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ACTIVE-SITE PROPERTIES OF THE BLUE COPPER PROTEINS

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I. Introduction

Metals at the active sites of metalloproteins display special properties and are generally impressively efficient in their functional roles. The metal or metals are said to be poised for catalytic action or in an entatic state, an expression first used by Vallee and Williams in their seminal paper of 1968 (*1*). The fine tuning or control of the peptide in many cases results in previously unobserved aspects of the coordination chemistry of the metal in question. There are many examples, almost as many as there are metalloproteins, from (in the case of Fe) the five-coordinate high-spin Fe(II) in myoglobin and hemoglobin

(ready to coordinate O_2), to the matching low-spin Fe(II) and Fe(III) structures of the heme prosthetic group of the cytochromes, which enables them to mediate readily in electron transport. The blue copper proteins are involved in electron transport, and provide one of the best examples of active site "design." Electron transfer reactions proceed by a transition state, in which the structures are intermediate between the reactant and product states. In many cases, formation of the intermediate involves a simple adjustment in metal–ligand bond lengths. However, in the case of Cu(I) and Cu(II), it is more complicated because the two states normally have different geometries, tetrahedral and tetragonal (square–planar or octahedral), respectively. In such instances redox interconversion is more demanding in terms of energy. In the case of the blue Cu proteins the existence of a compromise geometry acceptable to both oxidation states makes it easier to interconvert the two. From a not very favorable situation, therefore, nature has learnt how to use copper as a redox center. It is not, however, a simple change giving a symmetrical geometric form, nor a single unique structure that applies to all blue copper proteins, although certain prime features are retained.

The first crystal structure information on a blue copper protein, for poplar plastocyanin in the Cu(II) state, was published in 1978 (*2, 3*). Since then, the Cu(I) state and related apo and Hg(II) substituted forms (*5, 6*), the green algal plastocyanin from *Enteromorpha prolifera* [Cu(II)] (*7*), azurin from *Alcaligenes denitrificans* [Cu(II) and Cu(I)] (*8, 9*), azurin from *Pseudomonas aeruginosa* [Cu(II)] (*10, 11*), as well as pseudoazurin from *Alcaligenes faecalis* S-6 (*12*), and the cucumber basic protein, both in the Cu(II) state, have been published (*13*), making this one of the best-documented class of proteins. In addition, information as to three-dimensional structure in solution has been obtained from two-dimensional NMR studies on French bean and *Scenedesmus obliquus* plastocyanins (*14, 15*). This review is concerned in the main with the active site chemistry. Other recent reviews are listed (*16–20*).

II. Classification

Blue copper proteins have a single Cu atom at the active site, and three characteristic properties: (1) an intense blue color at ~600 nm, with absorption coefficients of 2000–6000 M^{-1} cm^{-1}, arising from S(Cys) → Cu(II) charge transfer; (b) an unusually narrow hyperfine coupling ($A_\parallel$ values of 0.0035–0.0063 cm^{-1}) in the EPR spectrum of the Cu(II) protein due to asymmetry at the metal; and (3) high reduction potentials (range 184–680 mV) as compared to the aqua Cu(II/I)

couple (115 mV). The proteins are often referred to collectively as Type 1 Cu proteins. Type 2 proteins also have a single copper atom, but the Cu(II) state is not characterized by an intense color or unusual EPR spectrum. Type 3 proteins are binuclear and therefore not EPR active. The classification is in need of some revision with the recent discovery that in ascorbate oxidase, Type 2 and Type 3 active sites in the same enzyme are present as a single trinuclear center (*21*), which possibly merits a further (Type 4) classification. Type 1 center(s) are also present in this and other multi-Cu enzymes. For example, laccase-like ascorbate oxidase has one Type 1 and a trimeric center, whereas ceruloplasmin appears to have three Type 1 centers in addition to a trimer unit (*22*).

III. Occurrence and Sequences

Well-characterized (Type 1) blue Cu proteins are as listed in Table I. By far the most extensive studies have been with plastocyanin (M_r ~10,500), which consists of 99 amino acids or in some cases (with deletions at 57 and 58) 97 amino acids (*18, 20*). There are 104 amino acids in the case of plastocyanin from the blue–green alga *Anabaena variabilis* (*23*), which has some quite different properties. The name was introduced by Katoh and Takamiya in 1961 at the time of their isolation of plastocyanin from spinach chloroplasts (*24*). In a 1977 review on the chemistry, function, and evolution of plastocyanin, Boulter *et al.* (*25*) listed 11 plastocyanins that had been fully sequenced, together with 55 other partial sequences (typically the first 40 residues). The latter information has enabled some comment on the rate of evolution of plastocyanin. The extensiveness of such studies is also an indication of the relative ease with which the protein can be isolated. The list of completed plastocyanin sequences now includes those from 20 higher plants, four green algae (*S. obliquus, Chlorella fusca, Enteromorpha prolifera,* and *Ulva arasakii*), and one blue–green alga (*A. variabilis*) (Fig. 1) (*20, 26*). Of the 20 higher plant sequences, 47 of the 99 residues are invariant. With the inclusion of the four green algal plastocyanins this number reduces to 28, and with plastocyanin from the blue–green algal source *A. variabilis* it is 23. The invariant residues include His 37, Cys 84, His 87, and Met 92, which coordinate the Cu at the active site.

The azurins have 128 or 129 amino acids (M_r ~14,000); at least 10 have been sequenced (*16*). Of the nine sequences considered by Rydon and Lundgren, 47 residues are invariant, including His 46, Cys 112, His 117, and Met 121, which coordinate the Cu, and a further 32 are conservatively substituted (*27*). There is an additional interaction of

TABLE I

PROPERTIES OF BLUE COPPER PROTEINS

Protein	Source	Amino acids	pI[a]	E^{0b} (mV)	λ_{max} (nm)	ε (M^{-1} cm)	$g_{\parallel}$	$A_{\parallel}$
Plastocyanin	Higher plants/green algae[c]	99	4.2	375	597	4500	2.23	0.0063
Azurin	Denitrifying bacteria	128	5.4	305	625	5200[d]	2.26	0.0060
Pseudoazurin	Denitrifying bacteria	123	7.65	—	593	2900	—	—
CBP[e]	Cucumber	96	10.5	317	597	3400	2.21	0.0055
Amicyanin[f]	Methylotropic bacteria	106	4.7	260	596	3900	2.26	0.0055
Rusticyanin	*Thiobacillus ferrooxidans* bacteria	144	9.1	680[g]	597	2240	2.23	0.0045
Stellacyanin	Lacquer tree[h]	107	9.9	184	608	4080	2.29	0.0035
Umecyanin	Horseradish roots	125	5.8	283	610	3400	2.23	0.0035

[a] PCu(II), where distinction is made.
[b] pH ~ 7.5
[c] Plastocyanin from blue–green algae *Anabaena variabilis* has some different properties.
[d] See Ref. 67; Ref. 66 gives a value of 5800 M^{-1} cm^{-1}.
[e] Cucumber basic protein, previously referred to as plantacyanin.
[f] *Thiobacillus versutus*.
[g] pH ~ 2.
[h] *Rhus vernicifera*.

