H-Bonding Maintains the Active Site of Type 1 Copper Proteins: Site-Directed Mutagenesis of Asn38 in *Poplar* Plastocyanin†

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ABSTRACT: Type I Cu proteins maintain a trigonal N_2S coordination group (with weak axial ligation) in both oxidation states of the $Cu^{2+/+}$ ion, thereby reducing the reorganization energy for electron transfer. Requirements for maintaining this coordination group were investigated in *poplar* plastocyanin (Pcy) by mutation of a conserved element of the type 1 architecture, an asparagine residue (Asn38) adjacent to one of the ligating histidines. The side chain of this asparagine forms an active site clasp via two H-bonds with the residue (Ser85) adjacent to the ligating cysteine (Cys84). In addition, the main chain NH of Asn38 donates an H-bond to the thiolate ligand. We have investigated the importance of these interactions by mutating Asn38 to Gln, Thr, and Leu. The mutant proteins are capable of folding and binding Cu^{2+}, but the blue color fades; the rate of fading increases in the order Gln < Thr < Leu. The color is not restored by ferricyanide, showing that the protein is modified irreversibly, probably by oxidation of Cys84. The more stable mutants N38Q and N38T were characterized spectroscopically. The wild-type properties are slightly perturbed for N38Q, but N38T shows remarkable similarity to another type 1 Cu protein, azurin (Azu) from *Pseudomonas aeruginosa*. The Cu−S(Cys) bond is longer in Azu than in Pcy, and the NH H-bond to the ligating S atom is shorter. Molecular modeling suggests a similar effect for N38T because the threonine residue shifts toward Ser85 in order to avoid a steric clash and to optimize H-bonding. These results demonstrate that H-bonding adjacent to the type 1 site stabilizes an architecture which both modulates the electronic properties of the Cu, and suppresses side reactions of the cysteine ligand.

Type 1 copper proteins are widely found in nature and function as electron-transfer agents (*1*−*3*). The spectroscopic properties of the oxidized form are unusual for Cu(II), as is the coordination geometry: a trigonal plane formed by one cysteine and two histidine side chains, with weak axial interaction from a distant methionine side chain, and sometimes a backbone carbonyl group (*4*). Most known Cu(II) complexes are tetragonal, because of the ligand field stabilization of the d^9 electronic configuration by four equatorial σ bonds. Cu(I) complexes, however, have d^{10} electronic configuration and prefer tetrahedral or trigonal to tetragonal coordination. Consequently, a trigonal Cu(II) coordination geometry minimizes the ligand reorganizational energy associated with electron transfer. X-ray crystallography has established that at physiological pH the coordination group is unaltered between oxidized and reduced protein (*5*−*7*). The same coordination geometry is also observed when metals other than Cu are bound at the site (*8*).

These considerations have produced the concepts of "entatic" (*9*) or "rack-induced" (*10*, *11*) states, in which the protein is seen as enforcing a high-energy coordination geometry on the Cu(II) ion in order to reduce the reorganization energy and accelerate electron transfer. However, Roos has questioned this concept on the basis of density functional theory (DFT) calculations (*12*) which show the type 1 Cu^{2+} coordination group (modeled as imidazole, methanethiolate, and dimethyl sulfide ligands at the positions defined by the protein X-ray crystal structure) to be stable toward distortion, even in the absence of protein restoring forces. Ligand sets consisting of four N/O atom ligands were found to prefer tetragonal geometry, as expected. The trigonal geometry of the type 1 site was inferred to result from the simultaneous π and σ donor propensity of the thiolate ligand. The π and σ donor orbitals are known to dominate the type 1 spectroscopic properties (*13*), including the small EPR hyperfine coupling constants and the low-energy S → Cu(II) charge transfer (CT) transition, which gives rise to the intense blue color. The π and σ thiolate orbitals can be viewed as occupying two corners of a virtual tetragonal plane(*13*).

