Cleavage of the iron-methionine bond in c-type cytochromes: Crystal structure of oxidized and reduced cytochrome c_2 from *Rhodopseudomonas palustris* and its ammonia complex

SILVANO GEREMIA,¹ GIANPIERO GARAU,¹ LISA VACCARI,¹ RICCARDO SGARRA,² MARIA SILVIA VIEZZOLI,³ MARIO CALLIGARIS,¹ AND LUCIO RANDACCIO¹

¹Centro di Eccellenza di Biocristallografia, Dipartimento di Scienze Chimiche, Università di Trieste, Trieste I-34127, Italy

²Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, Trieste I-34127, Italy

³Dipartimento di Chimica, Università di Firenze, Sesto Fiorentino (Fi) I-50019, Italy

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Abstract

The three-dimensional structures of the native cytochrome c_2 from *Rhodopseudomonas palustris* and of its ammonia complex have been obtained at pH 4.4 and pH 8.5, respectively. The structure of the native form has been refined in the oxidized state at 1.70 Å and in the reduced state at 1.95 Å resolution. These are the first high-resolution crystal structures in both oxidation states of a cytochrome c_2 with relatively high redox potential (+350 mV). The differences between the two oxidation states of the native form, including the position of internal water molecules, are small. The unusual six-residue insertion Gly82-Ala87, which precedes the heme binding Met93, forms an isolated 3_{10} -helix secondary structural element not previously observed in other c-type cytochromes. Furthermore, this cytochrome shows an external methionine residue involved in a strained folding near the exposed edge of the heme. The structural comparison of the present cytochrome c_2 with other c-type cytochromes has revealed that the presence of such a residue, with torsion angles ϕ and ψ of approximately -140 and -130° , respectively, is a typical feature of this family of proteins. The refined crystal structure of the ammonia complex, obtained at 1.15 Å resolution, shows that the sulphur atom of the Met93 axial ligand does not coordinate the heme iron atom, but is replaced by an exogenous ammonia molecule. This is the only example so far reported of an X-ray structure with the heme iron coordinated by an ammonia molecule. The detachment of Met93 is accompanied by a very localized change in backbone conformation, involving mainly the residues Lys92, Met93, and Thr94. Previous studies under typical denaturing conditions, including high-pH values and the presence of exogenous ligands, have shown that the detachment of the Met axial ligand is a basic step in the folding/unfolding process of c-type cytochromes. The ammonia adduct represents a structural model for this important step of the unfolding pathway. Factors proposed to be important for the methionine dissociation are the strength of the H-bond between the Met93 and Tyr66 residues that stabilizes the native form, and the presence in this bacterial cytochrome c_2 of the rare six-residue insertion in the helix 3_{10} conformation that increases Met loop flexibility.

Keywords: Cytochrome c_2 ; electron carrier; reduction potential; ammonia adduct; protein folding; conformational changes; X-ray structure

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Reprint requests to: Dr. Silvano Geremia, Centro di Eccellenza di Biocristallografia, Dipartimento di Scienze Chimiche, Università di Trieste, via L. Giorgieri 1, I-34127 Trieste, Italy; e-mail: geremia@univ.trieste.it; fax: +39-040-6763903.

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Bacterial cytochromes c_2 are biological electron shuttles involved in cyclic redox processes. In the photosynthetic process, they function as a water-soluble electron carrier between two membrane proteins: the photosynthetic reaction center and the cytochrome bc_1 complex (Michel et al. 1986). The molecular mass of cytochromes c_2 ranges from 12 kD to 13 – 14 kD (Ambler et al. 1979), and the percentage identity in amino acid sequence between any two similar species for these cytochromes c_2 ranges between 20% and 60%, with significant differences also observed between the primary sequence of proteins isolated from different strains of the same bacterium (Samyn et al. 1998). For example, the *Rhodospseudomonas palustris* strains 2.1.6 (ATCC 17001) and 2.1.37 (ATCC 17007) show only 89% homology (Ambler et al. 1979). These Class I cytochromes c have a single heme group attached covalently to the polypeptide chain through thioether linkages. The heme iron is axially coordinated by a histidine and by a methionine residue. To date, X-ray structures have been determined for the cytochromes c_2 isolated from *Rhodospirillum rubrum* (Salemme et al. 1973), *Rhodobacter capsulatus* (Benning et al. 1991), *Paracoccus denitrificans* (Timkovich and Dickerson 1976; Benning et al. 1994), *Rhodobacter sphaeroides* (Axelrod et al. 1994), *Rhodospseudomonas viridis* (Sogabe and Miki 1995), *Rhodopila globuliformis* (Benning et al. 1996), and *Methylobacterium extorquens* (Read et al. 1999). The prokaryotic cytochromes c_2 have been recognized to be structurally similar and evolutionarily related to the mitochondrial cytochromes c (Moore and Pettigrew 1990). The reduction potential of bacterial cytochromes c_2 varies from +250 to +450 mV (Benning et al. 1996), whereas it is approximately +260 mV in all eukaryotic cytochromes c (Pettigrew et al. 1978). Local structural differences among the cytochromes c_2 and comparisons with the eukaryotic cytochrome c analogs are of interest when investigating the factors controlling the redox potentials in c -type cytochromes.

