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Structure of the M148Q mutant of rusticyanin at 1.5 Å: a model for the copper site of stellacyanin

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The small blue copper protein rusticyanin from *Thiobacillus ferrooxidans* contains a type 1 Cu centre with a single axial ligand, Met148, which together with the His-Cys-His trigonal planar ligands produces a distorted trigonal pyramidal coordination geometry to copper. Type 1 Cu sites are found in cupredoxins and several multicopper proteins, including oxidases and nitrite reductases. The role of the axial ligand has been extensively debated in terms of its function in the fine tuning of the redox potential and spectroscopic properties of type 1 Cu sites. Numerous mutations of the Met ligand in azurins have been studied, but interpretation of the results has been complicated by the presence of the additional carbonyl oxygen ligand from Gly45, a neighbouring residue to the coordinating His46. The importance of the axial ligand has been further emphasized by the finding that the type 1 centre in *Rhus vernicifera* stellacyanin, with the lowest redox potential in a type 1 Cu site of 184 mV, has Gln as the axial ligand, whilst fungal laccase and ceruloplasmin, which have redox potentials of 550–800 mV, have a Leu in this position. Here, the crystal structure of the M148Q mutant of rusticyanin at 1.5 Å resolution is presented. This is a significantly higher resolution than that of the structures of native rusticyanin. In addition, the M148Q structure is that of the oxidized protein while the native structures to date are of the reduced protein. The mutant protein crystallizes with two molecules per asymmetric unit, in contrast to the one present in the native crystal form. This mutant's redox potential (550 mV at pH 3.2) is lowered compared with that of the native protein (∼670 mV at pH 3.2) by about 120 mV. The type 1 Cu site of M148Q closely mimics the structural characteristics of the equivalent site in non-glycosylated cucumber stellacyanin (redox potential ∼260 mV) and, owing to the absence in rusticyanin of the fifth, carbonyl ligand present in azurin, may provide a better model for the *R. vernicifera* stellacyanin (redox potential ∼184 mV) type 1 Cu site, which also lacks the fifth ligand. Furthermore, the presence of two molecules in the asymmetric unit cell indicates a potential binding region of the redox partners.

1. Introduction

The small blue copper protein rusticyanin is thought to be a principal component in the iron respiratory electron-transport chain of *T. ferrooxidans* (Ronk *et al.*, 1991; Ingledew, 1994). Crystallographic studies of native rusticyanin at ∼2 Å (Walter *et al.*, 1996; Harvey *et al.*, 1998) have confirmed that the Cu coordination is very similar to that of other cupredoxins; namely, a distorted trigonal planar geometry with three strong ligands, His85 $N^{\delta1}$, Cys138 S^{γ} and His143 $N^{\delta1}$, and a relatively distant Met148 S^{δ} ligand in the axial position. Despite this

structural similarity, rusticyanin possesses the highest redox potential of the type 1 Cu protein family (680 mV compared with more typical values of ~300 mV) and is extremely acid stable to pH 2. It is clear that factors outside of the immediate Cu coordination sphere are responsible for the elevated redox potential of rusticyanin.

It has been suggested that in rusticyanin Ser86 (the residue which is adjacent to the Cu ligand His85), rather than the conserved Asn found in all other structurally characterized type 1 Cu proteins, may provide some protection for the metal in highly acidic media (Grossmann *et al.*, 1995). This Asn is also fully conserved in Cu-containing nitrite reductases. The mutation of Asn to Leu in azurin results in an increase of the redox potential by 110 mV (Hoitink & Canters, 1992). Site-directed mutagenesis of rusticyanin has confirmed that substitution of Ser86 has a significant impact on the acid stability and that its mutation to Asn reduces the redox potential by ~80 mV (Hall *et al.*, 1998). The mutation of Asn to Gln, Thr and Leu in poplar plastocyanin (Dong *et al.*, 1999) has suggested the importance of this residue in hydrogen bonding and its role in suppressing the side reaction of the Cys ligand.

