Parsley Plastocyanin. The Possible Presence of Sulfhydryl and Tyrosine in the Copper Environment†

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ABSTRACT: Parsley plastocyanin has been isolated and characterized. It contains one copper atom per molecule of about 10,000 mol wt. The copper has an electron paramagnetic resonance (epr) spectrum very similar to that of other plastocyanins and simple blue proteins. The reactivity of the copper site was tested. *p*-Chloromercuribenzoate reversibly changes the epr spectrum to that of simple nitrogenous complexes of copper. Copper was reduced by cyanide and at alkaline pH values. In the latter case, before bleaching of the blue color, a new type of epr spectrum was observed, which was still typical of blue proteins, but had different parameters compared to that of plastocyanin at neutral pH. It was possible to obtain an apoprotein able to recombine about 75% of the copper in the native site. Aged preparations of apoprotein, with little or no recombination ability, showed a significant trend to dimerization. Parsley plastocyanin shows a fluorescence peak at 315 nm with excitation maximum at 275 nm. This fluorescence increases three times in the apoprotein without change in the shape. The maximum of emission is shifted reversibly to 304 nm, by lowering the pH to values below pH 2. This fluorescence is attributed to the phenolate ion in a low polarity environment.

Plastocyanins are deep blue copper proteins involved in electron transport during the photosynthetic process; they are contained in the chloroplast. Several plastocyanins have been extensively studied (Blumberg and Peisach, 1966; Katoh *et al.*, 1962; Katoh and Takamiya, 1964; Milne and Wells, 1970), but very little is known about the nature of copper ligands. Katoh and Takamiya (1964) provided evidence for the presence of a sulfhydryl as a copper ligand in spinach plastocyanin. Previous work from this laboratory demonstrated that other blue copper proteins contain a sulfhydryl, which is a possible ligand of copper, and an indole side chain of tryptophan very near to it (Finazzi Agrò *et al.*, 1970, 1973; Rotilio *et al.*, 1970; Morpurgo *et al.*, 1972), while superoxide dismutase, a light blue copper protein, has three nitrogen atoms and a water molecule as copper ligands (Rotilio *et al.*, 1971, 1972a,b; Gaber *et al.*, 1972). In view of these facts, our aim was to study many different blue proteins in order to elucidate the possible relationships between environment of the copper ion in the protein, its spectroscopic properties, and its catalytic role.

In the present paper we report the isolation and preliminary characterization of parsley plastocyanin.

Materials and Methods

All chemicals were reagent grade, and used without further purification.

The plastocyanin was purified from parsley leaves; 1 kg of washed leaves was homogenized with 900 g of crushed ice, 100 ml of 1 M Tris-HCl buffer (pH 7.6), and 1000 ml of cold acetone. The homogenate was filtered through a nylon cloth and then centrifuged for 30 min at 4000*g*. Next 1.16 volumes of cold acetone was added to the supernatant. The resulting suspension was allowed to stand at 4° until a white sediment became apparent. The precipitate was then collected by centrifuging 30 min at 4000*g*; afterwards it was dissolved in the smallest possible volume of 0.15 M Tris-HCl buffer (pH 7.6) and dialyzed overnight against the same buffer. After removing the insoluble material, the supernatant was placed on a DEAE-cellulose column (3 cm × 30 cm), previously equilibrated with the same buffer. The column was washed with the buffer until the absorbance of 280 nm was less than 0.15. A linear gradient of Tris buffer (0.15 M → 0.28 M) at pH 7.6 in a total volume of 1500 ml was then applied.

The plastocyanin was eluted in the reduced colorless form. Therefore the protein was reoxidized with a small excess of $K_3[Fe(CN)]_6$. Finally it was filtered through a Bio-Gel P 20 column (1 cm × 40 cm), equilibrated, and eluted with 0.05 M Tris-HCl buffer (pH 7.6). This purification procedure yielded about 5 mg of homogeneous plastocyanin from 1 kg of parsley leaves. The protein concentration was measured by a method of Goa (1953), and by optical absorption at 275 and 597 nm.

