The finding that numerous one-carbon-reduced folates (such as 5-methyltetrahydrofolate and 5-formyltetrahydrofolate) as well as MTX are all transported into L1210 cells via a common carrier was based upon influx inhibition studies. From these studies, we conclude that this carrier is the 46K–48K membrane-derived protein. The need for an intracellular reduced folate "shuttle" protein arises from the fact that, although the same carrier translocates different types of reduced folates (and MTX) across the membrane, the ultimate cytosolic target proteins for these compounds are different enzymes. The 38K protein, therefore, may mediate the distribution of folates, once inside the cell, to enzymes other than DHFR.

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Auracyanin, a Blue Copper Protein from the Green Photosynthetic Bacterium *Chloroflexus aurantiacus*†

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ABSTRACT: A small, type 1 blue copper protein has been isolated from the green photosynthetic bacterium *Chloroflexus aurantiacus*. This protein, named auracyanin, appears to be peripherally associated with the cytoplasmic membrane, with a midpoint potential of +240 mV and a molar extinction coefficient of ϵ_{596} = 2900 M^{-1} cm^{-1}. Auracyanin is isolated as a disulfide-bridged dimer with a monomer molecular mass of 12 800 Da. The isoelectric point of auracyanin is 4.0. ESR spectra exhibit rhombic distortion and give no indication of interaction between the coppers. The function of auracyanin is not yet known, although its redox properties are compatible with a role in photosynthetic and/or respiratory electron flow.

Copper-containing proteins have been isolated from a variety of sources both eukaryotic and bacterial. While the function of some of these copper proteins is unknown, many are thought to take part in some sort of electron-transfer process (Ryden, 1984). Copper proteins can have single or multiple copper centers with the copper in either the +1 or +2 state. Because Cu^{2+} has a d^9 electronic configuration, it is paramagnetic and

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displays an ESR spectrum. The ESR spectra of the type 1 or "blue copper" centers show unusually small hyperfine splitting constants (Boas, 1984). In the oxidized (Cu^{2+}) form, the type 1 copper center has a characteristic intense blue color due largely to a broad absorption peak centered at ~600 nm with an extinction coefficient (ϵ) between 1000 and 10000 M^{-1} cm^{-1}. This absorption is ~100 times larger than that of other Cu^{2+} compounds and is attributed to a charge-transfer transition from a cysteine ligand to the copper center (Solomon et al., 1980). Upon reduction of the copper center, the blue color and the visible absorption of the center are bleached. A large number of the small type 1 blue copper proteins have been studied by spectroscopic and physical techniques (Adman, 1985; Solomon et al., 1986).

The best characterized of the blue copper proteins is plastocyanin, which contains a single type 1 copper center. A 1.6-Å resolution X-ray crystal structure has been obtained for plastocyanin (Guss & Freeman, 1983). Plastocyanin transfers electrons from the cytochrome b_6/f complex to the oxidized primary donor of photosystem I in most oxygen-evolving photosynthetic organisms (Haehnel, 1986). A variety of algae and cyanobacteria may contain either plastocyanin or a soluble *c*-type cytochrome in this position in the photosynthetic electron-transfer chain depending on the availability of copper (Wood, 1978; Krogmann, 1986; Sandmann, 1986). Cytochrome c_2 functions in both respiratory and photosynthetic electron flow in the purple photosynthetic bacteria and rereduces the oxidized reaction center (Dutton, 1986).

Chloroflexus aurantiacus is a thermophilic green photosynthetic bacterium that contains a photosynthetic reaction center similar to the one found in the purple photosynthetic bacteria (Pierson & Castenholz, 1974; Blankenship, 1985; Amesz, 1987; Kirmaier & Holten, 1987). This organism is unusual in many ways including its apparent lack of soluble *c*-type cytochromes (Bartsch, 1978; Bruce et al., 1982; Wynn et al., 1987) and its lipid and carotenoid composition (Knudsen et al., 1982; Pierson & Castenholz, 1974). The pathway for carbon reduction is also novel (Holo & Sirevåg, 1986). While *C. aurantiacus* stains Gram negative, the composition of the cell wall is more similar to that found in Gram-positive organisms (Jürgens et al., 1987). Finally, *C. aurantiacus* is only distantly related to all other photosynthetic organisms, according to analysis of its 16S ribosomal RNA (Woese, 1987) and appears to have branched away from the other photosynthetic organisms at an early point. This suggests that *C. aurantiacus* is an important organism with which to investigate the evolution of photosynthesis.

