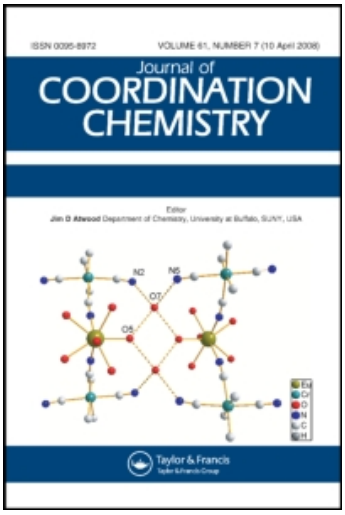

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A STRUCTURAL APPROACH TO THE COPPER SITES OF THE BLUE ELECTRON TRANSFER PROTEINS

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The copper-binding regions of plastocyanins and azurins are examined by the Chou-Fasman method. Similar structural features are found in the proposed copper-binding site for stellacyanin. These structural features are related to the copper-ligand bond lengths and to the reduction potentials of the blue copper proteins. The range of reduction potentials observed in these proteins is considered to be a function of the chelating peptide loop sizes and of the nature of the amino acid side chains in the loop region. A copper core of two histidines, a cysteine and a methionine is proposed for rusticyanin with a tighter chelate loop structure in the C-terminal region than is found in plastocyanin and azurin.

INTRODUCTION

In contrast to the cytochromes in which an iron ion bound to a porphyrin ring has only axial interactions with protein residues, the metal in the blue copper proteins is directly attached to functional groups of amino acid side chains.[1–3] The hydrophobic environment provided to the heme by protein side chains and the relative inaccessibility of the heme in the protein are thought to result in the more positive reduction potentials of heme proteins.[4–6] The blue copper proteins have high positive reduction potentials, seldom observed in low-molecular-weight copper complexes,[7,8] which make possible their electron transfer function. These potentials range from +184 mV for *Rhus vernificera* stellacyanin to +370 mV for spinach (*Spinacia oleracea*) plastocyanin at pH 7.1 and 7, respectively, for proteins from plant sources and also show wide variation among azurins isolated from a bacterial source.[9] For example, the reduction potential of *Pseudomonas aeruginosa* azurin is +300 mV while that of *Paracoccus denitrificans* azurin is +230 mV at pH 7.[9,10] The highest value observed for a blue copper protein is +680 mV for rusticyanin measured at pH 2.[11] No doubt the hydrophobic pocket in which the copper ion is located in plastocyanins and azurins and its relative inaccessibility accounts for a major portion of the value of the reduction potential in these proteins.

The crystal structure of an azurin to 3 Å resolution and that of poplar plastocyanin to 2.7 Å resolution have given an estimate of the distance from hydrophilic solvent to the copper ion as about 7.5 Å and about 6 Å, respectively, in the direction of their imidazole(histidine) ligand in the C-terminal region of the polypeptide chain.[2,3] This distance can also be estimated from the kinetic accessibility of the protein to redox reagents.[12] As the crystallographic estimate refers to the shortest distance from copper to solvent, however, while the electron transfer distances in redox reactions calculated by Gray *et al.*[12] refer to the distance from the coordination sphere of copper to the site on the protein surface at which electron transfer takes place, the two methods are only expected to converge to the same result when 3.7 Å is added to the values reported by Gray *et al.*[12] for the van der Waals contact diameter of imidazole (if this site is in the vicinity of the imidazole ligand of copper in the C-terminal region: histidine-87 in plastocyanin,[3] histidine-117 in azurin,[2] and probably histidine-92 in stellacyanin[13]). This histidine residue is in contact with solvent in plas-

tocyanin. The presence of a bulky phenylalanine residue in this region, surrounding the imidazole ring of histidine-117 and blocking access to copper, may result in a more hydrophobic environment in azurin.[14] There is no equivalent for this residue in plastocyanin.[14,15] Methionine-13 and methionine-44 of azurin (which are aligned with leucine-12 and phenylalanine-35 of plastocyanin[15] and which are at the rim of the hydrophobic copper pocket and away from it, respectively, in plastocyanin[16,17]) also shield the copper ion from solvent in this direction in azurin.[16] In contrast, the presence of the disulfide cystine-87-93 may make the analogous histidine ligand more accessible in stellacyanin and may allow for a less hydrophobic copper ion environment.[13]

The sites on the protein surface at which various redox reagents interact have not been thoroughly identified.[12,18-21] Positively charged reagents bind in a region of anionic residues in plastocyanin while negatively charged reagents bind close to the copper site.[21] The self-exchange rate constant, k_{11}^{∞}, of stellacyanin changes relatively little in its reactions with inorganic reagents. Using the average value for k_{11}^{∞} of stellacyanin to obtain an empirical estimate of the parameter C which appears in the expression for the distance of electron transfer, this distance can be calculated for the other blue copper proteins if the assumption is made that stellacyanin is in contact with all inorganic reagents through a peripheral carbon atom on the imidazole ring of its histidine ligand in the C-terminal region. This gives, as the distance from the imidazole ligand of copper to the protein surface, 2.8 Å in french bean (*Phaesolus vulgaris*) plastocyanin and 5.5 Å in *Pseudomonas aeruginosa* azurin in the reaction of the oxidized protein with (ethylenedinitrilotetraacetato)ferrate(2-).[12] To compare these distances to the crystallographic estimates measured from the copper ion, 3.7 Å must be added, giving 6.5 and 9.2 Å, respectively, for plastocyanin and azurin. Gray *et al.*[12] have estimated an error of the order of ±0.8 Å as a result of uncertainty in the values of rate constants. The distance in stellacyanin from copper to the surface of the protein has been estimated to be approximately 6 Å from ENDOR measurements.[22]

The comparable distances from the copper ion to hydrophilic solvent expected in different azurins and plastocyanins if their tertiary structures are the same does not provide an immediate answer to why the reduction potential varies considerably among azurins. The entatic state theory of Vallee and Williams[23] has attributed the high positive reduction potentials of all blue copper proteins to a tetrahedral-like geometry around the copper ion which lowers the potential barrier to electron transfer, but has not dealt with the variation of the reduction potential among the blue copper proteins. Thus, the inorganic chemist is confronted with the problem of a more extended description of the copper site in a blue copper protein (See the description of the crystal structure of popular plastocyanin at 1.6 Å resolution with 16.8% refinement[16]).

