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The 1.6 Å resolution crystal structure of a mutant plastocyanin bearing a 21–25 engineered disulfide bridge

Mario Milani,[a,b] Laura Andolfi,[c,d] Salvatore Cannistraro,[c] Martin Ph. Verbeet[d] and Martino Bolognesi[a]*

[a]Department of Physics-INFM, c/o Advanced Biotechnology Center-IST, University of Genova, Largo Rosanna Benzi 10, 16132 Genova, Italy, [b]Istituto Giannina Gaslini, Largo Gerolamo Gaslini 5, 16147 Genova, Italy, [c]Unità INFM, Dipartimento di Scienze Ambientali, Università della Tuscia, Largo dell'Università, 01100 Viterbo, Italy, and [d]Leiden Institute of Chemistry, Gorlaeus Laboratoria, Leiden University, Einsteinweg 55, 2300 RA, Leiden, The Netherlands

Correspondence e-mail: bolognes@fisica.unige.it

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Plastocyanin is an electron-transfer protein which has been largely used for biophysical studies as well as for protein-engineering experiments. A surface disulfide bridge has been engineered in poplar plastocyanin to allow protein chemisorption on gold substrates. The mutated plastocyanin crystal structure has been studied at 1.6 Å resolution (R factor = 0.145, $R_{\rm free}$ = 0.205) to characterize the effects of the engineered disulfide on the overall protein structure and on the Cu-coordination sphere in view of biophysical applications. The new orthorhombic crystal form isolated for the mutated plastocyanin displays two protein molecules per asymmetric unit.

1. Introduction

Plastocyanin is a key copper protein involved in electron transfer from photosystem II to photosystem I. Poplar (*Populus laminosum*) plastocyanin (PC) consists of 99 amino acids and includes one type I Cu atom, coordinated by His37 $N^{\delta1}$, His87 $N^{\delta1}$, Cys84 S^{γ} and Met92 S^{δ} atoms. Two regions on the protein surface are suggested to play a role in the electron-transfer reaction: a hydrophobic patch around the Cu ligand His87 and an acidic patch centred around Thr83 containing several negatively charged residues (Redinbo *et al.*, 1994). With the aim of studying PC electron-transfer properties at the single-molecule level using the scanning tunnelling microscope (STM), we engineered within PC a surface disulfide bridge by site-directed mutagenesis of residues Ile21 and Glu25. The disulfide bond, located in the 'southern pole' of PC as opposed to the type I Cu site located in the 'northern pole', is expected to provide an anchoring group for chemisorption onto gold substrates. Such a strategy of thiol gold immobilization has been previously exploited in azurin, an homologous Cu protein that also bears a native disulfide bond, although differently located to that engineered in PC (Friis *et al.*, 1997, 1998, 1999; Chi *et al.*, 2000; Facci *et al.*, 2001).

Spectroscopic and cyclic voltammetry analysis of the Ile21Cys, Glu25Cys PC mutant (mut-PC) suggested that no substantial change in the copper-site coordination had occurred as a result of disulfide-bridge engineering. Moreover, preliminary STM investigations indicated that mut-PC adsorbs onto gold substrates *via* the disulfide, retaining its redox properties (Andolfi *et al.*, 2001). However, molecular-dynamics (MD) simulations focusing on a comparison between mut-PC and wild-type PC indicated that the two proteins differ in their overall flexibility (Arcangeli *et al.*, 2001).

The present communication reports the 1.6 Å resolution crystallographic analysis of the oxidized mut-PC at pH 7.0. The refined structure shows the correct establishment of the 21–25 disulfide bridge and the conservation of the type I Cu coordination stereochemistry. Strikingly, the new crystal form obtained displays two mut-PC molecules per asymmetric unit. Structural perturbations induced by the engineered disulfide are discussed in light of the well characterized wild-type PC structure (Guss *et al.*, 1992).

2. Materials and methods

2.1. Protein expression and purification

Design and expression of mut-PC were carried out as reported previously (Andolfi *et al.*, 2001). Briefly, the pET-3a plasmid which contains the poplar plastocyanin gene (Ybe & Hecht, 1994) was used as template and the mutation was incorporated with a mutagenic primer carrying the mutated codons. The expression vector containing the mutated gene was reconstructed by a combination of restriction sites. The mutant protein was over-expressed in the cytoplasm of *Escherichia coli* HMS174 (DE3) and grown at 310 K in 2× YT medium supplemented with 100 µg ml^{-1} of ampicillin and 0.1 m*M* copper citrate. Protein production was induced by addition of 0.2 m*M* IPTG. Mut-PC was released by the freeze–thaw method (Johnson & Hecht, 1994). Subsequently, the protein was purified by anion-exchange chromatography (DEAE Sepharose Fast Flow) and using a Superdex 75 size-exclusion column in a Pharmacia FPLC setup. The purity of the sample was verified *via*

Table 1
Data-collection and refinement statistics.