The nature of the axial ligand has been extensively discussed in terms of the fine tuning of the redox potential of cupredoxins (Gray & Malmström, 1983). Type 1 Cu sites containing different ligands in the axial position show a highly variable redox potential. Specifically, stellacyanin, which has the lowest redox potential of the type 1 Cu proteins (184 mV), has a Gln in place of the usual Met ligand (Fields *et al.*, 1991; Thomann *et al.*, 1991; Strange *et al.*, 1995). Furthermore, the type 1 Cu centres in fungal laccase and ceruloplasmin, where the Met ligand is replaced by a Leu, have a redox potential in the range 550–800 mV (Zaitseva *et al.*, 1996; Ducros *et al.*, 1998). It has been argued that a variation of only 140 mV can be attributed to the strength of interaction between the axial ligand and Cu, based on density-function calculations (Olsson & Ryde, 1999). In rusticyanin, mutation of Met148 to Gln decreases the redox potential by ~110 mV, while a substitution with Leu leads to an increase of ~130 mV (Hall *et al.*, 1999).

In azurins, a fifth Cu ligand from a carbonyl O atom provides a second axial interaction in a somewhat counterbalancing manner to the Met (the Cu—O bond varies between 2.7 and 3.2 Å depending on the source). Rusticyanin lacks this second axial interaction (Cu—O is either 3.9 or 5.7 Å) and thus provides a better framework for studying the role of the axial ligand and its effect on the type 1 Cu site, including the 'fine-tuning' of the redox potential. In addition, rusticyanin is a superior model for the type 1 Cu site of stellacyanin, which also lacks the additional axial interaction.

Here, we describe the crystal structure of the site-directed mutant of Met148 to Gln in rusticyanin at 1.5 Å resolution. The structure is compared with the 1.6 Å structure of the non-glycosolated cucumber stellacyanin (Hart *et al.*, 1996), which has Gln as the axial ligand but has a redox potential of 260 mV. Further comparisons are made with EXAFS data for *R. vernicifera* stellacyanin, which has a redox potential of 184 mV, and also with a previous model for stellacyanin, the M121Q mutant of azurin (Romero *et al.*, 1993).

The current structure reveals two molecules in the asymmetric unit cell. Their interaction is discussed in terms of the electron self-exchange reaction and the potential interaction region of the redox-partner protein. An asymmetry between the two molecules in the asymmetric unit in the orientation of several important residues close to the copper site is demonstrated.

2. Materials and methods

2.1. Protein and crystal growth

M148Q mutant rusticyanin was cloned, expressed and purified as described previously (Hall *et al.*, 1999). Single crystals were grown by the hanging-drop vapour-diffusion method at 293 K. 5 µl of a solution containing 12 mg ml^{-1} protein in 10 m*M* H_2SO_4 was mixed with an equal volume of reservoir solution consisting of 30% PEG 8000, 100 m*M* MES, 50 m*M* citric acid pH 4 and suspended over a 1 ml reservoir. Large needle-like crystals grew within one week to maximal dimensions of 0.8 × 0.2 × 0.1 mm. The crystals were an intense blue in colour and thus represent the oxidized form of the protein.

2.2. X-ray data collection

Crystals of M148Q were flash-cooled to 100 K in a nitrogen Cryostream after being soaked for ~1 min in a cryoprotectant solution containing 12% glycerol, 25% PEG 8000, 100 m*M* MES, 50 m*M* citric acid pH 4. Data to 1.5 Å resolution were collected at beamline BL6A2 at the Photon Factory, KEK at a wavelength of 1.0 Å using the Weissenberg method (Sakabe, 1991). The detectors were twin Fuji image plates mounted on a cassette of 210 mm radius and were scanned off-line using a BAS-2000 scanner. Data were processed using *DENZO* and scaled and merged using *SCALEPACK* (Otwinowski, 1993). The M148Q mutant crystallizes in space group $P2_1$, with unit-cell parameters a = 42.9, b = 61.4, c = 53.3 Å, β = 96.2°. This differs from the crystals of native rusticyanin (a = 32.4, b = 60.7, c = 38.0 Å, β = 107.8°) and the increase in $P2_1$ cell volume indicates the presence of a second molecule in the asymmetric unit. The solvent content was 41.0% in comparison to the value of 44.4% for native rusticyanin (Harvey *et al.*, 1998). A total of 32 036 independent reflections were collected over the resolution range 52.7–1.50 Å. A high-resolution limit of 1.50 Å was set based on the observation that the outermost resolution shell (1.53–1.50 Å) was 70.7% complete with an $R_{\rm merge}$ of 20.3% and a $I/\sigma(I)$ of 3.0. Data-reduction parameters are summarized in Table 1.