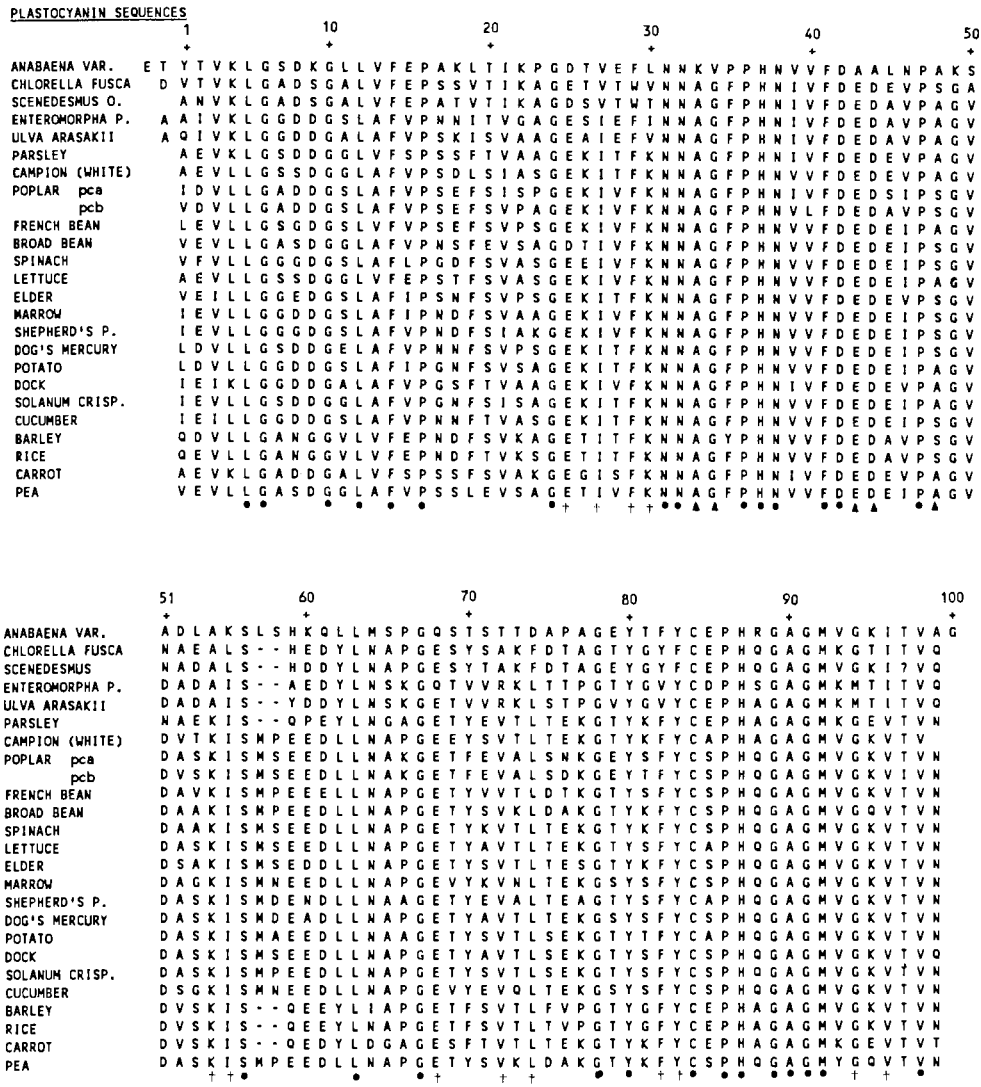

FIG. 1. Comparison of sequences of 25 plastocyanins from higher plants and algae. Amino acids are indicated by the one-letter abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine, L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine, T, threonine; V, valine, W, tryptophan; and Y, tyrosine. Deletions are indicated by gaps, in particular at 57 and 58, giving increased homology in the sequences. For recent reports, see Ref. *26*. Also denoted are invariant residues (●), additional residues that are invariant by considering only the higher plant and green algal sequences (†), and additional residues that are invariant by considering only the higher plant sequences (▲).

the Cu with Gly 45. Histidines at 35 and 83 are also invariant. There are homologies between plastocyanin and azurin, but only nine residues are invariant between the two families.

In 1985 the existence of two new classes of bacterial blue proteins, the pseudoazurins and amicyanins, was demonstrated (*28*). Sequence information is available for pseudoazurin from *Pseudomonas* AMI (*28*), *Achromobacter cycloclastes* (*29*), as well as *A. faecalis,* for which the structure has been determined (*12*). The name pseudoazurin rather than cupredoxin is used here. The sequence for amicyanin, also present in *Pseudomonas* AMI, has been reported, and that for amicyanin from *Thiobacillus versutus* is being determined. Preliminary X-ray crystallographic information has been reported for amicyanin from *T. versutus* (*30*).

The other categories listed in Table I are at present single examples (*16, 18, 20*).

IV. Functional Role

Plastocyanin is involved in electron transport between photosystems II and I of the chloroplast in higher plants and algae (*31–33*). More specifically, its function is to transfer electrons from cytochrome *f* (360 mV) to the chlorophyll-containing pigment $P700^+$ (520 mV), which is a component of photosystem I. Photosynthesis occurs at the highly convoluted thylakoid membranes inside the chloroplast. Cytochrome *f* (285 amino acids) is a component of the cytochrome b_6/f complex (also containing Rieske's Fe/S protein), which is located in the thylakoid membrane (*18, 20*). However, it has a major globular component (residues 1–250) located in the aqueous media of the inner thylakoid space (*34*). The latter also contains plastocyanin that, by means of a hydrophobic region, may associate with but is not permanently attached to the membrane. Plastocyanin is released from the chloroplast by mechanical damage, whereas cytochrome *f* requires treatment with detergent to detach it from the membrane. The 35-residue hydrophobic section of cytochrome *f* is removed by protease action in certain isolation procedures, thus preventing aggregation in detergent-free aqueous solution. Studies on the reactions of plastocyanin with isolated cytochrome *f* (*35–37*) and P700 (*38*) have been reported.

Azurins are found in the respiratory chains of various denitrifying bacteria, where their role is to transport electrons between cytochrome c_{551} and cytochrome oxidase. The kinetics of the reaction of *P. aeruginosa* azurin and cytochrome c_{551} has been studied (*39, 40*). The role of

the pseudoazurins is believed to be to transfer electrons to a Cu-containing nitrite reductase (*41*). Properties appear to be intermediate between those of plastocyanin and azurin.

Two amicyanins from the methylotropic bacteria *Pseudomonas* AMI and *T. versutus* have been a recent focus of attention (*42, 43*). Their function is to mediate electron transfer between bacterial cytochrome *c* and methylamine dehydrogenase in a relatively short electron transport chain.

Rusticyanin is a component in the respiratory chain of the bacterium *Thiobacillus ferrooxidans* (*44–46*). This bacterium is capable of growth solely on the energy available from the oxidation of aqua Fe(II) to Fe(III) by O_2. It is found in acid mine leachings, and is used commercially in the extraction of copper and uranium (see the review by Ewart and Hughes, this volume). Its ability to take into solution iron pyrites is particularly relevant. It has been suggested that an acid-stable cytochrome mediates electron transfer between rusticyanin and Fe^{2+} (*47, 48*). The working pH is ~2.0.

There is little information available regarding the function of stellacyanin and umecyanin, which are isolated from nonphotosynthetic plant tissue.

V. X-Ray Crystal Structures

A. Plastocyanin

Both the Cu(II) and Cu(I) poplar (pca sequence, Fig. 1) structures have been refined to resolutions of 1.6 Å (*3, 4*). The molecule has the shape of a slightly flattened barrel of approximate dimensions 40 × 32 × 28 Å, with the Cu buried ~6 Å close to the long axis. There are eight strands of polypeptide chain that are connected by seven loops at the ends of the barrel. Seven of the strands have substantial β character and strand five is irregular and contains the only helical structure (about 1.5 turns). Whereas the dimensions of the distorted tetrahedral Cu(II) active site do not vary for structures determined at pH 6.0 and 4.2, PCu(I) shows a change in geometry from four to three coordination as the pH is decreased from 7.8 to 3.8 (*4*). Table II lists information for both states at the higher pH, when the same distorted tetrahedral geometry is present. The two Cu—N(His) and the Cu—S(Cys) bond lengths are normal, and as observed in low-molecular coordination complexes, but the Cu—S(Met) distance is unusually long at 2.90 Å. This and other features have provoked much interest in the spectro-

TABLE II

BOND DISTANCES AND ANGLES FOR THE Cu ACTIVE SITE OF POPLAR PLASTOCYANIN[a]

Bond	Cu(I) (pH 7.8)	Cu(II)
Distances (Å)		
Cu—N(His 37)	2.12	2.04
Cu—S(Cys 84)	2.11	2.13
Cu—N(His 87)	2.25	2.10
Cu—S(Met 92)	2.90	2.90
Angles (degrees)		
N(His 37)—Cu—S(Cys 84)	141	132
N(His 37)—Cu—N(His 87)	92	97
N(His 37)—Cu—S(Met 92)	90	85
S(Cys 84)—Cu—N(His 87)	112	123
S(Cys 84)—Cu—S(Met 92)	114	108
N(His 87)—Cu—S(Met 92)	102	103

[a] See Refs. *3* and *4*.

scopic properties. The three residues (Gly-Ala-Gly) preceding Met 92 are invariant, and may have an influence on structure, in particular the unusually long Cu—S(Met) bond distance. The change in bond length on interconversion of PCu(I) and PCu(II) at pH 7.8 (Table II), averages (four bonds) 0.06 Å.

Inactivation of PCu(I) as the pH is decreased from pH 7.0 to below 5.0 was first identified in kinetic studies (*18, 20, 49*). This is now clearly documented as an equilibrium involving conversion of a four-coordinate distorted tetrahedral structure (at high pH) to a trigonal–planar form (at low pH) (see also p. 392). In the process, the Cu—N (His 87) bond dissociates, and the imidazole N, which was formerly coordinated, becomes protonated (Fig. 2). Interconversion of the two states by electron transfer is as a result more difficult to bring about.