In this paper the method of Chou-Fasman[24] for assigning protein secondary structures is utilized to define some of the characteristics of copper sites in the blue single-copper proteins. This method is based on the analysis of a number of crystal structures and is claimed to have about 77% over-all accuracy in assigning helical, beta strand and random coil conformation of residues in proteins.[24] The claimed accuracy is better in the prediction of the secondary structure of regions, 88% for helical segments and 95% for beta-regions.[24] It was previously applied to the whole polypeptide chain of potato (*Solanum tuberosum*) plastocyanin along with other predictive methods and did not give the correct copper environment.[25] However, as shown in the Appendix, it was possible to reproduce the structural regions of poplar plastocyanin whose crystal structure is now known[3,16,17] and to suggest a reasonable structure for *Pseudomonas aeruginosa* azurin which can be compared to its crystal structure which has also been reported.[2,14] In any case, the Chou-Fasman method is used in this paper not to establish the structure of the blue copper proteins as such, but to establish structural features that may be associated with the copper-binding sites of these electron transfer proteins. The method is applied to the copper sites of eight azurins, thirteen plastocyanins and to a previously proposed copper site for stellacyanin.[13]

PROCEDURE

The Chou-Fasman[24] method assigns values to amino acid side chains in a protein that are a measure of their probability to appear in helical, beta strand, reverse turn or random coil regions. This probability is further associated with the tendency of different residues to promote the formation of such structural regions. The nomenclature used in the Tables is the one-letter notation for amino acid side chains[26] and:

H_{helix} = strong helix-promoter (not necessarily alpha-helix)
H_{beta} = strong beta-strand-promoter (beta sheet is included)
h_{helix} = less strong helix-promoter
h_{beta} = less strong beta-strand-promoter
I_{helix} = intermediate helix-promoter
I_{beta} = intermediate beta-strand-promoter
i_{helix} = weak helix-promoter
i_{beta} = weak beta-strand-promoter
b_{helix} = helix-disrupter
b_{beta} = beta-strand-disrupter
B_{helix} = strong helix-disrupter
B_{beta} = strong beta-strand-disrupter

The remarkable feature of these assignments is that the dual ability of many residues to form either helical or other types of structural regions is recognized. Thus, leucine (a strong helix-promoter that is also found in beta-strand regions quite often) is assigned a helix-forming probability $H_{helix} = 1.34$ and a beta-strand-forming probability $h_{beta} = 1.22$. Isoleucine (a strong beta-strand-promoter that does not often appear in helical regions) is assigned $H_{beta} = 1.60$ and $I_{helix} = 1.00$. Tyrosine, which has a probability greater than 1 of occurring in a reverse turn, is a helix-disrupter ($b_{helix} = 0.61$) and a beta-strand-promoter ($h_{beta} = 1.29$). Thus, not the individual residues, but the clusters in which they appear, determine the secondary structure assignment for the region and this assignment is based on both qualitative and semi-quantitative considerations.[24]

The copper cores discussed in this paper are shown in the photographs. They were built with CPK molecular models (The Ealing Corporation, South Natick, Massachusetts).

RESULTS

The Secondary Structure Assignments for Potato Plastocyanin

The assignments of secondary structure to the polypeptide chain of potato plastocyanin shown in Table I differ in some regions from those of Wallace.[25] Some of Wallace's[25] assignments overlap. For example, at the C-terminal end of the chain, he suggests reverse turns at 6–9 or at 8–11 (7–10 and 9–12 in Wallace's[25] numbering, which will not be used any more in this paper). In potato plastocyanin residues 6 and 7 are both glycine and a reasonable assignment is a reverse turn at 7–10, with glycine-6 being assigned as the last residue of the first beta strand (Table I). In the crystal structure of poplar plastocyanin[17] both glycine-6 and glycine-10 are located at the bend between the first and second beta strands, as may have been expected.

Wallace[25] suggested a reverse turn at 15–18. Residues 14 and 15 are strong beta-strand-promoters (phenylalanine and isoleucine, respectively). Therefore, in Table I residues 16–18 are assigned to random coil while 14 and 15 are part of the preceding beta strand.

Residues 19–25 were considered to be random coil by Wallace.[25] An alternative to the helix assignment at 19–26 in Table I might be beta structure at 19–22 ($(beta)_n = 1.16$,

TABLE I

Chou-Fasman[24] assignments of secondary structure for potato plastocyanin. The assigned structure for the segment is shown in the last column. The amino acid sequence data is from Ref. 25. For the one-letter notation for amino acids see Ref. 26.

Segment	Side chains	Chou-Fasman assignments for structural type		Final assignment for the segment
		Helical	Beta sheet	
1–6	LDVLLG	HihHHB	hiHhhi	beta strand
7–10	GDDG	BiiB	iiii	reverse turn
11–15	SLAFI	iHHhI	bhIHH	beta strand
16–18	PGN	BBb	bib	random coil
19–26	FSVSAGEK	hihiHBHI	HbHbIiBb	helix
27–30	ITFK	IihI	HhHb	beta strand
31–36	NNAGFP	bbHBhB	bbIiHb	random coil
37–41	HNVVF	hbhhh	bbHHH	beta strand
42–49	DEDEIPAG	iHiHIBHB	iBiBHbIi	random coil
50–68	VDASKISM	hiHiIIih	HiIbbHbH	helix
	AEEDLLNAAGE	HHHiHHbHHBH	IBBihhbIIiB	helix
69–75	TYSVTLS	ibihiHi	hhbHhhb	beta strand
76–79	EKGT	HIBi	bbih	reverse turn
79–85	TYTFYCA	ibihbiH	hhhHhhI	beta strand
86–91	PHQGAG	BhhBHB	bbhiIi	random coil
92–99	MVGKVTVN	hhBIhihb	HHibHhHb	beta strand

where $(beta)_n$ is the average value assigned by chou-Fasman[24] for n consecutive residues and $(helix)_n$ is similarly defined), followed by a reverse turn at 23–26. However, $(helix)_n = 1.14$ for the last four residues and, therefore, a reverse turn can not be assigned. Nevertheless, in the crystal structure of poplar plastocyanin the invariant glycine-24 residue is found at the bend between beta strands 2 and 3 and there is a single turn of alpha-helix at 51–54.[17] As potato plastocyanin and poplar plastocyanin differ in their amino acid sequence,[17,25] some structural differences may be expected between them, particularly with respect to the beginning and end of beta strand regions and the connecting segments. Although some helical structure may be accommodated by the protein in this portion of the chain without affecting the copper-binding site, plastocyanins have almost no helical secondary structure.[27] As discussed in the Appendix, the assignment of H_{helix} and B_{beta} for glutamate consistently leads to a wrong secondary structure assignment to segments of potato plastocyanin, poplar plastocyanin and *P. a.* azurin.

The residues that are close to the copper ion from the C-terminal end are mostly invariant in all plastocyanins. These residues are glycine-10, leucine-12, phenylalanine-14 and alanine-13 (which may be substituted by valine), described as being at the rim and side of the hydrophobic copper pocket by Freeman.[17] Thus, the invariant proline-16 residue may make possible the near-approach of the residues listed above to the copper site by providing for a break in regular structure (described as a bend in beta strand 2 in the crystal structure;[17] assigned to a three-residue random coil segment in Table I).