2.3. Structure solution and refinement

The structure was solved using molecular replacement in *AMoRe* (Navaza, 1994). A monomer of the M148L mutant of rusticyanin, solved to 2.3 Å resolution (Kanbi, Hall, Dodd & Hasnain, unpublished results), was used as the search model. The solutions to the rotation and translation functions indi-

Table 1
Summary of data-collection statistics, final model and refinement results.

Values in parentheses are for the outer resolution shell, 1.53–1.50 Å.

Data collection and processing	
Resolution (Å)	52.7–1.50
Unit-cell parameters (Å,°)	a = 42.9, b = 61.4, c = 53.3, β = 96.2
Solvent content (%)	41
No. of observed reflections	62663
No. of unique reflections	32036
Completeness (%)	76.5 (70.7)
$R_{\rm merge}$ (%)	5.6 (20.3)
$I/\sigma(I)$	12.0 (3.0)
Refinement and model quality	
$R_{\rm work}$ (%)	18.2
$R_{\rm free}$† (%)	21.7
ESU (Å)	0.10
No. of atoms	
Protein	2320
Solvent	179
Metal	2
B factor	
Protein (Å^2)	10.3
Water (Å^2)	19.9
RMS deviations	
Bond distances (Å)	0.009
Bond-angle distances (Å)	0.027
Ramachandran plot‡ (non-Gly and non-Pro)	
Residues in most favoured regions (%)	89.7
Residues in additional allowed regions (%)	10.3
Overall G factor	−0.01

† $R_{\rm free}$ was calculated for 1665 reflections excluded until the final refinement cycle. ‡ Ramachandran *et al.* (1963).

cated a pair of molecules in the asymmetric unit. The molecular-replacement solution model was initially refined using *CNS* (Brunger *et al.*, 1998). Prior to any refinement, 5% (1665) of the reflections were set aside for calculation of the free R factor and the mutated residue 148 was truncated to alanine in both molecules. Following two cycles of *CNS* simulated-annealing refinement (4000–300 K) using data in the resolution range 8.0–1.7 Å, the R factor was reduced to 23.8% ($R_{\rm free}$ = 27.6%). The Gln148 residue was built into clear electron density at this stage for each molecule. Refinement proceeded in *REFMAC* using data in the resolution range 57.2–1.5 Å (Murshudov & Dodson, 1997) using the maximum-likelihood method with a bulk-solvent correction to an R factor of 20.9% ($R_{\rm free}$ = 24.1). At no point in the refinement were restraints applied to the copper–ligand distances or bond angles. A total of 179 water molecules were selected from a list of peaks greater than 4σ in the $F_o - F_c$ electron-density map. Stereochemical checks following each cycle of refinement included *PROCHECK* (Laskowski *et al.*, 1993) and *OOPS* (Jones *et al.*, 1991).

3. Results

3.1. Quality of the refined model

The refined model of the M148Q mutant has two molecules in the asymmetric unit, which together comprise 306 amino acids, 179 water molecules and two Cu ions, giving a total of 2501 atoms. The R factor was 18.2% and the free R factor was 21.7%. The small difference between these parameters indicates that the structure has not been over-refined. The estimated standard uncertainty (ESU; Cruickshank, 1996) as implemented in *REFMAC* was 0.10 Å, which may be taken as the data-only positional uncertainty of an atom with a B factor equal to that determined from the Wilson plot (10.5 Å^2). The 1σ $2F_o - F_c$ electron-density map was continuous over the entire main chain. Residues 1 and 2 at the N-terminus of each molecule were omitted from the model owing to a lack of electron density.

