Docking of cytochrome c₆ and plastocyanin to the aa₃-type cytochrome c oxidase in the cyanobacterium Phormidium laminosum

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The interactions between redox proteins are transient in nature. Therefore, very few crystal structures are available for the complexes formed between these proteins. Computational docking simulations thus provide a useful alternative method for studying the interactions between electron transfer proteins. In this paper, we have studied the interactions between the aa₃-type cytochrome c oxidase of the cyanobacterium Phormidium laminosum and its redox partners plastocyanin and cytochrome c₆ using a combination of comparative modelling techniques and docking simulations. Rigid-body docking orientations were scored with a combined energy function that accounts for electrostatics and desolvation. These simulations have identified two plausible docking sites, one of which appears to be unique to the binding of plastocyanin to the oxidase. This unique binding site may be due to the presence of a long loop region in the subunit II of cyano-bacterial oxidases. Control simulations were performed with the ba₃-type cytochrome c oxidase and its redox partner cytochrome c₅₅₂ from Thermus thermophilus. The docking between cytochrome c oxidase and its redox partners plastocyanin and cytochrome c₆ is dominated by hydrophobic residues, a feature already observed from kinetic and structural studies in other complexes of P. laminosum (e.g. plastocyanin or cytochrome c₆ with cytochrome f and photosystem I).

Keywords: aa₃-cytochrome c oxidase/electron transfer/homology modelling/protein–protein docking/redox proteins

Introduction

Electron transfer reactions are essential for many key biological processes, such as photosynthesis and the respiratory pathways, and require the formation of specific complexes between proteins that carry the redox centres. The interactions between such electron transfer proteins are weak and transient to ensure that dissociation does not limit overall turnover. In addition, the proteins are required to interact with multiple partners. However, the interactions also need to be specific to allow for a close apposition of the redox centres, because of the distance-dependence of electron transfer, and to avoid futile interactions (Bendall, 1996). Generally, the interfaces of transient complexes have a hydrophobic centre with charged and polar residues at the periphery, which seems to pre-orient the proteins and guide their association (Crowley and Ubbink, 2003; Prudencio and Ubbink, 2004).

The aa₃-cytochrome c oxidase (COX) belongs to the superfamily of haem-copper oxidases and is involved in the transfer of electrons from cytochrome c to oxygen. COX enzymes form a homologous family, including the (well-characterised) mitochondrial oxidases and those from Paracoccus denitrificans and from Rhodobacter sphaeroides. These oxidases reduce molecular oxygen to water and couple electron transfer to the pumping of protons, ultimately leading to the production of ATP.

The subunit composition of the COX enzymes varies between organisms, e.g. the mitochondrial enzyme has 13 subunits. However, all have three core subunits (subunits I–III). The redox centres are ligated by subunits I and II. Subunit I (CtaD) ligates haem a and the binuclear centre composed of haem a₁ and Cu₅₆. Subunit II (CtaC) ligates the Cu₅₆ centre. Subunit III (CtaE) does not ligate any redox centres, but may play a role in complex stability and assembly (Haltia et al., 1994; Bratton et al., 1999, 2000).

Given the general difficulties in obtaining the crystallographic three-dimensional (3D) structure of the complexes formed by electron transfer proteins (due to the existence of transmembrane regions and also to the transient nature of the interactions), Brownian dynamics simulations and docking studies are becoming increasingly important, since they provide important insights into the nature of these interactions (Gross, 2004). Actually, protein–protein docking is a very active research area, and many algorithms have been recently developed to predict the structure of a protein–protein complex from their unbound subunits. A good picture of the current state of docking prediction is given by the international experiment CAPRI (http://capri.ebi.ac.uk), which aims to provide objective tests to monitor the performance of current docking methods (Mendez et al., 2005).

Simulation of the docking of horse heart cytochrome c (Cyt c) to bovine COX revealed a complex with a large electrostatic contribution (Roberts and Pique, 1999). In spite of this, the actual docking site appears to be a hydrophobic region, which includes a loop lying over the Cu₅₆ site. A Trp residue (Trp104 in bovine numbering) in the Cu₅₆ site was involved in electrostatic interactions leading to the formation of the encounter complex, rather than at the interface of the final complex.
Another docking study was performed to examine the interaction between the soluble Cu₆ domain of the b₃₂ oxidase from *Thermus thermophilus* and its physiological donor Cyt c₅₅₂ (*Muresanu et al., 2006*). Unlike Cyt c₅₅₂ from *P. denitrificans*, *T. thermophilus* Cyt c₅₅₂ is a periplasmic protein and does not contain a transmembrane region. Restraints for the docking simulation were derived from chemical shift perturbation analysis, and the resulting docked complex was dominated by hydrophobic interactions, in agreement with kinetic measurements (*Maneg et al., 2004*). This is likely to reflect the thermophilic nature of the organism.

