

The 1.6 Å X-ray Structure of the Unusual c-type Cytochrome, Cytochrome c_L , from the Methylophilic Bacterium *Methylobacterium extorquens*

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The structure of cytochrome c_L from *Methylobacterium extorquens* has been determined by X-ray crystallography to a resolution of 1.6 Å. This unusually large, acidic cytochrome is the physiological electron acceptor for the quinoprotein methanol dehydrogenase in the periplasm of methylophilic bacteria. Its amino acid sequence is completely different from that of other cytochromes but its X-ray structure reveals a core that is typical of class I cytochromes c , having α -helices folded into a compact structure enclosing the single haem c prosthetic group and leaving one edge of the haem exposed. The haem is bound through thioether bonds to Cys65 and Cys68, and the fifth ligand to the haem iron is provided by His69. Remarkably, the sixth ligand is provided by His112, and not by Met109, which had been shown to be the sixth ligand in solution. Cytochrome c_L is unusual in having a disulphide bridge that tethers the long C-terminal extension to the body of the structure. The crystal structure reveals that, close to the inner haem propionate, there is tightly bound calcium ion that is likely to be involved in stabilization of the redox potential, and that may be important in the flow of electrons from reduced pyrroloquinoline quinone in methanol dehydrogenase to the haem of cytochrome c_L . As predicted, both haem propionates are exposed to solvent, accounting for the unusual influence of pH on the redox potential of this cytochrome.

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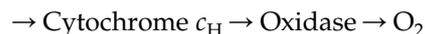
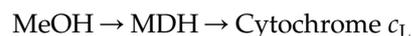
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Introduction

During the growth of methylophilic bacteria, methanol is oxidised by the large soluble, periplasmic quinoprotein methanol dehydrogenase (MDH).^{1,2} This initiates an electron transfer process in which the reduced MDH is oxidised by the unusual cytochrome c_L , which is in turn re-oxidised

by the typical cytochrome c_2 (called cytochrome c_H), which then donates electrons to the membrane-bound cytochrome oxidase, cytochrome aa_3 .^{3,4}



The water-soluble nature and the periplasmic location of the first three components of this pathway make this an ideal system for studying intra-molecular and inter-molecular electron transfer. We have solved and reported the three-dimensional structure of MDH^{5–7} and cytochrome c_H ⁸ from *Methylobacterium extorquens*. Here, we describe the structure of the remaining soluble protein in this electron transport sequence. MDH is a tetrameric protein consisting of two α -chains and

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Abbreviations used: PQQ, pyrroloquinoline quinone; MDH, methanol dehydrogenase; cytochrome c_M , mitochondrial cytochrome c ; HP6, outer haem propionate; HP7, inner haem propionate.

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two β -chains ($\alpha_2\beta_2$). The large (66 kDa) catalytic α -subunit contains the non-covalently bound pyrroloquinoline quinone (PQQ) prosthetic group coordinated to a calcium ion in the active site. The α -subunit is folded into a β -propeller superbarrel structure composed of eight twisted antiparallel β -sheets. The smaller β -subunit (8.5 kDa) wraps around one side of the α -subunit and is thought to have no catalytic function.

MDH passes electrons to cytochrome c_L in two separate single-electron transfer steps, creating a semi-quinone PQQ moiety after the first transfer.⁹ Cytochrome c_H then mediates electron transfer from cytochrome c_L to the oxidase. At no stage in this electron transport pathway does a three-protein complex exist. Cross-linking studies have shown that the same carboxylate residues from cytochrome c_L that interact with lysine residues from MDH, interact with lysine residues on cytochrome c_H .¹⁰ The three-dimensional structure of cytochrome c_H , the third component of the pathway, revealed that its tertiary structure most closely resembles that of mitochondrial cytochrome c , rather than that of other bacterial cytochromes c_2 .⁸ Cytochrome c_H possesses several interesting features that are not encountered in other class I c -type cytochromes; most strikingly, the residues around the haem pocket are particularly hydrophobic and the usual conserved water molecule in the left-hand side of the cleft is absent.⁸ Moreover, the crucial conserved tyrosine residue (Tyr67), which has been postulated to mediate the redox potential through the modulation of a hydrogen bond to the methionine residue ligated to the haem iron,¹¹ is replaced by tryptophan.

The specific cytochrome electron acceptor from MDH is the unusual cytochrome c_L ,^{3,12,13} whose amino acid sequence is completely different from that of other c -type cytochromes.¹⁴ As pointed out by Moore & Pettigrew in their extensive survey of cytochromes c ,¹⁵ this cytochrome does not fit into any established amino acid sequence class, although its features are closer to those of class I cytochromes c than to other classes. Except for the haem-binding motif (CSGCH), the sequence of cytochrome c_L displays no homology with any other protein, except for its counterpart from *Paracoccus denitrificans*, cytochrome c_{551i} .¹⁶ Class I cytochromes c include mitochondrial cytochromes c and bacterial cytochromes c_2 , which function in electron transport chains similar to those of mitochondria, or in photosynthetic electron transport systems. Typical of these cytochromes, cytochrome c_L has a single, low-spin, haem prosthetic group, bonded covalently to cysteine residues from the protein and a Met-Fe-His iron coordination.^{12,13} Its midpoint redox potential of 256 mV is also typical, although its response to pH changes is unusual. Also unusual is its large size (about 19 kDa), the presence of a disulphide bridge, its low isoelectric point of 3.5 (class I cytochromes c are typically basic) and its atypical reaction with carbon monoxide.

