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Gene synthesis, expression, and mutagenesis of the blue copper proteins azurin and plastocyanin

(copper center/synthetic gene)

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ABSTRACT **Genes for the blue copper proteins *Populus nigra* var. *italica* plastocyanin and *Pseudomonas aeruginosa* azurin have been constructed by a stepwise procedure. The leader sequence for azurin has been placed before the genes directing plastocyanin and azurin transport to the periplasmic space when the genes are expressed in *Escherichia coli*. Site-saturation mutagenesis has been used to alter two copper-binding residues of azurin (Met-121 and His-46) and Met-92 of plastocyanin. While the plastocyanin mutants do not appear to bind copper, the azurin variants all bind copper and show characteristic type I blue copper centers. In particular, the electronic spectra reflect the dominance of the charge transfer interaction between copper and the thiolate of Cys-112, being relatively insensitive to changes in Met-121 or His-46. In contrast, removal of Met-121 appreciably alters the EPR spectra of the mutants, although, to a first order, the spectra of all mutants are themselves similar, suggesting a more distorted geometry around copper in the mutants than in the wild type.**

The family of blue copper proteins includes plastocyanins (1) from green plants and some algae and azurin (2) from bacteria. These proteins perform essential roles as electron carriers (3, 4) in such important processes as photosynthesis and bacterial respiration. They provide a unique ligand environment (5–8) to their single type I copper atom that endows them with a rich blue color as well as an unusually high potential for the Cu(II)–Cu(I) couple (9). Furthermore, the ligation geometries are essentially identical for both Cu(II) and Cu(I) forms near neutral pH, giving these proteins the ability to transfer electrons very rapidly (10, 11). Lastly, the three-dimensional structures for a representative azurin at 1.8 Å (12) and plastocyanin at 1.6 Å (13) have been determined. For these reasons, the blue copper proteins provide attractive candidates for mutagenic structure–function studies (14) aimed at gaining insights into such diverse aspects of their behaviors as electronic spectra, paramagnetic properties, redox potentials, rates of electron transfer, transmission of electrons through the interior of the protein for subsequent transfer to redox partners, and surface sites involved in such protein–protein interactions. The close relationship in both structure and function of the plastocyanins and azurins provides an additional attraction for the concurrent study of these questions against these similar, but nevertheless significantly different, protein backgrounds.

We have approached this problem by the total synthesis of genes for poplar (*Populus nigra* var. *italica*) leaf plastocyanin (because of its highly refined three-dimensional structure; ref. 13) and *Pseudomonas aeruginosa* azurin (15). The synthetic genes were introduced adjacent to synthetic leader sequences for plastocyanin and azurin and the resulting constructs were expressed in *Escherichia coli*. When grown in the presence of 1 mM Cu(II), the properly processed and folded native proteins can be isolated from the periplasm. Because of the presence in the synthetic genes of relatively closely spaced sites for restriction endonuclease digestion, cassette mutagenesis (16) allows facile creation of specific mutants or families of mutants obtained by procedures such as site saturation (17). Using these approaches, we have created mutants at two of the copper ligation sites of azurin (His-46 and Met-121) and at one site of plastocyanin (Met-92).

MATERIALS AND METHODS

Materials. Most restriction enzymes were purchased from Boehringer Mannheim or New England Biolabs. Polynucleotide kinase was purchased from New England Biolabs. DNA ligase was purchased from either BRL or Boehringer Mannheim. Ampicillin was purchased from Sigma; isopropyl β-D-thiogalactopyranoside, Tris, and other buffer reagents came from Boehringer Mannheim. FMC provided the high purity, low melting point agarose (GTG grade) used in preparative gels. Plasmid pBR322 was purchased from BRL, while pUC18 and chromatographic material came from Pharmacia. The Vectastain Elite kit for Western blotting was purchased from Vector Laboratories. Rabbit anti-plastocyanin or anti-azurin antibody was obtained from Berkeley Antibody (Richmond, CA).

E. coli strain LS1, an HB101 derivative, was used in the construction of both genes. Cells harboring the plasmids with the partially constructed gene were grown in L broth (10 g of tryptone per liter/5 g of yeast extract per liter/5 g of NaCl per liter). *E. coli* strain TG1 (18), a JM101 derivative, was used during the expression experiments. This strain was grown in a richer medium such as modified XB (25 g of tryptone per liter/7.5 g of yeast extract per liter/20 mM $MgSO_4$/50 mM sodium phosphate, pH 7.5) containing 1 mM $CuSO_4$.