We have isolated a small type 1 blue copper protein from this organism that may be involved in photosynthetic or respiratory electron flow (Gabrielson & Blankenship, 1985; Trost et al., 1987; McManus et al., 1988).

Materials and Methods

Sources. All chemicals used were of reagent grade unless specified otherwise. Ultrapure grade SDS was obtained from Boehringer Mannheim, Indianapolis, IN. Acrylamide and bis(acrylamide) were from Bio-Rad, Richmond, CA.

Purification. C. aurantiacus strain J10-fl was grown under high-light conditions in the modified medium D of Pierson and Castenholz (1974). Cultures were routinely checked for purity by light microscopy. Approximately 200 g of wet-packed cell paste was brought to a volume of 300 mL with 20 mM Tris, pH 8.0. The cell suspension was cooled to 0 °C with an ice bath and sonicated three times for 3 min at a power setting of 8 on a Branson Model 350 sonifier cell disrupter. During the second sonication, the protease inhibitor phenylmethanesulfonyl fluoride was added to a concentration of 1 mM from a 100 mM stock solution in 2-propanol. Unbroken cells and cell debris were removed by centrifugation at 12100*g* for 15 min. Membranes were pelleted by centrifugation of the resultant supernatant liquid at 200000*g* for 120 min in a Beckman 50.2 Ti rotor. The pelleted membranes were resuspended with 20 mM Tris, pH 8.0, and diluted to an optical density of 15 at 865 nm.

Initially, auracyanin was obtained by detergent treatment of the diluted membranes [1.5% lauryldimethylamine *N*-oxide (LDAO)[1] for 1 h at 37 °C followed by ultracentrifugation and DEAE-Sephacel chromatography of the supernatant liquid]. In later experiments, auracyanin was obtained by salt treatment of isolated membranes. Diluted membranes were stirred with 200 mM NaCl/20 mM $MgCl_2$ overnight at 4 °C, followed by ultracentrifugation at 200000*g*. The supernatant liquid from the ultracentrifugation was fractionated by adding solid $(NH_4)_2SO_4$. Additions of 10% w/v were made with stirring at 0 °C, followed by a 15-min incubation period. Precipitated material was removed after each addition by centrifugation at 12100*g* for 10 min. Crude auracyanin precipitated at approximately 40% w/v ammonium sulfate, as assayed by oxidized (plus ferricyanide) minus reduced (plus ascorbate) visible absorbance difference spectroscopy. The pellet containing the auracyanin was resolubilized in 20 mM Tris, pH 8.0, and dialyzed against the same buffer to remove ammonium sulfate. After concentration using an Amicon PM-10 membrane filter, the sample was chromatographed on a 2.5 × 35 cm Sephadex G-100 column. The auracyanin-containing fractions were pooled, reconcentrated, and chromatographed on DEAE-Sephacel in 20 mM Tris, pH 9.0. In the ion-exchange columns, no added salt was needed to elute auracyanin if LDAO was present but a 0–100 mM NaCl gradient was required in the absence of detergent.

Characterization. Absorption spectra were taken on either a Shimadzu UV-160 or a Cary 219 recording spectrophotometer. The circular dichroism (CD) spectrum was recorded on an instrument described elsewhere (Brune et al., 1987) equipped with a Hinds International PEM-80 photoelastic modulator.

Molecular weights were determined by discontinuous SDS–PAGE using samples treated with 2% SDS and heated at 100 °C for 5 min either with or without 10 mM dithiothreitol (DTT). The monomer molecular weight was determined on a 15% T gel by using low molecular weight standards from Sigma.