Another overlapping assignment by Wallace[25] is beta strand at 38–42 and helix at 40–46. The assignment of Table I is beta strand at 37–41 followed by random coil at 42–49. This section includes the copper ligand histidine-37 and structural characteristics of the copper-binding regions, shown in Table II, are discussed in the next section for all plastocyanins and azurins that are listed. Wallace[25] assigned helical structure to residues at 57–66. The assignment of Table I is helix at 50–68. This long segment corresponds to irregular beta strand 5 and the bend between strands 5 and 6 in poplar plastocyanin.[3,16,17]

More significant differences from Wallace's[25] assignments occur closer to the C-terminal end of the chain. He did not report a reverse turn at 76–79 followed by a beta strand at 79–85 (Table I) that would correspond to strand 7 of poplar plastocyanin.[3,16,17] As a result, he found cysteine-84 and histidine-87 to be too far apart to bind to the same copper ion.[25] The overlapping reverse turns at 78–81 and 80–83, $((ihhh)_{beta}$ and $(hhHh)_{beta})$, are misassigned and indicate a beta strand ($(beta)_n$ = 1.22 for these six residues). In Table I residue-78 is assigned as part of a reverse turn at 76–79 and the beta strand ends at alanine-85 ($(beta)_n$ = 1.25). These assignments are in general agreement with the crystal structure of poplar plastocyanin.[3,16,17]

The Copper Sites of Azurins, Plastocyanins and Stellacyanin

The copper ligands in azurin are cysteine-112, histidine-117, methionine-121 (strands 7 and 8 and the 7–8 connection close to the C-terminal end) and histidine-46 from the middle portion of the chain brought close to the copper site by the tertiary structure of the azurin.[2,14] These residues are invariant and are located in highly conserved segments of the protein chain in all azurins.[15] Similarly, in plastocyanin three of the copper ligands are located close to the C-terminal end (cysteine-84, histidine-87, methionine-92 from strands 7 and 8 and the 7–8 connection) and the fourth copper ligand is histidine-37 from strand 4.[3,16,17] These segments of the chain are also highly conserved.[15,27] A copper core of histidine-46,

TABLE IIA

The side chains of plastocyanins (Pc) and azurins (Az) in the regions of the ligands of copper.[15,27] The homologous side chains of stellacyanin (St) are included for comparison.[28,31] Invariant side chains are underlined. For the one-letter notation employed see Ref. 26. Azurin numbers are used.

	42													108																		
(1)	A	G	F	P	H	N	V	V	F	D	.	.		Y	S	F	Y	C	A	-	P	-	H	Q	G	A	G	M	V	G	
(2)	A	G	F				V								S	F	Y		A					Q					V		
(3)	A	G	F				V								T	F	Y		A					Q					V		
(4)	A	G	F				I								S	F	Y		S					Q					V		
(5)	A	G	F				V								K	F	Y		S					Q					V		
(6)	A	G	F				V								S	F	Y		S					Q					V		
(7)	A	G	F				V								S	F	Y		S					Q					V		
(8)	A	G	F				V								K	F	Y		S					Q					V		
(9)	A	G	F				V								S	F	Y		S					Q					V		
(10)	A	G	F				I								S	F	Y		S					Q					V		
(11)	A	G	F				V								K	F	Y		S					Q					V		
(12)	K	V	P				V								T	F	Y		E					R					V		
(13)	A	G	F				I								G	Y	F		E					Q					K		
(14)	N	V	M	G	H	N	W	V	L	S	.	.		Y	M	F	F	C	T	F	P	G	H	-	S	A	L	M	K	G	. .
(15)	N	V					W		L	T					A	F			S						S	A	M		K		
(16)	N	V					W		L	T					T	F			S						G	A	L		K		
(17)	N	V					L		I	S					G	F			S						I	S	M		K		
(18)	N	V					W		L	T					M	F			S						I	A	M		K		
(19)	A	A					V		V	S					A	F			S						W	S	I		T		
(20)	N	V					W		L	T					A	Y			S						F	A	L		K		
(21)	N	V					W		L	T					E	F			S						N	S	M		K		
(22)	R	R	F	-	H	N	V	-	-	D	.	.		Y	-	Y	I	C	G	V	P	K	H	C	D	L	G	Q	K	V	. .

(1) Shepherd's purse Pc; (2) lettuce Pc; (3) potato Pc; (4) poplar Pc; (5) spinach Pc; (6) dog's mercury Pc; (7) french bean Pc; (8) broad bean Pc; (9) vegetable marrow Pc; (10) dock Pc; (11) elder Pc; (12) blue-green algal Pc; (13) green algal Pc; (14) *P. a.* Az; (15) *P. denitrificans* Az; (16) *Bordetella bronchiseptica* Az; (17) *P. fluorescens B* Az; (18) *P. fluorescens C* Az; (19) *A. faecalis* Az; (20) *Alcaligenes sp.* Az; (21) *P. fluorescens D* Az; (22) *Rhus vernificera* St.

TABLE IIB

Chou-Fasman[24] assignments for helical structure for the side chains of plastocyanins, azurins and stellacyanin shown in IIA. Azurin numbers are used

	42	108
(1)	HBhBhbhhhi . .	bihbiH-B-hhBHBhhB
(2)	HBhBhbhhhi . .	bihbiH-B-hhBHBhhB
(3)	HBhBhbhhhi . .	bihbiH-B-hhBHBhhB
(4)	HBhBhbIhhi . .	bihbii-B-hhBHBhhB
(5)	HBhBhbhhhi . .	bIhbii-B-hhBHBhhB
(6)	HBhBhbhhhi . .	bihbii-B-hhBHBhhB
(7)	HBhBhbhhhi . .	bihbii-B-hhBHBhhB
(8)	HBhBhbhhhi . .	bIhbii-B-hhBHBhhB
(9)	HBhBhbhhhi . .	bihbii-B-hhBHBhhB
(10)	HBhBhbIhhi . .	bihbii-B-hhBHBhhB
(11)	HBhBhbhhhi . .	bIhbii-B-hhBHBhhB
(12)	IhBBhbhhhi . .	bihbiH-B-hiBHBhhB
(13)	HbhBhbIhhi . .	bBbhiH-B-hhBHBhIB
(14)	bhhBhbhhHi . .	bhhhiihBBh-iHHhIB
(15)	bhhBhbhhHi . .	bHhhiiBBh-iHhhIB
(16)	bhhBhbhhHi . .	bihhiihBBh-BHHhIB
(17)	bhhBhbHIi . . .	bBhhiihBBh-IihhIB
(18)	bhhBhbhhHi . .	bhhhiihBBh-IHhhIB
(19)	HHhBhbhhi . .	bHhhiihBBh-hiIhiB
(20)	bhhBhbhhHi . .	bHbhiihBBh-hHHhIB
(21)	bhhBhbhhHi . .	bHhhiihBBh-bihhIB
(22)	iih-hbh--i	b-bIiBhBIhiiHBhIh

TABLE IIC

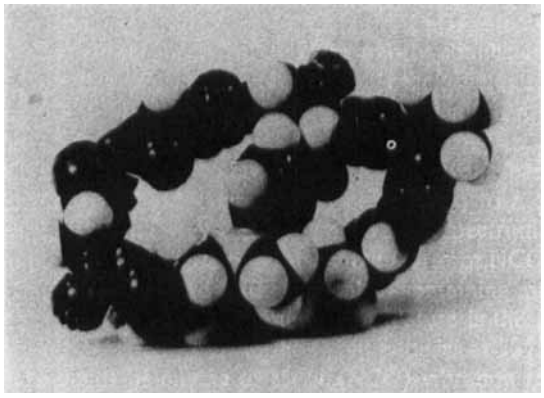
Chou-Fasman[24] assignments for beta-sheet for the side chains of plastocyanins, azurins and stellacyanin shown in IIA. Azurin numbers are used