Sulfhydryl groups were determined spectrophotometrically using *p*-chloromercuribenzoate (pCMB)[1] as described by Boyer (1954). The copper content was determined by atomic absorption spectroscopy, using a Hilger and Watts Atomspek atomic absorption spectrometer, equipped with a Varian Techtron carbon rod atomizer Model 61.

Starch gel electrophoresis was conducted according to Poulik (1957).

Disc electrophoresis was conducted as described by Davis (1964). A 7.5% gel system running at pH 9.5 was used.

The ultracentrifuge analysis was carried out in a Spinco Model E ultracentrifuge equipped with a rotor temperature indicator and a control unit. The experiments were conducted at 7–8° using a rotor speed of 59,780 rpm. Three different protein preparations were analyzed in the concentration range 2–6 mg/ml in 0.05 or 0.1 M potassium phosphate buffer (pH

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[1] Abbreviations used are: pCMB, *p*-chloromercuribenzoate; epr, electron paramagnetic resonance; ME, mercaptoethanol.

7.0). Sedimentation coefficients were evaluated from the movement of the maximum ordinate of the peak using a least-squares procedure and were reduced in a conventional way to $s_{20,w}$. In the sedimentation experiments the diffusion coefficients were calculated from the areas of the schlieren diagrams and corrected for radial dilution and for the movement of the boundary in the centrifugal field as described by Elias (1961). Molecular weights were obtained from s_{obsd} and D_{corr} on the basis of the relationship

$$M = RTs/D(1 - \bar{V}\rho)$$

The partial specific volume was obtained from the amino acid composition as described by Schachman (1957).

Copper was removed from the protein by dialysis at neutral pH against NaCN after reduction to Cu^+ with dithionite. Since the apoprotein is very unstable, different dialysis systems were used. The best results were obtained either by conducting the dialysis in anaerobiosis or by adding 30% glycerol to the medium.

After hydrolysis with 6 N HCl, the amino acid analysis was carried out with a Bio-Cal BC 200 instrument, using a single column system. The total cystine–cysteine content was determined as cysteic acid after performic acid oxidation (Hirs, 1967). The tryptophan content was estimated on the short column of the amino acid analyzer after alkaline hydrolysis in 4 N $Ba(OH)_2$ according to Davis *et al.* (1970), and after acid hydrolysis in 6 N HCl in the presence of 2% thioglycolic acid (Matsubare and Sasaki, 1969).

Optical absorption spectra were performed with a Beckman DK 2 A Ratio-recording spectrophotometer.

Fluorescence measurements were performed with a Turner Model 210 Spectro, at 25°. Phosphorescence experiments were performed with an Aminco Bowman spectrophosphorimeter, using liquid nitrogen as coolant.

Epr measurements at 9 and 35 GHz were conducted as previously described (Rotilio *et al.*, 1972b).

Results

Molecular Properties of Parsley Plastocyanin. Purified parsley plastocyanin, especially in the reduced form, at variance with other blue proteins, is rather unstable at room temperature and even at 4°. Reduction occurs on aging at 4° with disappearance of the blue color. The addition of oxidizing agents such as $K_3[Fe(CN)]_6$ restores the original color.

Freshly purified preparations of parsley plastocyanin showed always a single band, before and after staining, both on starch gel and on polyacrylamide disc electrophoresis at any concentration tested and at different pH values. Minimum molecular weight calculations, on the basis of copper content determined in different samples, gave a value of 10,100 ± 300.

The $s_{20,w}$ values obtained for electrophoretically homogeneous samples were constant at 1.4 S over the examined concentration range. Although the experiments were performed at 7–8°, some precipitate appeared at the bottom of the cell during the ultracentrifuge run. On the basis of the sedimentation coefficient and of the calculated partial specific volume $\bar{V} = 0.727$, assuming $f_1/f_0 = 1.2$ (Wyman and Ingalls, 1941), an approximate molecular weight of 10,000 can be calculated. An attempt was made to calculate the molecular weight from sedimentation diffusion measurements. However, the diffusion coefficient varied from 6 to 11 × 10^{-7} cm^2/sec. This phenomenon could be anticipated in view of the instability of the protein during the course of the experiment. Hence the values of the apparent molecular weight calculated from sedimentation diffusion ranged from 7200 to 12,900.