The stereochemistry of the model was generally good. The overall quality of the model is summarized in Table 1.

3.2. Overall protein fold, *B*-factor distribution and comparison with the native protein

The M148Q mutant protein folds as a Greek key β-sandwich core with two α-helical regions connected *via* several loops and random coils. The N-terminal helix is unusual in cupredoxins and has been suggested to be partly responsible for the extreme acid stability of rusticyanin (Walter *et al.*, 1996). The two molecules in the asymmetric unit superimpose with an RMSD of 0.42 Å for all C$^\alpha$ atoms. The overall B factor was 10.3 Å^2 for protein atoms and 19.9 Å^2 for solvent molecules. High temperature factors are generally confined to loop regions and solvent-accessible regions.

Comparison of the M148Q structure with the native structure (Walter *et al.*, 1996; Botuyan *et al.*, 1996; Harvey *et al.*, 1998) shows little change. A superposition with the 2.1 Å native structure (with one molecule in the asymmetric unit; Harvey *et al.*, 1998; PDB code 1a8z) shows an RMS deviation of 0.38 Å from the native C$^\alpha$ positions for molecule A and 0.43 Å for molecule B. The copper site shows small changes in the metal–ligand bond distances of −0.12, −0.04 and −0.08 Å for His85 N$^{\delta1}$, His143 N$^{\delta1}$ and Cys138 S$^\gamma$, respectively, in the mutant compared with the native (averaged between the two molecules in the asymmetric unit). These differences are likely to be of limited significance in comparison to an ESU of 0.10 Å for M148Q and confirm that little structural change occurs as a result of the transfer of a single electron. A superposition of the native and M148Q Cu centres is given in Fig. 1(*a*).

3.3. The M148Q copper site

The type 1 Cu site in the M148Q crystal structure occupies a distorted trigonal pyramidal geometry consisting of an NNS trigonal planar ligation from residues His85, His143 and Cys138 and the axial ligand from residue Gln148. The $2F_o - F_c$ electron density contoured at 2σ for the Cu site of molecule A is shown in Fig. 2. The Cu ion is ligated to His85 N$^{\delta1}$ at 2.01 Å, His143 N$^{\delta1}$ at 1.99 Å and Cys138 S$^\gamma$ at 2.17 Å. The Gln148 O coordinates at 2.33 Å. The corresponding values for the second molecule in the asymmetric unit are 2.04, 2.04, 2.19 and 2.39 Å, respectively. In comparison to the overall ESU of 0.10 Å, the sites are essentially identical (Fig. 1*a*). The Cu atom is displaced 0.31 Å from the NNS plane towards the

Table 2
Cu-centre parameters.

Copper-site ligand distances and angles for the M148Q rusticyanin mutant and for (*a*) native rusticyanin (Harvey *et al.*, 1998), (*b*) cucumber stellacyanin (Hart *et al.*, 1996) and (*c*) M121Q azurin (Romero *et al.*, 1993).

	M148Q (*A*) (1.5 Å)	M148Q (*B*) (1.5 Å)	Native rusticyanin (2.1 Å) (*a*)	Stella-cyanin (1.6 Å) (*b*)	M121Q azurin (1.9 Å) (*c*)
Distances (Å)					
Cu—His $N^{\delta2}$	2.01	2.04	2.14	1.96	1.91
Cu—His $N^{\delta2}$	1.99	2.04	2.06	2.04	2.06
Cu—Cys $S^{\delta1}$	2.17	2.19	2.26	2.18	2.13
Cu—axial ligating atom	2.33 (Gln)	2.39 (Gln)	2.90 (Met)	2.21 (Gln)	2.25 (Gln)
Angles (°)					
His $N^{\delta2}$(i)—Cu—Cys $S^{\delta1}$	133	129	124.7	137	140
His $N^{\delta2}$(i)—Cu—axial ligand	93	94	90	94	87
His $N^{\delta2}$(i)—Cu—His $N^{\delta2}$(ii)	101	102	102	101	97
His $N^{\delta2}$(ii)—Cu—Cys $S^{\delta1}$	118.5	124	122	118	116
His $N^{\delta2}$(ii)—Cu—axial ligand	96	92	105	102	98
Axial ligand—Cu—Cys $S^{\delta1}$	105	104	105	118	106