Despite these studies, little is known about the detailed interactions between cyanobacterial COX and its redox partners. COX has been found in all cyanobacteria characterised so far, including the moderate thermophile *Phormidium laminosum*, and is composed of the three core subunits only (*Peschek et al., 2004*). Cyanobacterial COX is found at both the cytoplasmic and the thylakoid membranes; the latter is a distinct internal membrane system. Only in cyanobacteria (and not in any other species), the type I copper protein plastocyanin (Pc) has been shown to be capable of reducing COX (*Paumann et al., 2005*). In cyanobacteria, Pc and c-type cytochrome *c₆* (Cyt *c₆*) interact with COX, predominantly at subunit II (CtaC). These proteins also transfer electrons between cytochrome *f* of the cytochrome *b₅* complex and photosystem I (PSI).

In this paper, we have studied the interactions between COX from *P. laminosum* and its redox partners Pc and Cyt *c₆* using a combination of comparative modelling techniques and docking simulations. A model of subunit II (CtaC) has revealed some distinct features of the cyanobacterial COX, and its docking to Pc and Cyt *c₆* has identified two plausible docking sites, one of which appears to be unique to the Pc binding.

**Materials and methods**

**Prediction of transmembrane regions and signal sequences**

The translated sequences of the *P. laminosum* genes were analysed for potential transmembrane regions (TMHMM and HMTOP) and signal sequences (SignalP, PSORT). The program TMHMM, version 2.0 (*Sonnhammer et al., 1998; Krogh et al., 2001*), is available from the Centre for Biological Sequence analysis (CBS) at http://www.cbs.dtu.dk/services/TMHMM/. The program HMTOP, version 2.0 (Tusnady and Simon, 1998, 2001), is available at http://www.enzim.hu/hmtop. The program SignalP, version 3.0 (*Nielsen et al., 1997; Nielsen and Krogh, 1998; Bendtsen et al., 2004*), is available from the CBS at http://www.cbs.dtu.dk/services/SignalP/. The programs PSORT (Nakai and Horton, 1999) and PSORT-B, which is recommended for gram-negative bacteria (*Gardy et al., 2003*), are available at http://psort.nibb.ac.jp/.

**Prediction of protein secondary structure and regions of disorder**

Secondary structure predictions were made using PSIPRED (*Jones, 1999*) at the UCL server, http://bioinf.cs.ucl.ac.uk/psipred/ ([McGuffin et al., 2000; Bryson et al., 2005]). PONDR (*Romero et al., 1997; Li et al., 1999; Romero et al., 2001*) and DISOPRED (*Ward et al., 2004*) at the UCL server, http://bioinf.cs.ucl.ac.uk/disopred/, were used to predict regions of disorder.

**Comparative models**

Possible templates for the modelling of truncated version of *P. laminosum* CtaC were selected, using FUGUE (*Shi et al., 2001*), from the HOMSTRAD database (*Mizuguchi et al., 1999b*). Alignments for the purposes of modelling were generated using FUGUE, and the most appropriate alignment was selected and subjected to manual editing. Modeller version 8.0 (*Sali and Blundell, 1993*) was used to generate models.

The quality of the models was assessed using JOY (*Mizuguchi et al., 1999a), Verify 3D (*Eisenberg et al., 1997*) at http://nihserver.mbi.ucla.edu/Verify_3D/, and Prosa2003 (*Hendlich et al., 1990; Sippl, 1990*). Estimates of the reliability of the models, their pG value (*Sanchez and Sali, 1998*), were obtained from the pG server at http://atlas.physbio.mssm.edu:8084/servers/pg/.

Models were visualised using MacPyMOL and PyMOLX11Hybrid ([Delano Scientific LLC; http://delsci.com/mac pymol/]).

APBS (*Baker et al., 2001*) was used to calculate and visualise the electrostatic potentials of the final model of CtaC. APBS was run as a plugin of PyMOLX11Hybrid using the default conditions.

**Docking simulations**

Docking calculations were performed with the pyDock method (*Man-Kuang Cheng et al., 2007*). In the first step, FTDOCK (*Gabb et al., 1997*) and ZDOCK (*Chen and Weng, 2003*) docking programs were run in order to generate a sufficient number of rigid-body docking poses (10 000 from FTDOCK and 2000 from ZDOCK). Then, the resulting orientations were scored with a combined energy function that accounted for electrostatics and desolvation (*Man-Kuang Cheng et al., 2007*). Intermolecular Coulomb electrostatics was computed with distance-dependent dielectric constant (*ε = 4.0/x*), with atomic pairwise interactions truncated between −1.0 and 1.0 kcal/mol. Accessible surface area-based desolvation accounted for the effective transfer of a solvent-exposed surface residue to the protein–protein interface, by using atomic solvation parameters optimised for rigid-body docking (*Fernandez-Recio et al., 2004*). A similar energy function implemented in the ICM-DISCO (*Fernandez-Recio et al., 2003*) docking program already gave excellent predictive rates in the CAPRI experiment (*Fernandez-Recio et al., 2005*).