TABLE I: Amino Acid Composition of Parsley Plastocyanin.[a]

Amino Acid	Calculated No. of Residues per 10,000 mol wt	Nearest Integer
Aspartic acid	8.2	8
Threonine[b]	5.4	5
Serine[b]	5.2	5
Glutamic acid	9.6	10
Proline	3.9	4
Glycine	10.2	10
Alanine	8.2	8
½-Cystine[c]	1.1	1
Valine	8.4	8
Methionine[c]	1.5	2
Isoleucine	2.4	2
Leucine	3.6	4
Tyrosine	3	3
Phenylalanine	4.5	5
Histidine	1.3	1
Lysine	5.1	5
Arginine	0	0
Tryptophan	0	0

[a] Hydrolysis times were 24, 36, 48, and 72 hr at 110°. Except when otherwise indicated, the values represent the average of determinations at different hydrolysis time. [b] Extrapolate to zero time. [c] Determined as cysteic acid and methionine sulfone after performic acid oxidation.

Table I shows the amino acid composition of parsley plastocyanin. It is worthnoting the presence of one sulfhydryl and one histidine per mole and the absence of arginine and tryptophan.

Absorption Spectra of Plastocyanin. Visible and ultraviolet spectra of parsley plastocyanin are reported in Figure 1 (· · · · ·). The visible spectrum is composed of three absorption bands at 460, 597, add 785 nm, with relative intensities 1:10:3 The molar extinction coefficient of the blue color is $\epsilon_{597} \sim 5000$. The ultraviolet absorption shows a peak at 275 nm with a

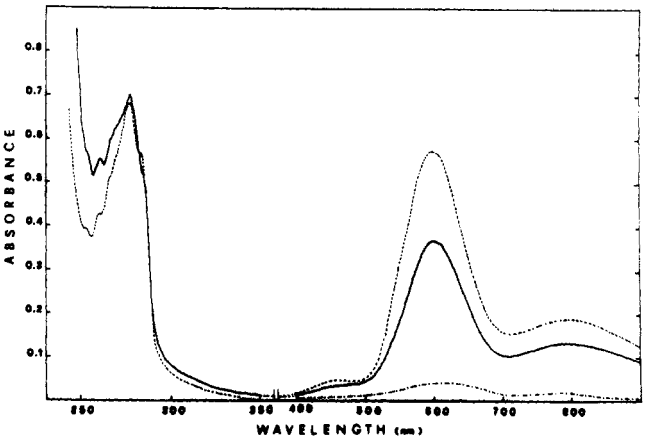

FIGURE 1: Absorption spectra of holoplastocyanin (· · ·), apoplastocyanin (---), and reconstituted plastocyanin (——). The uv spectrum of the apoprotein is identical with that of the holoprotein; uv path length, 0.5 cm; visual path length, 1 cm.

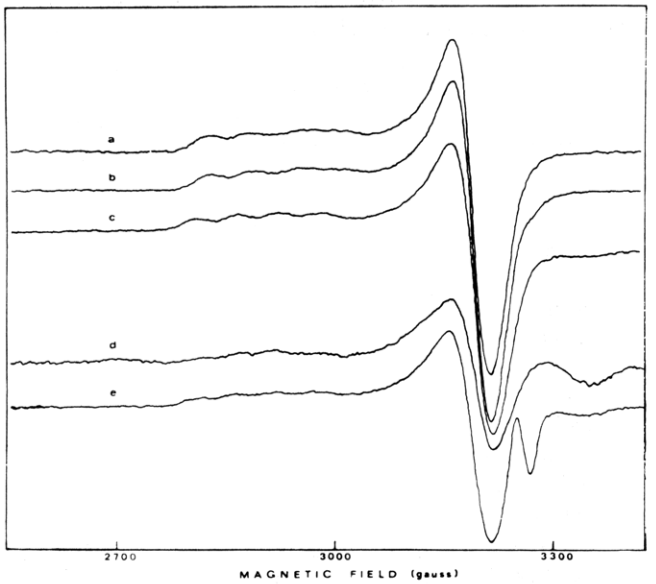

FIGURE 2: Epr spectra of plastocyanin at various pH values. Curve a): 8.5×10^{-4} M plastocyanin in 0.1 M KCl (pH 7.35). The pH of the solution was sequentially adjusted by small additions of KOH up to pH 10.2 (curve b), pH 11.15 (curve c), and pH 11.34 (curve d). To the sample of curve d, excess $K_3[Fe(CN)]_6$ was added and the solution frozen immediately for spectrum (curve e). Modulation amplitude, 10 G; microwave power, 20 mW; temperature, −160°. Gain of curve d is twice that of other curves.