ligating atom of Gln148 in both sites in comparison to a displacement of 0.4 Å towards Met148 in native rusticyanin. The copper-site parameters are given in Table 2.

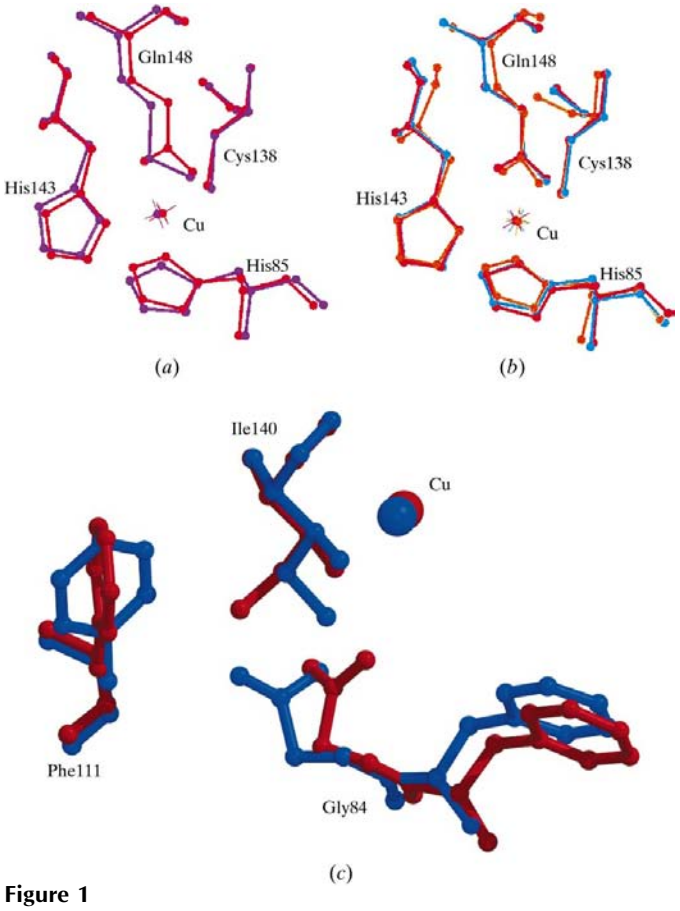

Figure 1
Least-squares superposition of the type 1 Cu site for (*a*) M148Q (red) *versus* native rusticyanin (purple) (Harvey *et al.*, 1998) and (*b*) M148Q rusticyanin (red) *versus* cucumber stellacyanin (blue) and M121Q azurin (orange) (Romero *et al.*, 1993). All superpositions were performed using least-squares fitting of all active-site atoms except those from the axial ligand in *INSIGHT*II. (*c*) Superposition of residues Gly84, Ile140, Phe111 and Cu for molecules *A* (red) and *B* (blue), showing the structural differences between the two monomers in the asymmetric unit.

In native rusticyanin, the side chain of Ile140 forms a close hydrophobic contact to the Cu ion. Walter et al. (1996) suggested that this arrangement forces the carbonyl O atom of Gly84 to point away from the Cu site. In the M148Q mutant structure, one molecule (*B*) of the pair forming the asymmetric unit conforms to this model, with a Cu to Ile140 $C^{\gamma1}$ separation of 3.7 Å and a Cu to Gly84 O separation of 5.8 Å. In the case of the remaining M148Q molecule (*A*) Ile140 occupies a different rotamer, such that the Cu—Ile140 $C^{\gamma1}$ distance is now 5.4 Å. The removal of the Ile140 close contact to Cu is associated with a peptide flip in residue Gly84, bringing its backbone O atom to a position some 3.9 Å from Cu. In addition, the Phe111 ring plane is rotated by ∼60°, increasing its distance from the new position of Ile140 $C^{\gamma1}$. A superposition of these three residues for molecules *A* and *B* is given in Fig. 1(*c*).

