin. Fluorescence spectrum of azurin (4.5×10^{-5} M) pH 6.5 (A); the same at pH 1.5 (B) and at pH 1.5 with 2% sodium dodecyl sulfate (C).

Electron paramagnetic resonance experiments were carried out at 133°K with a Varian V 4502.14 spectrometer, equipped with a 100-kHz field modulation unit and with the Varian variable-temperature accessory. Microwave frequency was 9.16 GHz and microwave power was 6.4 mW. The field modulation amplitudes were of the order of 5 gauss. pH measurements were made at room temperature in a pH 4 Radiometer equipped with a G-200B glass electrode.

Results

Fluorescence Spectra of Azurin at Neutral pH. The fluorescence spectra of azurin and apoazurin at neutral pH showed excitation maxima at 280 nm, and emission maxima at 308 nm (Figure 1). The only difference when the copper was removed appeared to be a three times higher quantum yield (*i.e.*, 0.1 in the case of apoprotein). When excited at either 275 or 292 nm the emission spectrum had the same shape. At all wavelengths the ratio of emission intensities was equal to the ratio of the optical densities at the two wavelengths of excitation. The reduction of the copper by dithionite did not change the fluorescence spectra. The very low wavelength of emission shown by this protein seemed to be in contrast with the presence of tryptophan in the protein claimed by Ambler (Ambler and Brown, 1964, 1967). However determinations with the Edelhoch method gave an average of 0.91 residue/mole of protein. Furthermore the fluorescence maximum in the presence of 6 M Gd·HCl shifted to 350 nm, as usual in tryptophan-containing proteins.

Effects of Low pH. When the pH of the medium was lowered below 2 the ultraviolet spectrum fine structure and the 292-nm peak of azurin disappeared (Figure 2). Moreover at this pH the protein loses its blue color.

Electron paramagnetic resonance spectra carried out at acid pH (Figure 3b) showed that the copper remains paramagnetic, although in an environment very different from that of the protein at neutral pH. The metal ion is however still bound to the protein, since the electron paramagnetic resonance spectrum of $CuCl_2$ at the same pH is completely different (Figure 3a). This conformational change is completely reversible (Figure 3c). Fluorescence spectra of azurin at pH 1.5 are shown in Figure 4. The emission maximum shifted to 345 nm and the quantum yield was approximately twice as much as at neutral pH. However, the addition of 2% sodium dodecyl sulfate to this sample restored the fluorescence spectrum present at neutral pH, whereas the quantum yield was not restored.

Effects of High pH. Bleaching of the blue color of azurin occurs also at pH values above 8 and is accompanied by a disappearance of the copper electron paramagnetic resonance signal (Brill *et al.*, 1968; Maria, 1966). In the case of the alkaline bleaching, however, no relevant fluorescence change was detected other than a decrease in intensity.

Figure 5 shows the changes of the electron paramagnetic resonance spectrum of azurin as pH rises. Up to about pH 10 there was only a decrease of the intensity of the signal without significant changes in the shape of the spectrum.

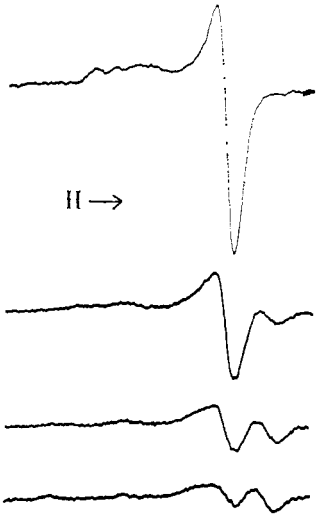

FIGURE 5: Electron paramagnetic resonance spectra of azurin 10^{-3} M. From the top: at pH 6.5; at pH 10.5; after 10 min at pH 12; after 30 min at pH 12.