The structure of apoplastocyanin obtained by soaking crystals of poplar PCu(I) in 0.15 *M* CN^- to remove the metal has been determined to 1.8 Å resolution (*5*). The structure closely resembles that of the holoprotein. In particular, the positions of the Cu-binding residues in the apo- and haloproteins differ by only 0.1–0.3 Å. This indicates that the irregular geometry of the Cu site is imposed on the metal by the polypeptide. By soaking crystals of poplar PCu(II) in Hg(II) acetate solution, replacement of the Cu(II) by Hg(II) is observed. The crystal structure of the Hg(II)-substituted derivative has been determined to 1.9 Å resolution (*6*). Only minor changes are observed at the active

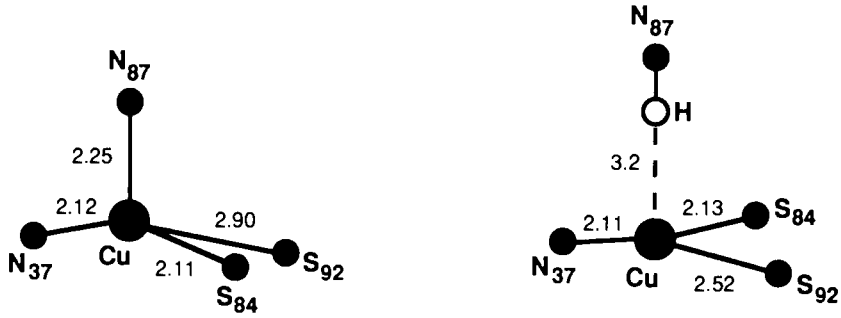

FIG. 2. Bond lengths for the Cu(I) active site of poplar plastocyanin: (left) the distorted tetrahedral coordination at pH > 7 and (right) the trigonal–planar form resulting from protonation of His 87 and present at pH < 4.5.

site, and it is again concluded that the geometry at the active site is determined by the polypeptide. In spite of the known affinity of Hg(II) for S donor groups, the weak metal–S(Met) bond in Cu(II) plastocyanin remains weak in the Hg(II)-substituted derivatives. The Hg(II)–ligand bond lengths [in angstroms; the corresponding Cu(II) values are given in parentheses] are as follows: N(His 37), 2.34 (2.04); S(Cys 84), 2.38 (2.13); N(His 87), 2.36 (2.10); S(Met 92), 3.02 (2.90). There is essentially the same displacement of the metal from the N(His 37), S(Cys 84), N(His 87) plane (0.36 Å) as in the Cu(II) case (0.34 Å). The side chain of Pro 36 close to the metal site has flipped over from the C^{γ}-exo conformation in PCu(II) to the C^{γ}-endo position. As in the Cu protein, the metal–S(Cys) bond is strong and an important feature.

In the most recent study, the crystal structure of plastocyanin from the green alga *Enteromorpha prolifera* PCu(II) has been reported to a resolution of 1.85 Å (*7*). The β-sandwich structure is virtually the same as that of poplar plastocyanin, with which it has a 56% sequence homology. Two of the residues (57 and 58), which are components of one of the two prominent kinks in poplar plastocyanin, are missing, and there is a resultant tightening up in the structure and change in position of the sole helical turn in the molecule. The dimensions of the Cu site in the two plastocyanins are, within the limits of precision, the same. An intramolecular H bond between two carboxylates, Glu 43 and Asp 53, has been noted and could explain the unusually high pK_a values (~5) obtained for all Cu(II) plastocyanins (*20*).

Interestingly, in all blue copper protein crystal structures so far determined it is the N^{δ} of the imidazole that coordinates to the Cu. Since a proton attached at the N^{ϵ} is more acidic (pK_a values 5.0–7.5) than the covalently bound H atom at N^{δ}, this observation is of considerable interest and will be returned to.

B. Azurin

The structures of three Cu(II) azurins from *A. denitrificans* (*8*), *P. aeruginosa* (*10, 11*), and *Pseudomonas denitrificans* (*50*) have been determined to 1.8, 2.7, and 3.0 Å resolution, respectively. In the case of *P. aeruginosa* there are four molecules in the asymmetric unit but only two in the case of *A. denitrificans,* which has yielded the most detailed information. Although these two azurins differ in their sequences at 49 positions, their three-dimensional structures are remarkably similar. The *A. denitrificans* Cu(I) structure has recently been determined, and relevant data are summarized in Table III (*9*).

The refinement of the Cu(II) state has shown that the geometry is close to that of a distorted trigonal–bipyramid geometry (with axial bonds elongated) rather than the tetrahedral arrangement as in plastocyanin (*8*). The two N(His) bonds and the S(Cys) bond are in a trigonal plane, and are supplemented by the much larger S(Met) bond (Cu—S of 3.13 Å) and the bond to the peptide carbonyl of Gly 45 (Cu—O of 3.11 Å), which occupy the two axial positions (Fig. 3). The Cu is 0.10 Å from the plane defined by His 46, Cys 112, and His 117.

TABLE III

Bond Distances and Angles for the Cu Active Site of Azurin from *Alcaligenes denitrificans*[a]

Bond	Cu(I)	Cu(II)
Distances (Å)		
Cu—O(Gly 45)	3.22	3.13
Cu—N(His 46)	2.13 (2.12)	2.08 (2.04)
Cu—S(Cys 112)	2.26 (2.11)	2.15 (2.13)
Cu—N(His 117)	2.05 (2.25)	2.00 (2.10)
Cu—S(Met 121)	3.23 (2.90)	3.11 (2.90)
Angles (degrees)		
N(His 46)—Cu—S(Cys 112)	132 (141)	135 (132)
N(His 46)—Cu—N(His 117)	104 (92)	105 (97)
N(His 46)—Cu—S(Met 121)	78 (90)	77 (85)
S(Cys 112)—Cu—N(His 117)	123 (112)	119 (123)
S(Cys 112)—Cu—S(Met 121)	109 (114)	107 (108)
N(His 117)—Cu—S(Met 121)	92 (102)	96 (103)
O(Gly 45)—Cu—N(His 46)	68	74
O(Gly 45)—Cu—S(Cys 112)	104	104
O(Gly 45)—Cu—N(His 117)	83	80
O(Gly 45)—Cu—S(Met 121)	143	147

[a] See Ref. *9*; numbers in parentheses are the corresponding values for poplar plastocyanin (see Refs. *3* and *4*).

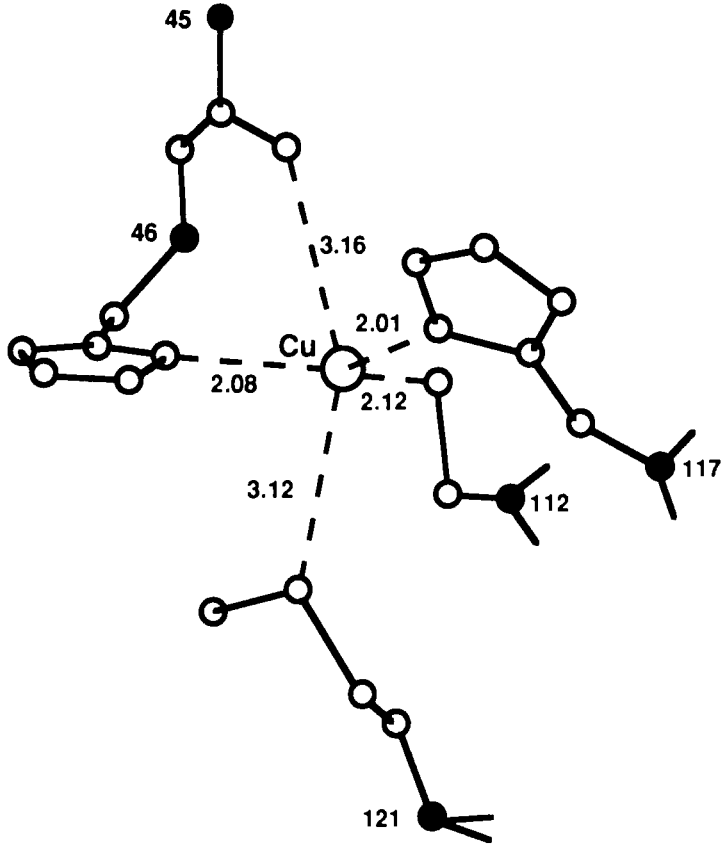

FIG. 3. The approximately trigonal–bypyramid active site of Cu(II) azurin, with long axial bonds to the polypeptide carbonyl O atom of Gly 45 and S atom of Met 121.

The same geometric arrangement applies in the case of the Cu(I) protein (*9*).

Homologies between azurin and plastocyanin have been considered. A schematic representation of the polypeptide folding of *A. denitrificans* azurin is shown in Fig. 4. Essential differences as compared to plastocyanin are the prominent 52–81 azurin flap, including an α-helix section (Fig. 5), which replaces the acidic residues 42–45 on plastocyanin. There is an extension of the plastocyanin 33–37 section, and the 59–61 acidic residues are also replaced.

The difference in bond lengths corresponding to interconversion of the two oxidation states averages 0.08 Å, and is the same whether the Cu—O bond is included or excluded.