	42	108
(1)	IiHbbbHHHi . .	hbHhhI-b-pbhiIiHHi
(2)	IiHbbbHHHi . .	hbHhhI-b-bhiIiHHi
(3)	IiHbbbHHHi . .	hhHhhI-b-bhiIiHHi
(4)	IiHbbbHHHi . .	hbHhhb-b-bhiIiHHi
(5)	IiHbbbHHHi . .	hbHhhb-b-bhiIiHHi
(6)	IiHbbbHHHi . .	hbHhhb-b-bhiIiHHi
(7)	IiHbbbHHHi . .	hbHhhb-b-bhiIiHHi
(8)	IiHbbbHHHi . .	hbHhhb-b-bhiIiHHi
(9)	IiHbbbHHHi . .	hbHhhb-b-bhiIiHHi
(10)	IiHbbbHHHi . .	hbHhhb-b-bhiIiHHi
(11)	IiHbbbHHHi . .	hbHhhb-b-bhiIiHHi
(12)	bHbbbbHHHi . .	hhHhhB-b-biiIiHHi
(13)	IiHbbbHHHi . .	hihHhB-b-bhiIiHbi
(14)	bHHibbhHhb . .	hHHHhhHbib-bIhHbi
(15)	bHHibbhHhh . .	hIHHhbHbib-bIHHbi
(16)	bHHibbhHhh . .	hhHHhbHbib-iIhHbi
(17)	bHHibbhHHb . .	hiHHhbHbib-HbHHbi
(18)	bHHibbhHhh . .	hHHHhbHbib-HIHHbi
(19)	IIHibbHHHb . .	hIHHhbHbib-hbHHhi
(20)	bHHibbhHhh . .	hIhHhbib-HIhHbi
(21)	bHHibbhHhh . .	hBHHhbHbib-bbHHbi
(22)	iiH-bbH--i	h-hHhiHbbbhihihbH

histidine-92, cysteine-59 and cystine-87-93 (with the sulfur atom from cysteine-87 coordinating) was proposed for stellacyanin.[13] The amino acid side chains in these segments[15,27,28] and the structural assignments for these segments are shown in Table II for thirteen plastocyanins, eight azurins and stellacyanin.

In all higher-plant plastocyanins listed, with the exception of plastocyanins from potato, shepherd's purse and lettuce, the region cysteine to methionine (shown in photograph 1) contains the residues cysteine-84, serine, proline, histidine, glutamine, glycine, alanine, glycine and methionine. In the case of plastocyanins from potato, shepherd's purse and lettuce serine-85 is replaced by alamine-85 and Chou-Fasman rules suggest no structural variation at the 7–8 connection. This allows the coordination of copper to the same side chains. With alanine after cysteine the assignment is random coil from proline-86 to glycine-91 (see Table I), whereas, in the other higher-plant plastocyanins, a random coil region is indicated starting at about serine-85 (Table III of the Appendix). In the crystal structure of poplar plastocyanin this region contains the cysteine-histidine-copper loop followed by a bend.[17] In fact, two consecutive reverse turns can be assigned at residues 85–88 $((bbbh)_{beta}; (iBhh)_{helix})$ and residues 89–92 $((iIiH)_{beta}; (BHBh)_{helix})$. The algal plastocyanins contain a glutamate residue in place of serine or alanine,[15,27] and a random coil region, as in the higher-plant plastocyanins, would be expected. Because of the presence of proline next to glutamate-85, a helix could not nucleate.

In the eight azurins listed the position after cysteine-112 contains either a serine or a threonine and is, in either case, probably the end residue or next to the end residue of beta strand 7. The next four residues are invariant but the residues between histidine-117 and methionine-121 show a large degree of variation in azurins.[15] In *P. a.* azurin, *P denitrificans* azurin and *Bordetella* azurin (entries 14–16) there is a helix- and beta-structure-breaking tetrapeptide at 122–125 and the assignment is helix from histidine-117 to methionine-121. In these azurins the proline and glycine residues preceding histidine-117 and the lysine and glycine residues following methionine-121 are assigned to random coil, with the last beta strand starting at residue-124 (See the Appendix for a complete assignment for *P. a.* azurin). In those azurins in which there is no structure-breaker at 122–125 a reverse turn is assigned

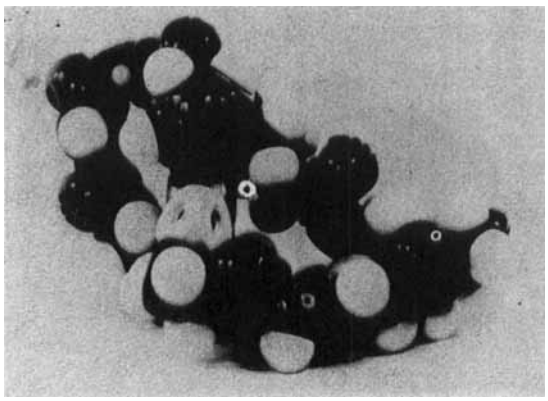


PHOTOGRAPH 1 *Plastocyanin.* The peptide backbone and the side chains that bind to copper are shown for the segment cysteine-84 to methionine-92. The copper ion is below the imidazole ring of histidine-87 at the center of the picture and it is blocked in this view by its sulfur(methionine-92) ligand. The methyl group of methionine-92 is partially blocking a view of the sulfur atom of cysteine-84. The ring that is partially visible at the top right is proline-86. The loops comprise a five- or six-residue random coil region (Tables I, IIC, III). The loop between cysteine-histidine-copper contains no bulky residues while the analogous larger loop in azurin has to accommodate a phenylalanine side chain.

at the invariant residues phenylalanine-114, proline-115, glycine-116 and histidine-117 ($(Hbib)_{beta}$; $(hBBh)_{helix}$). This assignment is made for *P. fluorescens B* azurin, *P. fluorescens C* azurin and *A. faecalis* agurin (entries 17–19). In *P. fluorescens D.* azurin (entry 21) the presence of two structure-breaking residues right after the right before histidine-117 result in a random coil assignment at 115–119 ($(BBhbi)_{helix}$; $(bibbb)_{beta}$). In all of these azurins the last beta strand starts after the reverse turn or random coil assignment. In *Alcaligenes sp.* azurin (entry 20) the segment from histidine-117 to the end of the polypeptide chain has $(helix)_n = 1.15$ and $(beta)_n = 1.09$. This would suggest the replacement of the last beta strand by two helixes in the azurin (Glycine-123 can not occur in the inner helix[24]). The copper-binding loops of azurins are shown in photograph 2.