Even if the trigonal Cu(II) geometry is intrinsically stable, the protein clearly plays a key role in preventing side reactions. This role have been highlighted by the protein engineering studies of Hellinga (*14*), who designed a binding site of the correct coordination geometry by site-directed mutation of a nonmetalloprotein, thioredoxin. Although Hg^{2+} bound to the site as expected, Cu^{2+} did not. The absence of any charge-transfer absorption was evidence that the Cu^{2+} bound to nonthiolate ligands only. When potentially coordinating aspartate residues near the designed site were mutated, Cu^{2+} addition produced disulfide formation, presumably reflecting local unfolding of the cysteine residue. This side reaction was suppressed by reengineering the site

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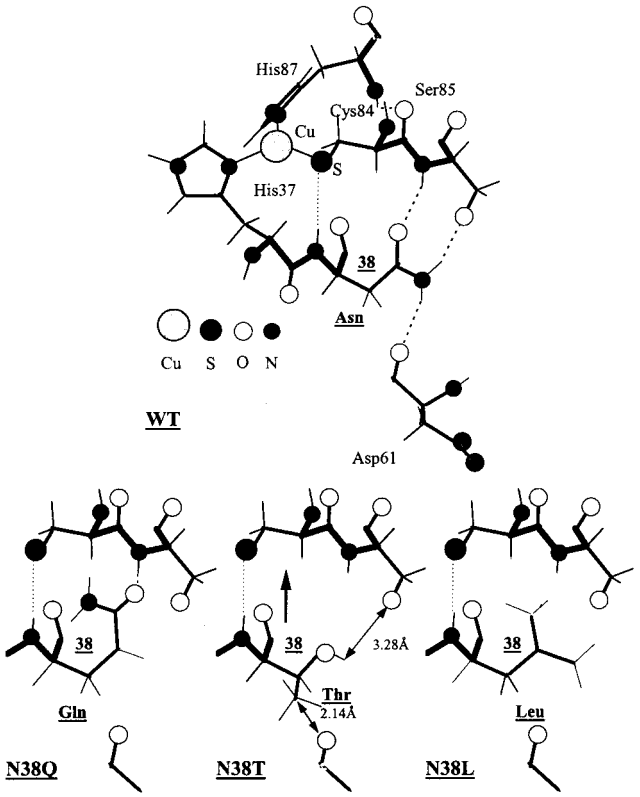

FIGURE 1: H-bonding network of Asn38 (top). Molecular modeling with InsightII (Molecular Simulation, Inc.) shows the expected local interactions for the Gln, Thr, and Leu mutants. Energy minimization was not performed. The methionine ligand (Met92), which is pointed out of the paper, is omitted for clarity (PDB 5 Pcy).

to anchor the cysteine on a β strand. But the desired trigonal coordination was still not obtained because a water molecule was able to expand the coordination group.

Thus the type 1 Cu site must (i) prevent metal ligation by competing side chains, (ii) exclude water, and (iii) inhibit thiolate oxidation by immobilizing the cysteine side chain. To accomplish the protection of the active site, type 1 proteins have evolved a rigid structure, as evidenced by the lack of change even when the copper is removed from plastocyanin (Pcy) (*15, 16*). Presumably, this rigidity results from a conservation of steric and electrostatic forces, but there is currently no information on which specific interactions are critical.

The availability of type 1 protein genes and expression systems provides an opportunity for systematic investigation of the native interactions through site-directed mutagenesis. This technique has been exploited to explore the roles of the ligating residues themselves in azurin (Azu) (*17*−*20*) and Pcy (*20*). Remarkably the histidine ligands of Azu can be mutated to other ligating residues (*19, 21, 22*) and even to glycine, which cannot itself ligate the metal but leaves a "hole", through which exogenous small molecule ligands can bind to the Cu^{2+} (*21, 22*). The cysteine remains bound to the Cu^{2+} in all these mutants, but spectroscopic changes signal significant changes in coordination geometry. The effects of substituting the axial methionine by glutamine and threonine have also been investigated and varying degrees of tetrahedrality have been achieved (*23*−*26*). Finally a "loop" mutant has been produced in which the seven-residue loop connecting three of the ligands in amicyanin was replaced by the nine-residue loop from Pcy (*27*). The resulting hybrid protein displayed spectroscopic indicators that resembled a third type 1 protein, pseudoazurin, more closely than Pcy.

In the present study we focus on the role of an invariant asparagine (Asn38 in Pcy), which follows one of the ligating histidine (His37 in Pcy) in the active site of type 1 copper proteins. In Pcy, this asparagine residue forms three H-bonds, which could be important in fixing the position of the cysteine ligand, Cys84 (Figure 1) (*5, 28*). Two of these H-bonds are formed between the Asn side chain and residue Ser85, which is adjacent to the key ligating residue, Cys84. It seems likely that these H-bonds play an important role in securing the native conformation of the Cys84 side chain. The third H-bond is from the Asn38 main chain NH to the ligating S atom of Cys84. Since this H-bond affects the electron density on the S atom, it must modulate the strength of the Cu−S bond.