The coordinates for CtaC structure were obtained by comparative modelling, as described above. The X-ray structures of Pc and Cyt *c₆* were available as Protein Data Bank (PDB) entries 1BAW (Bond et al., 1999) and 2V08 (Worrall et al., 2007), respectively. For the docking control of *T. thermophilus* CtaC and Cyt c₅₅₂, we have used the same structures as previously described in a recent docking experiment (*Muresanu et al., 2006*). For CtaC of *T. thermophilus*, we have used the PDB entry 1EHK, considering only the soluble part of chain B. For Cyt c₅₅₂, we have used the PDB entry 1DT1. We have compared our results with the model proposed by Muresanu et al. (2006), deposited as PDB entry 2FWL (only model 1 was considered for our comparison). The following residues, based on NMR data as previously described (*Muresanu et al., 2006*), have been included as
restraints in pyDockRST, using a protocol previously described (Chelliah et al., 2006). For CtaC: Ala87, Phe88, Arg146, Gly156 and Asn159. For Cyt c552: Gly15, Asn18, Gly24, Gln57, Val68, Ser70, Ala113, Lys115, Gln119 and Gln120. Redox centres were not considered in the docking scoring function.

Results and discussion

Comparative model of P. laminosum CtaC

No structural information is yet available for any cyanobacterial COX. However, high-resolution structures are available for a number of COX complexes, including those from P. denitrificans (Iwata et al., 1995; Ostermeier et al., 1997), R. sphaeroides (Svensson-Ek et al., 2002) and Bos taurus (Yoshikawa et al., 1998; Tomizaki et al., 1999). In addition, a crystal structure has been solved for the ubiquinol-oxidase from Escherichia coli (Abramson et al., 2000).

These structures are potential templates for a comparative model of the P. laminosum COX complex. Indeed, membrane-topographic models of subunits I and II of the COX from Synechocystis sp. PCC 6803 have been generated using the P. denitrificans complex (Paumann et al., 2004). In this study, focus has been placed on subunit II (CtaC) of P. laminosum COX, as this subunit dominates the interaction with its donors and contains the CuA centre where electrons are initially received.

The amino acid sequence of P. laminosum CtaC contains two putative transmembrane helices at residues 47–68 and 90–112, followed by a predicted region of disorder, at 136–175 by DISOPRED (Ward et al., 2004) or at 131–184 by PONDOR (Romero et al., 1997, 2001; Li et al., 1999) (Supplementary Fig. 1 available at PDBS online). A region from Pro195 to Ser352 was selected for modelling, which includes both the region thought to accept electrons and the CuA-binding site.

A FUGUE (Shi et al., 2001) search with the selected region of P. laminosum CtaC, Pro195-Ser352, against the HOMSTRAD database (Mizuguchi et al., 1998b) produced the highest scoring hit to the COX2 family of structures (with a Z-score of 25.9, >99% confidence). This family consists of the bovine complex (PDB entry 2OCC) (Yoshikawa et al., 1998), the two-subunit P. denitrificans complex (PDB entry 1AR1) (Ostermeier et al., 1997), and the soluble domain of E. coli CytoA with an engineered copper-site (PDB entry 1CYX) (Pickersgill, 1988). We searched the PDB and SCOP (Murzin et al., 1995) for other homologues and examined which of the available structures would be suitable templates for the comparative modelling of P. laminosum CtaC.

All these structures contain a periplasmic β sandwich domain, which is likely to be homologous to Pc, azurin and other electron-transfer proteins (known as the Cupredoxin superfamily in SCOP). Although the core of this domain adopts a Greek-key type seven-stranded β sandwich fold, some members have additional strands and show large structural variations.

Even among the closest homologues of P. laminosum CtaC, the percentage identities (PID), after the optimal FUGUE alignments, were relatively low between the selected region of P. laminosum CtaC and the aligned regions of the homologues (e.g. 28% with E. coli CytoA and 36% with P. denitrificans CtaC, respectively). Therefore, the PID alone was insufficient for selecting the most appropriate modelling template(s).

Several attempts at FUGUE alignments revealed that the region before the CuA-binding site was extended in P. denitrificans and bovine CtaC, relative to P. laminosum CtaC and E. coli CytoA (summarised in Supplementary Fig. 2 available at PEDS online). In addition, the P. laminosum protein was shown to have a C-terminal extension relative to P. denitrificans and bovine CtaC. An even greater C-terminal extension was observed in E. coli CytoA. This extension of E. coli CytoA contains two α-helices, separated by a short β-strand. In the C-terminal extension of P. laminosum CtaC, PSIPRED (Jones, 1999) predicted three relatively long helices (marked with medium grey asterisks in Fig. 1A), as well as a β-strand between the final two helices (marked with light grey asterisks in Fig. 1A). These predicted secondary structures aligned well with the observed secondary structures in E. coli CytoA.