Oligonucleotides were synthesized by phosphoramidite chemistry (19) on an Applied Biosystems automated DNA synthesizer (model 380A or 380B). They were then purified by electrophoresis on polyacrylamide gels followed by passage through NACS PREPAC columns from BRL. Alternatively, the dimethoxytrityl group could be left attached to the 5′-terminal nucleotide at the end of the synthesis and the oligonucleotides purified through OPC cartridges obtained from Applied Biosystems.

Gene Synthesis. Our approach involves synthesis of the gene in a stepwise fashion. The construction of the gene by this method proceeds from the ends toward the middle. Segments of the gene are sequentially cloned into an appropriate vector that allows amplification of the growing gene at intermediate stages of synthesis. After a segment has been inserted and the plasmid amplified, the resulting intermediate, containing two unique restriction sites within the segment last inserted, is opened at these sites, which then act as recipients for the next segment of the gene. Importantly, although all bases that

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define one of the particular sites used for opening must, of course, be present in that intermediate, the site(s) need not be reconstituted for incorporation of the next cassette; only those bases necessary to provide compatible overhangs or located upstream of the upstream site and downstream of the downstream site need to be retained in the final gene. Thus, any particular restriction endonuclease may in principle be used multiple times in a given synthesis.

Though a very conservative approach for the synthesis of genes that encode plastocyanin (297 base pairs) or azurin (384 base pairs), this general strategy has considerable flexibility and should prove particularly suited to the synthesis of larger genes that might be difficult to prepare by the consecutive annealing of segments followed by cloning. The approach also permits editing at intermediate stages. This strategy is shown in Fig. 1, which outlines the steps used in synthesis of the structural genes for plastocyanin and azurin. We used pBR322 as the vector for this synthesis and constructed the gene between the *Eco*RI and *Ava* I sites after removal of the Tet^R gene that occupies this region of pBR322. This removes a large number of unique restriction sites that can subsequently be utilized in gene synthesis and leaves the β-lactamase gene intact as a selectable marker. Fig. 2 shows the base sequences and restriction sites for the two synthetic genes. In a similar way, a ribosome binding site and plastocyanin (20, 21) or azurin (22, 23) leader sequences were prepared and introduced just in front of the structural genes and then ligated into the polylinker site of pUC18 for expression that is controlled by a *lac* promoter induced by isopropyl β-D-thiogalactopyranoside.

Expression. A pUC18 vector (24) into which the appropriate genetic information had been inserted (promoter, ribosome binding site, spacer, leader sequence, and structural gene) was used to transform *E. coli* (TG1), and the cells were grown at 37°C in medium (25 g of bactotryptone, 7.5 g of yeast extract, and 5 g of NaCl per liter) containing 1 mM $CuSO_4$ and 50 μg of ampicillin per ml. After reaching logarithmic phase (OD_{600}, 0.5–1.0), the cells were induced with isopropyl β-D-thiogalactopyranoside (0.5 mM) and allowed to grow an additional 3–5 hr.

Protein Isolation. For Western blot analysis, a small sample of cells (≈2 ml) was centrifuged in a microcentrifuge and suspended in 100 μl of buffer (10% glycine/5% 2-mercaptoethanol/3% SDS/62.5 mM Tris·HCl, pH 7.6/1 mM EDTA/0.05% bromophenol blue). The solution was heated at 95°C for 10 min. The resulting solution was vigorously mixed in a Vortex to reduce viscosity and a 5-μl aliquot was loaded onto a SDS/15% polyacrylamide gel with a 4% stack. After electrophoresis, protein from the gel was transferred to a nitrocellulose membrane using a Bio-Rad Trans-Blot cell equipped with a surface electrode. The protein (azurin or plastocyanin) was visualized by using rabbit antibody raised against the appropriate protein together with the Vectastain Western blotting kit.

Osmotic extrusion was used for isolation of protein. Cells were harvested in a Sorvall superspeed centrifuge and the resulting pellet was resuspended in a hyperosmotic solution (20% sucrose/30 mM Tris·HCl, pH 8). After sitting on ice for 10 min, the solution was centrifuged to a pellet and gently resuspended in a cold solution of 0.5 mM $MgCl_2$. (In the case of plastocyanin, this solution also contains 1 mM $CuSO_4$; see below.) Periplasmic proteins were extruded and the cell debris was removed by further centrifugation.