To test the role of the Asn38 side chain, we used PCR-mediated mutagenesis to replace this asparagine with leucine, glutamine, or threonine. Leu was chosen because it is isosteric with asparagine but cannot form H-bonds, while Gln and Thr were chosen because they retain H-bonding potential but position the H-bonding moieties in different locations. All the mutant proteins were found to decolorize much more rapidly than the WT protein, establishing the importance of the Asn side chain for maintaining the native metal binding site. The glutamine and threonine mutants were stable enough for spectroscopic characterization; the latter showed, unexpectedly, behavior quite similar to Azu. Molecular modeling suggests that a stronger NH H-bond to thiolate, with concomitant weakening of the S−Cu bond, is responsible for this behavior.

MATERIALS AND METHODS

Enzymes, Chemicals, Bacterial Strains, and Plasmids. Restriction enzymes and ligase were purchased from New England Biolabs. AmpliTaq Taq DNA polymerase and GeneAmp PCR reagent kits were purchased from Perkin-Elmer/Cetus. The PCR optimizing kit Optimer was purchased from Invitrogen. The GeneClean kit was bought from BIO 101 Inc. The Wizard Miniprep kit was purchased from Promega. Low-melting SeaPlaque agarose (FMC), NuSieve GTG agarose (FMC), ampicillin (Sigma), and IPTG (isopropyl β-thiogalactoside) (Sigma) were used as supplied. Primers for PCR were synthesized at the DNA synthesizing facility in the Department of Molecular Biology at Princeton University.

E. coli K strain ×90 (*ara*$^-$, Δ*lac-pro, nalA, argEam, rif*^{R}, *thil*$^-$ [F′, *lacI*^{q}, *pro*$^+$] (*29*) was used for cloning the Pcy gene and ×90(DE3) for heterologous expression. The Pcy gene was cloned into pET3a with *Nde* I and *BamH* I restriction sites and placed under the control of T7 promoter gene as described by Ybe and Hecht (*30*).

Mutagenesis. Mutant versions of the Pcy gene were constructed in plasmid pET3a by cassette replacement mutagenesis using a cassette encoding the first 53 amino acids of Pcy. This cassette is bracketed by a *Nde* I site in DNA encoding the initiator methionine and a *Mlu* I site in DNA encoding residue 53. Mutant version of this cassette were generated by a two-step PCR procedure (Figure 2). In

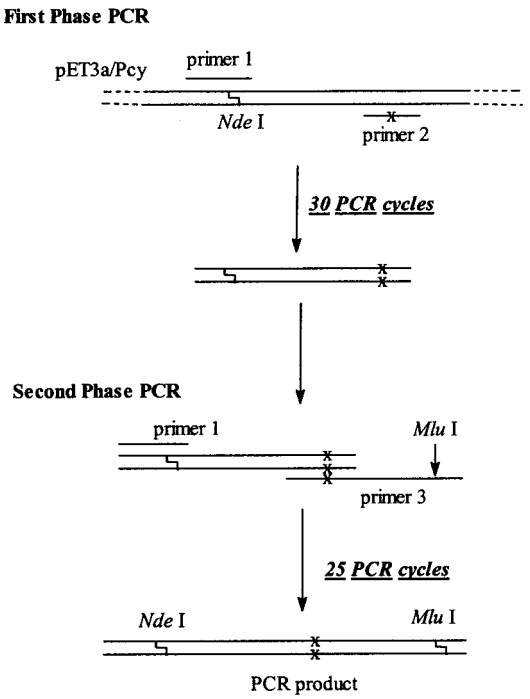

FIGURE 2: Scheme of the two-phase PCR reaction (see text).

the first step, the desired mutations were incorporated using a 23-base primer spanning the *Nde* I site (primer no. 1) and a 21-base mutagenic primer spanning the DNA encoding residue 38 (primer no. 2); the product of the first step was elongated in a second step of PCR using primer no. 1 and a 58-base primer (primer no. 3) that extended the DNA 5 bases beyond the *Mlu* I site.