The covalently attached heme group and its axial ligands not only are essential for the physiological function of the c -type cytochromes, but they play an important role in the folding/unfolding process (Stellwagen et al. 1972; Fisher et al. 1973; Myer 1984; Damaschun et al. 1991; Hamada et al. 1993; Elöve et al. 1994). The detachment of the Met ligand from iron coordination is the first step of cytochrome c unfolding observed on increasing pH and/or temperature (Elöve et al. 1994; Banci et al. 1998). The pH-induced protein conformational transitions and changes in the ligation state of the heme iron in cytochrome c_2 from *Rps. palustris* have been monitored by electrochemical and spectroscopic measurements (Battistuzzi et al. 1995; Bertini et al. 1998). At pH values >8, the appearance of an additional NMR signal pattern was interpreted as the result of the formation of new species with distinct structural properties (Bertini et al. 1998). In these and previous works (Timkov-

ich et al. 1984) on cytochrome c , it was hypothesized that the alkaline transition is probably because of the replacement of the methionine axially bound to the heme iron with a stronger (most probably N-donor) ligand (Ubbink et al. 1994; Battistuzzi et al. 1997). The axial Met displacement in c -type cytochromes has been studied widely also using antagonist exogenous ligands (George and Schejter 1964; Myer 1984; Shao et al. 1993 1995; Banci et al. 1998; Dumortier et al. 1998). Recently an NMR study of the conformational flexibility of the oxidized horse heart cytochrome c through its interaction with NH_3 has been reported (Banci et al. 1998). It was shown that NH_3 binds to iron(III) by displacing the axial Met with an affinity constant in the range $1.5\text{--}4\text{ M}^{-1}$. The 1H -NMR spectra of this ammonia adduct is similar to that obtained with alkaline transition. Despite the extensive studies in solution, the imidazole adduct of cytochrome c_2 from *R. Sphaeroides* is the only crystal structure (2.2 Å resolution) so far reported among c -type cytochromes with the axial Met ligand replaced (Axelrod et al. 1994).

This prompted us to undertake a structural investigation of the pH-dependent forms of cytochrome c_2 from *Rps. palustris* (Garau et al. 2000). We crystallized two different crystal forms of cytochrome c_2 from *Rps. palustris* (strain 42 OL) using ammonium sulfate as precipitant: a monoclinic form obtained at acid pH value (the native form) and a trigonal form (the ammonia adduct) obtained at basic pH value (Garau et al. 2000). In a preliminary account of this work, we have shown the first electron density map of the trigonal form obtained at 1.40 Å resolution, in which an important change in the iron coordination environment because of the substitution of the Met ligand by a single heavy atom was apparent. In this full paper we report the X-ray structures of the cytochrome c_2 from *Rps. palustris* in its native form and in both redox states (oxidized state at 1.70 Å and reduced state at 1.95 Å resolution), and of the oxidized ammonia adduct (at 1.15 Å resolution). The anisotropically refined structure of this complex has permitted the unambiguous identification of the exogenous ligand as well as the definitive assignment of the primary structure of this bacterial cytochrome c_2 . The primary sequence of the *Rps. palustris* cytochrome c_2 obtained from the strain 42OL was also checked by mass spectrometry analysis.