Protein Purification. *Azurin.* To the solution obtained after osmotic extrusion was added 1/10th vol of 0.5 M ammonium acetate buffer (pH 4.1). This causes some contaminating proteins to precipitate. The supernatant was filtered through a 0.22-μm filter and the pH was adjusted to 4.1 before being loaded onto a column (5 × 5 cm) of CM-Sepharose previously equilibrated with ammonium acetate (pH 4.1). The column was washed with the same buffer, and the rich blue azurin was then eluted with ammonium acetate (pH 5.1). The fractions containing azurin were concentrated by ultrafiltration (Amicon YM3) and dialyzed against ammonium acetate buffer (pH 4.1). The solution was applied to an FPLC Mono S cation-exchange column and the azurin eluted with a pH gradient of 4.1–9, and further purified using a Sepharose 12 gel-filtration column at pH 7.0.

Plastocyanin. For purification of plastocyanin, the osmotic extrusion buffer contained 0.5 mM $MgCl_2$, 1.0 mM $CuSO_4$, and 1 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)-methane (Bistris) (pH 7.5). Plastocyanin was purified by anion-exchange chromatography [Q-Sepharose, 20 mM Bistris (pH 6.5)], eluted in buffer containing 0.5 M NaCl followed by two consecutive FPLC gel filtrations [Sepharose 12, 16/50; run 1, 20 mM Bistris (pH 6.5); run 2, 60 mM Tris (pH 8.0)] and FPLC anion-exchange [Mono Q, 10/10; 20 mM Tris (pH 8.0) eluted with a 0–0.5 M NaCl gradient]. All buffers also contained 5 mM $K_3Fe(CN)_6$ to maintain the Cu(II) form of the protein.

Mutagenesis. Mutants were prepared by cassette mutagenesis, in which the DNA sequences were inserted between appropriate restriction sites. For preparation of families of mutants, as for example at Met-121 in azurin, the approach of site saturation was used with mixed oligonucleotide cassettes NN(G/C) (21) to generate all 20 amino acid substitutions at a site at one time.

Spectral Analysis. CD spectra were recorded on a Jasco J-600 spectrophotometer. EPR spectra were recorded on a Varian E-Line Century series X-band spectrometer at 77 K and 9.077 GHz.

RESULTS AND DISCUSSION

Before the successful approaches for expression outlined above, many other attempts to produce blue copper proteins were tried. Biosynthesis of full-length apoplastocyanin itself directly into the cytoplasm of *E. coli* seemed to cause death of the cells. However, fusion proteins such as protein A–apoplastocyanin could be isolated in good yields. Cleavage of this fusion protein by factor Xa (25–27), enterokinase (28–30), or formic acid (31) (with appropriately unique amino acid target sequences inserted between protein A and plastocyanin), although fraught with various technical difficulties, did

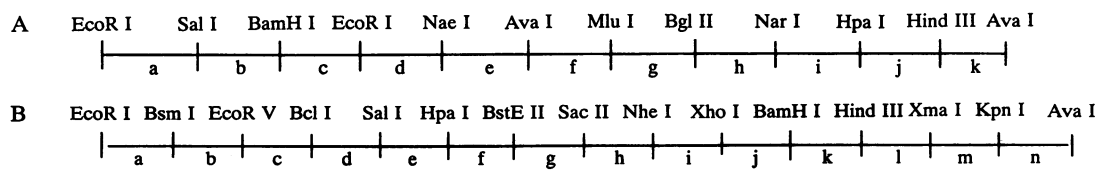

FIG. 1. General strategies used in the construction of plastocyanin (*A*) and azurin (*B*) genes. In each step of gene synthesis, the plasmid was cleaved at two adjacent restriction sites, and the next piece was ligated into that opening. This approach allows for isolation and amplification of a partially constructed gene for editing. (*A*) Step 1, a–c and k; step 2, d and e; step 3, f, g, i, and j; step 4, h. The *Eco*RI and *Ava* I sites of pBR322 between which this gene was constructed were destroyed during ligation, making the *Eco*RI and *Ava* I sites within the plastocyanin gene unique on the entire plasmid. (*B*) Step 1, l–n; step 2, a and k; step 3, b–e; step 4, f and j; step 5, g–i.