Protein assays were carried out with modified Lowry (Peterson, 1977), bicinchoninic acid (Pierce Chemical Co., Rockford, IL), and brilliant blue G250 binding (Sedmak & Grossberg, 1977) assays with bovine serum albumin as the standard.

Copper content was determined by graphite furnace atomic absorption using the method of standard additions on a Perkin-Elmer Model PE403 atomic absorption spectrophotometer with a H2A2100 furnace. Absorption was monitored at 216.5 nm with deuterium arc background correction. The auracyanin sample and all dilution buffers used for copper analysis were passed through a 1 × 10 cm column of Chelex 100 resin (Bio-Rad) to remove free copper, and the absorption spectrum vs 20 mM Tris/0.05% LDAO, pH 8.0, was recorded on a Cary

[1] Abbreviations: ESR, electron spin resonance; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; LDAO, lauryldimethylamine *N*-oxide; DEAE, diethylaminoethyl; DTT, dithiothreitol; ODV, optical density × volume in milliliters.

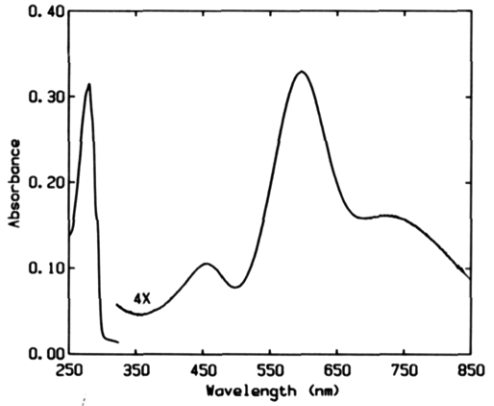

FIGURE 1: Room temperature UV/vis absorption spectrum of 28 μM oxidized auracyanin in 20 mM Tris, pH 8.0.

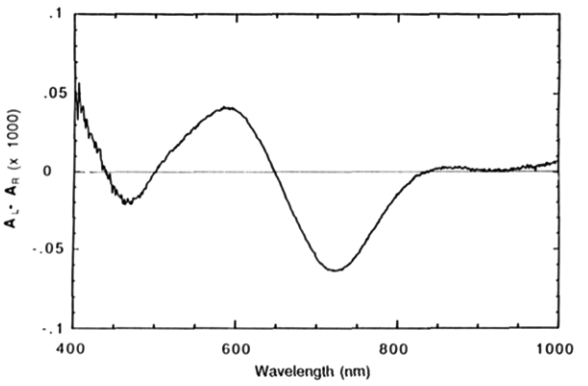

FIGURE 2: Room temperature circular dichroism spectrum of 117 μM oxidized auracyanin in 20 mM Tris, pH 8.0, with a 1-cm path length cell.

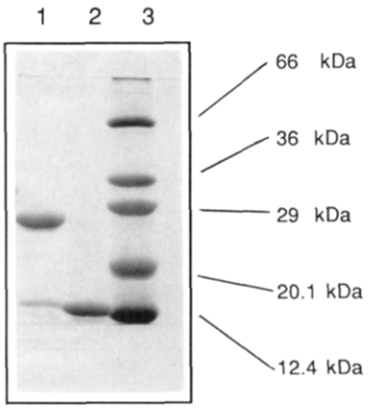

FIGURE 3: SDS–PAGE of auracyanin on 12.5% acrylamide. Lane 1: 10 μg of auracyanin treated with 2% SDS and heated at 100 °C for 5 min. Lane 2: the same as lane 1 but with 10 mM dithiothreitol. Lane 3: molecular weight standards.

219 spectrophotometer. Several different sample concentrations were used. No matrix effect was observed (Freedman & Peisach, 1984).

Potentiometric titrations of auracyanin were carried out on a sample with A_{596} = 0.5 in 20 mM Tris/0.05% LDAO, pH 8.0. The sample was titrated in a homemade three-neck 1.0-cm-path cuvette, and the absorbance at 596 nm was monitored with a Cary 219 spectrophotometer. The potential was referenced to a Radiometer K401 calomel electrode with a Radiometer P101 platinum working electrode. Sodium ascorbate and potassium ferricyanide were used as titrants. Redox mediators were 10 μM each of phenazine methosulfate and 2,3,5,6-tetramethylphenylenediamine.