Table II suggests that, although in plastocyanins and in azurins the ligands are the same, the cysteine(-84,112) to methionine(-92,121) region differs in that azurins appear to have a great deal more structure in this region in their description by Chou-Fasman assignments. This structural difference may be reflected in the different copper(II)-sulfur(methionine) bond lengths that have been observed in *P. a.* azurin by the EXAFS method[29] and in poplar plastocyanin by crystallography.[16] Furthermore, the substitutions in the histidine-methionine-copper loop of azurin may result in structural variations in the different azurins close to the copper site according to Chou-Fasman assignments. Helical character, perhaps one turn of helix, was reported in the crystal structure of *P. a.* azurin with a 3 Å resolution electron density map.[2,14] It was suggested that the structure of these residues might vary between an alpha-helix and a 3_{10}-helix or involve an intermediate character in different oxidation states of the protein. A recent experimental study of the peptide chains $(leu\text{-}gly)_{10}$ and of $((leu\text{-}gly)_4(ala)_{1\text{ or }2}(leu\text{-}gly)_4)$ has shown that the substitution of the middle (leu-gly) segment by one or two alanine residues results in a change from beta structure to helical.[30] For these chains $(helix)_n = 0.94$ and $(beta)_n = 1.02$ before substitution but $(helix)_n = 1.08\text{–}1.10$ and $(beta)_n = 1.02$ after substitution. Therefore, Chou-Fasman method would correctly predict that the alanine-substituted chains should be helical.[24]

If the side chain substitutions cause real structural variations in the different azurins in the histidine-methionine-copper loop, with varying degrees of helical character, and as Adman[14]



PHOTOGRAPH 2 *Azurin.* The peptide backbone and the side chains that bind to copper are shown for the segment cysteine-112 to methionine-121. The imidazole ring of histidine-117 is above the copper ion and the proline-115 ring is partially visible to the far right. The sulfur atom at the center front is that of cysteine-112. The region of many substitutions in the azurins between histidine-117 and methionine-121 (Table IIA) is the five-residue loop on the left. Only the beta-CH_2-group and a portion of one of the hydrogens on the gamma-CH_2-group of methionine-121 is visible in this view. This histidine-methionine-copper loop may have helical character (Tables IIC, IV) and is to be contrasted with the analogous six-residue loop in plastocyanin.

suggests, this loop may undergo some change in structure during oxidation-reduction, it may be inferred that the reduction potential of an azurin can be regulated to some extent by side chain substitutions in this loop.

In stellacyanin structural features similar to those of azurins and plastocyanins were searched for in those regions that show homology to these proteins. This provides some of the justification for the choice of histidine-92 and cysteine-87 end of the disulfide cystine-87-93 as copper ligands.[13] If cysteine-87 is assigned at the end of a beta strand, the residues between cysteine-87 and histidine-92 can be assigned to random coil ($(iHbbb)_{beta}$; $(BhBIh)_{helix}$). This places cysteine-93 at the start of the next (and last) beta strand. The cysteine-87 end of cystine-87-93 and histidine-92 can both bind to copper, forming, as in azurin, a six-residue loop.[13] The other two ligands in stellacyanin are suggested to be cysteine-59 and histidine-46.[13,28,31] A folding scheme has been suggested for stellacyanin which allows all four of these side chains to be located close to a copper core.[13] In the proposed structure cysteine-59 is the end residue of a beta strand and histidine-46 is part of a helical region.[13] This is a structural variation from azurins and plastocyanins.

As assigned in Table I, histidine-37 of plastocyanin is the first residue of a beta strand, preceded by a random coil region (see also Table III in the Appendix). Histidine-46 of azurin is located at a beta strand, between structure-disrupting glycine-45 and asparagine-47 residues (see Table IV in the Appendix). There is disruption is structure in the region of copper ligand in azurins and plastocyanins from different sources (Table II).

DISCUSSION

The blue electron transfer proteins are characterized by their high positive reduction potentials, strong charge transfer bands in the visible region and very small hyperfine splitting constants in the parallel region ($|A_{\parallel}|$) of predominantly axial EPR spectra.[9,32] The d-d transitions have been located between ~900 nm and 2000 nm,[33] and the small ligand field stabilization energy (LFSE) associated with these low-energy transitions suggest a distorted tetrahedral copper(II) site, in agreement with entatic theory.[23] A ligand field model with a 5–6° flattening of the tetrahedral angle was found to account for the d-d transitions of *P. a.* azurin, french bean plastocyanin and *Rhus vernificera* stellacyanin.[34] The calculated values of LFSE of $-6885\ cm^{-1}$ for azurin, $-6113\ cm^{-1}$ for plastocyanin and $-6152\ cm^{-1}$ for stellacyanin can not account for the large difference in the reduction potentials on the basis of the geometry of the copper ion, as proposed by entatic theory.[23] As pointed out by Bacci,[35] an approximately 6° flattening of a tetrahedral geometry around the copper ion also seems unrealistic. The crystal structure of poplar plastocyanin at 1.6 Å resolution to 16.8% refinement suggests much larger deviations from the tetrahedral value. A preliminary calculation in the angular overlap model that includes vibronic coupling and effects of covalency and d-p hybridization (Case III of reference 35), gives a 12.1° deviation from the tetrahedral angle along with a good fit to the d-d transitions of a generalized blue copper center. The LFSE is calculated to be $-6667\ cm^{-1}$ and is close to the value calculated by Gray et al[34] for azurin. The values of the EPR parameters, $g_{\parallel}$ and $g_{\perp}$, calculated by the angular overlap model are reasonable.[35] For $|A_{\parallel}|$ a value of 3.5 mK, somewhat low for azurin and plastocyanin but equal to the experimental value for stellacyanin[32] is obtained.[35] In contrast, the ligand field model gave a good fit to the values of $g_{\parallel}$ and $g_{\perp}$ but the calculated values of $|A_{\parallel}|$ were larger than 10 mK.[34] Values of $|A_{\parallel}|$ of this magnitude are observed in low-molecular-weight copper complexes when tetrahedral distortion of the copper(II) ion site occurs. In a definitive experiment the doping of two bis(N-alkylsalicylaldiminato)copper(II) complexes into planar palladium and tetrahedral zinc analogues of these complexes gave the result in both cases of reducing the $|A_{\perp}|$ value from 18.6 mK to 11.3 and 12.0 mK.[36] However, the blue

copper proteins have $|A_\perp|$ values lower than 10 mK (they are for *P. a.* azurin, french bean plastocyanin and *R. v.* stellacyanin, respectively, 6.0, 6.3 and 3.5 mK).[32,34] A value of $|A_\parallel|$ equal to 8 mK was obtained for a gamma-irradiated tetrakis(acetonitrile)copper(I) single crystal in which a tetrahedral copper(II) center is produced.[37] Thus, both a tetrahedrally-distorted geometry and soft ligands appear to be required to obtain $|A_\parallel|$ values lower than 10 mK. This is in agreement with Peisach and Blumberg's[38] analysis of the trends in the values of $g_\parallel$ and $|A_\parallel|$ when the ligands around a copper(II) ion is varied from a hard core (CuO_4) to a very soft core (CuS_4), with intermediate values for (CuN_xO_y), (CuN_xO_y), (CuN_xS_y) cores. A correlated study of the electronic spectrum and crystal structure of poplar plastocyanin attributes an elongated C_{3v} geometry and rhombic distortions to the copper(II) site.[39] Structural differences between the copper sites in plastocyanin and azurin were not explained.