(*a*) Data-collection statistics. Outer shell statistics (1.63–1.60 Å) are given in parentheses.

Wavelength (Å)	0.934
Resolution (Å)	35.0–1.6
Completeness (%)	98.1 (71.6)
Mosaicity (°)	0.44
R_{merge} (%)	4.5 (18.9)
Unique/total reflections	25069/135535
Average $I/\sigma(I)$	22 (4.1)

(*b*) Refinement statistics and model quality.

Resolution range (Å)	35.0–1.6
Total No. of non-H atoms	1778
No. of water molecules	279
R factor/R_{free}† (%)	14.5/20.5
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 47.38, b = 49.31, c = 76.58
R.m.s.d. from ideal geometry	
Bond lengths (Å)	0.012
Bond angles (°)	1.45
Ramachandran plot‡	
Most favoured region (%)	87.1
Additional allowed region (%)	12.9
Averaged B factors (Å^2)	
Molecule *A*	16.8
Molecule *B*	21.1
Solvent	30.8

† Calculated using 5% of the reflections. ‡ Data produced with the program *PROCHECK* (Laskowski *et al.*, 1993).

reducing SDS–PAGE and isoelectric focusing gel (PhastGel IEF 3–9 Pharmacia).

2.2. Crystallization, data collection and processing

Crystals of mut-PC were grown at 277 K using the hanging-drop setup from reservoirs containing 2.5 *M* ammonium sulfate and 0.05 *M* potassium phosphate pH 7.0. The droplet consisted of 1 µl protein (8.8 mg ml^{-1}) and 1 µl of reservoir solution. The blue crystals displayed a tabular shape (about 0.3 × 0.3 × 0.04 mm) and grew in about 10 d. X-ray diffraction data were collected at 100 K (cryoprotectant: 20% glycerol, 3.0 *M* ammonium sulfate pH 7.0) on beamline ID 14.1 at ESRF (Grenoble, France), λ = 0.934 Å, to 1.6 Å resolution. All data were integrated and scaled using the programs *DENZO* and *SCALEPACK* (Otwinowski, 1993). From analysis of the diffracted intensities, the crystal was shown to belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 47.38, b = 49.31, c = 76.58 Å and two mut-PC molecules per asymmetric unit (see Table 1).

2.3. Structure analysis and refinement

The mut-PC three-dimensional structure was solved by molecular replacement using the program *EPMR* (Kissinger *et al.*, 1999), and data in the 15–4 Å resolution range. The search model was the wild-type poplar PC solved at 1.33 Å resolution (PDB code 1plc; Guss *et al.*, 1992), whose residues 21 and 25 had been truncated at the C$^\beta$ atoms and the active-site Cu atom removed. The correlation coefficient after positioning the first molecule was 0.292, with an R factor of 0.566; after positioning the second molecule the correlation coefficient was 0.534, with an R factor of 0.469. Rigid-body refinement with *REFMAC* (Murshudov *et al.*, 1997), treating the two molecules as independent bodies, resulted in a R factor of 0.451 (R_{free} = 0.431) for data in the 20.0–2.0 Å resolution range. The subsequent refinement cycles were also based on the *REFMAC* suite (restrained refinement with maximum-likelihood residual and isotropic B-factor refinement) using all the data collected in the 35.0–1.6 Å resolution range. Map inspection and manual rebuilding were performed with *O* (Jones *et al.*, 1991). Following convergence of atomic coordinates with isotropic atomic B-factor refinement (R factor 0.187, R_{free} 0.230), nine cycles of atomic coordinate refinement with anisotropic B factors were run. Convergence was reached at an R factor of 0.145 (R_{free} = 0.205) for all data in the 35.0–1.6 Å resolution range, with ideal stereochemical parameters (Engh & Huber, 1991; Laskowski *et al.*, 1993) (see Table 1).