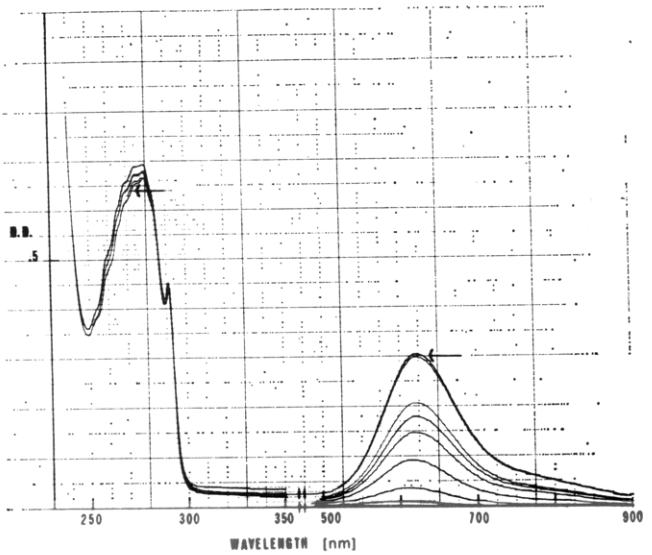

FIGURE 6: Absorption spectra of reconstituted azurin. Effect of addition of increasing amounts of $CuCl_2$ to apoazurin (6.6×10^{-5} M) in KCl 0.11 M pH 6.5. From the bottom (visible range): apoazurin; 3×10^{-6} M $CuCl_2$; 2×10^{-5} M $CuCl_2$; 3.6×10^{-5} M $CuCl_2$; 3.6×10^{-5} M $CuCl_2$ after 10 min; 3.6×10^{-5} M $CuCl_2$ after 30 min; 7×10^{-5} M $CuCl_2$; 7×10^{-5} M $CuCl_2$ after 15 min; 10^{-4} M $CuCl_2$ after 30 min. The arrows indicate the end points of titration.

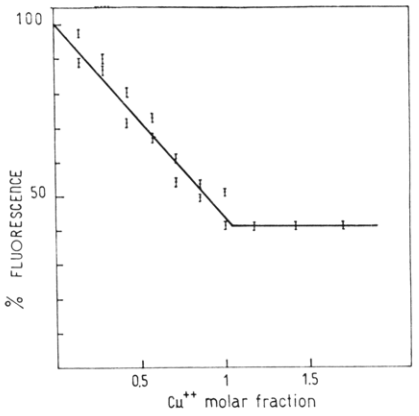

FIGURE 7: Effect of Cu^{2+} on fluorescence of apoazurin. Fluorescence intensities at 308 nm as a function of added $CuCl_2$.

Above pH 10 a gradual modification of the spectrum became evident, which may be related to a drastic change in the environment of part of the copper. When left to stand at pH 13 the copper form typical of the native protein disappeared completely and the residual spectrum was similar to that of the copper–biuret complex in alkaline solution. A further increase of pH did not change the electron paramagnetic resonance spectrum. The addition in alkali of an excess of ferricyanide restored the original electron paramagnetic resonance spectrum, but, on being left to stand, this again disappeared and only the signal of the biuret-type copper was present, indicating that the residual paramagnetic copper of the alkali-treated azurin is bound in a nonspecific way by the modified protein. If the protein is brought to pH 12 by dialysis in the presence of an equivalent concentration of external copper, the latter appears in the electron paramagnetic resonance spectrum as in a complex of biuret type.

Effects of Gd·HCl. When 6 M Gd·HCl was added to azurin the fine ultraviolet structure, the 292- and 625-nm peaks vanished. Electron paramagnetic resonance controls showed that in this condition the copper signal disappeared.

Spectrophotometric Titration of Tyrosine in Azurin and Apoazurin. The two tyrosines present in the protein showed an abnormal pK' (*i.e.*, $\simeq$12.4) when titrated spectrophotometrically. Some uncertainties were present in the upper part of the curve because the optical density measurements were performed immediately after the addition of alkali. Hysteresis phenomena were always present. A $\Delta\epsilon_{295}$ of 3.6×10^3 was observed instead of the more usual value of 2.33×10^3 (Beaven and Holiday, 1952). Such an abnormal pK' of tyrosines was obtained also in the titration of the apoprotein.

Reaction of the Apoprotein with Copper. Figure 6 shows the spectral changes obtained by addition of cupric chloride to a solution of apoprotein. The addition of an equivalent of copper to the apoprotein restored the full 625-nm absorption intensity. No change was found in the ultraviolet range. It was possible to follow the recombination of copper to the apoprotein by fluorescence quenching. When the protein was titrated with $CuCl_2$ (Figure 7), fluorescence decreased linearly until the copper added was stoichiometric with respect to the protein. Excess metal had no further effect on the quenching.

Reaction with PMB and Mercuric Ions. PMB did not react with azurin even in the presence of 6 M Gd·HCl. However the copper-free protein reacted readily with a sharp titration end point corresponding to one SH group per mole of protein. The addition of stoichiometric amounts of copper to the PMB-treated apoprotein did not restore the normal visible spectrum of the protein, whereas with a large excess of metal the 625-nm absorption band tended to reappear. The fluorescence of the apoprotein was unaffected by the addition of PMB whereas the stoichiometric addition of mercuric acetate lead to a 60–70% quenching (Figure 8). The mercuric acetate treated apoprotein did not turn blue even with an excess of copper.

Discussion

The spectroscopic data found in the literature on *P. fluorescens* azurin, together with those presented in this paper, allow a tentative picture of the relationship between the tertiary structure of the protein and the copper binding.

A well-demonstrated fact is, first of all, the presence in the interior of the protein of at least one strongly hydrophobic site. Many lines of evidence lead to this conclusion, chiefly those from absorption and fluorescence spectra.