It has been possible to prepare a copper complex that has all essential features of the copper core of the blue copper proteins by using an actual protein with a tetrahedral hole as the macrocyclic ligand.[40] The ligand in this case is the horse liver alcohol dehydrogenase and the tetrahedral hole is prepared by the removal of the catalytic zinc ion of the subunit.[40] For this copper(II)-reconstituted protein $|A_\parallel| = 3.0$ mK and the strongest charge transfer band is located at 620 nm ($\varepsilon = 2000$).[40] Interestingly, the value of $|A_\parallel|$ becomes 11.5 mK when pyrazole (a known inhibitor of the activity of horse liver alcohol dehydrogenase) is added. The ligands of the active-site zinc ion are two cysteines, a histidine and an exchangeable water molecule and it is thought that pyrazole coordinates to copper(II) as a fifth ligand.[41] The increase in the $|A_\parallel|$ value is consistent with a change in the geometry of the copper(II) ion in the copper(II)-reconstituted protein when pyrazole is added. Possibly, this is accompanied by other changes in the vicinity of the copper(II) ion.

The fact that the tetrahedral copper(II) site of the reconstituted horse liver alcohol dehydrogenase is unstable is also evidenced by autoreduction of this site in the absence of pyrazole in less than 24 hours.[40] As this zinc protein has no evolutionary kinship to the blue electron transfer proteins, it ought not be surprising that the special copper(II) ion environment of the electron transfer proteins can not be readily maintained even in a macrocycle as large as a protein. It was proposed that this is mainly accomplished by the four-to-six-residue peptide loops that form in the C-terminal region of the blue single-copper proteins and that the lower reduction potential of stellacyanin is a result mostly of its different peptide loop structure.[13] Such peptide loops are missing in the zinc enzyme (The side chain ligands of the active-site zinc ion are cysteine-46, cysteine-174 and histidine-67). Assuming that the copper(II) ion has the same ligands, in the absence of constraints by the protein it relaxes to a geometry more favorable to the copper(II) ion as the $Cu(NS_2O)$ core changes to a $Cu(N_2S_2O)$ core when pyrazole is added. Interestingly, a tetrahedrally distorted copper(II) ion environment can be stabilized in the protein in the presence of the coenzyme of horse liver alcohol dehydrogenase, oxidized nicotinamide adenine dinucleotide (NAD^+).[40] The exchangeable water molecule appears to be lost from the coordination sphere of the copper(II) ion in the presence of NAD^+, leaving behind a tetra-coordinated copper core.[41]

It now appears that neither a geometric description of the copper site as given by entatic theory[23] nor a description in terms of coordinating groups[33] is sufficient by itself to characterize this site. The same groups are bound to copper in azurin and plastocyanin.[2,3] The bond length of the copper(II)—nitrogen(imidazole) bond is 1.97 Å in oxidized *P. a.* azurin, 1.98 Å in oxidized french bean plastocyanin and 2.05 Å in reduced french bean plastocyanin, as determined by EXAFS.[29,42] These distances can be compared to the copper(II)—nitrogen(imidazole) bond distance of 2.00 Å found in tetrakis(imidazole)copper(II) diperchlorate used as the reference compound.[42] The equatorial copper(II)—nitrogen(imidazole) bond lengths in (1,8-diamino-3,6-dithiaoctane)(1-methylimidazole)copper(II) diperchlorate[43] and the sterically hindered dichlorotris(1,2-dimethylimidazole)copper(II)[43] are 2.011

and 2.145 Å, respectively. In the copper(I) dimeric complex with one histamine ligand bridging through imidazole and $-NH_2$ groups, dicarbonyltris(histamine)dicopper(I) ditetraphenylborate, the copper(I)—nitrogen(imidazole) bond lengths are 2.00 Å (chelating) and 1.97 Å (bridging).[45]

Two different copper—sulfur bond lengths, a somewhat short copper(II)—sulfur(methionine) bond length of 2.24 Å and a very short copper(II)—sulfur(cysteine) bond length of 2.10 Å is obtained by EXAFS for oxidized azurin.[29] This 2.10 Å copper(II)—sulfur(cysteine) bond length is also found in oxidized stellacyanin and plastocyanin,[46] and is corroborated by the crystal structure of poplar plastocyanin at 1.6 Å resolution to 16.8% refinement (2.13 Å).[16] However, the crystallographic bond length for the copper(II)—sulfur(methionine) bond is 2.90 Å in plastocyanin.[16] Clearly, despite the similarities in physicochemical properties, a difference exists in the coordination environments of copper in azurin and plastocyanin. Upon reduction, a 0.1 Å longer copper(I)—sulfur(cysteine) bond length, determined by EXAFS,[46,47] brings it closer to the normal range for copper—sulfur bonds.[48] The crystallographic bond lengths for reduced poplar plastocyanin[16] are 2.2 and 2.9 Å, respectively, for copper(II)—sulfur(cysteine-84) and —sulfur(methionine-92) bonds at pH ~7.8 (extrapolated from lower pH) (An average value for copper(I)—sulfur bond distance is ~2.34 Å[48]). This region is described as random coil by Chou-Fasman assignments in most plastocyanins and in all plastocyanins contains two invariant glycine residues and an invariant alanine residue (Table II). It may accommodate a change in the copper environment upon reduction at lower pH larger than the expected increase in the radius of the copper ion.

It appears that the peptide loops that chelate the copper ion in plastocyanin undergo some change at high and low pH. At acidic pH histidine-87 of poplar plastocyanin which is exposed to solvent becomes protonated and the copper(II)—imidazole(histidine-87) bond is broken, leading to the formation of a trigonal copper(I) complex.[16] Autoreduction was also observed in spinach plastocyanin at alkaline pH, accompanied by a gradual increase in the intensity of the hydrophobic fluorescent probe, 2-*p*-toluidino-naphthalene-6-sulfonate.[49] Takabe *et al.*[49] suggest that the hydrophobic region near cysteine-84 becomes exposed to solvent when spinach plastocyanin is incubated in alkaline solutions. In poplar plastocyanin there are hydrogen bonds between the side chain carbonyl oxygen of asparagine-38 (next to the copper ligand, histidine-37) and the backbone amide of serine-85 (next to the copper ligand, cysteine-84) and between the side chain of tyrosine-80 (at the start of beta strand 7) and the backbone carbonyl of asparagine-76 (at the bend between strands 6 and 7).[16,17] If there are similar hydrogen bonds in spinach plastocyanin, the removal of these constraints at alkaline pH may result in the reduction of the copper(II) ion as it relaxes from a distorted geometry to an expected tetrahedral geometry. This would imply that the 2.1 Å copper(II)—sulfur(cysteine) bond in oxidized plastocyanin is maintained by the chelating peptide loops of the C-terminal region and by hydrogen bonds. In the biochemical reduction of plastocyanin changes in the conformation of the side chains in the peptide loops around copper and adjustments in dihedral angles may be expected to occur. The integrity of the copper site itself must be maintained for facile outer-sphere electron transfer.[3,16]

In the azurins one turn of helix between histidine-117 and methionine-121 would place the histidine and methionine alpha-carbons along the same border with the alpha-carbon atoms about 6 Å apart.[50] The side chains may then be in a favourable position to form bonds to the copper(II) ion and the length of the copper(II)—sulfur(methionine) bond in *P. a.* azurin may result from this structural feature. It was suggested in the *Results* section that the many substitutions in the side chains between histidine-117 and methionine-121 might cause changes in the secondary structure of this loop. Structural variation may also be expected upon reduction according to Adman.[14] The relaxation time observed for the copper(II) center upon passing a laser beam through a solution of azurin is less than 5×10^{-13}

seconds and suggests that ligand rearrangement is not rate-determining for electron transfer.[51] That the copper(II) ion is poised by its protein environment is also indicated by the time constant for reverse charge transfer (1.6×10^{-12} seconds).[51] It seems reasonable to conclude that the copper(II)-sulfur(cysteine) bond in azurin, as in plastocyanin, is maintained at the short bond distance of 2.1 Å by the peptide loops that form around the copper ion in the C-terminal region.