Electron spin resonance (ESR) spectra were taken on a Bruker ER200D spectrophotometer in quartz sample tubes at both X and Q bands. A sample with A_{596} = 3.0 in 20 mM Tris (pH 8.0)/0.05% LDAO was thoroughly degassed by using three successive freezing, evacuation, and thawing cycles after which the sample tubes were filled with argon before sealing. The temperature of the samples was maintained at either 77 or 4 K. Computer simulations of the ESR spectra were done with a program adapted from Daul et al. (1981).

Amino acid analysis was performed by the Biotechnology Instrumentation Facility at the University of California—Riverside. The purity of the sample analyzed was greater than 96% as determined by SDS–PAGE. Cysteine and methionine were determined on a separate performic acid oxidized sample, and the composition shown is the composite from both the oxidized and nonoxidized determinations. Integer residue values were calculated on the basis of a monomer molecular mass of 12 800 Da.

Isoelectric point determination was by direct measurement using a Ross combination pH electrode (Corning) on samples recovered from preparative isoelectric focusing in a Rotofor preparative isoelectric focusing unit (Bio-Rad). The sample was first focused by using 3-10 Pharmalytes (Pharmacia). The blue fractions were then recovered, combined, and refocused without any additional ampholytes.

RESULTS

Auracyanin shows an absorption spectrum typical of blue copper proteins (Figure 1) with a broad peak at 596 nm and a wide shoulder at 715 nm. In addition, there is a small absorption peak at 420 nm and a peak at 280 nm due to aromatic amino acids. The purity of auracyanin can be determined from the ratio of A_{280} to A_{596}. The best ratio we have obtained is 3.67 (20 mM Tris, pH 8.0). Assignments of the visible absorption peaks should be similar to those found for the other small type 1 blue copper proteins (Solomon et al., 1980; Penfield et al., 1985). The CD spectrum of auracyanin (Figure 2) is most similar to that of plastocyanin and azurin. The large negative CD band seen at 450 nm in stellacyanin is not present in auracyanin.

The yield of pure auracyanin from salt-washed membranes varied from 2.0 to 5.0 A_{596} × volume in milliliters (ODV) from 200 g of wet cell paste (corresponding to 8.0–20 mg of pure protein). Higher yields have been obtained from the LDAO-treated membranes, as much as 20 ODV/200 g of cells. However, due to the large amount of interfering pigments that are also released by this treatment, the spectral assay for auracyanin is unreliable and it is difficult to recover the pure protein. For these reasons the salt-washed preparative method was used in the majority of the experiments.

Discontinuous SDS–PAGE (Figure 3, lane 1) of purified auracyanin in the absence of the disulfide reducing agent DTT results in two bands, a major band at a molecular mass of 25 kDa and a minor band at 13 kDa. In the presence of DTT, SDS–PAGE results in a single band with a molecular mass of 12.8 kDa (lane 2). This suggests that auracyanin exists as a disulfide-linked dimer when isolated. Preliminary sequence analysis indicates it is a homodimer (McManus et al., 1988). Whether the dimer is present in vivo is not certain and may be due to dimerization during purification or preparation for SDS–PAGE. A similar result has been found for plastocyanin (Yocum et al., 1975). The apparent monomer molecular weight is 12 800. Preparative isoelectric focusing results in a p*I* of 4.0. The pHs of the sample chambers adjacent to the chamber containing auracyanin were 3.8 and 4.1.

Atomic absorption analysis showed little if any matrix effect, and the average of all samples assayed gives an extinction

Table I: Amino Acid Composition of Auracyanin

amino acid	mol %	residues/mol	integer res/mol
Asx	8.67	11.0	11
Thr	8.59	10.9	11
Ser	7.09	9.0	9
Glx	9.46	12.0	12
Pro	5.12	6.5	6
Gly	14.81	18.8	19
Ala	12.37	15.7	16
Cys	1.97	2.5	3
Val	6.38	8.1	8
Met	1.97	2.5	3
Ile	4.57	5.8	6
Leu	6.78	8.6	9
Tyr	2.68	3.4	3
Phe	2.60	3.3	3
Trp	NA[a]	NA	NA
Lys	3.94	5.0	5
His	1.42	1.8	2
Arg	1.58	2.0	2
total	100.00	126.9	128

[a] Not available.

coefficient at 596 nm of $\epsilon_{596} = 2900\ M^{-1}\ cm^{-1}$ relative to the copper concentration with a standard deviation of 7.5%.