The ultraviolet absorption spectrum of azurin shows a sharp maximum at 292 nm in addition to the peak at 280 nm which presents an evident fine structure. By general agreement a well-resolved 292-nm peak is typical of tryptophan in an apolar environment (Ambler and Brown, 1967; Wetlaufer, 1962), whereas it is still matter of controversy if it arises from the same transition as that responsible for the 280-nm peak. A very sharp fine structure on the main peak is shown by phenylalanine in nonpolar solvents (Wetlaufer, 1962). The disappearance of both these features below pH

2 (Figure 2) indicates a change from a hydrophobic environment to a more hydrophylic one.

The fluorescence maximum of azurin excited at 280 nm is at 308 nm. This wavelength is just the position of the first vibrational band (1–0) for tryptophan in nonpolar environments. However in the fluorescence spectrum of azurin (Figure 1) a well-defined 0–0 band is not evident, but there is only an asymmetry on the longer wavelength side of the peak. The presence of the 292-nm peak in the excitation spectrum is a further evidence in assigning this fluorescence to the tryptophan. So far no example has been reported of tryptophan-containing protein with the fluorescence maximum at such a low wavelength. In the case of bovine growth hormone, Edelhoch *et al.* (1966) have reported that the native protein has an emission maximum at 315 nm when excited at 280 nm. They attribute this feature to a large contribution by tyrosine. In the case of azurin we have found that the emission spectra obtained with 275- and 292-nm exciting light have the same shape and this fact would exclude, according to Weber (1961a,b), a contribution of tyrosine to tryptophan fluorescence. Even a tyrosine–tryptophan energy transfer seems to be excluded in this way. In fact the presence of such a transfer would have produced a relatively higher intensity of emission when the sample was excited at 275 nm where both tryptophan and tyrosine absorb (Longworth, 1968). Such an unusual tryptophan fluorescence is certainly related to the hydrophobic character of its surroundings. In fact conditions which alter the tertiary structure of the protein (6 M Gd·HCl, low pH) shift the fluorescence maximum to wavelengths typical of tryptophan in aqueous solution. Conversely the lowering of the dielectric constant of the environment, produced by the addition of sodium dodecyl sulfate in acidic solution (Konev, 1967) brings back the emission maximum to 310 nm (Figure 3). In this context it should be recalled that acidic pH (*i.e.*, pH 1.5) leads to the disappearance (Tang and Coleman, 1967; Tang *et al.*, 1968) of the multiple Cotton effects which are present in azurin and laccase at neutral pH (Tang and Coleman, 1967; Tang *et al.*, 1968; Bossa *et al.*, 1969) in the region of aromatic amino acids (270–300 nm). This indicates that dissymmetry and hydrophobic character are strictly associated in the tryptophan-containing part of the protein. It is worth noting that when the hydrophobic environment around the tryptophan is destroyed, the blue color of the protein also disappears. The bleaching of the blue color occurs also above pH 8, but in this case the copper becomes diamagnetic. Furthermore, the alkaline bleaching is reflected neither in tryptophan fluorescence changes, nor in modification of the fine structure in the ultraviolet spectrum.

The copper-free protein has absorption and fluorescence spectra identical with those of azurin. The binding of copper to the apoprotein leads to a quenching of the fluorescence, which can be ascribed to the "heavy metal effect:" it is well known that the interaction of a heavy atom with a π electron causes a rise in singlet–triplet mixing and in intersystem crossing (McClure, 1948). The metal can achieve this effect either by direct action on the fluorescent cromophore or by forming an "energy sink" at some distance from it. Recently Lehrer (1969) has described the fluorescence quenching produced by the binding of iron and copper on transferrin. This author ascribes it to a mechanism of long-range energy transfer, *via* Förster resonance coupling (Förster, 1959; Wilkinson, 1964). In the case of azurin, however, this mechanism cannot be involved. In fact the condition for a long-range energy transfer is the overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor. Thus one should have found a change in the absorption spectrum caused by addition of copper to the apoprotein, but in Figure 5 it is evident that the only absorption difference is in a region far removed from the emission of the tryptophan. Specificity and extent of tryptophan fluorescence quenching would lead to the conclusion that copper binds to or at least very near tryptophan. The implication of tryptophan in copper binding has already been pointed out for conalbumin (Tan and Woodworth, 1968). The possible involvement of tyrosines in copper binding cannot be ruled out; their anomalous pK' suggests that they are also in a strongly hydrophobic environment.