On the basis of these observations, we decided to use the E. coli CytoA as the template for the P. laminosum CtaC model. The selected region of P. laminosum CtaC (Pro195 to Ser352) was realigned with a region of E. coli CytoA (PDB 1CYX) from Lys125 to Phe281 using FUGUE (Wilmanns et al., 1995). Multiple models were generated from this initial alignment with MODELLER (Sali and Blundell, 1993) and evaluated using Verify3D (Eisenberg et al., 1990; Sippl, 1990) and JOY (Mizuguchi et al., 1998a). To improve the result, the alignment was manually edited and further models were generated. The final alignment, formatted with JOY, is shown in Fig. 1A.

The generally positive 3D–1D compatibility scores from Verify3D confirmed the quality of the models. We also examined whether the secondary structures were properly formed in the C-terminal extension. The final model had two short β-strand regions (Gln312–Asn313 and Gln336–Glu337) that were hydrogen bonded to each other. The Prosa2003 Z-score of this model was −5.91, which was within the expected region of a correct model of this size (158 amino acids) (and at the edge of the scatter observed for the Z-scores calculated from experimentally determined structures). This result was consistent with a pG value (Sanchez and Sali, 1998) of 1.0 (certain).

In this final model, a large β-sheet region (B1–B10) is present at the N-terminus (Fig. 1B and C). A single 3₁₀-helix separates B9 and B10. Three α-helices are present in the C-terminal half of the model, with short β-strands interpersed between them. These helices correspond reasonably well to the helices predicted by PSIPRED (discussed above).

The helices α1 and α2 are separated by β11, whereas the helices α2 and α3 are separated by β12 and β13. A β-sheet forms between β3 (Gly216–Ala219) and β12 (Ala332–Tyr334). A similar interaction is seen between the corresponding β-strands of the E. coli CytoA template, where the β-strand at residues 147–150 interacts with the β-strand at residues 264–266.

Electrostatic potential shows a predominantly hydrophobic docking surface but also reveals some acidic residues that may orient the docking partner

APBS (Baker et al., 2001) was used to predict the surface electrostatic potentials of the model of P. laminosum CtaC.
The surface exposed ligands of the CuA-centre (His241, His284, Cys280 and Glu278) and the residue proposed to accept electrons (Tyr206) are on a relatively non-polar surface (Fig. 2). Therefore, the docking site of \(\text{P. laminosum}\) CtaC is likely to be dominated by hydrophobic residues. On the reverse side of CtaC, with respect to Tyr206, there are a number of acidic residues: Glu221, Asp297, Glu298, Asp300, Asp329 and Asp342 (Fig. 2). However, another group of acidic residues sits close to Tyr206: Asp238, Glu278 (a CuA ligand), Asp324 and Asp327 (Fig. 2). These acidic residues might be used to guide the proteins to the correct docking site via long-range electrostatic interactions. Although most of the acidic residues of CtaC that are important for the docking of the interacting proteins are not conserved in cyanobacterial CtaC proteins (Paumann et al., 2005), the residue Asp238 (in \(\text{P. laminosum}\) numbering) appears to be conserved in cyanobacteria. The conservation of this residue has been highlighted for other cyanobacterial species, including \(\text{Thermosynechococcus vulcanus}\) (Sone et al., 1993) and \(\text{Synechocystis}\) sp. PCC 6803 (Alge and Peschek, 1993). It is therefore interesting that this conserved acidic residue sits close to Tyr206. The corresponding residue of the bovine CtaC, Asp158, is the only acidic residue at the interface with horse Cyt c whose side chain lies along the protein surface and does not point out into the solvent (Roberts and Pique, 1999). This residue is also a second sphere ligand of the CuA site and interacts with His161. Therefore, the conservation of this residue among cyanobacterial proteins may reflect its role in the orientation of the CuA ligand and not in the docking of electron donors.

**Docking of COX and Pc**

Docking simulations were performed between the extrinsic domain of \(\text{P. laminosum}\) CtaC (using the structural model described above) and Pc (PDB entry 1BAW) (Bond et al., 1999). We used the pyDock suite of programs (see Materials and methods) for the rigid-body docking calculations. The rigid-body treatment of the proteins is justified, since the electron transfer reactions are fast and therefore the proteins are unlikely to undergo large-scale conformational changes (Flock and Helms, 2002).
We used for docking the extrinsic domain of *P. laminosum* CtaC. Previous results indicate that soluble versions of CtaC are a good model system in kinetic analysis *in vitro* for the entire COX complex (Maneg *et al.*, 2003). Indeed, a similar approach was successfully used to dock redox proteins of *T. thermophilus* (Muresanu *et al.*, 2006).