3.4. Intermolecular interface and possible electron self-exchange pathway

The M148Q mutant crystallizes with two molecules in the asymmetric unit in contrast to the single molecule present in the native structure. The two molecules pack in a head-to-head arrangement similar to that observed in azurin, with a Cu–Cu separation of some 15 Å. Such a packing arrangement has been suggested to be physiologically important in the electron self-exchange reaction of azurin (Nar *et al.*, 1991; Dodd *et al.*, 1995). An interesting water-mediated hydrogen-bonding network is formed at the interface. His143 $N^{\varepsilon2}$ in molecule *A* is hydrogen bonded to water 166 at a distance of 2.7 Å. This water hydrogen bonds to water 110 at a distance of 3.4 Å, which in turn completes the chain with a bond of 2.8 Å to Pro141 O of molecule *B*. In addition, the equivalent proline oxygen in molecule *A* is hydrogen bonded to water 67 at 3.3 Å, which is also hydrogen bonded to His143 $N^{\varepsilon2}$ of molecule *B* at a distance of 3.4 Å. This network is shown in Fig. 3(*a*). In addition, there are a number of non-bonded interactions at the interface (Fig. 3*b*). This observed crystalline interface may suggest a possible docking site for the redox partner of rusticyanin.

3.5. Comparison with stellacyanins

No crystal structure exists for the most commonly studied stellacyanin, that from *R. vernicifera* (Rv-STC). However, a crystal structure has been determined for the 109 amino-acid non-glycosylated cucumber stellacyanin to 1.9 Å (C-STC;

Hart *et al.*, 1996). The Cu ion in the C-STC crystal structure was ligated by two histidine N atoms at 1.96 and 2.04 Å and a cysteine sulfur at 2.18 Å. The axial glutamine ligand ligates at a distance of 2.21 Å. This site is very similar to that observed for M148Q rusticyanin (Fig. 1*b*). The differences in Cu–ligand distances between M148Q and C-STC are +0.06, −0.02, +0.00 and +0.15 Å for His, His, Cys and Gln ligands, respectively. The estimated error in atomic positions for C-STC is 0.17 Å, so these differences must be considered marginal. The Cu ion lies some 0.3 Å out of the plane in both M148Q and in C-STC.

Structural information has been obtained for *R. vernicifera* stellacyanin from an EXAFS study (Strange *et al.*, 1995). This showed a variability of the copper site as a function of redox state. At pH 6.5, the copper coordination in the oxidized protein was determined to be two histidine N atoms at 1.93 Å, a cysteine sulfur at 2.17 Å and a fourth, axial ligand (either O or N) at ∼2.7 Å. In the case of reduced stellacyanin, the best EXAFS fit was obtained with two histidine N atoms at 1.91 Å, a cysteine S atom at 2.16 Å and an oxygen or nitrogen ligand at 2.4 Å. The axial ligand has been assumed to be glutamine from sequence-alignment studies (Fields *et al.*, 1991).

Thus, the site of reduced Rv-STC at low pH is similar to M148Q-Rc and C-STC (axial ligand distance of 2.4 *versus* 2.35 and 2.21 Å), but that of oxidized Rv-STC is significantly different. The redox potential of Rv-STC is 184 mV, while that of C-STC is 260 mV. The two proteins share only 34% sequence homology. It seems likely from a comparison of the crystallographic results for C-STC and the EXAFS results for Rv-STC that the Cu sites of the two proteins are somewhat different.