A

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       RBS              Met Leu Arg Lys Leu Ala Ala Val Ser Leu Leu Ser Leu
GTCGACAGGAGG TAAATTAAT ATG CTG CGC AAG CTA GCT GCA GTG TCT CTG CTG TCT CTG
                                      Nhe I  Pst I
                                    ⇩
Leu Ser Ala Pro Leu Leu Ala Ile Asp Val Leu Leu Gly Ala Asp Asp Gly Ser Leu
CTG TCT GCT CCG CTG CTG GCT ATC GAC GTA CTC CTT GGT GCT GAC GAC GGA TCC CTG
                                          Sty I                 BamH I

Ala Phe Val Pro Ser Glu Phe Ser Ile Ser Pro Gly Glu Lys Ile Val Phe Lys Asn
GCA TTC GTT CCG TCC GAA TTC TCT ATC TCT CCG GGC GAA AAA ATC GTA TTC AAA AAC
                     EcoR I

Asn Ala Gly Phe Pro His Asn Ile Val Phe Asp Glu Asp Ser Ile Pro Ser Gly Val
AAC GCC GGC TTC CCG CAC AAC ATC GTA TTT GAC GAA GAC TCC ATC CCG AGT GGC GTT
     Nae I                                                 Ava I

Asp Ala Ser Lys Ile Ser Met Ser Glu Glu Asp Leu Leu Asn Ala Lys Gly Glu Thr
GAC GCG TCC AAA ATC TCC ATG TCC GAA GAA GAT CTG CTG AAC GCA AAA GGT GAA ACT
                                    Bgl II

Phe Glu Val Ala Leu Ser Asn Lys Gly Glu Tyr Ser Phe Tyr Cys Ser Pro His Gln
TTT GAA GTA GCA CTG TCC AAC AAA GGT GAA TAC TCC TTC TAC TGC TCC CCG CAC CAG


Gly Ala Gly Met Val Gly Lys Val Thr Val Asn Trm
GGC GCC GGT ATG GTT GGT AAA GTA ACC GTT AAC TAG
 Nar I                              Hpa I
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B

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   RBS             Met Leu Arg Lys Leu Ala Ala Val Ser Leu Leu Ser Leu Leu
CAAGGAGG AATAACATA ATG CTG CGT AAG CTG GCT GCA GTG TCT CTG CTG TCT CTG CTG
                                       Pst I
                                  ⇩
Ser Ala Pro Leu Leu Ala Ala Glu Cys Ser Val Asp Ile Gln Gly Asn Asp Gln Met
TCT GCT CCG CTG CTG GCT GCA GAA TGC TCC GTT GAT ATC CAG GGT AAT GAT CAG ATG
                            Bsm I          EcoR V             Bcl I

Gln Phe Asn Thr Asn Ala Ile Thr Val Asp Lys Ser Cys Lys Gln Phe Thr Val Asn
CAG TTC AAC ACC AAC GCC ATC ACC GTC GAC AAG AGC TGC AAG CAG TTC ACT GTT AAC
                              Sal I                              Hpa I

Leu Ser His Pro Gly Asn Leu Pro Lys Asn Val Met Gly His Asn Trp Val Leu Ser
CTG TCT CAC CCA GGT AAC CTG CCG AAG AAC GTT ATG GGT CAC AAC TGG GTT CTG TCC
               BstE II

Thr Ala Ala Asp Met Gln Gly Val Val Thr Asp Gly Met Ala Ser Gly Leu Asp Lys
ACC GCG GCT GAC ATG CAA GGC GTT GTC ACT GAC GGT ATG GCT AGC GGT CTG GAT AAA
  Sac II                                              Nhe I

Asp Tyr Leu Lys Pro Asp Asp Ser Arg Val Ile Ala His Thr Lys Leu Ile Gly Ser
GAC TAC CTG AAG CCG GAT GAC TCT CGA GTT ATC GCC CAC ACC AAG CTG ATC GGA TCC
                                Xho I                                BamH I

Gly Glu Lys Asp Ser Val Thr Phe Asp Val Ser Lys Leu Lys Glu Gly Glu Gln Tyr
GGT GAA AAA GAC TCC GTT ACT TTC GAC GTT TCC AAG CTT AAA GAA GGT GAA CAG TAC
                                      HinD III

Met Phe Phe Cys Thr Phe Pro Gly His Ser Ala Leu Met Lys Gly Thr Leu Thr Leu
ATG TTC TTC TGC ACT TTC CCG GGT CAC TCC GCA CTG ATG AAA GGT ACC CTG ACT CTG
                       Xma I                              Kpn I