The unrestricted docking calculations yielded interesting results. Around 40% of the lowest-energy docking solutions had the redox centres at a reasonable distance for electron transfer (Page *et al.*, 2003). However, we proceeded to check whether these docking solutions would clash with the transmembrane domains of the entire COX complex (subunits I, II and III). Given that the transmembrane regions of COX subunits I, II and III are structurally conserved in other organisms, we simply superimposed our *P. laminosum* CtaC model onto the corresponding domain of *R. sphaeroides* COX (PDB 1M56) (Svensson-Ek *et al.*, 2002), took the coordinates of the transmembrane regions from this crystal structure and used them as a reference structure for the entire COX complex. We filtered the docking solutions, checking for clashing residues in Pc (defined as those whose centre of coordinates would be located within 3 Å from any atom of the entire COX complex). Around half of the lowest-energy docking solutions would have more than 20% of Pc residues clashing with the complete COX complex, so these solutions were excluded from our analysis. This threshold of 20% of clashing residues was defined because: (i) we used the structure of the homologous *R. sphaeroides* COX, so we can only use this reference structure as a rough criterion for clashing residues and (ii) we cannot disregard the possibility of small conformational rearrangements in the extrinsic domain of CtaC and/or the COX transmembrane domains, which could make feasible some of the current docking solutions with small number of clashes. The five lowest-energy docking orientations with close redox centres and <20% of COX-clashing residues are shown in Table I.

The electron transfer is mediated by a Trp residue of CtaC in *R. sphaeroides*, *P. denitrificans* and *B. taurus*. In cyanobacteria, this Trp residue is replaced by Phe/Tyr (Tyr206 of *P. laminosum* CtaC, marked with a black arrow in Fig. 1A) (Paumann *et al.*, 2005). The site of electron donation from Pc is likely to be the exposed His ligand of the copper centre (His92 of *P. laminosum* Pc) (Bendall *et al.*, 1999). Table I also shows the distance between CtaC Tyr206 and Pc His92 of the best docking solutions.

As can be seen in Fig. 3, the docking results gave two distinct orientations, one energetically more favourable and the other more populated in the conditions of the docking calculations. In both orientations, the redox groups are at a similar distance. In the first orientation (as in solution rank 8), the binding interface is not too far from that proposed by Muresanu *et al.* (2006) for the interaction between

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**Fig. 2.** (A) Electrostatic surface potentials of the final CtaC model predicted by APBS. The colour-scale for the potentials is from −5 k_BT e (red) to +5 k_BT e (blue). (B) View rotated 90º along the z-axis (vertical axis parallel to the image plane). (C) Same orientation as in (A), indicating the position of the acidic residues. (D) Same orientation as in (B); the positions of the surface exposed copper ligands His241, His284 (magenta), Cys280 (yellow) and Glu278 (red) and Tyr206 (green) are shown in relation to the surface-exposed acidic residues (red). Image produced using MacPyMOL.
T. thermophilus CtaC and Cyt c_552 (see later for a docking analysis of this system). This binding mode is also similar to that observed for the docking of Cyt c_6 (see next section).

The second orientation (as in the remaining docking solutions) may indicate a specific binding site for Pc. Several lines of evidence support this hypothesis. First, this binding mode would involve CtaC Asp238, a residue conserved in cyanobacteria. Second, cyanobacterial CtaC is the only COX capable of binding Pc (Paumann et al., 2005). Finally, no docking solution for Cyt c_6 was found around this site (see next section). In Fig. 4 are shown the possible electron pathways that can be deduced from docking solution ranked 23, which had the shortest distance between the redox groups, and that between the residues expected to be involved in the electron transfer: Pc His92 and CtaC Tyr206 and Asp238 (Table I).

Although this second orientation involves the C-terminal extension unique in cyanobacterial CtaC, the modelled structure of this region was carried over from the template E. coli CyoA and it appears to be of reasonable quality. Finally, the fact that no solutions accumulate in this area in the Cyt c_6 docking (see below) rules out the possibility of an artificial hydrophobic exposure due to inaccurate modelling.

**Docking of Cox and Cyt c_6**

Docking simulations were performed between the extrinsic domain of P. laminosum CtaC and Cyt c_6 (PDB 2V08) (Worrall et al., 2007). We used pyDock in the same fashion as for the CtaC–Pc docking.