superimposed fine structure, which is most probably due to phenylalanines. The apparent A_{275}/A_{597} ratio ranges between 2.1 and 2.5 in purified samples, which corresponds to an approximate extinction coefficient at 280 nm of 10,000–12,000. This figure was reduced to about one-half by adjusting the protein to a pH below 2 and dialyzing it against a solution at the same pH. Dialysis or gel filtration at higher pH did not lower the uv absorption. The absorbing species bound to the protein at a pH higher than 2 and lost by dialysis at low pH

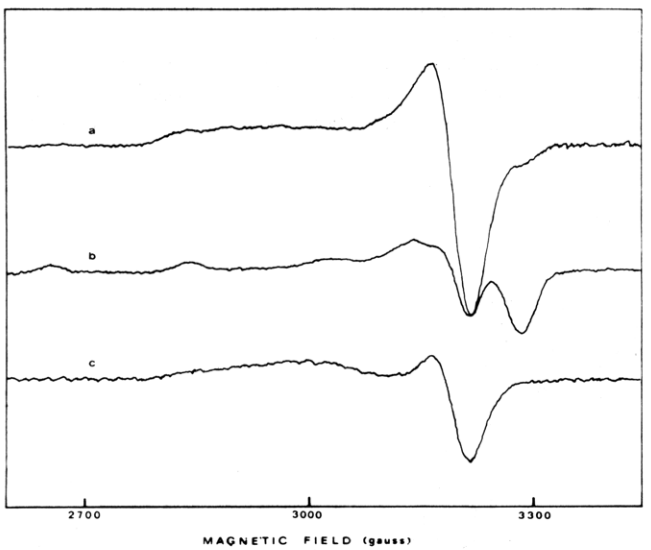

FIGURE 3: Epr spectra of the reaction of plastocyanin with pCMB. To 8.5×10^{-4} M plastocyanin, in phosphate buffer pH 7.6 (curve a), a twofold excess of pCMB was added; the sample was frozen after 25 min for spectrum (curve b). The sample was dialyzed against ME and then treated with excess $K_3[Fe(CN)]_6$ (curve c). Instrumental setting as in Figure 4. Gain of curve c is twice that of the other curve to normalize for dilution during dialysis.

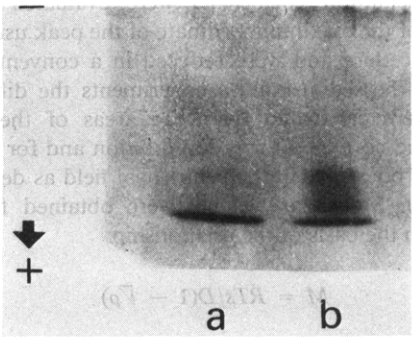

FIGURE 4: Starch gel electrophoresis pattern of holoplastocyanin (a) and of an aged preparation of apoprotein (b).

appeared to be ferrocyanide since untreated, purified samples of plastocyanin gave a positive Prussian Blue test, when treated with $FeCl_3$ in concentrated HCl.

Denaturation of the protein causes the disappearance of the blue color and minor modifications in the ultraviolet spectrum.

It was found that *N*-acetyltyrosine ethyl ester in a low polarity medium (90% dioxane, 10% H_2O) can reproduce the absorption spectrum of parsley plastocyanin quite well, whereas the spectrum of the same compound in water is very similar to that of the denatured protein.