Primer no. 1 has the sequence 5′-TTTAAGAAGGAG-ATATA*CATATG*-3′ with the *Nde* I site italicized. The ATG in this site encoded the initiator methionine. Primer no. 2 spans the region from Asp 42 to Phe 35 with the codon for position 38 changed from GTT (Asn) to TTG (Gln), AGT (Thr), or CAG (Leu). Primer no. 3 spans from Ser53 to Phe35 with the same 3′ end as primer no. 2. The sequences of these oligonucleotides are shown in Table 1. The 5′ end of primer no. 3 extends five bases beyond the *Mlu* I site to facilitate restriction digestion.

Reaction conditions were optimized with Optimer PCR kit. For the first PCR step, each reaction contained 1 ng (10^{-15} mol) of template (pET3a/PCY), 100 pmol each of primer no. 1 and no. 2, 800 pmol dNTPs, and 2.5 units of AmpliTaq polymerase in 100 μL of buffer at pH = 9.5 containing 30 mM Tris-HCl, 7.5 mM $(NH_4)_2SO_4$, and 1.75 mM $MgCl_2$. The reaction mixture was subjected to 30 cycles of the following program: 95 °C, 1 min; 47 °C, 1 min; 72 °C, 1 min. Unreacted primers were removed by filtration with a Spinfilter (Millipore Ultrafree-MAC, 30 000 NMWL). In the second PCR step, primer no. 1 and primer no. 3 were used to amplify the product of the first step. Reaction mixtures contained 10^{-13} mol of template, 100 pmol of each primer, 800 pmol of dNTPs, and 2.5 units of AmpliTaq polymerase in the same buffer as described above. The reaction mixture was subjected to 25 cycles of the following program: 95 °C, 1 min; 45 °C, 1 min; 72 °C, 1 min. The final product was digested with *Nde* I and *Mlu* I, purified with 3% NuSieve agarose gel or filtration with Spinfilter (Millipore) and cloned into the *Nde* I and *Mlu* I sites of the Pcy gene in pET3a (*30*). Transformants were picked, plasmid DNA was isolated using a Wizard kit, and DNA was sequenced by the Sanger dideoxy method (N38Q) (*31*) or at the DNA sequencing facility at Joslin Diabetes center, Boston, MA (N38T and N38L).

Protein Expression and Purification. WT and mutant Pcy were expressed in *Escherichia coli* strain ×90(DE3) and isolated as described (*30*, *32*−*34*). For WT Pcy, 1 mM Cu-(II) citrate was added to the growth medium as described previously (*32*). The N38Q mutant produced Pcy satisfactorily under these conditions, but for the N38T and N38L mutants, the protein yield was much lower. For these mutants, an optimal expression level was obtained with 0.1 mM cupric citrate. A similar Cu concentration dependence has been observed for the expression of pseudoazurin mutants (*35*).

E. coli cells were harvested by centrifugation, and plastocyanin was released from the cells by three cycles of freeze/thaw (*32*, *33*) and dialyzed into 0.5 mM $MgCl_2$ in H_2O at 4 °C until a maximum amount of blue-colored Pcy was obtained. The protein solution was concentrated with an Amicon centriprep and filtered through a 0.2 μm Acrodisc (Gelman Sciences). The protein was purified by HPLC with a POROS 20HQ anion exchange column (Perseptive Biosystems) as previously described (*30*, *34*). Protein purity was assessed by running samples on 8−25% acrylamide PhastGels run on a PhastSystem (Pharmacia) with SDS denaturing buffer strips. Gels were stained with PhastGel Blue R.

Sample Preparation. For RR experiments with WT, N38Q, and N38T Pcy, protein samples were exchanged to pH 6.0 with 20 mM Bis-Tris buffer in 150 mM NaCl and frozen with liquid nitrogen in a coldfinger (*36*). For EPR experiment with WT and N38Q mutant, the samples were washed with 150 mM NaCl solution in pH 6.0, 20 mM Bis-Tris buffer in an Amicon concentrator (molecular weight cutoff = 3K) to remove nonspecifically bound Cu ions and then mixed with glycerol to a final concentration of 10% glycerol.