The Lowry and bicinchoninic protein assays, coupled with the absorption at 596 nm and the SDS–PAGE monomer molecular mass, indicate that there are 0.8 and 1.1 copper atoms per protein monomer, respectively. The protein content indicated by the Bradford dye binding assay was anomalously low and would suggest that there are 3.2 coppers per monomer. In light of the relatively small size of the protein, the apparent lack of interaction of the copper centers (see below), and what is known about other proteins in this class, we feel that this assay is probably unreliable for this protein. The amino acid sequence of auracyanin is presently being determined and should give a conclusive determination of the number of coppers per protein monomer. The amino acid composition of auracyanin is shown in Table I. The presence of only two histidine residues also supports the assignment of a single copper center per monomer (see Discussion).

The X- and Q-band ESR spectra of auracyanin are shown in Figure 4 along with computer simulations. The parameters from the computer-simulated spectra are compared with those of other type 1 copper proteins in Table II. The parameters most closely match those of rusticyanin (Cox, 1978) and cucumber basic blue protein (Aikazyan & Nalbandyan, 1979). The resolution of three g values indicates that the geometry about the copper center has rhombic rather than the axial distortion seen in type 1 copper centers such as azurin and plastocyanin.

An optically monitored potentiometric titration of auracyanin (Figure 5) is best fit to the Nernst equation with an $n = 1$ curve and a midpoint potential of +240 mV with respect to the standard hydrogen electrode.

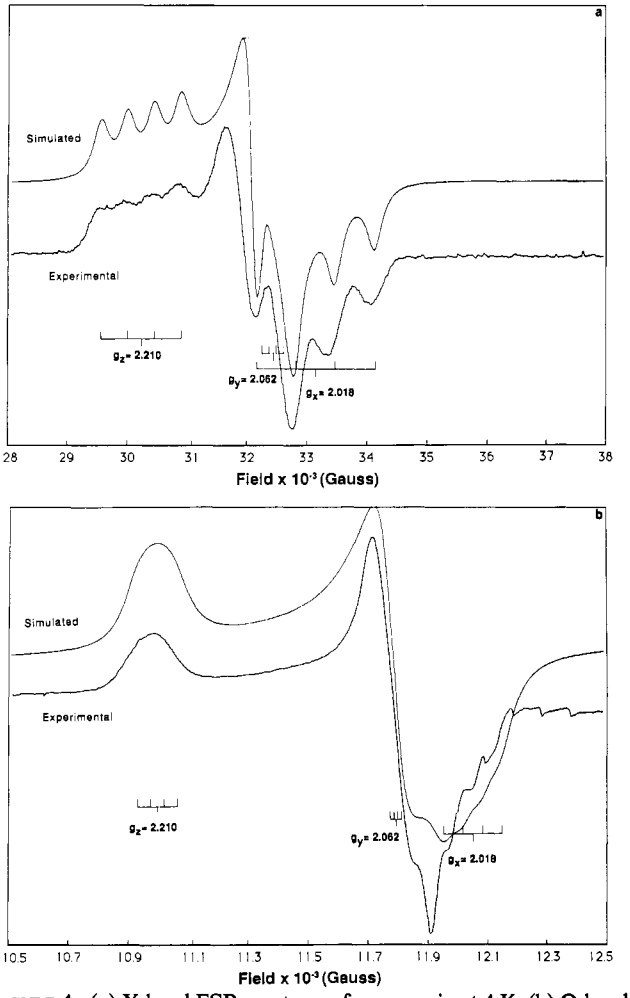

FIGURE 4: (a) X-band ESR spectrum of auracyanin at 4 K; (b) Q-band ESR spectrum of auracyanin at 77 K. Both samples are in 20 mM Tris/0.05% LDAO, pH 8.0. Simulated spectra used Lorentzian line shape with g values of 2.210, 2.062, and 2.018 and A values of 0.0047, 0.0012, and 0.0062 cm^{-1}. The high-field features seen in the Q-band ESR spectrum are due to manganese impurities in the quartz sample tube.