We describe the structure of oxidized cytochrome c_L from *M. extorquens* determined at 1.6 Å resolution

by X-ray crystallography. Previously, only the structure of the homologous cytochrome c_{551i} from *P. denitrificans* had been determined at 2.4 Å, as part of a ternary crystal complex with the blue copper protein amicyanin, and the tryptophan tryptophylquinone (TTQ)-containing protein, methylamine dehydrogenase.¹⁶

Results

Comparison of the sequences of cytochrome c_L , cytochrome c_{551i} and class I cytochromes c

Figure 1(a) depicts the sequences of cytochrome c_L and the homologous cytochrome c_{551i} from *P. denitrificans*, which shares 55% identity but is 17 residues shorter than cytochrome c_L , the differences being in the C-terminal and N-terminal loop regions. These cytochromes share no sequence identity with any other cytochrome, although the crystal structure shows that they possess a central core with the three-helix bundle (helices A, C and E) surrounding the haem that is characteristic of all cytochromes c . Figure 1(b) shows that they have 40–50 additional residues at the N terminus and a C-terminal extension of at least 20 residues compared with a typical class I cytochrome c . They also have about 20 fewer residues in the core region between the end of helix A and helix E (Figure 1(b)). The main structural features of cytochrome c_L are given in Table 1 along with those of mitochondrial cytochrome c from tuna,¹⁵ a typical class I cytochrome c (abbreviated here to cytochrome cM).

The structure of cytochrome c_L

The 1.6 Å crystal structure was determined by molecular replacement and refined to an R -factor of 20.9%, with a corresponding R -free of 25.1% (see Table 2 for the full refinement statistics). Mass spectrometry of the cytochrome dissolved from a crystal showed that it was about 1.4 kDa smaller than freshly purified protein, suggesting that some N-terminal proteolysis had occurred during the crystallization period (four weeks), yielding a protein lacking the first 13 residues. This might explain why the following ten residues in the sequence possessed insufficient structure to be seen in the crystal. With the exception of these first 23 residues, there was continuous electron density for all of the residues in the amino acid sequence; the electron density also completely covered the haem prosthetic group.

The final model consists of 1133 protein atoms, one haem group, one calcium ion and 173 water molecules. The Ramachandran plot displays two residues in disallowed regions; these are Lys27, which is in the relatively flexible N-terminal loop, and Ala105, which lies in the exceptionally flexible loop 3 (see below). In a comparison of the

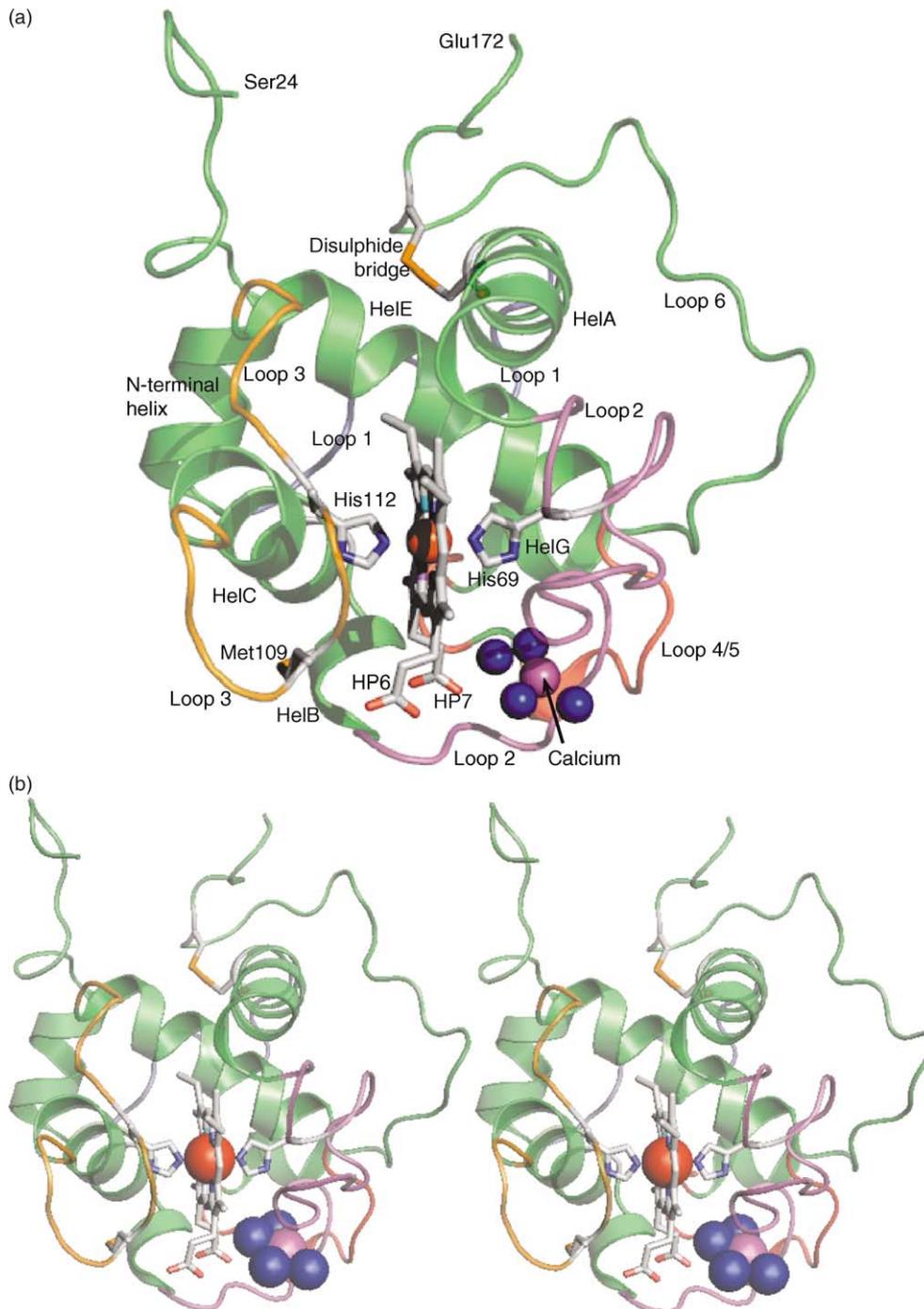


Figure 2. (a) The main structural features of cytochrome c_L . Although there is no sequence identity with other cytochromes, the helices A, C and E constitute the typical haem-enclosing fold seen in all cytochromes c . Helices are labelled HelA, HelB, etc. Loop 1 (grey) joins the N-terminal helix and helix A; loop 2 (purple) between helix A and helix B, carries the haem-binding sequence and the amino acid residues that coordinate to the calcium ion; loop 3 (orange) is the exceptionally flexible loop that joins helix C to helix E, and carries the sixth ligand to the haem (His112) and the methionine (Met109) that is the sixth ligand in solution. The red sphere is the iron atom at the centre of the haem prosthetic group. HP6 is the outer haem propionate group and HP7 is the inner haem propionate group. The blue spheres are the water molecules (Wat6-Wat9) that coordinate to the calcium ion (magenta sphere). Met109 is the residue that forms the sixth ligand to the haem in solutions of the cytochrome. (b) The main structural features of cytochrome c_L (stereo view). The elements of this structure are as indicated in (a).

ion (identified as Ca^{2+} , see below) close to the inner haem propionate, and a disulphide bridge between Cys53 (helix A) and Cys167, which is near the C terminus.