Spectroscopic Measurements. UV−vis spectra were collected with a computer-interfaced Hewlett-Packard 8452A diode array UV−vis photospectrometer using ∼1 mM protein solution in a 1 mm path length quartz cuvette at room temperature. Absorption coefficients of WT Pcy and N38Q mutant were determined by titrating 2 mL protein solutions (sealed in a cuvette with a septum and purged with Ar gas) with freshly made 1.09 mM ascorbic acid through an airtight microsyringe. Absorption changes were followed with the spectrophotometer at 597 nm (for WT Pcy) or 588 nm (for N38Q Pcy). The absorption coefficient ϵ was taken as the slope of the linear regression of absorbance change vs moles of added ascorbic acid. Identical conditions were used for the WT and mutant proteins to facilitate direct comparison.

X-band EPR spectra were taken with a Bruker ES300 X-band EPR spectrometer using ∼0.1 mM protein solution at 100 K. Parameters for recording EPR spectra were 12.5 mT/min sweep rate, 0.32 mT field modulation width, 9.099 GHz frequency, and 4 mW microwave power.

RR scattering was excited at 647.1 nm (Kr^+ laser Coherent-Innova 100-3a). RR spectra were collected with a coldfinger (77 K) (*36*) to minimize the degradation of protein and to improve resolution. Raman scattering was dispersed with either a Spex1404 double monochromator or a Spex1877

Table 1: Primer Sequences and Mutant Properties

Mutant	Sequence of Single Stranded DNA[a]	A_{max}(nm)	ε ($M^{-1}cm^{-1}$)	$g_{\perp}$	$A_{//}$
	Asn38				
WT	5'-CGAAAACGAT***GTT***GTGTGGG -3'	596	4760	2.053	0.0063
	Primer 2: 5'-CGAAAACGAT***TTG***GTGTGGG -3'				
N38Q	*Mlu* I	588	2360	2.057	0.0066
	Primer 3: 5'-ATCGCACGCGTCGACACCTGAAGGTATA CTGTCTTCGTCGAAAACGAT***TTG***GTGTGGG - 3'				
	Primer 2: 5'-CGAAAACGAT***AGT***GTGTGGG -3'				
N38T	Primer 3: 5'-ATCGCACGCGTCGACACCTGAAGGTATA CTGTCTTCGTCGAAAACGAT***AGT***GTGTGGG -3'	622	ND	ND	ND
	Primer 2: 5'-CGAAAACGAT***CAG***GTGTGGG -3'				
N38L	Primer 3: 5'-ATCGCACGCGTCGACACCTGAAGGTATA CTGTCTTCGTCGAAAACGAT***CAG***GTGTGGG -3'	596	ND	ND	ND

[a] ***Italic***: mutated bases; ____: restriction site; A_{max}: maximum absorption position; ε: absorption coefficient; $A_{//}$: hyperfine coupling consatnt; ND: not determined.

triple monochromator and detected with an intensified RCA31034A photomultiplier tube or with a diode array detector.

Thermal denaturation of the proteins was investigated by following absorption at 597 nm (WT) or 588 nm (N38Q) as a function of temperature with a Cary spectrophotometer equipped with a Cary temperature controller. A protein solution containing ~0.1 mM protein in pH 6.0, 20 mM Tris-HCl buffer, in a 10 mm cuvette covered with mineral oil, was heated from 30 to 80 °C, at a rate of 0.5 °C/min. Absorption was measured against a reference buffer solution which was heated along with the protein solutions.

RESULTS

Construction of Mutant and Protein Expression. To test the role of H-bonding by the Asn38 side chain, we replaced this Asn with leucine, glutamine, or threonine. Leu was chosen because it is isosteric with asparagine but cannot form H-bonds, while Gln and Thr were chosen because they retain H-bonding potential but position the H-bonding moieties in different locations. Due to lack of appropriate restriction sites in the Pcy gene around Asn38, we devised a two-step PCR method to construct the point mutants (see Figure 2 and Materials and Methods).

Protein Stability. The mutants are all less stable than WT Pcy, which is unaffected by extended periods of storage or repeated freeze−thaw cycles. The N38Q and N38T mutants were decolorized by storage at 4 °C for less than 5 days or by one freeze−thaw cycle. The color faded more rapidly for N38T than N38Q. The N38L protein was isolated as colorless protein and displayed only a transient blue color upon addition of Cu(II) citrate. In no case could the color of the mutants be restored by ferricyanide addition.