Lys Trm
AAA TAG
```

FIG. 2. Base sequences and restriction sites for plastocyanin (*A*) and azurin (*B*), including the ribosomal binding sites (RBS) and the azurin signal sequence from *P. aeruginosa*. Arrow indicates the junction between the signal sequence and the structural gene of plastocyanin or azurin that is correctly cleaved by *E. coli* signal peptidase upon cell membrane translocation. Each of the restriction sites is unique when these genes are located between the *Eco*RI and *Ava* I sites of pBR322.

produce apoplastocyanin whose efficient refolding in the presence of Cu(II) has yet to be achieved.

Far more successful expression was achieved when the blue copper proteins were transported to the periplasm as constructs involving the leader sequence for *P. aeruginosa* azurin (23, 32) or white campion plastocyanin (20, 21) followed by a structural gene. In these experiments, vectors incorporating the azurin leader followed by either the plastocyanin or azurin gene under control of the isopropyl β-D-thiogalactopyranoside-inducible *lac* promoter and grown in medium containing 1 mM Cu(II) led to the blue copper protein, properly processed and folded, being present in the periplasm. Use of a construct involving the entire plastocyanin leader (from white campion) followed by the structural gene for poplar plastocyanin also produced properly processed and folded plastocyanin, although in considerably lower yields than were obtained with a vector containing the azurin leader/plastocyanin sequence. The correct removal of the plastocyanin sequence during processing to generate mature plastocyanin upon translocation to the *E. coli* periplasm is an intriguing result as the complete plastocyanin leader (66 amino acids) is in fact two concatenated signal sequences; the first governs transport of plastocyanin into the chloroplast stroma while the second controls the subsequent translocation into the thylakoid lumen. The junction between these two sequences is unknown (21). Moreover, growth in the presence of 1 mM Cu(II) gave considerably higher yields of blue copper proteins than growths without Cu(II), presumably because of the greater resistance to proteolysis of the holoproteins (33). Our constructs contained a synthetic ribosome binding site designed from a consensus sequence (34–36); higher yields will likely be possible with more efficient ribosome binding sites as, for example, from that of the native azurin gene (23).

Azurin and plastocyanin were characterized by amino acid analyses, N-terminal sequencing, determination of the mass

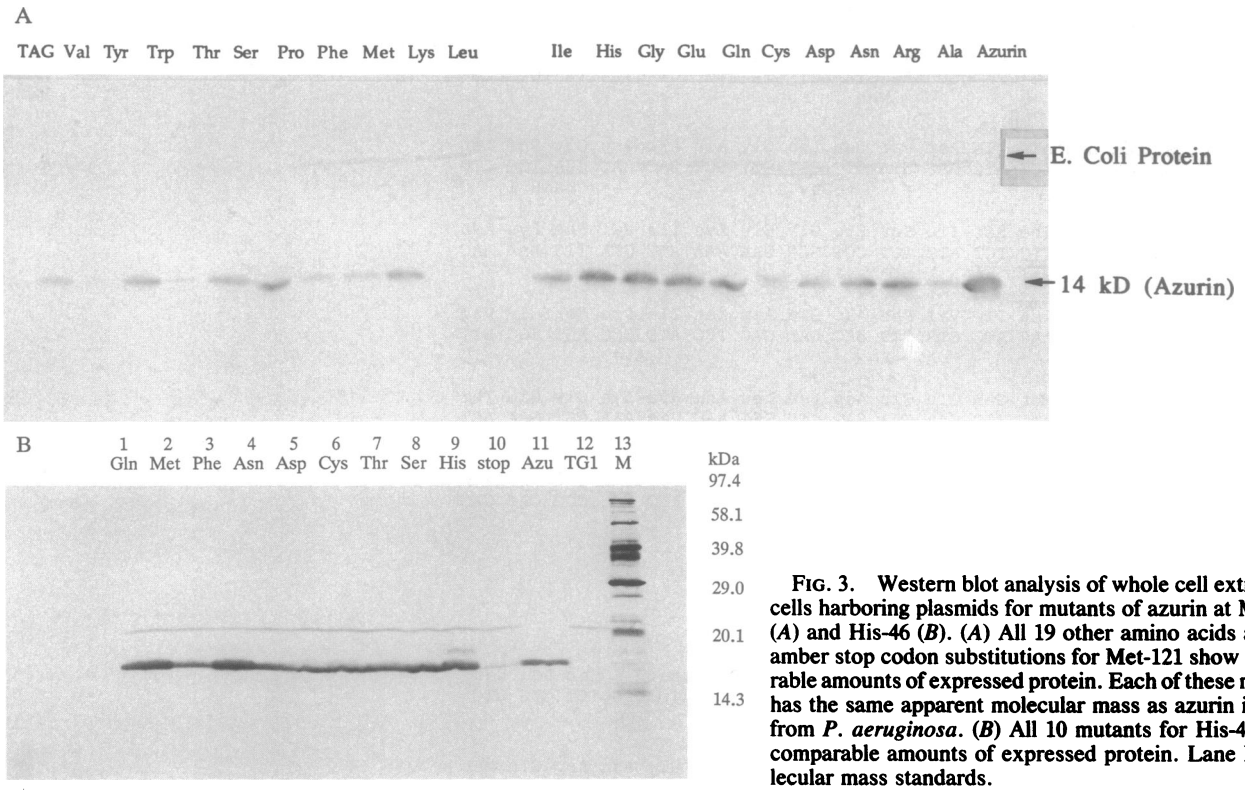

FIG. 3. Western blot analysis of whole cell extracts of cells harboring plasmids for mutants of azurin at Met-121 (*A*) and His-46 (*B*). (*A*) All 19 other amino acids and the amber stop codon substitutions for Met-121 show comparable amounts of expressed protein. Each of these mutants has the same apparent molecular mass as azurin isolated from *P. aeruginosa*. (*B*) All 10 mutants for His-46 show comparable amounts of expressed protein. Lane M, molecular mass standards.

of the molecular ion by time-of-flight mass spectrometry, UV-visible spectra, and CD. In all cases, the proteins from *E. coli* were indistinguishable from analogous proteins from native sources.