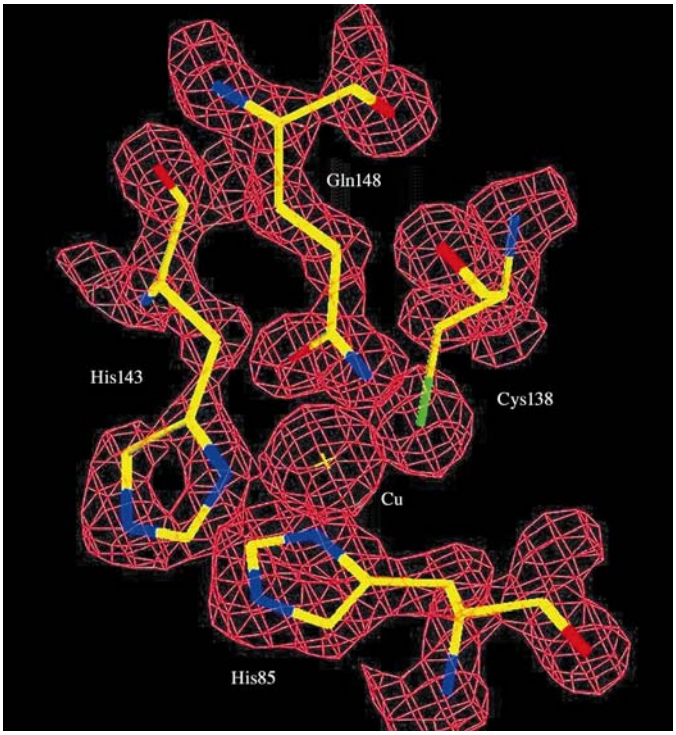

Figure 2
The $2F_o - Fc$ electron density at the M148Q rusticyanin type 1 Cu site contoured at 2σ.

3.6. Modelling the active site of stellacyanins: comparison with M121Q azurin

A superposition of the crystal structures of M148Q rusticyanin, M121Q azurin (Romero *et al.*, 1993) and C-STC shows that both of the mutant structures model the copper coordination of C-STC to some extent (Fig. 1*b*). However, azurin possesses a fifth Cu ligand, a carbonyl O atom from a glycine residue at ∼3.4 Å. In C-STC, this atom lies some 4.0 Å from