Eight residues from the visible N terminus (Ser24) there is a well-defined α -helix (the N-terminal helix) that is connected by loop 1 to helix A. This helix terminates in the first residue of the haem-binding

motif (Cys65) whose main-chain amide group forms a hydrogen bond with the main-chain carbonyl group of Phe61 in helix A (Phe10 in tuna). Helix A is linked to helix E by a hydrogen bond between Ser64 and the side-chain of Asn115, just prior to helix E. In cytochrome c_M , there is an equivalent hydrogen bond between Lys13 and a glutamate side-chain (Glu90) from helix E. The haem in cytochrome c_L also interacts with Ile128 in helix E (Leu94 in tuna).

Loop 2 passes from the end of helix A around the right-hand side and the base of cytochrome c_L , connecting the haem-binding motif (CSGCH) to helix B (Figure 2). Within this loop, Pro79 (Pro30 in tuna) is hydrogen bonded through its main-chain carbonyl group to the side-chain of the haem ligand His69, maintaining its correct orientation with respect to the haem. This loop also includes the amino acid residues that are coordinated to the calcium ion unique to cytochrome c_L (Gly80, Asp83 and Tyr85).

Helix C is stabilized by Phe98 and Phe102, which form a stacking interaction within the helix. The first residue of helix C (Asp94) participates in a salt-bridge with Arg129 from helix E, and there is a close van der Waal's contact between Leu97 (helix C) and Ile128 (helix E). These interactions help to stabilize the central three-helix core of cytochrome c_L .

Helix C and helix E are connected by loop 3 (103–117), which contains Met109 (the sixth ligand in solution) and His112 (the sixth ligand coordinated to the haem iron in the crystal structure). This sequence was particularly difficult to build into the model, requiring several rounds of rebuilding and refinement until the residues within the loop occupied the available electron density satisfactorily. The finding that cytochrome c_L has a bis-histidine arrangement in the crystal structure was entirely unexpected; in the crystal structure of

cytochrome c_{551i} the methionine equivalent of Met109 (Met101) remains ligated to the haem iron.¹⁶

The large helix E (residues 119–130) is part of the three-helix fold that partially encloses the haem and forms a hydrophobic interaction with helix A, involving aromatic residues on the two helices (between Phe61 and Trp127); the distance (4.2 Å) between the side-chains is similar to that in cytochrome c_M (4.1 Å). As in cytochrome c_M , there is an internal interaction within helix E; in cytochrome c_L this is a stacking interaction between Trp127 and His130. The helices are held together also by a relatively strong hydrogen bond between the side-chains of Asn56 in helix A and Gln123 in helix E; a similar interaction is seen in cytochrome c_{551i} (between Glu48 and Arg115). Whereas class I cytochromes c typically terminate shortly after the end of helix E, in cytochrome c_L there is a 40 residue C-terminal extension that loops around the side of the protein and forms many interactions with the N-terminal region (Figure 2). For example, the C-terminal loop 6 connects to the N-terminal loop 1 by hydrogen bonds between the side-chain of Gln166 and the carbonyl oxygen atom of Leu46 and the side-chain of Asp50, and by an ionic interaction between the side-chain of the penultimate amino acid residue Asp171 and Arg55 in helix A.

Loop 6 in the long C-terminal extension after the final large helix E is also held in place by a disulphide bridge between Cys167 and Cys53 in helix A (Figure 2).

The flexibility of cytochrome c_L

Figure 3 depicts the mean main-chain thermal parameters (B -values) for cytochrome c_L ; the average for the whole protein was 32.2 Å², the

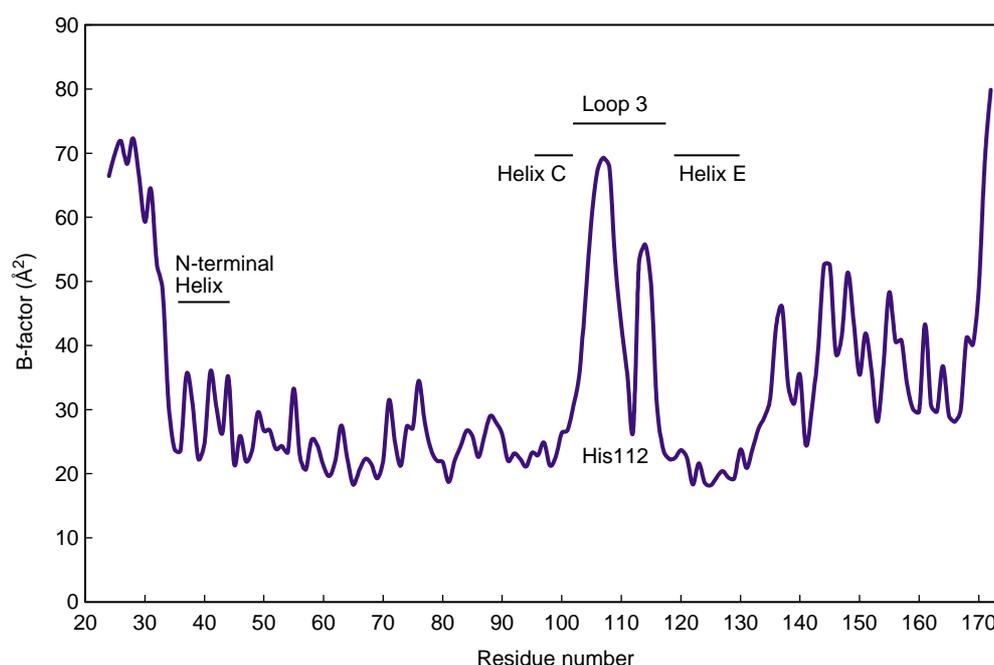


Figure 3. The B -factors (Å²) for the main-chain atoms of cytochrome c_L .