The ability to develop a blue color, even transiently, is evidence that the mutant proteins are capable of acquiring the native fold. Consistent with correct global folding, the mutant proteins eluted from ion exchange columns with the same elution volume as the WT protein. The N38Q mutant precipitated upon heating to 52 °C, the same temperature as WT Pcy (*37*). However, N38Q lost its blue color at a substantially lower temperature, ~40 °C, whereas WT Pcy retained its color up to the onset of precipitation.

These observations suggest that although the mutant proteins are capable of folding into the correct global structure, the stability of the native blue copper site is compromised to varying degrees by our mutations. The order of stability is WT > N38Q > N38T > N38L.

Spectroscopic Properties. The mutants all displayed absorption bands near 600 nm (Figure 3, Table 1), but the wavelength maxima (λ_{max}) were variable. Interestingly, λ_{max} = 596 nm for N38L, the same as WT Pcy. However the color was fleeting, and the measured absorbance was ~2% of the WT absorbance. The wavelength was shorter for N38Q, 588 nm, and longer for N38T, 622 nm. N38Q was stable enough to be titrated with ascorbic acid and yielded a molar absorbance, $\epsilon = 2460\ M^{-1}\ cm^{-1}$, half that of WT Pcy ($4760\ M^{-1}\ cm^{-1}$). It is uncertain whether this result means that ϵ is halved for N38Q or that only half the sites are occupied by Cu(II). The absorption band was fully recovered by ferricyanide addition, indicating that the binding site is relatively stable. Therefore, a lowered ϵ for N38Q is possible.

Frozen solution EPR spectra of WT Pcy gave the reported parameters (Table 1). $g_{||}$ was unaltered for N38Q, but $g_{\perp}$ and $A_{||}$ were slightly elevated (Table 1). The other two mutants, being unstable and available in only small quantities, were not characterized.

Low-temperature RR spectra were almost the same for N38Q as for WT Pcy (Figure 4; Table 2), but all bands were slightly ($1-3\ cm^{-1}$) lower in frequency. However, the N38T RR spectrum was dramatically altered (Figure 4; Table 2), with substantially downshifted frequencies and altered intensity distribution. The N38T RR spectrum is strikingly similar to published spectra of azurin (*38*). N38L was too unstable to obtain a RR spectrum.

DISCUSSION

The Asn38 side chain forms two H-bonds with Ser85; its amide NH_2 is a donor to the Ser85 OH group, while its amide C═O is an acceptor to the Ser85 backbone NH (Figure 1). Thus Asn38 and Ser85 form a clasp, securing the relative orientations of the adjacent His37 and Cys84 ligands to Cu.

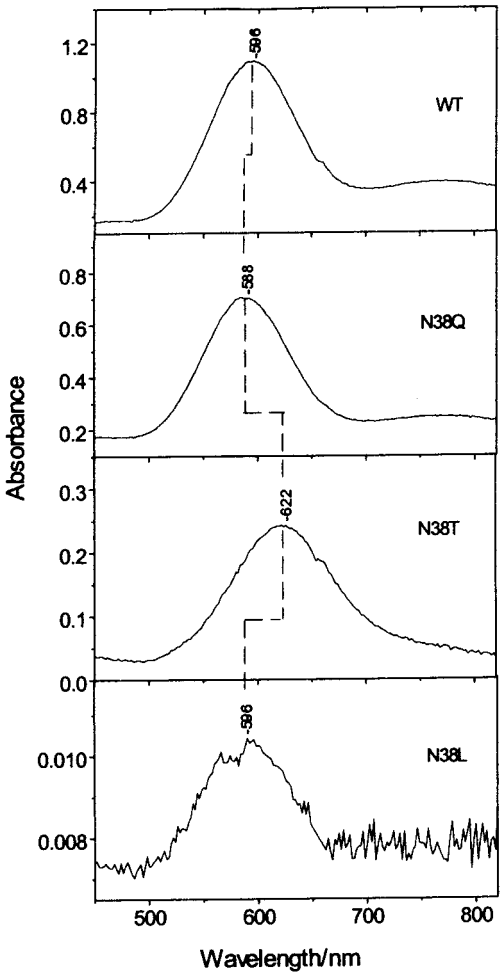

FIGURE 3: Absorption spectra of WT, N38Q, N38T, and N38L Pcy.

The importance of this clasp is revealed by the N38L mutant, whose Cu^{2+} complex is extremely unstable. Leucine is isosteric with asparagine, but its side chain is unable to form H-bonds.