It may now be asked if the similarities and differences in structure noted for azurin and plastocyanin do not also apply to stellacyanin. Assuming a general beta-barrel shape for the tertiary structure of stellacyanin also, it is possible, by means of Chou-Fasman rules, to suggest a secondary structure for this protein and a folding pattern that places the single cysteine residue of stellacyanin (cysteine-59), histidine-46 and histidine-92 in the same region of space.[13] The coordination of cystine-87-93 to copper along with histidine-92 would form a chelate loop involving six residues, which is a loop size also observed in azurin and plastocyanin. Resonance Raman data on stellacyanin and model compounds also support the presence of a disulfide ligand in stellacyanin.[52] It may be possible to attribute both the higher kinetic accessibility[12,18] and lower reduction potential[9] of stellacyanin to the presence of a disulfide ligand and to the difference in the peptide loops that form in this protein.[13]

At the other end of the range of reduction potentials observed for the blue copper proteins is rusticyanin, which has a potential of +680 mV.[11] It was proposed by Cass and Hill[53] that rusticyanin may have a copper core of two histidine and two methionine residues, as low-molecular-weight copper(II)-thioether complexes have been synthesized with such high reduction potentials.[54] It would seem, however, that the single cysteine residue that is found to be present in all blue copper proteins[9] is almost an obligatory ligand, bound to the copper(II) ion at a distinctively short bond distance[16,29] and giving rise to the intense charge transfer band in the 600-nm region.[33] Although highly intense low-energy charge transfer bands are also observed in copper(II)-thioether complexes,[54] the thiolato sulfur is redox-active while the thioether sulfur itself is not.[55]

It is the thesis of this paper that the function of the chelate loops (cysteine)$(x)_{4,6}$(histidine)$(x)_{5,6}$(methionine) is to keep the copper(II) ion from being reduced by its cysteine ligand while maintaining it in a constrained environment that gives it its high positive reduction potential of 0.2–0.6 V. The disruption from regular structure in the region of copper ligands is necessary for such peptide loops to form. A copper core of two histidines, one cysteine and one methionine may be a reasonable choice of ligands for rusticyanin if a tighter chelate loop structure than those in azurin and plastocyanin accounts for a portion of its reduction potential. This could be accomplished, for example, by a (cysteine)$(x)_4$(histidine)(x_5)(methionine) loop (or a variation) or by the substitution of bulky side chains.

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Appendix

STRUCTURAL ASSIGNMENTS FOR POPLAR PLASTOCYANIN

The assignments of secondary structure to segments of black poplar plastocyanin (*Populus nigra var. italica iso-I*) are shown in Table III and are in general agreement with its crystal structure when small differences, particularly with respect to the first or final residues of assigned segments, are ignored (see particularly reference 16 for a detailed description of the crystal structure).[3,16,17] It is not expected that these details of structure can be assigned precisely by any semi-empirical rules, such as Chou-Fasman rules.

In Table III proline-16 and the following residues to 26 are assigned to random coil. The crystal structure of poplar plastocyanin shows this proline to form a bend in beta strand 2, and, therefore, the beta strand residues at 19–22 are the continuation of strand 2. Both invariant glycine-34 and proline-36 are assigned to a six-residue bend between strands 3 and 4 as in potato plastocyanin (see Table I) and the copper ligand, histidine-37, is assigned to the beginning of beta strand 4. In the crystal structure proline-36 is said to be at the beginning of strand 4. The invariant asparagine-38 residue was found to form a hydrogen bond to the backbone amide of serine-85 through its side chain carbonyl oxygen. As this hydrogen bond does not involve the functional group of serine-85 (replaced by alanine in potato, lettuce and shepherd's purse plastocyanins, by glutamate in algal plastocyanins), it could exist in all plastocyanins.

TABLE III

Chou-Fasman[24] assignments of secondary structure for poplar plastocyanin. The assigned structure for the segment is shown in the last column. The amino acid sequence data is from Ref. 16. For the one-letter notation for amino acids see Ref. 26

Segment	Side chains	Chou-Fasman assignments for structural type		Final assignment for the segment
		Helical	Beta sheet	
1–6	IDVLLG	IihHHi	HiHhhi	beta strand
7–10	ADDG	HiiB	Iiii	reverse turn
11–16	SLAFVP	iHHhhB	bhIHHb	beta strand
17–26	SEFSISPGEK	iHhiIiBBHI	bBHbHbbiBb	random coil
27–30	IVFK	IhhI	HHHb	beta strand
31–36	NNAGFP	bbHBhB	bbIiHb	random coil
37–41	HNIVF	hbIhh	bbHHH	beta strand
42–49	DEDSIPSG	iHiiIBiB	iBibHbbi	random coil
50–63	VDASKISM	hiHiIIih	HiIbbHbH	helix
	SEEDLL	iHHiHH	bBBihh	
64–67	NAKG	nHIB	bIbi	reverse turn
68–75	ETFEVALS	HihHhHHi	BhHBHIIhb	helix
76–79	NKGE	bIBH	bbiB	reverse turn
80–84	YSFYC	bihbi	hbHhh	beta strand
85–91	SPHQGAG	iBhhBHB	bbbhiIi	random coil
92–99	MVGKVTVN	hhBIhihb	IIHibIHhIb	beta strand

The major disagreement between the assignments of Table III and the crystal structure of poplar plastocyanin is with regard to helical segments. According to the crystal structure there is only one turn of alpha-helix involving the residues 51–54.[3] However, Chou-Fasman method would assign 50–63 and 68–75 to helical structure.[24] These segments roughly correspond to strands 5 and 6.[17] The detached nature of these strands[3] and the lack of beta-character in "irregular strand 5"[16] were noted by Freeman. The helix assignments result from the presence of glutamate residues which are strong beta-strand-disrupters and strong helix-promoters according to Chou and Fasman.[24] However, H_{helix}, B_{beta} assignments for glutamate consistently give incorrect results for poplar plastocyanin, potato plastocyanin and *P. a.* azurin. It appears that there were hardly any charged residues in the beta-sheet regions of proteins from which the Chou-Fasman parameters were derived.[22] However, a number of aspartate and glutamate residues are present in the beta-barrel structures of poplar plastocyanin and *P. a.* azurin [2,3,14,16,17] As aspartate has similar Chou-Fasman helix and beta-sheet assignments (i_{helix} = 0.98; i_{beta} = 0.80)[24] it does not have a very large effect on the final assignment for a segment in general. In addition, aspartate residues have a high probability of occurring at a bend in the structure, where i_{helix} and i_{beta} assignments are appropriate.[24] In contrast, glutamate residues have a low probability of occurring at bends[24] and most of the glutamate residues of blue copper proteins are, therefore, expected to be located at beta strand regions. As a result the assignments of Chou-Fasman for glutamate (H_{helix} = 1.53; B_{beta} = 0.26)[24] give the wrong secondary structure assignment for regions in blue copper proteins particularly when they appear in clusters. An appropriate assignment which appears to make it possible to correctly assign almost all regions of secondary structure to different plastocyanins analogous to those of poplar plastocyanin is I_{helix} = 1.00 and I_{beta} = 1.00 for glutamate.[56]

In the neighborhood of the copper ligands in the C-terminal region (photograph 1) the invariant residues of the cysteine-histidine-copper and histidine-methionine-copper loops (proline-86, glycine-89, alanine-90, glycine-91) line the rim of the hydrophobic copper pocket in the crystal structure. The polar glutamine-88 residue is in contact with solvent and is near the 'negative patch' formed by the invariant acidic residues aspartate-42, glutamate-43 and aspartate-44.