loop regions showing the greatest flexibility (higher B -values). As expected, the sequence preceding the N-terminal helix is the most flexible and the entire C-terminal region after helix E is relatively flexible. This region is in effect an extension of the typical class I cytochrome c structure that normally terminates at the end of the conserved helix E (Figure 1). The most rigid part of the structure corresponds to part of the typical cytochrome c structure, from the start of helix A up to the end of helix C. Most of loop 3 (residues 103–118), which connects the large conserved helices C and E_g is extraordinarily flexible (average B -value of 52.2 Å²); this loop includes His112, which has replaced Met109 as the sixth haem ligand in the crystal structure (the B -factor for His112 itself is low because it is bonded to the haem iron). In cytochrome c_{551i} the mean B -value for the whole protein was 24.0 Å², and the value for loop 3 bearing the methionine ligand was 16.6 Å², indicating that the loop bearing the sixth haem ligand is the most rigid part of that structure. Although the primary sequences share 45% identity in this loop, the rmsd is 6.5 Å, by contrast with the much lower value of 0.95 Å determined for other parts of cytochrome c_L and cytochrome c_{551i} .

The unique calcium-binding site in cytochrome c_L

The 1.6 Å resolution structure of cytochrome c_L reveals the presence of a metal ion close to the inner haem propionate (HP7). The coordination sphere involves seven oxygen atoms with metal–oxygen bond lengths between 2.2 Å and 2.6 Å; all B -factors for the oxygen ligands and for the metal ion itself are below 25 Å². These bond lengths are typical for coordination to Na⁺,¹⁷ but when a Na⁺ was built into the electron density, the refined B -factor was unfeasibly low and the emergence of a large peak of positive $F_o - F_c$ electron density suggested the presence of a metal ion with a greater atomic number than sodium, such as calcium or potassium. It is very unlikely that a K⁺ occupies the "pocket", as its typical coordination bond lengths are much too high (2.6–3.2 Å).¹⁷ All the features of coordination of the metal, including the bond lengths (2.2–2.6 Å),^{17,18} and the binding geometry, are consistent with it being a calcium ion. The protein sequence is almost identical with that of cytochrome c_{551i} and cytochrome c_L (Figure 1) and the structures superimpose well (rmsd 0.95 Å). It can be concluded therefore that cytochrome c_{551i} possesses the same binding pocket as cytochrome c_L although the density peak in this pocket was assigned originally to a water molecule (or a sodium or potassium ion) in the relatively low-resolution structure (2.4 Å) of the ternary complex of cytochrome c_{551i} , amicyanin and methylamine dehydrogenase.¹⁶

To address the possibility that the calcium ion is an artefact of the crystallization conditions (the crystal was grown in the presence of calcium acetate), a second data set was collected from a

Table 3. The data processing statistics for the 2.8 Å resolution structure of cytochrome c_L prepared in the absence of added calcium ions

Total number of reflections	7902
Number of unique reflections	2612
Resolution (Å)	2.8
Completeness (%)	91.1
R_{merge}^a	0.115 (0.290)
Multiplicity	2.4
Average $I/\sigma(I)^a$	5.7 (2.0)

^a The values in parentheses refer to the outer resolution shell between 2.8 Å and 2.9 Å.

crystal grown in the absence of any added metal ions; the data-processing statistics for this lower 2.8 Å resolution structure are given in Table 3. Initial electron density maps revealed a strong peak of $F_o - F_c$ and $2F_o - F_c$ electron density in the binding pocket, which was interpreted as Ca²⁺ in the previous structure; consequently a calcium ion was added to the model and refined using programs from the CNS suite. After several rounds of refinement, the B -factor for the Ca²⁺ was 30.7 Å² and, after visual inspections of the electron density maps, no significant positive or negative $F_o - F_c$ electron density surrounded the metal ion. It was therefore concluded that the Ca²⁺ modelled in the 1.6 Å structure is indeed a genuine component of the cytochrome structure.

The Ca²⁺ is bonded to seven oxygen ligands in a typical pentagonal bipyramidal geometry, the axial ligands being provided by the carbonyl oxygen atoms of Gly80 and Tyr85 (Figure 4). The radial ligands are provided by four water molecules (Wat6–Wat9) and the side-chain of Asp83 (OD1); the second oxygen atom (OD2) of Asp83 is bonded to Wat6, while its main-chain carbonyl group is hydrogen bonded to Wat9, and to the side-chain nitrogen atom of Trp86. The other two water ligands (Wat7 and Wat8) are bonded to the O2A oxygen atom of the inner haem propionate (HP7). In class I cytochromes c , this inner haem propionate (HP7) is usually protected from bulk solvent by interaction with a bulky arginine side-chain (Arg38 in tuna).¹⁵ In cytochrome c_L the Ca²⁺ takes the position of Arg38, leaving HP7 exposed to bulk solvent by way of Wat7 (Figure 4). The O2A atom of HP7 is also hydrogen bonded to the main-chain amide group of Thr87, while the O1A atom of this propionate group forms a hydrogen bond with the main-chain amide group of Tyr88 (Figure 4). The outer haem propionate (HP6) is hydrogen bonded (by O1D) to the side-chain oxygen atom of Tyr88 and it is fully exposed to bulk solvent by way of hydrogen bonding of the second oxygen atom, O2D, to an external water molecule (Wat115); O2D is also hydrogen bonded to a second water molecule (Wat127), which is hydrogen bonded to Wat115 (Figure 4).