Epr Spectra of Parsley Plastocyanin. The epr spectrum of the oxidized plastocyanin at 9.2 GHz (Figure 2a) is typical of blue proteins and very similar to that of *Chenopodium album plastocyanin*. $A_\parallel$ is 197 MHz, $g_\parallel = 2.23$, and $g_{max} = 2.06$ compared with $A_\parallel = 190$ MHz, $g_\parallel = 2.26$, and $g_\perp = 2.053$ of the *Chenopodium album* protein (Blumberg and Peisach, 1966). Measurements at higher frequency (approximately 35 GHz) do not show any splitting attributable to the resolution of g_x and g_y. The reduced protein does not show epr signal. The effect of alkaline pH on the epr spectrum was studied. Figure 2 shows that at approximately pH 11 (Figure 2c) the epr parameter changed significantly ($A_\parallel = 228$ MHz, $g_\parallel = 2.26$, $g_{max} = 2.07$). Above this pH the intensity of the epr signal decreased and a new spectral form appeared (Figure 2d). This was probably due to alkali, denatured molecules. Addition of excess ferricyanide (Figure 2e) partially restored the original spectrum, and gave rise to a new signal at high field attributable to a free radical. Addition of cyanide to the native protein at neutral pH resulted in the loss of the blue color. After prolonged incubation in the presence of cyanide, the epr spectrum showed no copper signal, but the addition of ferricyanide restored both the blue color and the typical epr spectrum. One SH group per mole of protein was titrated with pCMB according to Boyer (1954). The increase of the 255-nm absorption, due to the mercury–mercaptide formation, was concomitant with the loss of the blue color. The same experiment followed by epr demonstrated (Figure 3) that pCMB eliminated the typical epr spectrum of copper without reducing or irreversibly denaturing the protein. In fact the addition of mercaptoethanol in order to remove the bound pCMB and then of excess ferricyanide partially restored the original spectrum. In this context, it is worth recalling that while freshly prepared apoplastocyanin has a sulfhydryl per mole, reactive with pCMB, aged or mistreated apoplastocyanin samples were unable to bind copper at the native site and did not react with pCMB. They recovered the recombination ability after treatment with ME. At pH 2 the blue color disappeared and the epr spectrum showed a copper signal of the type usual for the low molecular weight copper

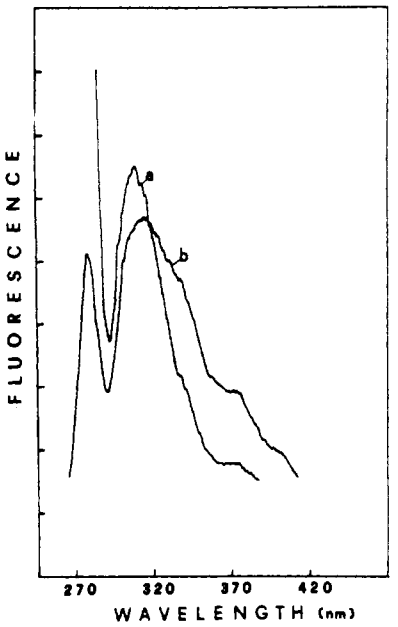

FIGURE 5: Effect of low pH on fluorescence of plastocyanin. Native plastocyanin (b) (3×10^{-6} M) was acidified with HCl down to pH ~2 (a). The amplification of (b) is 3.3 times that of (a). Excitation wavelength, 275 nm; temperature, 20°.

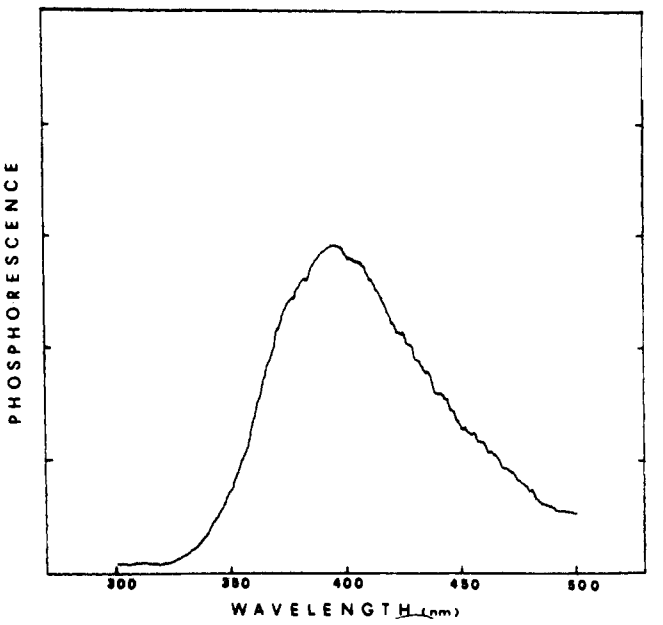

FIGURE 6: Phosphorescence spectrum of plastocyanin. Excitation wavelength, 275 nm; temperature, −196°.

complexes. The intensity of this signal, calculated by double integration of the derivative curve, accounted for all the copper present in the protein.