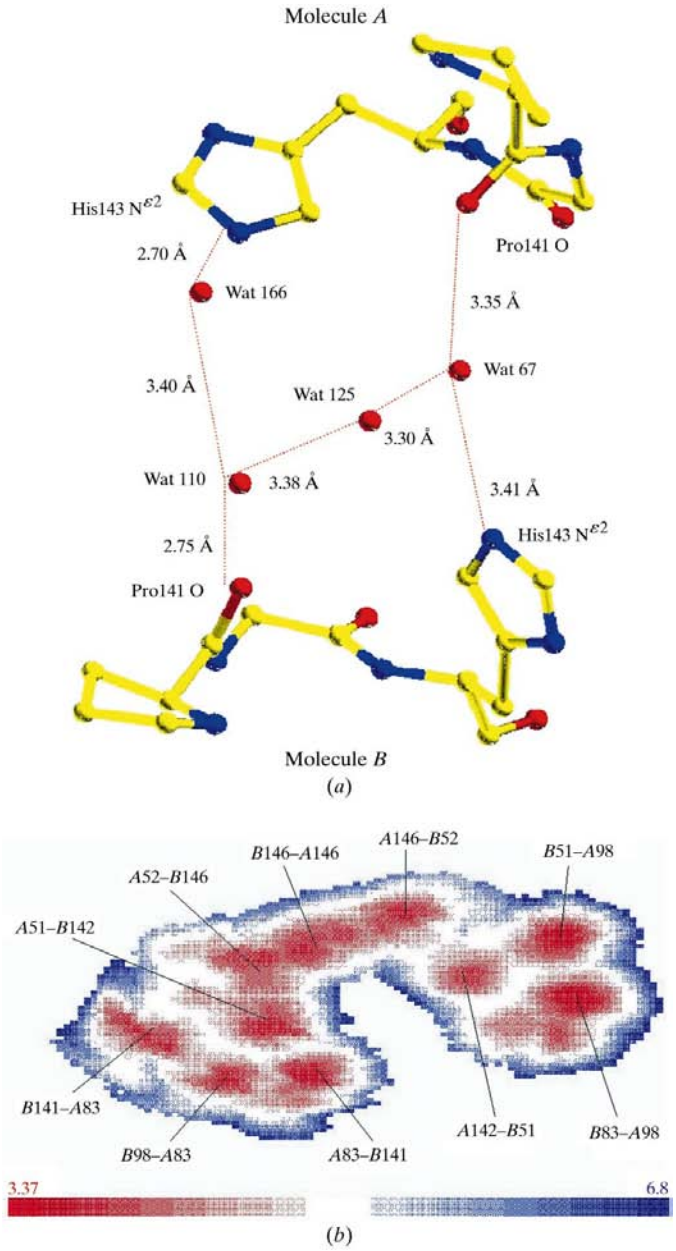

Figure 3
(*a*) The hydrogen-bonding network at the dimer interface of M148Q rusticyanin. Hydrogen bonds are shown as red dotted lines. C atoms are depicted in yellow, O atoms in red and N atoms in blue. This network may indicate a possible binding site for the redox partner of rusticyanin. (*b*) Buried surface plot of the interface between molecules *A* and *B* of M148Q rusticyanin. The plot is colour coded according to interatomic distances. Image generated using *MOLSURFER* (Gabdoulline *et al.*, 1999).

copper and does not interact with the metal. In M148Q rusticyanin also, as in the native, the Gly carbonyl is not coordinated to copper and is separated from the metal ion by 5.8 Å. In this respect, four-coordinate M148Q rusticyanin may represent a better structural model for stellacyanin than the five-coordinate M121Q azurin. Parameters for the Cu sites of M148Q rusticyanin, C-STC and M121Q azurin are given in Table 2.

3.7. Influence of the axial ligand on redox potentials in cupredoxins

The redox potential of rusticyanin (680 mV) is very high in comparison with other members of the cupredoxin family. This redox potential may be manipulated by mutation of the axial ligand; specifically, reduced by 110 mV by the mutation of Met to Gln. However, an equivalent mutation carried out on azurin (M121Q) resulted in a in lowering of the redox potential by only 25 mV to 263 mV. In contrast, a 'reverse' mutation in C-STC (Q99M) produced an increase in redox potential from 260 to 420 mV (Nersissian *et al.*, 1998). The nature of the axial ligand clearly contributes significantly to the redox potential of cupredoxins but only in the range ∼100–200 mV. This is consistent with density-function calculations, which determined that the maximum variation in redox potential for the range of Cu–axial ligand distances present in cupredoxins was 140 mV (Olsson & Ryde, 1999).

4. Conclusions

The X-ray structure of the M148Q point mutant has been solved to 1.5 Å resolution. The mutant protein has a near-identical tertiary structure to that of the native rusticyanin, thereby suggesting that the decrease in redox potential (110 mV) is a direct consequence of this specific change to the axial ligand in the Cu centre. The mutated type 1 copper site is similar to that found in cucumber stellacyanin, but somewhat different to that determined by EXAFS in *R. vernicifera* stellacyanin. The site is also similar to the M121Q mutant of azurin, but lacks the fifth, carbonyl ligand present in azurin but not in stellacyanin and therefore represents a better model for the stellacyanin site. This finding is further reinforced by the observations (i) that mutation of the axial ligand from Met to Gln produces only a 25 mV reduction in redox potential in azurin in comparison to 110 mV in the case of rusticyanin and (ii) a 'reverse' mutation in C-STC (Q99M) produced an increase in redox potential of 160 mV. The interface between the two molecules of M148Q indicates a potential docking site for the redox partner. A peptide flip of Ile140 is observed in molecule *B* resulting in removal of steric hindrance allowing the Gly840 to form a weak axial interaction with Cu at 3.7 Å compared with 5.8 Å in molecule *A*.

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