Discussion

The ESR parameters of auracyanin suggest that it should be grouped with the stellacyanins, including stellacyanin, mavicyanin, rusticyanin, and plantacyanin (Adman, 1985). This is a diverse subgroup of the small type 1 blue copper proteins, which have $A_x > A_z$ and a rhombic g tensor. The ESR parameters may be a reflection of a unique type of copper coordination about the copper center in these proteins. While the ESR parameters reflect the geometry and ligand field stabilization, they cannot be used to assign the particular ligands to the copper. The X-ray crystal structures of poplar leaf plastocyanin and azurin from *Pseudomonas aeruginosa*

Table II: ESR Parameters and Redox Potentials of Some Small Blue Copper Proteins[a]

		ESR parameters						
		$g_\parallel$		$g_\perp$	$A_\parallel$[b]		$A_\perp$[b]	redox potential (mV)
protein	source	g_z	g_y	g_x	A_z	A_y	A_x	
azurin	*Pseudomonas aeruginosa*		2.260	2.052		6.0	0.0	300
azurin	*Paracoccus denitrificans*		2.29	2.052		7.7	0.0	230
plastocyanin	french bean		2.226	2.053		6.3	<1.7	347
stellacyanin	lacquer tree latex	2.287	2.077	2.025	3.5	2.9	5.7	184
plantacyanin[c]	cucumber seedlings	2.207	2.08	2.02	5.5	1.0	6.0	317
rusticyanin	*Thiobacillus ferroxidans*	2.229	2.064	2.019	4.5	2.0	6.5	680
auracyanin	*Chloroflexus aurantiacus*	2.210	2.062	2.018	4.7	1.2	6.2	240

[a] Values taken from Ryden (1984), Cox (1978), and Adman (1985). [b] A values in $cm^{-1} \times 10^3$. [c] Also known as cucumber basic blue protein.

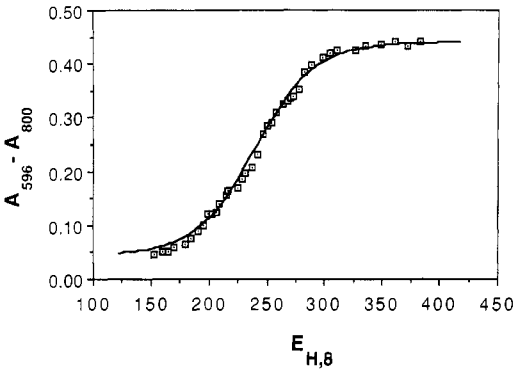

FIGURE 5: Optically monitored potentiometric titration of 170 μM auracyanin in 50 mM Tris/0.05% LDAO, pH 8.0. Mediators used were phenazine methosulfate and 2,3,5,6-tetramethylphenylenediamine at 10 μM. The solid line is a theoretical Nernst curve with a midpoint potential of 240 mV and $n = 1$.

show that the single copper atom is coordinated by two histidine imidazole nitrogens, a cysteine thiolate, and a more distant methionine thioether (Colman et al., 1978; Adman et al., 1978). In addition, azurin from *Alcaligenes denitrificans* has a weak fifth ligand from a peptide carbonyl oxygen (Norris et al., 1986). In contrast to azurin and plastocyanin, stellacyanin contains no methionine, so the fourth ligand must be some other group. From the amino acid sequence and spectroscopic studies it has been suggested that the fourth ligand is a cystine disulfide (Bergman et al., 1977; Solomon et al., 1980; Engeseth et al., 1984; Farver et al., 1987), although no direct structural data are available to confirm this.