Despite this instability, the N38L mutant is capable of acquiring the native fold, since a transient blue color is seen upon Cu^{2+} addition. However, the rapid fading of the color implies reduction and/or loss of Cu^{2+}, and the failure of ferricyanide to recover the blue color shows that the type 1 site is no longer intact. We infer that the cysteine has been oxidized via Cu^{2+} reduction *(39)*. The likely reaction is the highly exergonic formation of disulfide:

$$2Cu^{2+} + 2RS^{-} = RSSR + 2Cu^{+}$$

Since there is only one cysteine per plastocyanin monomer, this disulfide bond must either link two protein chains, or link a protein to a small molecule thiol, such as glutathione. Intermolecular disulfide formation has previously been observed for unstable turn mutants of Pcy (*30*) and also for type I sites engineered into thioredoxin (*14*). A principal role of the Asn38−Ser85 clasp is therefore to inhibit internal redox inactivation by preventing motion of the Cys84 side chain. Thiol oxidation is prevented because electron transfer produces the high-energy thiyl radical (this is the CT process which gives rise to the blue color).

The N38Q mutant is much more stable than N38L but is still labile relative to wild type. The blue color is lost upon heating to ~40 °C, upon storage for 5 days at 4 °C, or upon a single freeze−thaw cycle, although the wild-type protein is unaffected by these treatments. Like asparagine, glutamine has a primary amide side chain and, therefore, has the same H-bonding capability, but the side chain is lengthened by a methylene group. Molecular modeling shows that the methylene group precludes formation of both amide H-bonds with Ser85, but one of them (carbonyl to backbone NH) can still be attained without displacement of the backbone (Figure 1). As a result, a type 1 coordination group is found which is similar, but not identical, to the wild-type site. The RR spectrum is nearly the same (Figure 4), but the band frequencies are 2−3 cm^{-1} lower. The charge transfer band (Figure 3) is slightly blue shifted and appears to be only half as strong as the wild-type band. Thus the Cu^{2+}−thiolate chromophore is slightly but distinctly perturbed.

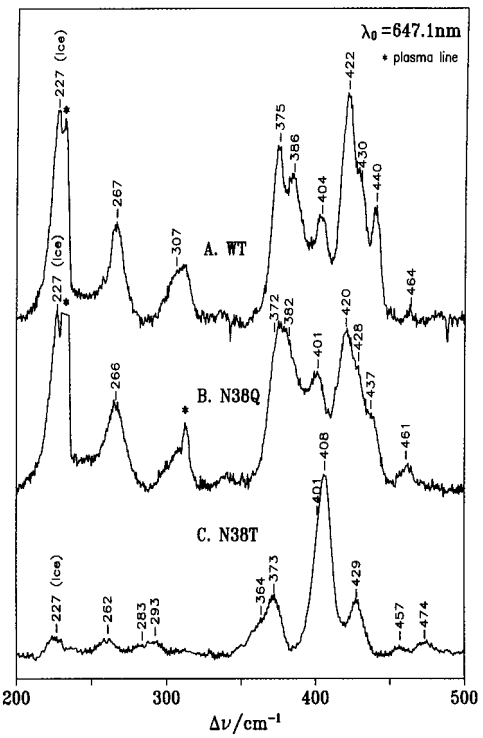

FIGURE 4: RR spectra of WT, N38Q, and N38T Pcy. Spectra were collected with the double monochromator under the following conditions: spectral width = 3 cm^{-1}; scan rate = 0.5 cm^{-1}/s; laser power = 90 mW; temperature = 77 K.

Table 2: RR Peak Positions (cm^{-1}) and Relative Intensities

WT Pcy		N38Q Pcy		N38T Pcy		Azu(PA)[b]	
cm^{-1}	rel[a] intensity	cm^{-1}	rel intensity	cm^{-1}	rel intensity	cm^{-1}	rel intensity
267	33.4	266	51.1	261	6.1	266.1	6.9
374	66.0	373	61.2	289	9.6	286.7	10.2
385	64.8	382	55.0	361	15.2		
394	35.9	392	34.9	372	24.9	372.6	34.5
403	24.5	401	71.9				
422	100	420	100	405.5	100	400.5/408.6	100
430	58.1	429	47.7	428	28.0	427.9	38.2
440	25.3	439	25.3	443	3.3	441	9.0
464		461	16.0	456	7.1	454.6	
				473	9.3	474	

[a] The strongest peak is taken as 100. For N38T and Azu, the total intensity of the peak at 400−408 cm^{-1} is taken as 100. [b] Reference 36.