Mutagenesis. *General.* At least three aspects of the function of blue copper proteins hold interest and should be amenable to analysis by mutagenic approaches: (*i*) the copper center, including the nature of ligands and their geometry; (*ii*) intramolecular electron transmission; (*iii*) docking sites involved in interactions between proteins and transfer of electrons along the redox chain.

Most of our work has so far focused on the first question with preliminary attention to two of the four copper ligands (Met-121 and His-46 in azurin). (The other two ligands in azurin are His-117 and Cys-112.) By site saturation (17), we have prepared genes for all 19 possible mutants at Met-121 and His-46. Expression of the Met-121 mutants has shown that protein for 20 of these can be demonstrated in the periplasm by Western blot analysis (Fig. 3). Of these, seven Met-121 mutants have been isolated as copper-containing proteins and characterized in a preliminary way. Of particular interest is the observation that all manifest the characteristic rich blue color originating most likely in the interaction between the copper and the thiolate of Cys-112. Nevertheless, replacement of Met-121 does result in clear changes in the electronic spectra as summarized in Table 1. Another mutant at this site, Met-121 → Leu, increases the redox potential by 70 mV and shifts the peak at 625 nm by 5 nm (37). Of the 19 mutants at His-46, one has been purified (His-46 → Asp). It is also a deep blue protein with the spectral characteristics outlined in Table 1. CD spectra of the azurin mutants show all of the previously identified peaks, although at slightly altered frequencies. These results, along with the UV-visible data, indicate that the essential integrity of the copper site has been retained in these mutants.

Table 1. Spectroscopic characteristics of azurin mutants based on UV-visible and CD observations

	Major peak, nm	Minor peak, nm
Wild type	625	445
Met-121 → Val	630	459
Met-121 → Ile	626	459
Met-121 → Asn	622	447
Met-121 → Asp	622	445
Met-121 → His	612	449
His-46 → Asp	616	458

The absorbance maxima of the two peaks within the visible region for the six mutants shown are all shifted by relatively small amounts due to both the absence of methionine and probably slightly perturbed geometries of the copper site.

Frozen solution EPR spectra for the wild-type azurin and three Met-121 mutants were recorded and the g and A values were determined (Table 2). Preliminary examination of the data suggests that the spectra of the mutants are more rhombic than the spectrum of the wild-type protein.

Fascinatingly, a great latitude in ligands, almost universally conserved throughout the blue copper family, can be accommodated in these mutants of azurin, while preserving the ability to bind copper and apparently normal, stable protein folding. [Some exceptional proteins with ligands other than the four commonly observed probably exist in nature—for example, stellacyanin (38), amicyanin (39), and rusticyanin (40).] The ability to generate analogues of these ligand environments at will should prove particularly useful.

Other examples of substituting either a conserved or a semiconserved residue of azurin have recently been reported. Both His-35 → Lys and Glu-91 → Gln have unchanged spectroscopic and redox properties, while in Phe-114 → Ala the optical band is downshifted by 7 nm and the