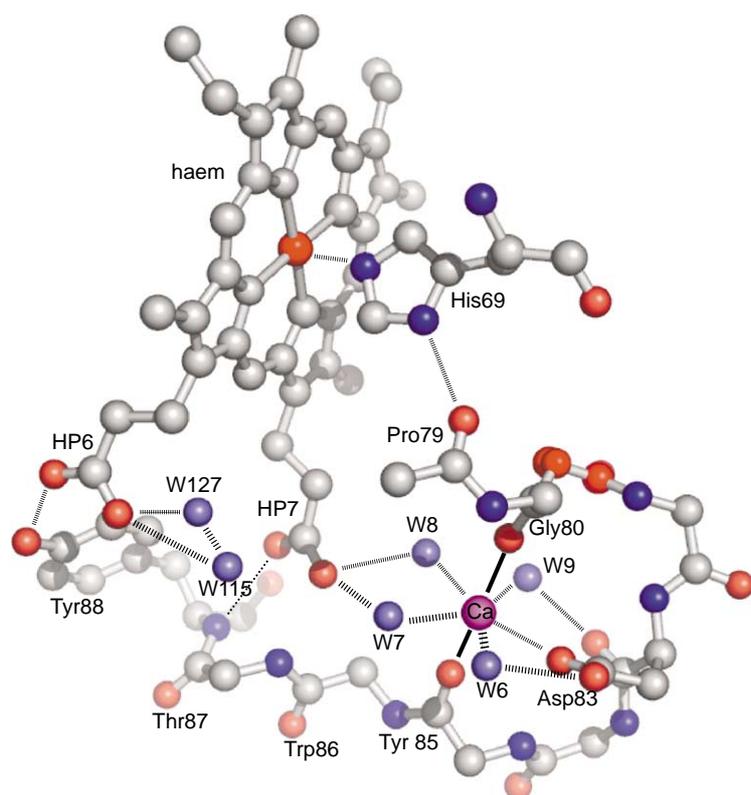


Figure 4. The calcium ion and the interactions of the two haem propionates, in cytochrome c_L . HP6 is the outer haem propionate and HP7 is the inner haem propionate. The lines indicate hydrogen bonds or coordinations. Water molecules (Wat6–Wat9, Wat115, and Wat127) are indicated by blue spheres; the calcium ion is indicated by a magenta sphere; the haem iron is indicated by a red sphere. The calcium ion and the oxygen atoms of the four water molecules (Wat6–Wat9) and the side-chain of Asp83 lie in one plane; the bonds to the oxygen atoms of Gly80 and Tyr85 (shown as continuous lines) are perpendicular to this plane. In addition to the bonds indicated here, the main-chain carbonyl group of Asp83 is also hydrogen bonded to the side-chain nitrogen atom of Trp86, (not shown). The water molecules Wat6, Wat7 and Wat115 are exposed to bulk solvent (Figure 7).

Ligands to the haem iron

The absorption spectrum of the oxidised cytochrome c_L in solution has the typical near-infrared absorption band at 695 nm,⁶ which is the signature of a methionine–iron coordination, present in all c -type cytochromes. Although the absorption spectrum of the crystal (Figure 5) confirms that the haem iron is in the oxidised form, it also indicates that the ligand to the haem in the crystal is not methionine. In the crystal structure, the sixth ligand to the haem iron is provided by a histidine residue (His112), three residues downstream from Met109. The coordination by the two histidine side-chains (His69 and His112) to the haem iron is extremely well defined by the high-resolution electron density map. The average B -factors for the atoms in His69 and His112 were 19.36 Å² and 26.69 Å², respectively, indicating the relative rigidity of their positions, and there was no $F_o - F_c$ electron density surrounding either residue after several rounds of refinement. The distances from the His69 and the His112 N^{ε2} atoms to the Fe³⁺ were 2.0–2.05 Å. The N^{ε1} atom of His69 (the fifth ligand) is hydrogen bonded to the carbonyl group of a nearby proline residue (Pro79), which serves to orientate the imidazole ring perpendicular to the plane of the haem, as seen in other cytochromes c (Figure 4).

The sixth haem ligand (His112) is hydrogen bonded by way of its second side-chain nitrogen (N^{ε1}) atom to a nearby water molecule (Wat84), which is further bonded to Wat149, and to the

main-chain oxygen atom of Gly110; both water molecules are exposed to bulk solvent. The B -factor of Wat84 is relatively high (31 Å²), indicating that it is relatively mobile.

The residues surrounding His69 (Ph61, Gly78, Leu81 and Ile128) are mainly hydrophobic, which is typical of c -type cytochromes, the hydrophobic environment contributing to their relatively high redox potentials.¹⁵

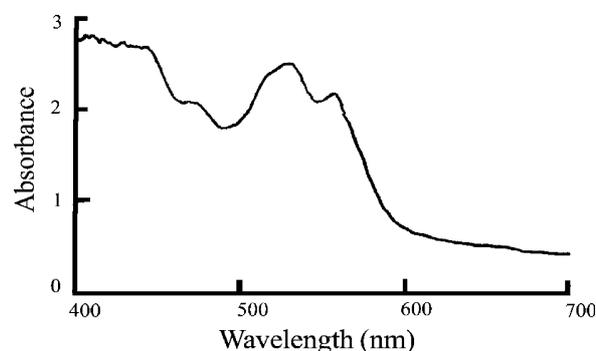


Figure 5. The absorption spectrum of a cytochrome c_L crystal at 100 K obtained using the cryobench microspectrophotometer 30 at ESRF. The relative heights of the peaks at 530 nm and 550 nm are characteristic of the oxidized cytochrome. The absence of any peak between 690 nm and 700 nm shows that there is no methionine–iron coordination.

Discussion

The disulphide bridge in cytochrome c_L

Cytochrome c_L is unusual in having a disulphide bridge, which bonds the long C-terminal extension to helix A (Figure 2). There is none in cytochrome c_{551i} , which has eight fewer residues in the C-terminal sequence after helix G. Its presence in cytochrome c_L had been inferred from the observation that Cys53 in helix A and Cys167 near the C terminus could not be carboxymethylated with [^{14}C]iodoacetate in native or denatured cytochrome, whereas after denaturation and reduction with dithiothreitol both cysteine residues could be carboxymethylated.⁶

Such a bridge occurs only once in more than 100 examples of class I cytochromes c ,¹⁵ this being in the cytochrome of bullfrog heart. When an equivalent disulphide bridge, linking the N and C termini, was introduced into yeast cytochrome c , its stability was enhanced considerably.¹⁹ It is likely that the disulphide bridge in cytochrome c_L also helps to maintain the stability of this exceptionally large cytochrome.