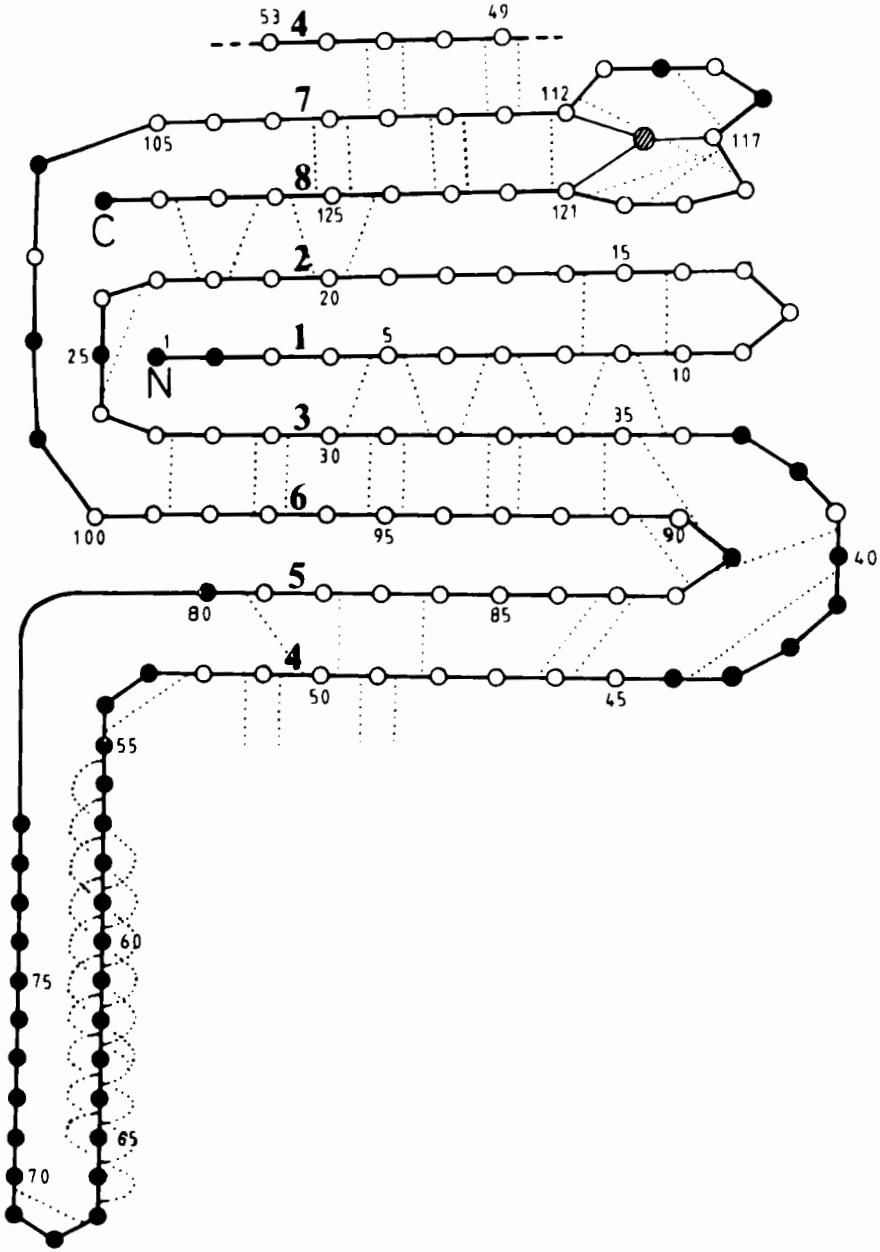

FIG. 4. The polypeptide chain folding of *Alcaligenes denitrificans* azurin. Solid circles are residues inserted for comparison with plastocyanin. Probable H bonds are shown by dotted lines. Strands of β structures are numbered according to their positions in the amino acid sequence; the inserted flap 52–81 contains an α-helix section that is seen on the right-hand side in Fig. 5. The cross-hatched circle denotes the position of the Cu atom. (Reproduced with permission from Ref. *8*.)

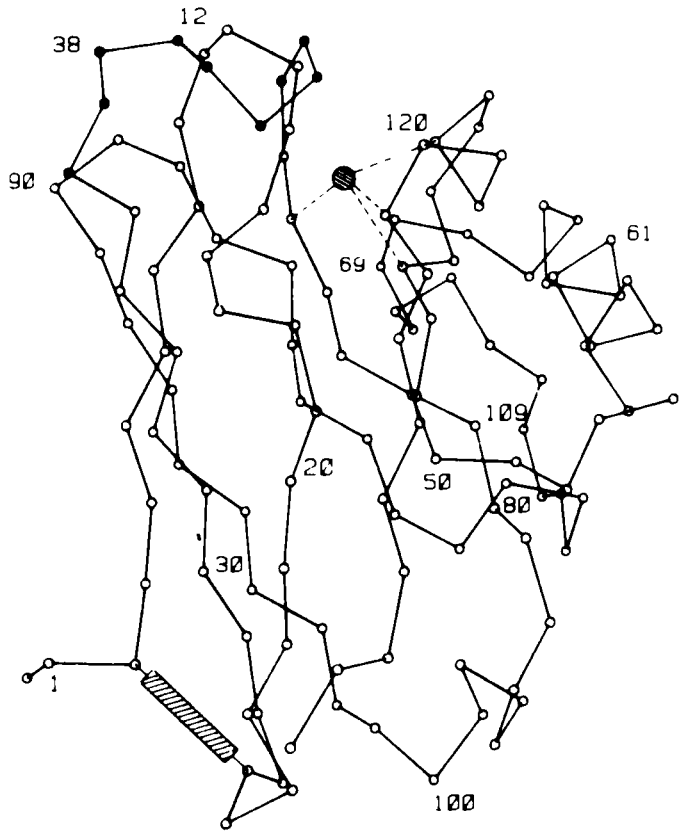

FIG. 5. The α-carbon positions in the structures of *Alcaligenes denitrificans* azurin. The cross-hatched circle denotes the Cu atom. A disulfide bridge links Cys 3 and Cys 26. Two important insertions are observed as compared to plastocyanin. The flap region is shown on the right, and an extra loop is at the top of the molecule. (Reproduced with permission from Ref. *8*.)

C. PSEUDOAZURIN

The crystal structure of pseudoazurin from *A. faecalis* S-6 has been reported for the Cu(II) state to 2.0 Å (*12*). The protein folds in a β sandwich that is described as being similar to plastocyanin and azurin. The distances to the Cu from its ligands are as follows: Cu—N(His 40), 2.10 Å; Cu—S(Cys 78), 2.07 Å; Cu—N(His 81), 2.21 Å; and Cu—S (Met 86), 2.69 Å. The latter distance is shorter than that found in plastocyanin and azurin. The analogous backbone carbonyl oxygen to that which is weakly coordinated in azurin is 4 Å from the Cu. The geometry around the Cu can be regarded as trigonal–bipyrimidal with the

Cu, now 0.3 Å from the plane defined by the two N(His) and S(Cys) ligating groups, and toward the S(Met).

D. Cucumber Basic Protein

Some information has been published on the cucumber basic protein (CBP) structure (*13*). The amino acids coordinating the Cu are His 39, Cys 79, His 84, and Met 89. The folds in plastocyanin, azurin, and CBP are distinctly different (*13*). In the case of azurin, strands four and five of the polypeptide backbone are part of the β sandwich, and connecting these ends is a flap comprising 30 residues and including three turns of an α helix hanging off the main body of the molecule. In plastocyanin, strand five is too irregular to be part of the β sandwich. In CBP, the β sandwich structure is further depleted by a bend and a twist in strands four and five that place these strands at a large angle from the others. As in stellacyanin, there are two additional cysteines. In CBP these are Cys 52 and Cys 85, which are joined as a disulfide bridge.

VI. EXAFS Studies

These have been quite extensive and results have been published for plastocyanin (*51*), azurin (*52*), stellacyanin (*53*), umecyanin (*54*), and rusticyanin (*55*) in both oxidation states. Fitting procedures are not sensitive to the inclusion of the Cu—S(Met) bond, and if the latter does contribute the best fit the bond distance obtained is not always reliable. In the case of azurin, the axial carbonyl group is not included in the fit. No information is obtained from EXAFS concerning bond angles. Table IV is a listing of data reported. It is noted that (average) Cu—N(His) bonds are persistently shorter than those obtained from

TABLE IV

Bond Distances (Å) Obtained from EXAFS Studies[a]

Protein	Cu(I)—N	Cu(II)—N	Cu(I)—S	Cu(II)—S	Ref.
Plastocyanin	2.05	1.97	2.22	2.11	*51*
Azurin	1.96	1.95	2.21	2.23	*52*
	2.00	1.97	2.22	2.12	*54*
Stellacyanin	1.98	1.93	2.25	2.21	*53*
Umecyanin	2.03	1.99	2.21	2.13	*54*
Rusticyanin	2.07	1.99	2.17	2.16	*55*

[a] Two identical Cu—N(His) bonds are assumed.

X-ray crystallography for Cu(II)—N (~0.09 Å) and Cu(I)—N (~0.12 Å), whereas agreement is to ~0.03 Å in the case of the Cu—S(Cys) bonds (the crystallographic values are smaller). An explanation of these differences is not clear, but may stem from the difficulties encountered in taking the Cu—S(Met) bond into account. It would be surprising if the difference stemmed in any way from the solid and solution state phases present in these studies. The crystallographic distances are generally regarded as more reliable.

VII. UV–VIS Spectra

Details of the position of the ~600-nm peak and absorption coefficients for blue copper proteins are indicated in Table I. There are some differences in the peak position, e.g., 597 nm for plastocyanin and 625 nm for azurin (Fig. 6). These may be related to the existence of a fifth ligand in the case of azurin (*8*), giving rise to trigonal–bipyramidal rather than tetrahedral coordination, but the same trend is not observed with pseudoazurin (*12*), which also appears to have a weak Cu—O interaction.