Properties of Apoplastocyanin. The apoprotein has the same ultraviolet spectrum of the holoprotein (see Figure 1) while the visible absorption is completely abolished. The addition of a stoichiometric amount of copper caused a slow reappearance of the blue color, with minor modification in the ultraviolet spectrum. Greater changes in ultraviolet absorption reflected unspecific copper binding to denaturated apoprotein, as indicated by epr controls. The addition of excess copper led to the precipitation of most unreactive apoprotein. Figure 4 shows the starch gel electrophoresis pattern of holoplastocyanin and of an aged apoprotein. The apoprotein sample showed slower moving components, which were absent in the presence of reducing agents.

Fluorescence and Phosphorescence of Plastocyanin. Parsley plastocyanin showed a fluorescence maximum at 315 nm when excited at 275 nm. The excitation spectrum is structureless and shows a maximum at 275 nm. At 295 nm it has only 6% of residual intensity. The shape of the emission is not affected by the wavelength of excitation. At pH values ≤ 2 this maximum shifted to 304 nm with an increase in quantum yield of about three times. The lowering of the pH also caused an evident increase of scattered light (Figure 5) and the disappearance of the blue color. After neutralization, the protein fluorescence spectrum returned slowly to its original energy, whereas the quantum yield was still higher than that of untreated protein samples and the protein solution was still colorless. After minutes to hours, depending upon the conditions used, the protein regained its color, while both the quantum yield of fluorescence and the amount of scattered light approached their original value. The fluorescence excitation and emission maxima were unaffected by lowering the temperature down to −196°.

The apoprotein showed a fluorescence identical with that of holoprotein, but with a higher quantum yield. During the recombination of apoprotein with copper, the fluorescence decrease paralleled the return of blue color. The reduction of the copper to Cu^+ did not affect the fluorescence of the protein. *N*-acetyltyrosine ethyl ester in dioxane–water (90:10 v/v) in the presence of increasing amounts of NaOH was again used as model compound. It appeared that a mixture of ionized and un-ionized tyrosines may produce fluorescence spectra very similar to that of plastocyanin.

Figure 6 shows the phosphorescence spectrum of plastocyanin obtained when it was excited with 275-nm light. The phosphorescence appeared as a structureless peak centered at about 400 nm. The lifetime of this phosphorescence is 2 ± 0.2 sec.

Discussion

Among the proteins of the same class parsley plastocyanin appears to have the same molecular weight of French bean plastocyanin (Milne and Wells, 1970) and to be more unstable than other blue proteins. This protein contains a single sulfhydryl group which seems in some way related with the properties of the copper site. In fact the reaction of the SH group in the holoprotein with pCMB bleaches the blue color without reduction of the copper (Figure 3). The resulting epr spectrum is typical of copper complexes with predominantly nitrogenous ligands. Its disappearance on treatment with ME and ferricyanide seems to rule out an aspecific copper binding to denaturated protein. Instead it is conceivable that some of the ligands which were originally binding the copper are still retained in the new binding site. Cyanide and alkaline pH values cause the blue color to disappear by reducing copper. During the alkaline bleaching an intermediate form becomes apparent with an epr spectrum significantly different from the native one. Optical and/or epr modifications during alkaline treatment have been reported for azurin (Avigliano *et al.*, 1970), umecyanin (Stigbrand and Sjöholm, 1972), and stellacyanin (Malmström *et al.*, 1970).

The absorption spectrum in the ultraviolet region of parsley plastocyanin is very similar to that of tyrosines in media of low polarity. Denaturation is often followed by changes in the

ultraviolet absorption which becomes similar to that of *N*-acetyltyrosine ethyl ester in water.