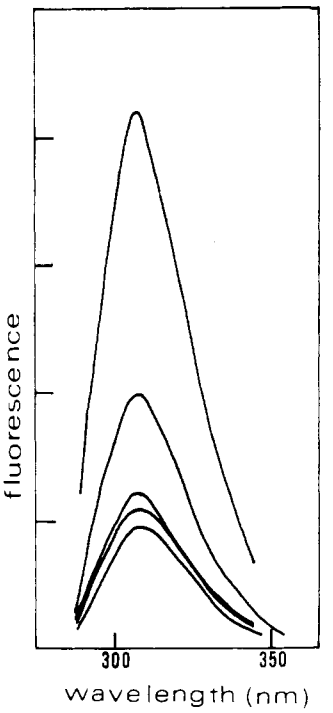

FIGURE 8: Effect of Hg^{2+} on fluorescence of apoazurin. Fluorescence spectrum of 1.26×10^{-5} M apoazurin in KCl (0.1 M, pH 6.5). From the top: (1) protein alone; (2) + 7.5×10^{-6} M; (3) + 1.05×10^{-5} M; (4) + 1.35×10^{-5} M; (5) + 1.65×10^{-5} M mercuric acetate.

There are two other possible explanations for the fluorescence quenching produced by copper binding to the apoprotein. The first one is that the metal causes a drop in efficiency of the energy transfer between tyrosines and tryptophan, but in the case of azurin this phenomenon seems to be absent. The other possibility is a conformational change concomitant to the binding of the copper; however, it seems unlikely in view of the identity of absorption and fluorescence spectra of holo- and apoproteins. Furthermore also the titration of the tyrosines gives the same result in both cases.

The sulfhydryl group appears to be another possible ligand of copper in azurin, the reaction of apoprotein with PMB and Hg^{2+} being remarkable evidence for this. Moreover

Hg^{2+} quenches the tryptophan fluorescence, whereas PMB does not; this is a reasonable result as the ion can exchange more than one bond with the protein, and thus might interact with tryptophan in a complex which should resemble very much that obtained with copper. In fact the copper seems unable to reconstitute the holoprotein in the presence of mercuric ions, whereas in the presence of PMB it can.

The disappearance of the copper signal in the presence of Gd·HCl and above pH 8, might therefore be related to the presence of a Cu–SH bond in a strongly hydrophobic environment. Reduction of the metal would occur when different conditions (exposure of the site to the solvent, high pH) produce the oxidation of the sulfur. On the other hand, the fact that copper is not reduced at low pH in spite of the exposure of that site to the solvent is in agreement with the pH dependence of the sulfhydryl oxidation.

The interaction of copper with the SH group at a specific site is further indicated by the results of the electron paramagnetic resonance experiments at alkaline pH, which show that the metal bound to the protein in a nonspecific way is never reduced. This SH group could be the pathway followed by electrons traveling from an external donor to the copper. A similar outer sphere mechanism has already been proposed for stellacyanin (Peisach *et al.*, 1967) and plastocyanin (Blumberg and Peisach, 1966).

The bleaching at low pH and the electron paramagnetic resonance spectra performed under the same conditions show a half-point at pH 1.9. It is hard to say if this can be attributed to the dissociation of a carboxyl group more or less directly involved in the metal binding.

No evidence is available as yet for the presence of nitrogen ligands in the binding site of enzyme. Not even the arguments advanced in the case of stellacyanin (Gould and Ehrenberg, 1968) can be invoked, owing to the reduction of the native copper in alkaline solution.

It can be concluded that *P. fluorescens* azurin has the only tryptophan, the copper, the SH group and the two tyrosines in strongly hydrophobic regions and that the tryptophan and the sulfydryl residue appear as candidates for copper binding. The perfect parallelism between the bleaching of the blue color (when it is not due to a valence change of copper) and the disappearance of the hydrophobic region may suggest that only in the absence of water (*viz.*, in a medium with a lower dielectric constant and dipolar moment) could copper bind in such an unusual way. This fact might explain the lack of model compounds for "blue" proteins. The generalization is justified by the large number of observations consistent with this view, made on similar proteins. In plastocyanin Blumberg and Peisach (1966) demonstrated by proton relaxation rate experiments, that copper is inaccessible to the solvent. The absorption spectra of most "blue" proteins show the characteristic ripples on the aromatic peak. Some of them have been found to have abnormal Cotton effects in the aromatic region (Tang and Coleman, 1967; Tang *et al.*, 1968; Bossa *et al.*, 1969).

The primary structure of *P. fluorescens* azurin is known (Ambler and Brown, 1964, 1967). The only SH group is that in position 112 among a sequence of phenylalanines (probably those responsible for the difference spectrum at acidic pH shown in Figure 2), and tryptophan is residue 48. If the hypothesis outlined in this paper is correct all these regions of the polypeptide chain should be grouped together with copper in the hydrophobic interior of the protein. A definitive answer to the problems posed in this paper may come from X-ray diffraction studies, and these are already in progress with the azurin of *Pseudomonas denitrificans* (Strahs, 1969).

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