Absorption coefficients (ε) at the peak show considerable variation, e.g., 2240 M^{-1} cm^{-1} for rusticyanin to 5200 M^{-1} cm^{-1} for azurin. It is generally assumed that ε is invariant for all plastocyanins and

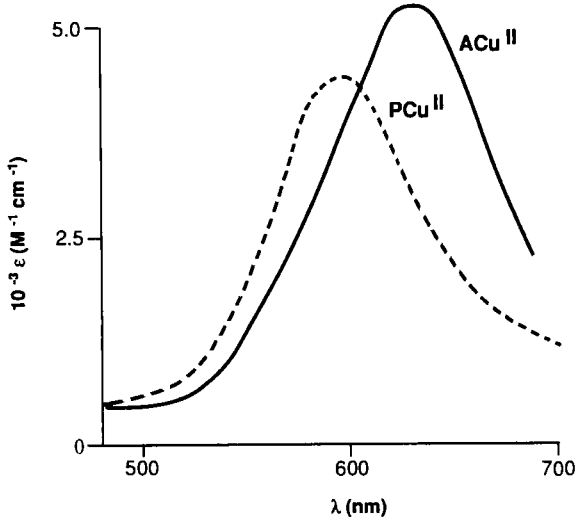

FIG. 6. A comparison of UV–VIS absorption spectra for the Cu(II) forms of *Pseudomonas aeruginosa* azurin (—) and spinach plastocyanin (---).

azurins, respectively, but this does not appear to have been rigorously checked. Although the value of 4500 M^{-1} cm^{-1} is assumed to apply, values as high as 5160 M^{-1} cm^{-1} have been reported for spinach plastocyanin in experiments at 25 K (*56*).

A detailed analysis of the UV–VIS spectrum of (spinach) plastocyanin in the Cu(II) state has been reported (*56*). A Gaussian resolution of bands at 427, 468, 535, 599, 717, 781, and 926 nm is indicated in Fig. 7. Detailed assignments have been made from low-temperature optical absorption and magnetic circular dichroic (MCD) and CD spectra in conjunction with self-consistent field Xα-scattered wave calculations. The intense blue band at 600 nm is due to the S(Cys) $p\pi \rightarrow d_{x^2-y^2}$ transition, which is intense because of the very good overlap between ground- and excited-state wave functions. Other transitions which are observed implicate, for example, the Met (427 nm) and His (468 nm) residues. These bonds are much less intense. The low energy of the d_{z^2} orbital indicates a reasonable interaction between the Cu and S(Met), even at 2.9 Å. It is concluded that the S(Cys)—Cu(II) bond makes a dominant contribution to the electronic structure of the active site, which is strongly influenced by the orientation of this residue by the

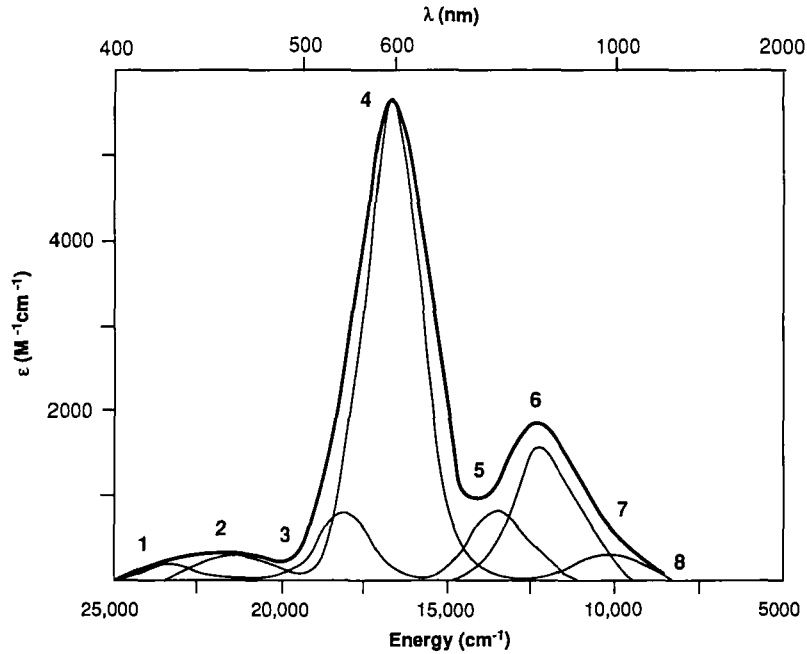

FIG. 7. The Gaussian resolution of the Cu(II) UV–VIS absorption spectrum (25 K) of spinach plastocyanin, as in Ref. *56*. The eighth band is not resolved in this spectrum.

protein backbone. Such changes affect spectroscopic features, and may also contribute to variations in reduction potentials and electron transfer reactivity of the different proteins.

VIII. Reduction Potentials

The reduction potentials of different plastocyanins increase as the pH is decreased below 7 (Fig. 8), due to protonation of His 87 at the Cu(I) active site and resultant redox inactivation. Much of this information has been obtained from rate constants (*18, 57, 58*). The reduction potential of the basic *A. variabilis* plastocyanin is noticeably smaller than for plastocyanins from higher plant and green algal

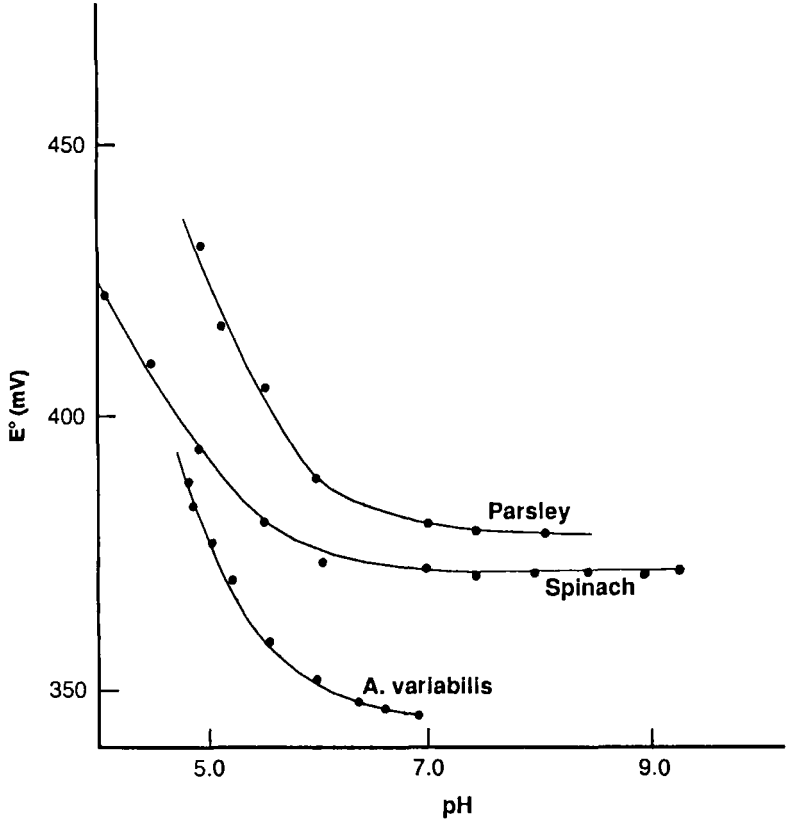

FIG. 8. Variation of reduction potential with pH for the spinach, parsley, and *Anabaena variabilis* plastocyanins, the PCu(II)/PCu(I) couple.

sources. At pH 5.0, reduction potentials are >400 mV (still increasing). There is little remaining redox reactivity of the Cu(I) protein at pH 4.5.

The variation of midpoint potentials of *P. aeruginosa* azurin with pH (Fig. 9) has been determined using the $[Fe(CN)_6]^{3-/4-}$ couple (*59*). From a fitting of E^0 values at different $[H^+]$ to Eq. (1),

$$E^0 = E' + \frac{0.059}{n} \log\left(\frac{K_{red} + [H^+]}{K_{ox} + [H^+]}\right) \quad (1)$$

where E' is the reduction potential limit at low pH, and K_{red} and K_{ox} are acid dissociation constants for the Cu(I) and Cu(II) protein, respectively, values of pK_{red} of 7.4 and pK_{ox} of 6.1 are obtained. Corresponding values, determined from the variation of rate constants with $[H^+]$ for the reactions of *P. aeruginosa* azurin, i.e., ACu(I) + $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ + ACu(II), are pK_{red} of 7.1 and pK_{ox} of 6.1 (*60*). Values of E^0 obtained from such kinetic studies indicated in Fig. 9 are in satisfactory agreement with the titration results. It has been shown by NMR that protonation/deprotonation of the nonligated His 35 residue, giving an independently determined pK_{red} of ~7, results in conformational changes in the vicinity of the Cu (*61*). Effects of pH on the EPR spectrum of the Cu(II) protein have also been noted (*62*). The conserved His 35 is near to but not at the surface of the protein (*8*). Two strands of

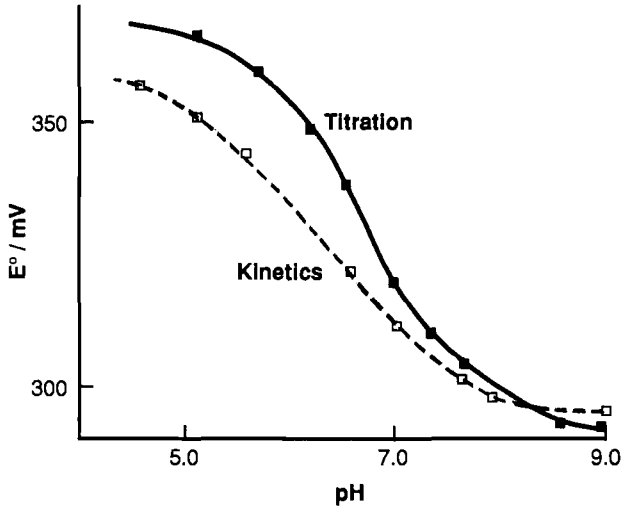

FIG. 9. Variation of reduction potential with pH for the *Pseudomonas aeruginosa* azurin ACu(II)/ACu(I) couple from titrations with $[Fe(CN)_6]^{3-/4-}$ (■), and from rate constants for ACu(I) + $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ + ACu(II) (□).