The protein binds quite strongly with ferrocyanide. Other proteins, like hemoglobin (Steinhardt, 1957; Benesch *et al.*, 1964), have been shown to bind strongly with ferrocyanide. Neither exhaustive dialysis nor gel filtration on Bio-Gel column removes all the ferrocyanide from plastocyanin. This fact makes it quite difficult or even impossible to determine the real ultraviolet extinction coefficient of the protein. The apparent molar extinction coefficient was about 12,000. After acid denaturation of the protein, which seems to release the bound ferrocyanide, this value is decreased by about 50%. The last figure matches the value calculated from the amino acid composition.

The fluorescence of plastocyanin at 315 nm seems to be caused by the phenolic side chain of tyrosine, as demonstrated by the shift obtained on denaturation of the protein. The resulting emission at 304 nm is typical of tyrosines as it is the phosphorescence of the native protein at 400 nm with a lifetime of about 2 sec (see Figure 6). Adrenodoxin, which also contains tyrosine and no tryptophan, shows an emission at 331 nm which shifts to 315 nm at 77°K (Kimura and Ting, 1971). This behavior has been interpreted in terms of formation of an exciplex (excited state complex) between tyrosine and an unknown partner (Kimura *et al.*, 1972). Parsley plastocyanin does not show any dependence of maximum emission as a function of temperature. Instead the quantum yield of fluorescence is affected by copper binding, being quenched in the presence of the metal. The latter finding recalls previous results obtained with other blue copper proteins (Finazzi Agrò *et al.*, 1970, 1973; Morpurgo *et al.*, 1972).

The experiments carried out with the *N*-acetyltyrosine indicate that the fluorescence emission of parsley plastocyanin can be interpreted as due to the phenolate form of tyrosine in a solvent of low polarity. In fact in 90% dioxane ionized tyrosines, practically nonfluorescent in water, show a fluorescence whose emissione maximum and quantum yield are very similar to those of plastocyanin. These parameters are dependent on the amount of NaOH added and on the dielectric constant of the medium, the variation of which can generate spectroscopically different species. It is important to point out that the deprotonated tyrosine is not involved in copper binding. Copper removal in fact does not affect the shape of fluorescence emission. It is impossible to state if every tyrosine of the three present in plastocyanin contributes in the same way to the fluorescence. On the other hand, the presence of the metal greatly reduces the quantum yield of the whole fluorescence. This quenching cannot be explained in terms of dipole–dipole energy transfer since in the reduced holoprotein which contains Cu^+ and does not show any absorption above 300 nm, the fluorescence is still quenched to the same extent. A similar behavior was observed in the other mononuclear blue copper proteins previously studied (Finazzi Agrò *et al.*, 1970, 1973; Morpurgo *et al.*, 1972).

As a conclusion, we want to point out analogies among blue copper proteins which were confirmed by the results reported in this paper. First, plastocyanin, as previously found in the cases of azurin, stellacyanin, and spinach plastocyanin (Katoh and Takamiya, 1964) contains a sulfhydryl which is in some way related to the blue chromophore. Till now no blue protein was reported to lack sulfhydryl groups. Thus we obtained additional evidence that sulfur could be an invariant copper ligand in blue proteins.

Another striking analogy among the blue proteins is the influence of copper on the intrinsic fluorescence of the protein. This feature might be related to the electron transfer mechanism from the exterior of the molecule to the copper. Also in plastocyanin the aromatic amino acids responsible for the fluorescence seem not to be exposed to the solvent. The phenylalanines might be the cause of the hydrophobic environment because their fine structure was blurred whenever the blue color and the anomalous fluorescence were lost.