Midpoint potentials of several of the small blue copper proteins are also shown in Table II. While the midpoint potential of +240 mV for auracyanin is somewhat low, it is not anomalously so. All of the copper proteins display higher potentials than the standard potential for the Cu^{2+}/Cu^{1+} pair of +158 mV. Most of the copper proteins are in the range of +300 mV, yet there is a remarkable variability, with the lowest potential of +184 mV for stellacyanin and the highest at +680 mV for rusticyanin. It has been suggested that the "tuning" of this potential is controlled by the relative position of the copper and the axial ligand(s) (Norris et al., 1986).

The amino acid composition of auracyanin indicates that there are probably only two histidine residues. By analogy to other small copper proteins, these must be involved in coordinating the copper, considering the absorption and CD spectra. In addition, there are only three cysteine residues. As in the case of the histidine residues, at least one of the cysteines should be involved in coordination of the copper. Because a second cysteine appears to be involved with an interchain disulfide bridge, there is only one remaining cysteine, making it impossible for the fourth ligand to be a cystine disulfide. It therefore seems likely that the fourth ligand is probably one of the methionines.

Although the function of auracyanin is not certain, preliminary kinetic data suggest that the functional role of auracyanin is similar to plastocyanin or a soluble *c*-type cytochrome (McManus et al., 1988). This is consistent with the midpoint potential of auracyanin and the fact that *C. aurantiacus* apparently contains no soluble *c*-type cytochromes.

In the purple bacterial photosystems, cytochrome c_2, a soluble *c*-type cytochrome, shuttles electrons between the cytochrome b/c_1 complex and either the oxidized reaction center or a *c*-type cytochrome that is tightly bound to the reaction center. This tightly bound cytochrome then rereduces the oxidized reaction center. In oxygenic photosynthetic organisms, plastocyanin has the analogous function of carrying electrons from the cytochrome b_6/f complex to the oxidized photosystem I reaction center, although soluble *c*-type cytochromes sometimes serve this function. The midpoint potential of +240 mV makes it thermodynamically possible for auracyanin to donate electrons either directly to the oxidized reaction center (+340 mV) or to the membrane-bound cytochrome *c*-554 (+260 mV), which is known to be involved in photosynthesis in *C. aurantiacus* (Bruce et al., 1982). In the latter case, cytochrome *c*-554 would be functioning in a manner analogous to that of the tightly bound cytochromes found in many of the purple bacterial photosynthetic reaction centers. It should be noted though that in the purple bacterium *Rhodobacter capsulatus* cytochrome c_2 deletion mutants can still grow photosynthetically, suggesting that a mobile carrier may not be absolutely essential (Daldal et al., 1986). However, cytochrome c_2 deletion mutants of the closely related organism *Rhodobacter sphaeroides* do not grow photosynthetically, making it difficult to generalize on this point (Donohue et al., 1988).

Auracyanin appears to be a peripheral membrane protein, because it is released from purified membranes by salt washing. We are currently investigating the cellular localization of auracyanin using immunoelectron microscopy. If auracyanin has a function analogous to cytochrome c_2 in the purple photosynthetic bacteria, then it should be localized on the periplasmic side of the cytoplasmic membrane.

Further kinetic studies are needed to determine if auracyanin is indeed involved in photosynthetic electron flow. Possible functions that have been suggested for other small type 1 copper proteins are Fe^{2+} oxidation during respiratory iron oxidation for rusticyanin (Cox & Boxer, 1978) or methylamine oxidation for amicyanin (Gray et al., 1986). If auracyanin does function as a "bacterial plastocyanin", it then has important consequences for theories concerning the evolution of photosynthetic electron-transfer pathways (Olson & Pierson, 1987). Studies of 16S ribosomal RNA have indicated that *C. aurantiacus* is a member of one of the earliest branching lines of the eubacteria (Woese, 1987). This suggests that *C. aurantiacus* may be a descendent of the *Chlorobium*-like green bacterium that produced the ancestral blue copper protein, as suggested by Ryden (1984). In summary, auracyanin is a unique protein that may provide insight into the evolution of photosynthesis and the nature of blue copper proteins.

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