3. Discussion

The overall structure of mut-PC matches closely the β-barrel of wild-type PC, where the 'northern pole' of the molecule hosts the type I Cu binding site and the 'southern pole' hosts the engineered disulfide (Fig. 1*a*). However, the C$^\alpha$ r.m.s.d. between mut-PC and wild-type PC (0.49 Å) is somewhat larger than expected, reflecting localized structural deviations (Fig. 1*b*). The two mut-

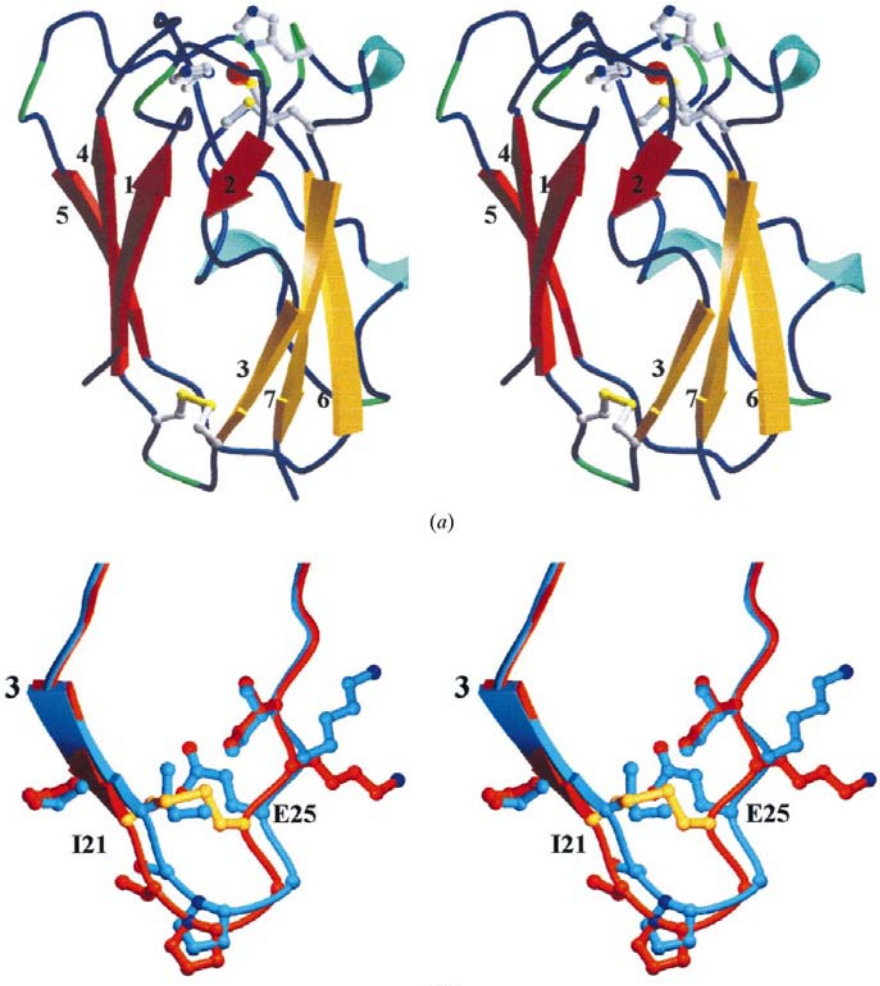

Figure 1
(*a*) Stereoview of the overall structure of mut-PC. Ball-and-stick models of the Cu site ('northern pole') and of the disulfide-bridge site ('southern pole') have been added. The two facing β-sheets are coloured red and yellow, the helical regions cyan and the hydrogen-bonded turns green. (*b*) Superposition of wild-type PC (blue) and mut-PC (orange) structures in the 20–27 region where the 21–25 disulfide (yellow) has been engineered. All figures were drawn with *Molscript* (Kraulis, 1991) and *Raster3D* (Merrit & Murphy, 1994).

PC molecules in the asymmetric unit display a C^{α} r.m.s.d. of 0.47 Å, the most remarkable difference being located in the 48–49 segment, where electron density of the mut-PC *B* molecule is available only for the protein backbone and temperature factors are at a maximum (see Fig. 2). In agreement with this observation, the extended 32–68 polypeptide loop is completely devoid of crystal contact in mut-PC molecule *B*, whereas it is involved in seven crystal contacts in molecule *A* (see Fig. 2). Remarkably, the 32–68 loop in wild-type PC hosts most of the negatively charged patch involved in intermolecular contacts with cytochrome *f* (Guss *et al.*, 1992; Redimbo *et al.*, 1994). Therefore, such a transition from a flexible loop structure (in the absence of intermolecular contacts) to a rigid protein structure (in the presence of crystal contacts) may reflect a functional property specifically coded in the PC structure to promote/mediate interactions with molecular partners.

As anticipated by the spectroscopic studies and molecular-dynamics simulations, the type I Cu coordination is well preserved in mut-PC, in keeping with the relative distance of the mutated residues from the Cu site (~20 Å). The coordination bond lengths for the four Cu ligands are shown in Table 2 related to the reference coordination parameters measured in the 1.33 Å structure of oxidized wild-type PC.