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Allosteric Effectors of Hemoglobin. Interaction of Human Adult and Fetal Hemoglobins with Poly(carboxylic acids)†

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ABSTRACT: Aliphatic and aromatic poly(carboxylic acids) interacted with hemoglobin lowering its oxygen affinity. The magnitude of the effect appeared unrelated to the structure of the polyacids and proportional to the number of carboxyl groups present in the compounds. The largest effect was produced by benzenepentacarboxylic acid which at 20° in 0.05 M bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane buffer at pH 7.3 increased log $P_{1/2}$ of human adult hemoglobin from about 0.3 to *ca.* 1.10 and that of human fetal hemoglobin from *ca.* 0.5 to *ca.* 0.95. Its affinity for fetal hemoglobin appeared less than that for adult hemoglobin. It increased the Bohr effect of both hemoglobins, indicating the release, in its presence, of extra protons in addition to those of the alkaline Bohr effect. The number of extra protons liberated in this way upon oxygenation was similar for adult and fetal hemoglobin. The value of the Hill parameter n for fetal hemoglobin appeared to be pH dependent both in the presence and absence of benzenepentacarboxylate, being near 2 below and near 3 above pH 7 with a sharp transition around this pH. The combined effect of benzenepentacarboxylate and CO_2 on adult hemoglobin was much less than the sum of the separate effects, suggesting a competition between the two reagents. The great similarity of the effects of benzenepentacarboxylate and 2,3-diphosphoglycerate on human adult and fetal hemoglobin would suggest that the two compounds bind to hemoglobin in a similar way.

The models proposed by Perutz (1970) and Arnone (1972) for the interaction of 2,3-diphosphoglycerate with human hemoglobin show that the 1β-valines, the 143β-histidines, and one of the 82β-lysines form salt bridges with the five negative charges of 2,3-diphosphoglycerate. The 82β-lysine would interact with the carboxyl group of 2,3-diphosphoglycerate and, according to Arnone (1972), the 2β-histidines can also participate in the binding, in this case substituting the 1β-valines. These groups are present in a crevice, between the two β chains, which only in deoxyhemoglobin is large enough to accommodate 2,3-diphosphoglycerate, thus explaining the preferential binding of 2,3-diphosphoglycerate to this form of hemoglobin (Perutz, 1970).

The electrostatic interaction is bound to modify the proton binding behavior of the groups involved, increasing the pK of the positive charges and decreasing that of the negative groups, so that within certain pH ranges hemoglobin is expected to absorb protons upon interaction with 2,3-diphosphoglycerate. This phenomenon contains much information. As it will be discussed in another manuscript, it might help to measure the affinity constant of the effector for hemoglobin, the number of groups which in hemoglobin participate in the interaction, their pK, and their shift to higher pK values in the presence of 2,3-diphosphoglycerate. This absorption of protons also affects the oxygen equilibrium; in fact the preferential binding of the effector to deoxyhemoglobin implies that they will be released all or in part upon oxygenation, in addition to those liberated by the alkaline Bohr effect groups. In this way the pH dependency of oxygen affinity in hemoglobin is increased. This phenomenon can be defined as the "additional Bohr effect" (ABE)[1] and is present in the complex 2,3-diphosphoglycerate–hemoglobin as demonstrated by Benesch *et al.* (1969), Bailey *et al.* (1970), Riggs and Imamura (1972), and DeBruin and Janssen (1973). A comparison between the number of ABE protons released and the number of protons absorbed by deoxyhemoglobin upon the interaction with 2,3-diphosphoglycerate would constitute a measure of the amount of effector displaced from hemoglobin upon oxygenation.

In the case of 2,3-diphosphoglycerate a direct measurement of these protons is made difficult by the high pK (Kumler and Eiler, 1943) of some of the negative charges of 2,3-diphosphoglycerate, which makes the ionization of these groups overlap with that of the positive groups of hemoglobin, so that simultaneous absorption and liberation of protons occur upon their interaction. Large corrections would have to be introduced in the experimental data in order to calculate the number of protons absorbed by hemoglobin. On the other hand it appears that the specificity of binding of effectors by hemoglobin is not limited to 2,3-diphosphoglycerate and that other chemicals can interact in a similar way (Chanutin and Curnish, 1967; Benesch and Benesch, 1967). Following this reasoning we began searching 2,3-diphosphoglycerate substitutes whose negative charges would be completely ionized below pH 7.

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[1] Abbreviations used are: bis-tris, bis(2-hydroxyethyl)iminotris-(hydroxymethyl)methane; Tris, tris(hydroxymethyl)aminomethane; ABE, additional Bohr effect.