The primary structure of an isoplastocyanin from the leaves of another hybrid black poplar tree has also been determined.[57] The residues, of this plastocyanin (black poplar *iso-2*) differ from those shown in Table III at ten positions. In this isoplastocyanin these residues are: valine-1, valine-21, proline-22, alanine-23, alanine-45, valine-46, valine-52, aspartate-76, threonine-81 and isoleucine-97.[57]

STRUCTURAL ASSIGNMENTS FOR AZURIN

The crystal structure[2,14] of *P. a.* azurin, secondary structural assignments for which are given in Table IV, shows it to have eight beta strands with a similar positioning of the copper ion and its ligands close to one end of a beta-barrel to that in poplar plastocyanin. Azurin differs from plastocyanin in having an invariant cystine-3-26 between the beginning of strand 1 and end of strand 2. The unequal number of residues in the first two beta strands leads to reverse turn assignments at 8–11 and 16–19 in Table IV (In the crystal structure there is a kink in strand 2 at 15–18).[16]

Two-to-three turns of helix were suggested to be present in the long 'flap' between strands labeled 5 and 6. The assignments of Table IV mare helix at 53–57 ($(helix)_n$ = 1.25; $(beta)_n$ = 1.13) but beta strand at 58–64 ($(helix)_n$ = 0.91; $(beta)_n$ = 1.23) and random coil at 65–75 ($(helix)_n$ = 0.98; $(beta)_n$ = 0.90) followed by a reverse turn at 76–79 ($(iii)_{helix}$; $(iibi)_{beta}$), or, alternatively, random coil at 65–79 ($(helix)_n$, $(beta)_n$ < 1), in approximate agreement with the crystal structure.

TABLE IV

Chou-Fasman[24] assignments of secondary structure for *Pseudomonas aerunginosa* azurin. The assigned structure for the segment is shown in the last column. The amino acid sequence data is from Ref. 15. For the one-letter notation for amino acids see Ref. 26. There is a disulfide bond between cysteine-3 and cystein-26

Segment	Side chains	Chou-Fasman assignments for structural type		Final assignment for the segment
		Helical	Beta sheet	
1–2	AE	HH	IB	random coil
3–8	CSVDIQ	iihiIh	hbHiHh	beta strand
9–12	GNDQ	Bbih	ibih	reverse turn
12–15	QMQF	hhhh	hHhH	beta strand
16–19	NTNA	bibH	bhbI	reverse turn
20–23	ITVD	Iihi	HhHi	beta strand
24–27	KSCK	IiiI	bbhb	reverse turn
28–33	QFTVNL	hhihbH	hHhHbh	beta strand
34–45	SHPGNLPKN	ihBBbHBIb	bbbibhbbb	random coil
	VMG	hhB	HHi	
46–52	HNWVLST	hbhhHii	bbhHhbh	beta strand
53–57	AADMQ	HHihh	IIiHh	helix
58–64	GVVTDGM	BhhiiBh	iHHhiiH	beta strand
65–75	ASGLDKDYLKP	HiBHiIibHIB	Ibihibihhbb	random coil
76–79	DDSR	iiii	iibi	reverse turn
80–87	VIAHTKLI	hIHhiIHI	HHIbhbhH	beta strand
88–93	GSGEKD	BiBHIi	ibiBbi	random coil
94–100	SVTFDVS	ihihihi	bHhHiHb	beta strand
101–106	KLKEGE	IHIHBH	bhbBiB	helix
107–114	QYMFFCTF	hbhhhiib	hhHHHhhH	beta strand
115–116	PG	BB	bi	random coil
117–121	HSALM	hiHHh	bbIhH	helix
122–123	KG	IB	bi	random coil
124–128	TLTLK	iHiHI	hhhhb	beta strand

In the segment 80–87 the central residues 82–85 have the assignment $(Ibhb)_{beta}$ with $(beta)_n < 1$. This segment can be broken up into two tetrapeptides at 80–83 and 84–87. In these tetrapeptides $(helix)_n = 1.21$, $(beta)_n = 1.23$ and $(helix)_n = 1.06$, $(beta)_n = 1.19$, in order. These numbers reflect the effect of the strong beta-structure-promotoers at both ends of the segment and the assignment of Table IV is beta strand in agreement with the crystal structure.

A helix assignment that is contrary to the crystal structure occurs at the segment 100–106 ($(helix)_n = 1.12$; $(beta)_n < 1$). In the crystal structure this segment is part of an eight-residue connecting segment between strands labeled 4 and 7.

In the regions of copper ligands the homology noted for plastocyanins and azurins[15] is borne out by similar positioning of some of these residues in poplar plastocyanin and *P. a.* azurin.[2,3,14,16,17] In both proteins there are invariant hydrophobic residues in the C-terminal region just before the single cysteine residue. Cysteine-112, histidine-117 and methionine-121, which are all invariant in the azurins as are the copper ligands in plastocyanin,[15] are brought close together in the assignments of Table IV by the random coil residues proline-115 and glycine-116, and by the helical structure of the histidine-117 to methionine-121 segment. A formal turn of alpha-helix would place the side chains of histidine and methionine residues in a favorable position to coordinate to copper.[50] The copper-binding region is shown in photograph 2. The six-residue cysteine-histidine-copper loop involves the bulky invariant phenylalanine-114 side chain and may be contrasted with the four-residue

loop in plastocyanin. The large number of substitutions in the histidine-methionine-copper loop make it likely that the turn of helix observed in the crystal structure of *P. a.* azurin may not exist in all azurins (see Table II).

Histidine-35 and histidine-83 are located too far away from the copper ion to bind to it according to the crystal structure. The fourth copper ligand is histidine-46 in a more conserved region that shows homology to plastocyanin.[15] The homologous residues are histidine-46, asparagine-47, valine-49 in azurin and histidine-37, asparagine-38, valine-40 in plastocyanin.[15] The two residues preceding histidine-46 (methionine-44 and glycine-45) are invariant in azurins.[15] As in plastocyanins,[3,16] the invariance of certain residues suggests that the general shape of *P. a.* azurin is conserved in all azurins. This is particularly expected of the regions that bind copper.