The unusual ligand to the haem iron in cytochrome c_L

The observation that the sixth ligand to the haem iron is His112 was most unexpected but was consistent with the lack of a near-infrared absorption band at 695 nm in the crystal. It had been shown that Met109 is the haem ligand.⁶ Consistent with this identification is the observation that the corresponding loop region in cytochrome c_{551i} contains an equivalent methionine residue (Met101) (Figure 1), which is seen to be coordinated

to the haem iron in the crystal structure of that cytochrome.¹⁶ The properties of a site-directed mutant in which Met109 was replaced by alanine confirmed the role of Met109 in cytochrome c_L ; the cytochrome was no longer able to act as electron acceptor from methanol dehydrogenase, and the 695 nm band was absent from the spectrum.⁶ The unchanged spectrum between 500 nm and 600 nm of the reduced mutant cytochrome showed that the Met109 ligand had been replaced by an alternative low-spin ligand, probably lysine or histidine.⁶

Most of loop 3, containing Met109 and His112, is highly mobile (Figure 3), permitting the switch in the sixth haem iron ligand from Met109 to the alternative His112 during crystallization. During this process, some of the N-terminal sequence was proteolysed; in the related cytochrome c_{551i} this sequence interacts with the loop region containing the methionine ligand, presumably stabilizing it and protecting it from bulk solvent (Figure 6).

That the haem iron readily dissociates from Met109 has been shown by the exceptional reactivity of cytochrome c_L with carbon monoxide, about 72% of the molecules binding CO in a CO-saturated solution.¹² Furthermore, preliminary NMR studies on cytochrome c_L suggested that the methionine residue coordinated to the haem has an unusual configuration when compared with other cytochromes c .²⁰ Magnetic circular dichroism spectroscopy has demonstrated that the Met109 ligand in cytochrome c_L is replaced readily by an alternative strong-field ligand such as histidine at high pH values.¹³ Another cytochrome in which the known methionine ligand to the haem iron was replaced during crystallization is the cytochrome c_2 from *Rhodospseudomonas palustris*. In this case, the crystals were grown in the presence of ammonium sulphate, which led to replacement of

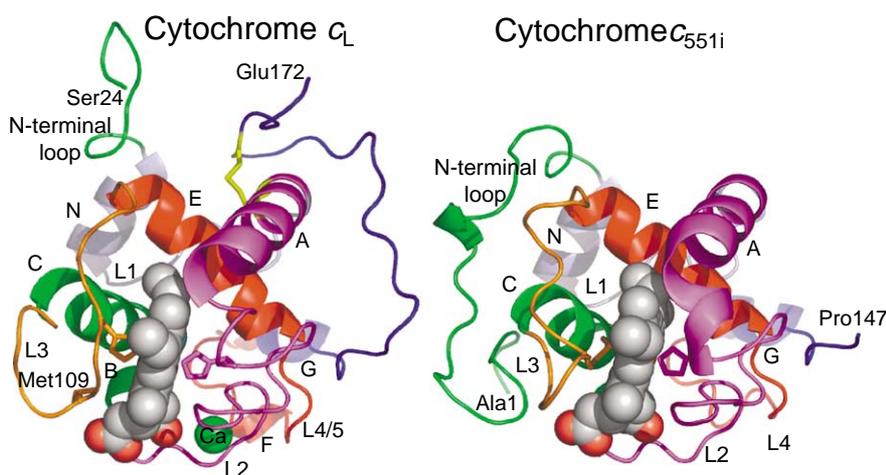


Figure 6. Comparison of the main structural components in cytochrome c_L and in its homologue cytochrome c_{551i} . The single letters (A–G, and N) indicate the α helices. The haem is represented with space-filling atoms. The loops are labelled L1–L6. The first 13 residues are missing from the cytochrome c_L and the next 11 residues are not seen in the crystal structure. In the homologous cytochrome c_{551i} all residues are present in the N-terminal loop (shown in green) in the crystal structure, in which they interact with loop 3 (L3; orange) protecting it from exposure to solvent; it is this interaction that is absent from cytochrome c_L , leading to an increased flexibility of loop 3 (Figure 3) and the replacement of Met109 by His112 in the crystal structure. The last eight residues of cytochrome c_{551i} are not seen in the structure.

the methionine residue by an ammonia molecule, a weaker than usual hydrogen bond from the methionine residue to the conserved tyrosine residue being proposed to be a contributing factor to a labile, easily dissociable iron–methionine bond.²¹

The unique calcium-binding site and the exposed propionates of cytochrome c_L (Figure 4)

It has been postulated that the positive charge of the arginine residue (Arg38 in tuna) that bonds to the inner haem propionate (HP7) contributes to the high redox potential of the c -type cytochromes.¹⁵ Replacing this arginine by site-directed mutagenesis with less electropositive amino acids leads to a lowering of the redox potential.²² The oxidised haem iron is in an energetically unfavourable environment, and stabilisation of the positively charged Fe^{3+} is achieved, in part, by the interaction with the ionized propionate. As the protein becomes reduced, the iron no longer requires electrostatic stabilisation, the charge on the propionate being balanced by the electropositive Arg38 or its equivalent. Cytochrome c_L appears to be the first c -type cytochrome in which the positive charge, which contributes to the observed characteristics mentioned above, is provided by a metal ion (Figure 4) and not by the side-chain of an amino acid.