peptide cross over and separate it from solvent, and these would have to move apart to allow access to the imidazole ring. This could of course conceivably occur on interaction with a redox partner at this site. However, what is probably most influential is that His 35 and ligated His 46 are in close proximity with some overlapping of the imidazole rings. Following protonation of His 35, a conformational change is reported to give a ~1-Å variation in the distance of the Cu to protons on His 35 (*63*). It appears that protonated His 35 transforms from a conformation where it cannot ionize to one in which it can. On present evidence, changes in redox reactivity occurring as a result of protonation are not major. Rate constants for the reaction with cytochrome c_{551} are, for example, only weakly dependent on pH. However, the dependence of E^0 for the cytochrome c_{551}(III)/(II) couple on pH is very like that shown for ACu(II)/(I). As in the case of plastocyanin cytochrome *f*, the reaction is remarkable for its rapidity in both directions, with the rate constant for ACu(I) + cytochrome c_{551}(III) of $6.1 \times 10^6\ M^{-1}\ \text{sec}^{-1}$, and for the reverse $7.8 \times 10^6\ M^{-1}\ \text{sec}^{-1}$ (*64*). Also, the self-exchange reaction of ACu(I) and ACu(II) shows only a small (~25%) dependence on pH over a wide range (4.5–9.0) (*63*). Interestingly, the His 35 of azurins from *A. faecilis* and *A. denitrificans* do not protonate (*64*). In the latter case, from the crystal structure (*8*), it has been noted (*63*) that the cleft in which the His 35 residue is located is closed off by a salt bridge and is less accessible to solvent.

Substitution of Se for S in Met 121 results in an increase in reduction potential of azurin from *P. aeruginosa* by 30 mV (*65*). On changing Met 121 to Leu 121 by site-directed mutagenesis, the reduction potential is increased by 70 mV (*66*). Also by site-directed mutagenesis, replacement of the conserved Met 44 by Lys in the hydrophobic region results in a 40- to 60-mV increase in reduction potential (*67*).

Although the Cu(II) form of *T. versutus* amicyanin is stable down to pH 4, the reduced form begins to denature below pH 6.4 (*68*). An active site protonation of the Cu(I) protein similar to that of plastocyanin, but with pK_a 7.18, affects the reduction potential.

Stellacyanin (184 mV) (*69*) and umecyanin (283 mV) (*70*) have the smallest reduction potentials in Table I. As yet there is no X-ray structural information for either protein. However, stellacyanin has no methionine in its sequence (*71*), and umecyanin has no methionine after position 74 (*72*), indicating coordination of some other group. From recent ^{1}H NMR relaxation measurements on Co(II)-substituted stellacyanin, further information has been obtained regarding the structure of the metal-binding region 71. There are strong indications that a second S atom is binding the metal, this being the long sought

after fourth ligand. The model suggests that this belongs to Cys 59, which with Cys 93 forms a disulfide bridge. A potential fifth ligand, the amide O atom from Asn 47, is also proposed. A case had been made earlier for involvement of Gln 97 (*13*).

Rusticyanin has a high reduction potential (680 mV), which is similar to that for the Type 1 Cu center in fungal as opposed to tree laccase (785 mV) (*73*). This trend is so far unexplained. From the sequence and EXAFS studies, His-Cys-His-Met coordination is a reasonable possibility for rusticyanin (*55*). It may well be that the reduction potential is determined by effects of a polypeptide backbone on Cu—S(Cys) and Cu—S(Met) bond distances and the Cu ligand field (*74*). If this is the case, however, rusticyanin would be expected to have one or both Cu—S distances shorter than in other blue copper proteins, which is not borne out by information from EXAFS (Table IV). A further possibility that the Cu(I) form is three-coordinate, as in the case of plastocyanin at low pH (Fig. 2), has no strong support at present (*75*).

It is also noted that rusticyanin has three additional histidines (five in all). An increase in reduction potential for Rieske's [2Fe–2S] protein (350 mV) as compared to that for chloroplast [2Fe–2S] ferrodoxins (−400 mV) has been explained by the coordination of two histidines instead of two cysteines (*76*). In the case of the high-potential [4Fe–4S] protein, the reduction potential of 350 mV, compared to that for [4Fe–4S] centers in bacterial ferrodoxins (−400 mV), is accounted for by a different redox state change. This is made possible by H bonding and/or the more buried nature of the [4Fe–4S] cluster (*77, 78*). On present evidence, neither of these possibilities would seem to explain the high E^0 of rusticyanin. Another so far unexplained difference in the case of rusticyanin is its stability at pH 2, which is the working pH in its *in vivo* reaction with an acid-resistant cytochrome and aqua Fe^{2+} (*47, 48*). An X-ray crystal structure of rusticyanin is required to help understand these different properties.

IX. Active-Site Protonations

As already indicated, at pH <7 the Cu(I) form of plastocyanin becomes less reactive (*49, 57, 58*). At pH 4.5 the reactivity is <10%. Crystallographic studies on the Cu(I) form of poplar plastocyanin at six different pH values in the 3.8–7.8 range have confirmed this effect as an H^+-induced dissociation of His 87 from the Cu(I) (Fig. 10) (*4*). At low pH values the Cu(I) becomes trigonally coordinated by His 37, Cys 84, and Met 92. The Cu—S(Met) distance decreases to 2.52 Å, a respect-

able bonding distance. Most of the rearrangement can be attributed to the movement of the Cu(I) by 0.68 Å within the Cu site. The net effect is to increase E^0 as illustrated in Fig. 8. Values of pK_a for this protonation have been determined by NMR and kinetic studies, and are <5.0 for spinach (average 4.9), French bean (4.7), and poplar (4.7), but ≥5.0 for parsley (5.6) and *S. obliquus* (5.2) plastocyanin, which have deletions at positions 57 and 58 (*57*).

With $[Co(phen)_3]^{3+}$ as oxidant for PCu(I), rate constants determined by the stopped-flow method approach zero at low pH, consistent with zero reactivity of the trigonally coordinated Cu(I) form. However, with $[Fe(CN)_6]^{3-}$ as oxidant there is sometimes difficulty in fitting rate constants to the relevant $[H^+]$ dependence, which leaves open the question as to whether the rates actually become zero (*57*). Whereas $[Co(phen)_3]^{3+}$ is believed to react with PCu(I) at both the remote (acidic) and the adjacent (hydrophobic) patches, $[Fe(CN)_6]^{3-}$ reacts predominantly at the latter. The instability of PCu(I) in solution at pH < 4.5 makes it difficult to settle this issue conclusively. The range of studies has been extended using the pH-jump method in which protein, at high pH (with relatively small concentration of buffer), is stopped-flow mixed with the redox reagent at low pH (with excess buffer), and this approach has been used more extensively in recent studies.

Of the other blue copper proteins, only amicyanin shows a similar effect of pH (*79*), and a pK_a of 7.18 has been obtained for the Cu(I) state. As with plastocyanin, no corresponding effect is observed for Cu(II) amicyanin, at least down to pH 4.5. The physiological relevance in the case of both proteins is at present unclear. Because in photosynthesis the pH of the inner thylakoid is less than 5.0, one possibility is that this is related to proton transport. Alternatively, it quite simply may be a control mechanism for electron transport.

Crystal structure information (*4*) for poplar plastocyanin in the Cu(I) state at low pH has indicated the existence of two conformers. The

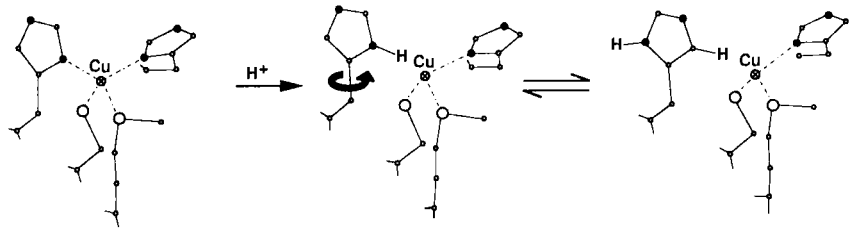

FIG. 10. The H^+-induced dissociation of Cu(I)—N(His 87) of plastocyanin (and amicyanin), and existence of two conformers of the protonated form (*4*).

second form is generated by rotation of the uncoordinated N^{δ}-protonated imidazole ring of His 87 by 180° about the C^{β}—C^{γ} bond. The detection of the second conformer is the result of accurate determination of two different positions of a solvent H_2O in the crystal structure. At pH 3.8 the interpretation requires the imidazole of His 87 to have rotated and the positions of the H_2O to shift so that it can H bond to the newly acquired proton of imidazole N^{δ}. The precision of the crystal structure at pH 3.8 is not sufficient to distinguish whether a mixture of the two conformers is present or exclusively the second conformer is present. Rotation of the imidazole is also favored by the formation of an N—H·····O (Pro 36) H bond. As a part of the pH-dependent changes, the Pro 36 side chain flips from the C^{γ}-exo to C^{γ}-endo conformation. Similar changes are observed when the Cu of PCu(II) is replaced by Hg(II), and also when the apoplastocyanin form is generated. The flexibility of Pro 36 is able to explain an apparent inconsistency, namely, the existence of a tightly packed active site structure, and the occurrence of the His 87 rotation as described.