In 20% of the lowest-energy solutions, the CtaC copper and the Cyt c_6 haeme group were located within 18Å. Again, we checked whether these solutions would clash with the complete COX complex. In comparison with the docking of Pc, where ~50% of the low-energy solutions accumulated around the region of CtaC in contact with the transmembrane domains, here <10% of the lowest-energy solutions would clash with the complete COX complex. Therefore, after removing these clashing solutions, seven docking orientations with close redox centres were kept (Table II). The haem to copper electron transfer is likely to be mediated by Tyr206 of CtaC, and the site of electron donation from Cyt c_6 is likely to be the exposed haem-edge (Roberts and Pique, 1999; Diaz-Moreno et al., 2005a, 2005b). Table II shows the distance between CtaC Tyr 206 and Cyt c_6 haem-edge in the best docking solutions.

As can be seen in Supplementary Fig. 3 available at PEDS online, the best-ranked docking solution with close cofactors (ranked 3) placed the redox groups at a reasonable distance for electron transfer. Interestingly, the haem group is located almost in the same spatial location as the Pc copper atom in solution ranked 8 of the Pc docking. We have also compared the conformation of this Cyt c_6 docking solution with the model proposed by Muresanu et al. (2006) for the interaction between CtaC and Cyt c_552. Although a direct comparison between these two models is impossible (P. laminosum CtaC is larger than T. thermophilus CtaC and, therefore, the orientation of Cyt c_552 in the Muresanu model would clash with CtaC in the P. laminosum system), the haem groups are roughly located in the same region of space but with different orientations (not shown). Moreover, the second best-ranked solution with close-distance cofactors (ranked 28) has the same orientation of the haem group as in the model of Muresanu et al. (2006), although it is spatially located at a larger distance due to the greater size of P. laminosum CtaC (not shown).

No good docking solution was found in the exact region that was most populated for the Pc binding. In the few docking solutions found near this region (in which the haem group was close to Tyr206 or Asp238 of CtaC), either their

| Table I. Best docking orientations of CtaC and Pc interaction according to distance between their redox centres |
|---|---|---|---|
| Rank | Distance (Å) | Cu–Cu | CtaC Tyr206–Pc His92 | CtaC Asp238–Pc His92 |
| 8 | 16.1 | 10.2 | 18.1 |
| 13 | 15.9 | 7.4 | 7.0 |
| 14 | 15.1 | 5.2 | 6.0 |
| 23 | 13.9 | 3.5 | 5.3 |
| 25 | 15.7 | 4.6 | 7.6 |

Rank is defined according to binding energy, after removing solutions that would clash with the entire COX complex (see text).
binding energies were poor or Cyt c₆ polypeptide had many clashes (>20% of its residues) with the complete COX complex, or Cyt c₆ haem group would clash (>20% of its atoms) with the CtaC molecule. These observations reinforce the hypothesis that the second binding site found for the Pc docking is specific to Pc, and not optimal for Cyt c₆.

Docking models of homologous systems

To examine whether the unique features observed in the docking results above reflect the intrinsic nature of the cyanobacterial system and not an artefact from our docking approach, we performed additional docking simulations with CtaC and Cyt c₅₅₂ from *T. thermophilus*, for which previous docking studies were available for comparison (Muresanu et al., 2006).

We ran docking calculations under the same conditions as the docking of cyanobacterial CtaC with Pc and Cyt c₆. As in the case of Pc docking, many of the low-energy docking solutions (as much as 70%) would clash with the transmembrane domains of COX. After removing these artificial clashing solutions, Table III shows those ones that have an RMSD < 15Å from the model previously proposed (PDB entry 2FWL, model 1) (Muresanu et al., 2006).

As can be seen in Fig. 5A, the lowest-energy docking solution (ranked 1) is similar to the model proposed by Muresanu et al. (2006) based on NMR restraints. The haem groups are basically in the same spatial location, with a small difference in orientation.

These results validate our docking strategy for identifying the binding mode of Pc and Cyt c₆ with CtaC, as it shows that, in spite of the rigid-body approach, we are able to find a reasonable docking orientation among the lowest-energy solutions based solely on energy scoring. Nevertheless, as a further exercise for our docking tools, we have also applied the same NMR restraints that Muresanu et al. (2006) used for generating their model with the HADDOCK program. After scoring solutions with the binding energy plus restraints (pyDockRST; see Materials and methods), some of the original docking solutions were still among the top-ranked solutions. However, some orientations, different from the Muresanu et al. (2006) model (RMSD > 20Å), appear to satisfy the restraints even better than the Muresanu et al. (2006) model (73% satisfied restraints calculated for PDB entry 2FWL, model 1), and therefore are found within the top-ranked solutions according to the binding energy plus restraints.