In most class I cytochromes c , neither of the haem propionates has any direct interaction with bulk solvent.¹⁵ The exceptional exposure of both propionates in cytochrome c_L (Figures 4 and 7) is consistent with previous predictions based on the unusual response of its midpoint redox potential to changes in pH. It was proposed that this arises from the presence in the cytochrome of two ionizing groups that have some interaction with the haem. The measured pK values are 3.6 and 5.6 in the oxidised form, and 4.4 and 6.4 in the reduced form, and it was concluded that the likely candidates for the dissociating group were the haem propionate groups themselves, and that these must be unusually exposed to bulk solvent.¹²

The interaction of helix A and helix E, and a comment on evolution

It is well known that there are families of functionally and evolutionarily related proteins that have similar overall 3-D structures but quite different amino acid sequences, and this has been discussed extensively in relation to the cytochromes c .²³ In a study of 164 sequences of the seven subfamilies there is only very weak homology (although high homology within subfamilies) and yet they share the common core structure of three central helices enfolding the covalently bound haem. Beside the three haem-binding residues, there are only four positions that are conserved in all subfamilies; these include two positions in helix A immediately preceding the haem-binding motif

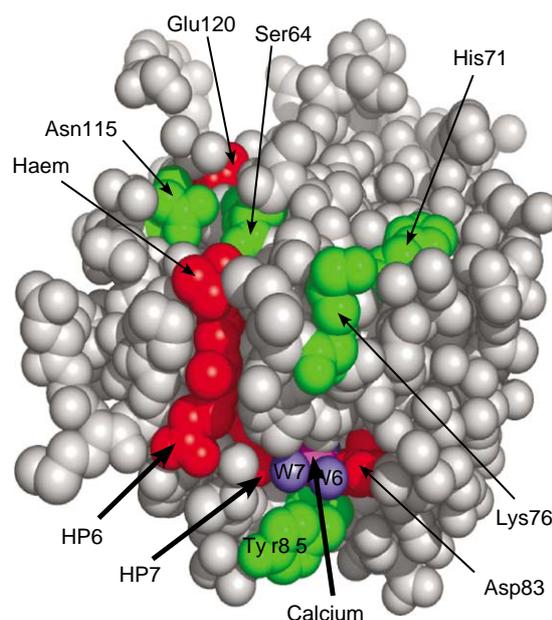


Figure 7. Surface view (space-filling) of cytochrome c_L . The outer haem propionate (HP6) is clearly exposed to solvent, as are the two water molecules (Wat6 and Wat7). Wat7 is bonded to the inner haem propionate (HP7). Asp83, one of the ligands to the calcium ion, has been proposed to be involved in interaction with methanol dehydrogenase together with Glu120, with the outer haem propionate (HP6) and with those residues coloured green.²⁴

(CxxCH). Using the numbering from tuna cytochrome c , the four conserved positions are 6 (Gly or Ala) and 10 (Phe or Tyr) in helix A; and 94 (Phe, Leu or Val) and 97 (Tyr, Trp or Phe) in helix E (Figure 1(b)). The residues at these positions form contacts with the haem-binding residues (Cys14, Cys17, and His18), and with each other, holding together helices A and E. These interactions are present also in both equilibrium and kinetic molten globule-like folding intermediates, and it has been concluded that residues at these conserved positions play a critical role in forming a common folding nucleus in all subfamilies of c -type cytochromes.²³ In cytochrome c_L , the positions in helix A immediately preceding the haem-binding residues are conserved (Gly57 and Phe61), as is one of the positions in helix E (Trp127; Tyr97 in tuna) (Figure 1(b)). The position occupied by Leu94 in tuna cytochrome c is not conserved but the interaction of Leu94 with Gly6 in helix A is replaced in cytochrome c_L by interactions of Gln123 and Thr124 in helix E and Gly57 in helix A.

In addition to the seven conserved positions, there are two further residues, Glu90 and Leu98 (tuna), present in all cytochrome structures except for the membrane cytochromes c_1 and c_f . These positions are conserved also in cytochrome c_L ; these are occupied by Glu88 and Ile128, which is in van der Waals contact with Phe61 (Figure 1(b)).²³

Thus, the core of cytochrome c_L is essentially similar in structure to those of other c -type

cytochromes, although it has negligible sequence identity and it is unique in lacking one of the seven conserved positions found in all other subfamilies. It should be noted that the typical cytochrome c_2 of *M. extorquens* (cytochrome c_H) has a high degree of sequence identity with typical class I cytochromes c (46% identity with that of tuna),⁸ which raises interesting questions about the apparently separate evolution of the two c -type cytochromes that function together in the electron transport chain for methanol oxidation in the methylophilic *M. extorquens*.

The interaction of cytochrome c_L with electron transfer partners

The edge of the haem in cytochrome c_L is exposed at its front face, and the residues immediately surrounding this haem edge are mainly hydrophobic; those on the right-hand side of the haem are Leu77, Gly78 and Pro79, and these residues are conserved in cytochrome c_{551i} . On the left-hand side of the haem cleft the residues are in the "relatively disordered" loop 3, which has a configuration different from that in cytochrome c_{551i} (Figure 6).

The interaction of class I cytochromes c with redox partners is usually by way of ionic interactions involving lysine residues on the front face of the cytochrome,¹⁵ and the structure of cytochrome c_H implies that this is the case also for its interactions with cytochrome c_L and with cytochrome oxidase.⁸ By contrast, cross-linking and chemical modification studies have demonstrated that carboxylate groups on cytochrome c_L interact with lysine or arginine residues on MDH, and that the same (or a similar) sites on cytochrome c_L are involved in its subsequent interaction with lysine or arginine residues on cytochrome c_H .¹⁰

The interaction between MDH and its electron acceptor cytochrome c_L has been modelled using the related proteins (MDH and cytochrome c_{551i}) from *P. denitrificans*;²⁴ this study yielded two models, both consistent with our previous chemical studies with cytochrome c_L .¹⁰ The carboxylate residues proposed to be involved in the interaction with MDH are conserved in cytochrome c_L ; in model I these corresponded to Asp83 and Asp138, together with the outer haem propionate (HP6); in model II (the model favoured by the original authors²⁰) these carboxylate groups corresponded to Asp83 and Glu120. The two aspartate residues are located at the lower front face of the cytochrome, whereas Glu120 is on the top face (Figure 7). It can be concluded that interaction with the redox partners of cytochrome c_L is by bonding between hydrophobic residues around the haem edge, and by way of ionic interaction involving the conserved carboxylate groups as shown by chemical modification, cross-linking and fluorescence studies.^{10,25}

It is worth noting that one of the carboxylate groups involved in binding to MDH is Asp83 (Asp75 in cytochrome c_{551i}), which is bonded through the Ca^{2+} and water molecules to the

inner haem propionate (HP7) (see below); whether or not this would affect the binding to MDH or more importantly be part of the route for electron transfer between the proteins requires further modelling of this system or determination of the MDH/cytochrome c_L complex from *M. extorquens*.