Table 2. Spin hamiltonian parameters for wild-type azurin [50 mM ammonium acetate (pH 7)] and Met-121 mutants [pH9]

	$g_{\parallel}$	$A_{\parallel}$, $\times 10^4$ cm^{-1}	$g_{\perp}$
Wild type	2.271	61	2.054
Met-121 → Asn	2.249	36	2.056
Met-121 → Ile	2.246	35	2.060
Met-121 → Val	2.243	34	2.064

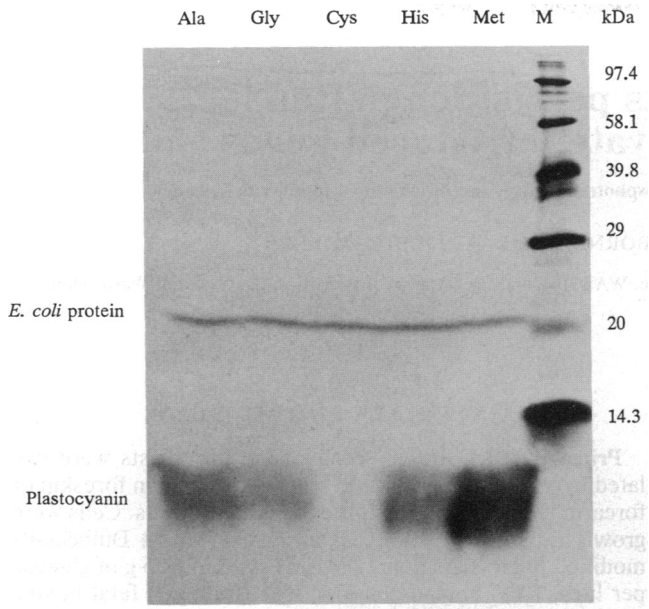

FIG. 4. Western blot analysis of plastocyanin and four mutants at Met-92. Three of the mutants (His, Gly, and Ala) show a band at the same molecular mass as the native plastocyanin. No protein can be visualized for the Met-92 → Cys mutant. Lane M, molecular mass standards.

reduction potential is lowered by 20–24 mV (41). Another mutation at a conserved residue within the hydrophobic patch, Met-44 → Lys, causes only a minimal effect on the spectroscopic properties but significantly affects the electron self-exchange rate (42).

Plastocyanin. The ability to create a particular mutation against both the azurin and plastocyanin backgrounds provides one of the rationales for the concurrent study of both families of proteins. To this end, we have prepared mutants at Met-92 of plastocyanin. (This is the ligand in plastocyanin analogous to Met-121 in azurin.) Site saturation generated all 19 mutant genes. Of these mutants, Met-92 → Cys, Met-92 → His, Met-92 → Ala, and Met-92 → Gly have been studied. On producing these mutants, *E. coli* has a frustrating tendency to lyse (behavior not observed with analogous mutants of azurin). This problem can be circumvented if the cells are aerated but not agitated during expression. Western blot analysis (Fig. 4) shows protein for the mutants Met-92 → His, Met-92 → Ala, and Met-92 → Gly present in the periplasm; no protein for the Met-92 → Cys has been observed. In no case has it so far been possible to isolate a blue copper-containing protein with any of these mutants. This may be due to a kinetic difficulty these proteins have in acquiring the copper or because the thermodynamic affinity for a copper ligand has been sharply reduced. Thus, although azurin and plastocyanin share very similar overall three-dimensional structures and almost identical copper sites, analogous mutations in these two systems seem to behave differently.

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1. Boulter, D., Haslett, B. G., Peacock, D., Ramshaw, J. A. M. & Scawen, M. D. (1977) in *International Review of Biochemistry, Plant Biochemistry II*, ed. Northcote, D. H. (University Park Press, Baltimore), Vol. 13, pp. 1–40.
2. Ryden, L. & Lungren, J. (1976) *Nature (London)* **261,** 344–346.
3. Farver, O. & Pecht, I. (1984) in *Copper Proteins and Copper Enzymes,* ed. Lontie, R. (CRC, Boca Raton, FL), Vol. 1, pp. 183–214.
4. Adman, E. T. (1985) in *Topics in Molecular and Structural Biology, Metalloproteins,* ed. Harrison, P. M. (Verlag Chemie, Weinheim, F.R.G.), Vol. 6, pp. 1–42.
5. McMillin, D. R. & Morris, M. C. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 6567–6570.
6. McMillin, D. R. (1985) *J. Chem. Educ.* **62,** 997–1001.
7. Penfield, K. W., Gay, R. R., Himmelwright, R. S., Eickman, N. C., Norris, V. A., Freeman, H. C. & Solomon, E. I. (1981) *J. Am. Chem. Soc.* **103,** 4382–4388.
8. Penfield, K. W., Gerwirth, A. A. & Solomon, E. I. (1985) *J. Am. Chem. Soc.* **107,** 4519–4529.