Although in previous tests no significant differences were found between pyDockRST and HADDOCK (Chelliah et al., 2006), current results might arise from the balance between binding energy and restraint ‘pseudo-energy’ in both programs. One can hypothesise that the ‘restraint-based’ solutions (detected by restraint-based pyDockRST) represent better the native complex state, whereas the ‘energy-based’ solutions (detected both by unrestricted pyDock and by restraint-based HADDOCK) could represent the encounter complex for a rigid-body approach. On the other hand, this could also indicate the existence of alternative orientations in the native state, which may explain why a single conformation is not able to satisfy all the restraints. In our docking, the best solution can satisfy only 80% of the restraints (Table IV) and actually, Muresanu et al. (2006) already mentioned the difficulties of satisfying all the restraints simultaneously.

As can be seen in Fig. 5B, this best docking solution after restraints is different from the lowest-energy solution (Fig. 5A), and from the model in PDB entry 2FWL (Muresanu et al., 2006). Interestingly, this docking solution after restraints has the redox group in a similar position as the Cu atom in solution ranked 8 for the Pc docking, and as

Table II. Best docking orientations of the CtaC and Cyt c₆ interaction according to distance between their redox centres

<table>
<thead>
<tr>
<th>Rank</th>
<th>Distance (Å)</th>
<th>Cu—haeme</th>
<th>CtaC Tyr206—haem-edge</th>
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<tr>
<td>3</td>
<td>13.2</td>
<td>8.6</td>
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<tr>
<td>28</td>
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<td>34</td>
<td>16.7</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>16.4</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>16.4</td>
<td>9.8</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>17.4</td>
<td>11.1</td>
<td></td>
</tr>
</tbody>
</table>

Rank is defined after filtering by COX clashing (see text).

Table III. Best docking orientations of CtaC and Cyt c₅₅₂ interaction, when compared with the model in PDB entry 2FWL, model 1 (Muresanu et al., 2006)

<table>
<thead>
<tr>
<th>Rank</th>
<th>RMSD (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.9</td>
</tr>
<tr>
<td>2</td>
<td>14.5</td>
</tr>
<tr>
<td>10</td>
<td>10.8</td>
</tr>
<tr>
<td>11</td>
<td>12.8</td>
</tr>
<tr>
<td>12</td>
<td>9.6</td>
</tr>
<tr>
<td>15</td>
<td>8.0</td>
</tr>
<tr>
<td>17</td>
<td>8.3</td>
</tr>
<tr>
<td>19</td>
<td>5.9</td>
</tr>
<tr>
<td>22</td>
<td>7.4</td>
</tr>
<tr>
<td>28</td>
<td>10.0</td>
</tr>
</tbody>
</table>
the haem group in the best solution for the Cyt c₆ docking. This suggests the existence of a general binding mode in this CtaC region and simultaneously opens the possibility of more than one single orientation contributing to the electron transfer in the complexed state. The fact of finding the same binding mode when using either an X-ray structure (in T. thermophilus CtaC) or a homology-based model (in P. laminosum CtaC) justifies the use of comparative modelling in our docking.

A new specific site in CtaC for Pc binding

In a number of organisms, including P. denitrificans and R. sphaeroides, and in mitochondria, the interactions between COX and its donors are dominated by an electrostatic attraction between lysine residues which line the haem edge of Cyt c, and acidic residues of CtaC, such as Asp206, Asp187, Glu154, Asp174 and Asp163 in P. denitrificans numbering (Witt et al., 1998; Drosou et al., 2002a, 2002b). Of these acidic residues, only Asp206 in P. denitrificans numbering (Asp238 in P. laminosum numbering; Fig. 1A) is conserved in cyanobacteria (Paumann et al., 2005). Consistent with this, a pronounced basic region around the haem edge of the mitochondrial Cyt c is not seen in cyanobacterial Cyt c₆ or Pc. The interactions of Pc/Cyt c₆ with cyanobacterial COX are likely to be dominated by hydrophobic interactions as has been seen from kinetic and structural studies for the interactions of Pc/Cyt c₆ with cytochrome f (Clackson and Wells, 1995) and PSI (Schlarb-Ridley et al., 2002). This role of hydrophobic binding is especially consistent with the docking of CtaC and Pc, where desolvation energy is the most dominant factor. The most populated binding orientation in the Pc docking would involve the CtaC residue Asp238, conserved in cyanobacteria, as mentioned before. This suggests a new docking site for Pc that has not been described before, and hence could be specific for this protein. However, we have not found a similar orientation among the low-energy docking solutions for Cyt c₆, which seems to indicate that the Asp238 is only involved in the interaction with Pc and not Cyt c₆.

An ensemble of conformations in the complex state?