The left-hand side of the haem cleft and the electron transfer mechanism

Class I c -type cytochromes typically have a relatively hydrophilic region in the left-hand side of the haem cleft; this contains a conserved water molecule and a conserved tyrosine residue in helix C (Tyr67 in tuna), which forms a hydrogen bond with the sulphur atom of the methionine sixth ligand in the ferrocyanide c . Tyr67 also forms a hydrogen bond to the conserved water molecule, which in turn is hydrogen bonded to a conserved threonine residue (Thr78 in tuna). It has been proposed that this highly conserved hydrophilic network plays an essential role in the process of electron transfer in these cytochromes.^{26,27} However, this conclusion has been challenged by the demonstration that in cytochrome c_H of *M. extorquens*, the left-hand side of the haem cleft is completely hydrophobic, the "conserved water" being absent and the key residue Tyr67 being replaced by a hydrophobic tryptophan residue.⁸

Helix C in cytochromes c_L is predominantly hydrophobic (DVGLFATIF) and it lacks the conserved tyrosine residue. Helix C in cytochrome c_{551i} is similar (DVGLFSTLY) and, although it does contain a tyrosine residue, this points away from the haem cleft and is involved in a stacking interaction with phenylalanine. In neither cytochrome is there any residue in helix C able to form a hydrogen bond to the haem ligand and neither of these cytochromes has the conserved water. The structures of cytochromes c_L and c_{551i} thus provide a further challenge to the suggestion that a hydrophilic network in the left-hand side of the haem cleft plays an essential role in the mechanism of c -type cytochromes.

Materials and Methods

Crystallization and data collection

The protein was purified as described.²⁸ Initial crystallization conditions were found using the Molecular Dimensions crystal screens I and II (screen I, condition no. 20: 0.1 M sodium cacodylate (pH 6.5), 0.2 M calcium acetate, 18% (w/v) PEG 8000). Crystal growth conditions were then optimized using the hanging-drop, vapour-diffusion method. The final conditions were 20 mg/ml of protein, 0.14–0.28 M sodium cacodylate (pH 6.5), 0.2 M calcium acetate, 45% PEG 8000. Crystals were grown over the course of one month, in the dark, at room temperature. The crystals were red and had a rod-shaped appearance, with dimensions of approximately $900\ \mu\text{m} \times 100\ \mu\text{m} \times 100\ \mu\text{m}$. A high-resolution and a low-resolution X-ray data set were collected on an ADSC Quantum CCD

detector at the ESRF (Grenoble, France, ID-29). The crystal diffracted X-rays to a maximum resolution of 1.6 Å. Indexing and processing of the reflections was performed using MOSFLM,²⁹ which indicated that the crystal was in a tetragonal ($P4$) point group, with unit cell dimensions of $a=66.5$ Å, $b=66.5$ Å, $c=39.0$ Å; α , β and γ were 90°. The reflection data were subsequently scaled and merged using programs from the CCP4 suite.³⁰ Inspection of the reflection intensities and systematic absences indicated that the crystals were of the $P4_1$ or $P4_3$ space group. This crystal form has one molecule per asymmetric unit; the final data processing statistics are shown in Table 1.

Structural determination and refinement

Initial phases for the cytochrome c_L structure were obtained by molecular replacement, using the program MOLREP.³¹ The initial coordinate model used in molecular replacement was that of cytochrome c_{551i} from *P. denitrificans* (PDB code 2MTA);¹⁶ the coordinates for the haem and water molecules were removed from the search model. MOLREP generated a strong rotation solution of 6.54σ above the noise level (with the next peak being 4.14σ). The data processed in $P4_3$ gave a strong translation function of 7.95σ above the noise (with the next peak being 3.80σ); no significant translation function peak was found with the data processed in $P4_1$. Having applied the appropriate rotation and translation vectors to the cytochrome c_{551i} coordinates, the model was subjected to rigid-body refinement, simulated annealing and B -factor refinement, using the programs from the CNS suite.³²

The initial electron density maps were visualized in QUANTA³³ and subsequent modelling was carried out using the X-Autofit-Autobuild option. The initial $F_o - F_c$ and $2F_o - F_c$ electron density maps obtained by molecular replacement revealed strong haem-shaped electron density in the region close to the residues that were expected to form interactions with the haem vinyl groups (Cys65 and Cys68) and coordinate to the haem iron (His69); this was taken as further support for the correct molecular replacement solution. The fitting of the haem into the cytochrome c_L model was achieved using the haem coordinates from the cytochrome c_{551i} structure and with appropriate rotation and translation values applied from the LSQKAB (least-squares) program.

Although good electron density was found in most regions, four regions were less well-defined; the C-terminal region Lys154-Glu172, the first 25 residues of the N terminus, residues Gly57-Ser67 and the loop containing the residues that interact with the left-hand side of the haem, Gly103-Asn115. All of these regions required substantial rebuilding. After several rounds of model rebuilding and refinement using QUANTA and the programs from the CNS suite, the electron density for these regions was satisfactory, except for the 25 residues at the N terminus of the protein. The Ramachandran plot showed two residues, Ala105 and Lys27, in disallowed regions. Lys27 is in the N-terminal region of the protein where the electron density is poor. It was therefore difficult to build this residue into the available electron density. Ala105 is in a solvent-exposed flexible loop region that connects helix C and helix E. Altering Ala105 and its neighbours such that it adopted an allowed conformation in the Ramachandran plot moved these residues out of the electron density; consequently, Ala105 was left in an unconventional conformation.

A second data set was collected from a crystal grown in the absence of any added metal ions (0.1 M Mes (pH 6.5),

30% PEG-MME 5 K, 0.2 M ammonium sulphate). Although the crystal was smaller than previous cytochrome c_L crystals, it was large enough to collect data at the ESRF, where it diffracted X-rays to 2.8 Å. The reflections were indexed using MOSFLM, which gave a unit cell and space group identical with the previous cytochrome c_L structure.

The absorption spectrum of a cytochrome c_L crystal at 100 K was obtained using the cryobench microspectrophotometer at ESRF.³⁴

Protein Data Bank accession numbers

The co-ordinates and structure factors have been deposited in the RCSB Protein Data Bank with PDB ID code 2c8s for the coordinate entry and code r2c8ssf for the structure factors.

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