A much greater perturbation is seen for the N38T mutant. The CT absorption band is red-shifted by 36 nm, and the RR spectrum is substantially altered with respect to band frequencies and relative intensities. The absorption and RR spectra of the N38T mutant are quite similar to those of azurin from *Pseudomonas aeruginosa* (*38*). The spectral differences between Pcy and Azu have been previously noted and discussed (*40*−*42*). The spectra have been modeled with a "chromophore-within-protein" normal-mode analysis (*43*). It seems probable that an important factor in the differences lies in a weakened Cu−S bond in azurin. The X-ray structure gives 2.07 Å for the Cu−S bond in Pcy (*5*) but 2.12 Å for the corresponding bond in azurin (*44, 45*). Although this metric difference is within the uncertainty of protein crystallography (*5*), the RR spectra also suggest a weakening of the Cu−S bond in azurin. The complex spectra reflect intimate mixing of Cu−S stretching and ligand bending coordinates (*34, 42*), but since the resonant excited state is S → Cu CT in character, weakening of the Cu−S bond results in shifting of RR intensity into lower frequency modes, as is observed for Azu, relative to Pcy (*41, 47*).

The Cu−S weakening in Azu has been suggested to result from an extra axial interaction with a backbone carbonyl group, located 3.11 Å from the Cu (*43*). However, we call attention to another mechanism namely modulation of the Cu−S strength by H-bond donation to the ligating S atom. In Azu, the calculated N−H···S distance (Figure 1) is 3.30 Å (*44*), compared to 3.41 Å in Pcy (*5*). The stronger H-bond is expected to diminish the negative charge on the thiolate, thereby weakening the Cu−S bond.

We suggest that a similar mechanism operates in the N38T mutant of plastocyanin. Because the threonine side chain of the mutant is shorter than the wild-type asparagine side chain, formation of a H-bond to Ser85 would require the backbone at position 38 to move up (in Figure 1) toward Ser85 and concomitantly toward Cys84. Indeed, molecular modeling (Figure 1) reveals that the Thr38 OH can accept an H-bond from the Ser85 backbone NH, only if the C_β atom is moved 0.8 Å in the direction of Ser85. Such movement would be reinforced by a steric clash between the Thr38 methyl group and the carbonyl group of a nearby residue, Asp61 (Figure 1). As a result of this upward movement, the Thr38 backbone NH would be closer to Cys84 S, thereby strengthening the H-bond between this NH and the S atom of Cys84. This would reduce the negative charge on the thiolate, which would weaken the Cu−S bond, thereby mimicking the structure of this region in azurin and accounting for the azurin-like spectroscopic properties.

Although this H-bond between the backbone NH of residue 38 and the sulfur is strengthened in the N38T mutant, this is not sufficient to compensate for the disruption of the clasp formed between the Asn38 side chain and Ser85. In the absence of this clasp, the Cys84 side chain is more mobile and, hence, more available to competing reactions, such as intermolecular disulfide formation. Consequently, for the N38T mutant, the color fades somewhat faster than for N38Q (although much more slowly than for N38L). Thus, while the strength of the N−H···S H-bond has an impact on the S → Cu CT, this H-bond is not sufficient to inhibit Cys84 side reactions. Prevention of these side reactions is accomplished by limiting the Cys84 mobility via the additional H-bonds provided by the clasp between Asn38 and Ser85.

CONCLUSIONS

Site-directed mutagenesis reveals the critical importance of intermolecular H-bonds adjacent to the binding site of type I Cu proteins. The side chain of Asn38 adjacent to the Cu ligand His37 forms a pair of H-bonds with Ser85, which is adjacent to the sulfur ligand at Cys84. The importance of this clasp is supported by the conservation of residue 85 as serine or threonine in all type I Cu proteins (*3*). Elimination of these H-bonds does not prevent protein folding but greatly destabilizes the Cu site and facilitates alternative reactions of the Cu^{2+}-bound thiolate. In addition, the H-bond from the asparagine mainchain NH to the ligating thiolate modulates the strength of the Cu−S bond. Thus H-bonding is one of the factors (*29*) that preserves the integrity of the type 1 site even if the coordination group is not intrinsically unstable on electronic grounds (*12, 48*).

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