The H^+-induced changes at the amicyanin active site have been studied kinetically by NMR (*68*). The reaction scheme (in D_2O) can be written as in Eq. (2),

$$\mathrm{A} \underset{k_{-1}}{\overset{k_1[\mathrm{D}^+]}{\rightleftharpoons}} \mathrm{B} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{C} \qquad (2)$$

where B and C are the two protonated forms, and is illustrated in Fig. 10. From the numerical analysis described, $k_1 = 3.7 \times 10^{12}\ M^{-1}\ \mathrm{sec}^{-1}$, $k_{-1} = 2.7 \times 10^5\ \mathrm{sec}^{-1}$, $k_2 = 250\ \mathrm{sec}^{-1}$, and $k_{-2} = 2100\ \mathrm{sec}^{-1}$. Activation parameters are also reported. The negative $\Delta H_1^{\ddagger}$ value of -25.0 kJ mol^{-1} for k_1 and the large numerical value of k_1 indicate that the reaction is not a single process, but is composite and involves at least one prior equilibrium.

X. Charge on Proteins

Blue copper proteins are globular and water soluble, with charged residues at the surface. Isoelectric (p*I*) values are an indication as to the extent (and sign) of the overall charge. Assuming no H bonding, the charge on proteins at pH > 7 can be estimated from the number of acidic (aspartate and glutamate), and basic (lysine, arginine, and histidine) residues at pH ~7. The pK_a of surface histidines, which can be in the range 5.0–8.0, is sometimes a complication leading to some uncer-

tainty in charge assignment. The active site charge has also to be considered. Higher plant and green algal plastocyanins are, for example, acidic, with p*I* values close to 4.2, and overall charge at pH > 7 is conserved at -9 ± 1 for PCu(I). An inspection of the sequences in Fig. 1 reveals that the positions of (for example) aspartate and glutamate residues (D and E) do vary. However, there is high conservation in the range 31–45, which includes the acidic patch 42–45, and at the acidic patch 59–61, which with 42–45 flanks a conserved (surface) tyrosine at position 83. Substitution of Tyr 83 by Phe in the case of *S. obliquus* does not appear to produce any change in reactivity. These various residues define the remote site that is believed to be functionally important. Even so, there are variations in the number of acidic residues, e.g., for spinach, Asp-Glu-Asp-Glu at 42–45 and Glu-Glu-Asp at 59–61 (4 and 3); for poplar, Asp-Glu-Asp and Glu-Glu-Asp (3 and 3); and for parsley, Asp-Glu-Asp-Glu and Glu 61 (4 and 1). In the latter case there are nearby deletions at positions 57 and 58. In spite of these changes, rate constants for a variety of redox partners, including the reduction of PCu(II) with $(Co(phen)_3]^{2+}$ and cytochrome *f*(II) (*80, 81*), which are believed to occur at least 50% and probably in the case of cytochrome *f* close to 100% at the remote site, show little variation (Table V). With $[Co(phen)_3]^{3+}$ as oxidant for PCu(I), some discrimination is apparent in that saturation (or limiting) kinetics, which is consistent with a favorable charge interaction to give the noncovalently bound adduct PCu(I), $[Co(phen)_3]^{3+}$, are observed with parsley (charges 4 and 1 at the remote

TABLE V

SPACING OF COORDINATED AMINO ACIDS IN THE SEQUENCES OF DIFFERENT BLUE COPPER PROTEINS

Protein	Coordinating amino acids				Intervening amino acids
Plastocyanin	His 37	Cys 84	His 87	Met 92	46, 2, 4
Azurin	His 96	Cys 112	His 117	Met 121	65, 4, 3
Pseudoazurin	His 40	Cys 78	His 81	Met 86	37, 2, 4
CBP[a]	His 39	Cys 79	His 84	Met 89	39, 4, 4
Amicyanin	His 47	Cys 86	His 89	Met 92	38, 2, 2
Rusticyanin	His 74[b]	Cys 127	His 132	Met 137	52, 4, 4
Stellacyanin	His 46	Cys 87	His 92	Glu 97[c]	40, 4, 4

[a] Cucumber basic protein.

[b] Alternatives are His 39 or His 45. Sequence information is from R. P. Ambler and J. W. Ingledew (unpublished work).

[c] As proposed in Ref. *13*.

site) (*49, 80*), spinach (4, 3) (*49*), and French bean (4, 3) (*82*), but not with poplar (3, 3) (*83*), *S. obliquus* (3, 2) (*57*), and *A. variabilis* (1, 0) (*58*) forms. This suggests that the four acidic residues at 42–45 are important in giving rise to saturation kinetics at $I = 0.10\ M$ (NaCl). At lower ionic strengths, with the increased tendency of two oppositely charged centers to associate, it is likely that saturation kinetics is observed in all cases. This has been illustrated in the case of the plastocyanin PCu(II) reduction by cytochrome *c*(II), which, in spite of favorable overall charges of −8 and +8, does not give evidence for saturation kinetics at $I = 0.10\ M$ (NaCl), but does so at lower ionic strengths (*82*).

The p*I* of the basic plastocyanin from the blue–green algae *A. variabilis* is 8.5 in the Cu(II) state (*58*). There are approximately the same number of charged residues in this protein, but fewer negative charges. There is no acidic patch, as observed in the case of all other plastocyanins, or other localities of high charge. In the case of *A. variabilis, S. obliquus,* and other algae, there is a soluble *c*-type cytochrome, rather confusingly referred to in the literature as c_{552}, c_{553}, c_{554}, and *small* cytochrome *f*. This protein, referred to here as cytochrome c_{553} ($M_r \sim 11{,}000$) (*84*), has the same role and functions alongside plastocyanin. The cytochrome is unknown in higher plants, where it appears to have been entirely replaced by plastocyanin. Its role, equivalent to that of plastocyanin, is reflected in the similar size, isoelectric point, and reduction potential. Thus the p*I* values vary in a parallel fashion for the cytochrome c_{553}'s (8.9 and 4.1 for *A. variabilis* and *S. obliquus,* respectively) and plastocyanins (8.5 and 4.2) from the same source. If during growth Cu is not sufficient to support plastocyanin accumulation in amounts required for photosynthesis, the cell makes use of Fe in the form of cytochrome c_{553} instead. Comparisons in the reactivity of cytochrome c_{553} and plastocyanin have been made (*84*).

The distribution of charge on the azurins is quite different from that of the plastocyanins, a negative residue generally having a positive residue nearby.

A final point concerns the variability in number of amino acids separating the residues coordinating the Cu active site, and the possible controlling influence this might have. Available information is summarized in Table VI. In the case of azurin and pseudoazurin, there is the additional influence of the peptide C=O on coordination at the Cu. All of the blue copper proteins have similar features, with coordination of the copper to one His from the first part of the sequence, and three other residues from the latter part. At present there appears to be no

TABLE VI

COMPARISON OF RATE CONSTANTS (25°C) FOR THE REDUCTION OF DIFFERENT PLASTOCYANINS PCu(II) WITH $[Co(phen)_3]^{2+}$ AND *Brassica* CYTOCHROME f^a

Source	$10^{-5}\ k_{Co}$ (M^{-1} sec^{-1})[b]	$10^{-5}\ k_{cyt}$ (M^{-1} sec^{-1})[c]	Ref.
Parsley	0.025	200 (110)	*35, 49*
Spinach	0.032	163 (115)	*49, 83*
Poplar	0.031	—	*82*
Scenedesmus obliquus	0.022	— (78)	*57, 83*
Anabaena variabilis	0.0021	4.0 (2.8)	*58, 83*

[a] I = 0.10 M (NaCl).
[b] pH 7.0
[c] pH 7.5 (pH 5.0 values in parentheses).

relationship between the number of amino acids bridging the ligating residues and various properties (e.g., E^0) of the proteins. Six of the nine residues, Cys 84 to Met 92, and not only the Cys-His-Met residues, are invariant in the 25 plastocyanin sequences (Fig. 1). One of these is the proline at position 86. Moreover, Tyr (or Phe) is conserved at 83. A particularly striking feature in the case of amicyanin is the tighter spacing of the coordinating residues.

From X-ray crystal structures the N^δ of the imidazole rings of the two histidine residues are coordinated to the Cu in plastocyanin (*3, 4, 7*), azurin (*8*), pseudoazurin (*12*), and CBP (*13*). However, in studies on Co(II)-substituted stellacyanin (*71*), it has been demonstrated that both histidines bind the metal via the N^ε atom. Similar differences have been observed in the case of binuclear Fe proteins for example. Thus in ribonucleotide reductase the N^δ of histidine is coordinated, whereas in hemerythrin it is the N^ε atom which is involved (*85*). In carbonic anhydrase the two coordinated imidazoles have N^δ and N^ε atoms respectively bonded to the same Zn (*85*). The differences are most likely attributable to steric factors involving the polypeptide.