9. Gray, H. B. & Solomon, E. I. (1981) in *Metal Ions in Biology,* ed. Spiro, T. G. (Wiley, New York), Vol. 3. pp. 1–39.
10. Kostic, N. M., Margalit, R., Che, C.-M. & Gray, H. B. (1983) *J. Am. Chem. Soc.* **105,** 7765–7767.
11. Marcus, R. A. & Sutin, N. (1985) *Biochim. Biophys. Acta* **811,** 265–322.
12. Guss, J. M., Harrowell, P. R., Murata, M., Norris, V. A. & Freeman, H. C. (1986) *J. Mol. Biol.* **192,** 361–387.
13. Baker, E. N. (1988) *J. Mol. Biol.* **203,** 1071–1095.
14. Dalbadie-McFarland, G., Cohen, L. W., Riggs, A. D., Morin, A. D., Itakura, K. & Richards, J. H. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6409–6413.
15. Adman, E. T. & Jensen, L. H. (1981) *Isr. J. Chem.* **21,** 8–12.
16. Richards, J. H. (1986) *Nature (London)* **323,** 187.
17. Schultz, S. C. & Richards, J. H. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1588–1592.
18. Gibson, T. J. (1984) Ph.D. thesis (Cambridge Univ., U.K.).
19. Beaucage, S. L. & Carruthers, M. H. (1986) *Tetrahedron Lett.* **22,** 1859–1862.
20. Smeekens, S., de Groot, M., van Binsbergen, J. & Weisbeek, P. (1985) *Nature (London)* **317,** 456–458.
21. Smeekens, S., Bauerle, C., Hageman, J., Keegstra, K. & Weisbeek, P. (1986) *Cell* **46,** 365–375.
22. Canters, G. W. (1987) *FEBS Lett.* **212,** 168–172.
23. Arvidsson, R. H. A., Nordling, M. & Lundberg, L. G. (1989) *Eur. J. Biochem.* **179,** 195–200.
24. Vieira, J. & Messing, J. (1982) *Gene* **19,** 259–268.
25. Titani, K., Hermodson, M. A., Fujikawa, K., Ericsson, L. H., Walsh, K. A., Neurath, H. & Davie, E. W. (1972) *Biochemistry* **11,** 4899–4903.
26. Fujikawa, K., Legaz, M. E. & Davie, E. W. (1972) *Biochemistry* **11,** 4892–4898.
27. Nagai, K. & Thøgersen, H. C. (1984) *Nature (London)* **309,** 810–812.
28. Anderson, L. E., Walsh, K. A. & Neurath, H. (1977) *Biochemistry* **16,** 3354–3360.
29. Baratti, J., Maroux, S., Louvard, D. & Desnuelle, P. (1973) *Biochim. Biophys. Acta* **315,** 147–161.
30. Maroux, S., Baratti, J. & Desnuelle (1971) *J. Biol. Chem.* **246,** 5031–5039.
31. Nilsson, B., Holmgren, E., Josephson, S., Gatenbeck, S., Philipson, L. & Uhlen, M. (1985) *Nucleic Acids Res.* **13,** 1151–1162.
32. Karlsson, B. G., Pascher, T., Nordling, M., Arvidsson, R. H. A. & Lundberg, L. G. (1989) *FEBS Lett.* **246,** 211–217.
33. Merchant, S. & Bogorad, L. (1986) *J. Biol. Chem.* **261,** 15850–15853.
34. Gold, L. M. & Stormo, G. D. (1987) in *Escherichia coli and Salmonella typhimurium,* ed. Neidhardt, F. C. (Am. Soc. Microbiol., Washington), pp. 1302–1307.
35. Gren, E. J. (1984) *Biochimie* **66,** 1–29.
36. Stormo, G. D. (1986) in *Maximizing Gene Expression,* ed. Reznikoff, W. & Gold, L. (Butterworth, Boston), pp. 195–224.
37. Karlsson, B. G., Aasa, R., Malmström, B. G. & Lundberg, L. G. (1989) *FEBS Lett.* **253,** 99–102.
38. Engeseth, H. R., Hermodson, M. A. & McMillin, D. R. (1984) *FEBS Lett.* **171,** 257–261.
39. Tobari, J. & Harada, Y. (1981) *Biochem. Biophys. Res. Commun.* **101,** 502–508.
40. Ingledew, W. J. & Cobley, J. C. (1980) *Biochim. Biophys. Acta* **590,** 14–23.
41. Pascher, T., Bergström, J., Malmström, B. G., Vänngård, T. & Lundberg, L. G. (1989) *FEBS Lett.* **258,** 266–268.
42. van de Kamp, M., Floris, R., Hali, F. C. & Canters, G. W. (1990) *J. Am. Chem. Soc.* **112,** 907–908.