The engineered Ile21Cys and Glu25Cys mutations lead to formation of the expected disulfide bridge, with left-handed chiriality. In wild-type PC the $21C^{\alpha}$–$25C^{\alpha}$ distance is 6.3 Å. This value drops moderately (5.8 Å) in mut-PC, indicating that the wild-type PC structure is properly arranged to host the engineered disulfide, while simultaneously allowing some structural adaptability. In agreement with these considerations, analysis of the stereochemical parameters characterizing the disulfide (see Table 2) indicates an absence of significant sterical strain on the cystine bridge. Nevertheless, the new intermolecular constraint imposed by the disulfide bridge has a perceivable influence on the enclosed protein loop structure (the r.m.s.d. calculated for C^{α} atoms in the 20–26 segment of wild-type PC relative to mut-PC is 1.10 Å; see Fig. 1*b*).

Analysis of the *B*-factor profiles indicates a clear difference for the 20–26 segments of mut-PC molecules *A versus B* which can be related to local crystal contacts. Specifically, the mut-PC molecule *A* displays five crystal-packing contacts in this region (at residues Phe19, Ser20, Ser22, Pro23 and Lys26), as opposed to molecule *B*, which has only one crystal-packing contact (at residue Ser22; see Fig. 2). It should be noted that no residues from the 20–26 loop region are part of the crystal-packing contacts in wild-type PC.

Table 2
Copper-site and disulfide-bridge geometry.

(*a*) Copper-site geometry.

Coordination bonds (Å)	Molecule *A*	Molecule *B*	Wild-type PC†
$Cu-N^{\delta1}$ (His87)	2.00	2.09	2.06
$Cu-N^{\delta1}$ (His37)	2.00	2.00	1.91
$Cu-S^{\gamma}$ (Cys84)	2.17	2.20	2.07
$Cu-S^{\delta}$ (Met92)	2.76	2.67	2.82

Disulfide-bridge geometry.

	Ref.‡	Molecule *A*	Molecule *B*	Azurin§
Distance S–S (Å)	2.03	2.03	2.02	2.02
Angle $S-S-C^{\beta}25$ (°)	103.9	102.5	102.9	103.6
Angle $S-S-C^{\beta}21$ (°)	103.9	104.2	103.0	106.2
Dihedral $C^{\beta}-S-S-C^{\beta}$ (°)	−90	−81.6	−83.1	−88.4

† Guss *et al.* (1992). ‡ *S*-(thioethylhydroxy)cystine (Kleywegt & Jones, 1998). § Nar *et al.* (1991); PDB code 4azu.

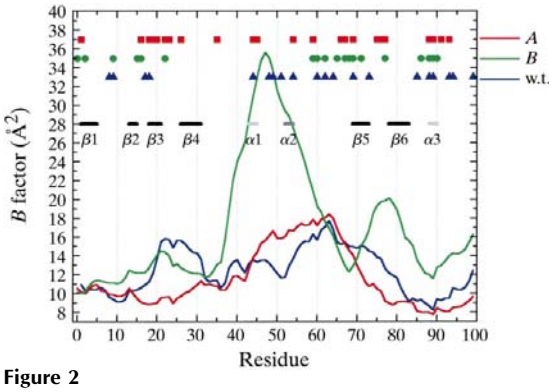

Figure 2
Smoothed *B* factors for wild-type PC (blue trace) and for mut-PC molecules *A* and *B* (red and green traces, respectively). Residues involved in crystal contacts are indicated by coloured symbols in the upper part of the figure; secondary-structure elements are identified by black and grey bars. The *B*-factor profiles of mut-PC molecules *A* and *B* have been normalized to that of wild-type PC by assigning the same *B*-factor value to the respective N-terminal residues.

4. Conclusions

The wild-type PC residues Ile21 and Glu25 fall in a short loop between β-strands 3 and 4, with a C^{β}–C^{β} distance of 4.1 Å. This starting structure, and some adaptation within the enclosed polypeptide loop, offered the opportunity to engineer a disulfide bridge in a straightforward manner. Such favourable local structure allows the establishment of an unstrained disulfide, as shown in Table 2 by comparison with a model compound and with the naturally occurring disulfide bridge in azurin. Nevertheless, the structural fluctuations localized in the 20–26 polypeptide loop enclosing the disulfide appear to be sufficient to produce a new crystal packing compared with wild-type PC, hosting two independent molecules in the asymmetric unit. From the gold chemisorption application viewpoint, PC proved an ideal protein molecule capable of accepting radical mutations while maintaining the Cu-coordination sphere and its redox potential virtually unperturbed.

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