The orientation and manner of coordination of imidazole rings is a further possible control which may determine active-site properties.

XI. Self-Exchange Rate Constants

Self-exchange rate constants have been determined for *P. aeruginosa* and *A. dinitrificans* azurin (*63*) and *T. versutus* amicyanin (*68*) by NMR line broadening, and for *Rhus vernicifera* stellacyanin by EPR

TABLE VII

SELF-EXCHANGE RATE CONSTANTS FOR Cu(I) AND Cu(II) STATES OF DIFFERENT BLUE COPPER PROTEINS[a]

Source	k_{exp} (M^{-1} sec^{-1})	Technique	Comments
Azurin (*P. aeuriginosa*)	9.6×10^5 (pH 4.5) 7.0×10^5 (pH 9.0)	NMR	I not specified; Refs. *63, 64*
Azurin (*P. aeuriginosa*)	2.4×10^6 (pH 5.0)[b]	EPR	22°C; Ref. *87*
Azurin (*A. denitrificans*)	4.0×10^5 (pH 6.7)	NMR	Ref. *68*
Amicyanin (*T. versutus*)	1.3×10^5 (pH 8.6)[c]	NMR	I = 0.05 M; small dependence only on I
Stellacyanin	1.2×10^5 (pH 7)	EPR	I = 0.22 M, 20°C; Ref. *86*
Plastocyanin	$<3 \times 10^3$ (pH 6)	NMR	$I \rightarrow 0$ (see text); Ref. *88*

[a] At 25°C, except as stated.
[b] Error ± 1.0×10^6.
[c] Protonated Cu(I) form, pK_a 7.18, gives $k < 1 \times 10^3$ M^{-1} sec^{-1}; see Ref. *68*.

(*86*) (Table VII) (*86–88*). The *P. aeruginosa* azurin self-exchange has also been determined by EPR (*85*). The value obtained for spinach plastocyanin from NMR studies at pH 6.0 (25°C) is an upper limit (*88*). The latter is slow because of the repulsion of negative charge on the two reactants (typically −8 and −9). The self-exchange is catalyzed by addition of $[Co(NH_3)_6]^{3+}$ or Mg^{2+} (or indeed K^+). Thus it is faster, 7×10^4 M^{-1} sec^{-1} (8 mM $[Co(NH_3)_6]^{3+}$), 2.7×10^4 M^{-1} sec^{-1} (20 mM $MgCl_2$), and 4×10^3 M^{-1} sec^{-1} (0.1 M KCl) with the screening provided by these reagents. The charge on stellacyanin, estimated at +7 for the Cu(I) state, is also high. Here, however, there are substantial (~40%) amounts of carbohydrate that may have the effect of decreasing the effect of the charge (*71*).

The situation with plastocyanin is similar to that observed for cytochrome *c*. Horse heart cytochrome *c* (M_r 12,400) has an estimated net charge of +8 and +9 for the Fe(II) and Fe(III) forms at pH 7, and the self-exchange rate constant (30°C) is 3.1×10^3 M^{-1} sec^{-1} at pH 7.5, I = 0.10 M (*89, 90*). In the case of cytochrome c_{551} on the other hand the net charge is much less. Thus for both *P. aeruginosa* and *Pseudomonas stutzeri* cytochrome c_{551} the charge is estimated at −1 for the Fe(II) protein. Self-exchange rate constants for *P. aeruginosa* determined by NMR are 1.2×10^7 M^{-1} sec^{-1} (at pH 7.0, 0.95 M phosphate, 42°C) (*91*) and 6×10^6 M^{-1} sec^{-1} (at pH 5.5, unbuffered, 27°C), respectively (*92*).

The rate constant for self-exchange between aqua Cu^+ and Cu^{2+} has not been determined, but by indirect means is estimated at 1×10^{-5} M^{-1} sec^{-1} (*93*). A slow self-exchange is expected for two ions of different geometry (here tetrahedral and tetragonally distorted octahedral), resulting in high reorganization energy requirements. Self-exchange

rate constants for the Cu proteins (Table VII) are more favorable because of the matching geometries of the two states. From the plastocyanin and azurin crystal structure information (Tables II and III), the Cu(I) to Cu(II) change is accompanied by an average decrease in bond length (Δd) of 0.06 and 0.08 Å, respectively. In the case of the often quoted $[Ru(NH_3)_6]^{2+/3+}$ (t_{2g} transfer) and $[Co(NH_3)_6]^{2+/3+}$ (e_g transfer) self-exchange processes, the Δd values are 0.04 and 0.22 Å (for each of the six bonds), and rate constants are 3.0×10^3 M^{-1} sec^{-1} and $<10^{-5}$ M^{-1} sec^{-1} (the latter in 10 M NH_3), respectively (*93, 94*).

A further influence on electron exchange is the ligand type present. Thus with bidentate aromatic 2,2′-bipyridine and 1,10-phenanthroline ligands (L), the $[RuL_3]^{2+/3+}$ self-exchange rate constants are $\sim 1 \times 10^8$ M^{-1} sec^{-1} (*95, 96*). In the case of the copper proteins the imidazole and S-donor ligands presumably have similar beneficial effects.

XII. Electron Transfer Routes

In the case of azurin the copper atom is some 7 Å from the surface of the molecule, and is separated from it by the imidazole ring of His 117 (*8*). The self-exchange between Cu(I) and Cu(II) forms is believed to occur via these two hydrophobic regions, the total separation of the Cu atoms being ~14 Å. Support for this mechanism has been obtained by replacing the conserved nonpolar Met 44 of the hydrophobic region by Lys (*67*), NMR, EPR, and UV–VIS spectra have shown that the three-dimensional structure is not significantly changed by the mutation. The electron self-exchange is significantly affected, and the rate constant of 1×10^5 M^{-1} sec^{-1} at pH 9 decreases to $<1 \times 10^{-3}$ M^{-1} sec^{-1}.

Plastocyanin reactions are believed to occur at two different sites. The first is adjacent to the active site, and the second at the more remote acidic patch (*18*). It would be surprising if plastocyanin self-exchange were to occur by interaction of two highly charged remote acid patch regions, and a mechanism similar to that of azurin is more likely. The net charge on each reactant results in a less favorable electron transfer rate than in the case of azurin.

The remote site on plastocyanin consists of acidic residues 42–45 and 59–61 on either side of Tyr 83. Typically, positively charged complexes react ~50% at this site. Evidence has been obtained for cytochrome *c* (*97*) and cytochrome *f* (*35*) reacting more extensively (possibly exclusively) at this site.

The X-ray crystal structure of ascorbate oxidase (*21*) defines a route for electron transfer from the Type 1 Cu center to the Cu_3 site via connecting Cys and His residues. The Type 1 domain has structural

similarities to plastocyanin, in which Cys 84 and Tyr 83 define the corresponding electron-transfer route. It is not yet clear whether electron transfer proceeds via the connecting atoms in a through-bond process (involving superexchange), or by the more direct route involving through-space electron transfer. In other instances, wherein intramolecular electron transfer is observed to occur, for example, between a chemically attached Ru center and the active site (*98, 99*), there is not always a direct bonded route to consider. Paths can be identified with some short through space or through H bond contributions, in addition to through-bond electron transfer (*100*). Distance and driving force alone does not seem at this time to explain the magnitude of rate constants, which argues against the through-space mechanism (*101, 102*). Assessment of these various factors is still at an early stage.

XIII. Comparison with Rubredoxin

A brief comparison with rubredoxin is appropriate because this is the only other tetrahedral single-metal electron transport protein. The tetrahedral high-spin Fe is coordinated by four S(Cys) atoms (M_r 8000). Oxidation states Fe(II) and Fe(III) are implicated in the redox cycle (reduction potential −60 mV) (*103*). From early crystallographic information (*104*), it was reported that three of the Fe(III)—S bonds averaged 2.24 Å, with the fourth substantially shorter at 2.05 Å. Subsequent EXAFS studies indicated a spread of only 0.04 ± 0.05 Å (*105*). The Fe—S distance of 2.26 Å was reported to increase to 2.32 Å on reduction (Δd = 0.06 Å). More recently, further X-ray diffraction studies on the Fe(III) protein have indicated a much smaller spread of 0.10 Å in Fe—S bond lengths, which is possibly close to the accuracy of measurement (*106*). The self-exchange rate constant has not been determined by direct measurement, but is calculated to be in the range $(4–20) \times 10^6\ M^{-1}\ \text{sec}^{-1}$ using Marcus theory (*107*). There is no apparent conflict of geometries for Fe(II) and Fe(III), and this is a more straightforward case to consider than that of the blue copper proteins, although one that nature does not appear to use very extensively.

XIV. Summary

A number of techniques have contributed to the present understanding of the active site chemistry of blue copper proteins. Perhaps foremost is that involving X-ray crystallography. A wide range of physical

techniques including kinetic/mechanistic studies has also played a vital role. Further studies on some of the less extensively studied proteins in this class are now required for a fuller understanding of the variability and fine tuning of the blue copper active site.

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