The finding of different alternative docking orientations in all the systems analysed in our study could be due to the possibility that proteins might not need to interact via a fixed complex; instead, electron transfer could take place from a weak association between the two proteins as long as the distance between the redox centres is favourable for electron transfer. This type of interaction has been proposed for other electron transfer systems, such as myoglobin: cytochrome b₅ (Worrall et al., 2002), cytochrome c: cytochrome b₅ (Volkov et al., 2005) or FNR: flavodoxin (Medina et al., 2008). Indeed, a pseudo-specific docking surface that allows a single protein to interact with multiple partners (Williams et al., 1995) has already been proposed for the interaction between horse heart Cyt c and bovine COX (Roberts and Pique, 1999). The possibility has also been discussed that several productive conformations, rather than a single fixed docking conformation, exist for the interaction between Cyt c₅₅₂ and COX of P. denitrificans (Flock and Helms, 2002).

Table IV. Ten lowest-energy docking orientations of CtaC and Cyt c₅₅₂ interaction, after introduction of restraints [defined by Muresanu et al. (2006)]

<table>
<thead>
<tr>
<th>Rank</th>
<th>RMSD (Å)</th>
<th>Restraints (%)</th>
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</thead>
<tbody>
<tr>
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<td>5</td>
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<tr>
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<td>14.5</td>
<td>67</td>
</tr>
<tr>
<td>8</td>
<td>22.6</td>
<td>73</td>
</tr>
<tr>
<td>9</td>
<td>21.2</td>
<td>67</td>
</tr>
</tbody>
</table>
We should not disregard other reasons to explain the heterogeneity in the docking orientations. For instance, the long loop region characterised for the cyanobacterial CtaC proteins, which is not seen in other CtaC proteins (Pereira et al., 2001), may allow for an interaction between Pc/Cyt \( c_6 \) and a region of the CtaC which is obscured by the membrane in the current docking simulation. The orientation of the cyanobacterial Cu\(_A\)-domain relative to the membrane may be different from that seen in other complexes, for example, \( P. \) denitrificans COX. A large region of disorder (>40 amino acids) within this loop is predicted by PONDR in a number of cyanobacterial CtaC proteins including CtaC from \( P. \) laminationosum, \( A. \) sp. PCC 7120, \( T. \) elongatus BP-1 and \( S. \) sp. PCC 6803 (data not shown). In contrast, no regions of disorder are described in the crystal structures of COX from \( B. \) taurus, \( P. \) denitrificans and \( R. \) sphaeroides. The disordered region within the loop may reflect a region of high flexibility, which could allow the protein to fold back on itself such that regions of the CtaC protein which lie next to the membrane in this docking simulation are fully exposed to the lumen, and the reverse for regions exposed to the lumen. However, the controls carried out in this study indicate that is very unlikely that this loop has a strong effect on the docking solutions.

It is also possible that the cyanobacterial loop between the transmembrane regions and the Cu\(_A\)-domain is itself involved in the interaction of CtaC with its electron donors perhaps via a disorder-to-order transition upon binding. Such a transition has been found to be a key to several protein–protein interactions and a way of uncoupling affinity and specificity (Dunker et al., 1998). This is interesting in the context of the transient-interactions between redox proteins, which are required to be weak and yet specific due to the distance-dependent nature of electron transfer.

There have been suggestions that a small c-type cytochrome, cytochrome \( c_M \), may interact with cyanobacterial COX, perhaps as an additional subunit. The absence of this protein from the current docking simulations could account for some of the differences observed between these results and previous ones. As a future work, simulation of the docking of cytochrome \( c_M \) could provide information as to whether such an interaction is likely.

One of the challenges of the current study is that the extrinsic domain of \( P. \) laminationosum CtaC used in the docking is a theoretical model and not an experimental 3D structure. However, from the evaluation carried out in this study our model does seem to be a valid one. In the future, further restraints for docking simulations should be derived from kinetic analysis of site-directed mutants or NMR studies providing that good expression systems can be developed.

In conclusion, from our modelling studies, the docking sites of \( P. \) laminationosum CtaC appear to be dominated by hydrophobic residues. This is consistent with the interactions of \( Pc \) or Cyt \( c_6 \) with cytochrome \( f \) (Crowley et al., 2001) and PSI (Schlarb-Ridley et al., 2002). Nevertheless, charged patches present in the CtaC model could have a role in pre-orientation of the proteins and guiding of its interactions. The results from docking calculations suggest that the long loop region of cyanobacterial CtaC may result in an additional new interaction site between COX and Pc, which differs in orientation to the complexes characterised previously for other organisms. Alternatively, electron transfer may not occur via a fixed complex but from different orientations.

The prediction of residues likely to be involved in the interaction based on the comparative model of the \( P. \) laminationosum CtaC and the docking simulations may give useful hints for alternative methods of analysis, e.g. site-directed mutagenesis and kinetic studies, provided that a suitable expression system can be developed. An obvious target for site-directed mutagenesis will be Asp238, one of the acidic residues close to the proposed docking site. In addition, it would be interesting to see whether any of the mutants of \( P. \) laminationosum Pc seen to affect the interactions with PSI and cytochrome \( f \) also affect the interaction with COX.

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**References**