Results and Discussion

Primary sequence

The primary structure of *Rps. palustris* cytochrome c_2 has been determined previously by Ambler et al. (1979) for two different strains: 2.1.6 (ATCC 17001) and 2.1.37 (ATCC 17007). For our structural studies, we have used a cytochrome c_2 protein expressed from the 42OL strain of the same bacteria isolated from a sugar-refinery soil and whose

amino acid sequence was unknown (Bertini et al. 1998). In the fitting process of the electron density maps obtained from the data at 1.15 Å resolution, we used the sequence of the *Rps. palustris* cytochrome c_2 from the 2.1.6 strain (Ambler et al. 1979) as starting reference of the primary sequence for the strain 42OL. During manual model inspection and refinement cycles, a total of 16 residues were mutated with respect to this starting sequence. The sequence changes (with the starting assignment in parentheses) are Lys4(Ala), Ala5(Lys), Ala29(Gly), Ala60(Gln), Asp61(Glu), Val64(Ile), Pro65(Ala), Ala68(Pro), Phe73(Tyr), Glu80(Asp), Lys83(Gln), Gln86(Lys), Val88(Thr), Val90(Cys), Glu100(Asp), and Val107(Ala). Thus, the resulting primary sequence of the protein obtained from the strain 42OL has a much higher identity with that of the 2.1.37 strain protein sequence. In fact, only five amino acids are different in the two primary sequences: Ala29(Gly), Val64(Ile), Pro65(Asn), Ala68(Asn), and Glu80(Asp). Further information from electron density maps of the monoclinic form were used to confirm all amino acid assignments. In particular, ambiguities on Asx, Glx, and Thr-Val residues were resolved by checking the H-bond network. Only the ambiguities for Asx61, Glx80, and Asx85 residues could not be resolved definitively. They were assigned to Asp61, Glu80, and Asp85 to maintain the charge residue of the corresponding amino acids of the protein expressed from 2.1.6 and 2.1.37 strains.

The purified *Rps. palustris* cytochrome c_2 used for crystallization was a mixture of several mass forms as determined by mass spectrometry. The main peak (12.783.5 daltons) of the mass spectra is in good agreement with the value of 12784.5 daltons, calculated from the primary sequence obtained by X-ray analysis.

Molecular structure of the native form

The crystal structures of oxidized and reduced cytochrome c_2 from *Rps. palustris* obtained at acid pH are isomorphous, and four independent polypeptide chains (Mol A, Mol B, Mol C, and Mol D) are present in the asymmetric unit. In addition, two sulphate ions, one glycerol molecule, and 653 water molecules are detected in the asymmetric unit of the oxidized structure and a total of 613 water molecules in the reduced structure. In the two native structures, the four independent peptide chains have the same folding, with the exclusion of Thr94 and Phe95 residues in the reduced and oxidized states and of Lys21, Ala87, Val88, and Gly89 residues in the reduced state, as shown in the Kleywegt NCS plots (Kleywegt and Jones 1996) of the main-chain torsion angles (ϕ and ψ) (Fig. 1). In the oxidized state, the α -carbon atoms of Mol A superimposes on those of Mol B, Mol C, and Mol D with a root mean square deviation (RMSD) of 0.17, 0.06, and 0.10 Å, respectively. A similar behavior is found in the reduced state. For the sake of simplicity, the following discussion on protein folding refers only to Mol A

Fig. 1. Kleywegt NCS plot of the main-chain torsion angles (ϕ , ψ) of the native *Rps. palustris* cytochrome c_2 structure in the oxidized (A) and reduced (B) state. The NCS-related residues are joined by straight lines.

of the oxidized state unless otherwise indicated. The polypeptide chain is composed by five α -helices, one 3_{10} -helix, and tight turns that wrap around the prosthetic group (Table 1). A cartoon representation of the polypeptide chain backbone, with heme and iron ligands in stick, is illustrated in Figure 2. The B and C edges of the heme group are bound covalently to the Cys13 and Cys16 residues, respectively (Fig. 3). The Cys13 O forms two H-bonds with Cys16 N and His17 N. The His17 residue is the fifth iron-coordinating ligand, whereas the sixth ligand, Met93, is part of a

Table 1. List of secondary structural elements in the native form of *Rps. palustris* cytochrome *c*₂

Amino acid residues	Structural element
Ala3–Cys13	α-Helix (A)
Met14–His17	β-Turn (type I)
Arg18–Lys21	β-Turn (type I)
Met23–Gly25	γ-Turn
Leu28–Val31	β-Turn (type II)
Val31–Arg34	β-Turn (type II)
Lys35–Thr38	β-Turn (type II)
Ala39–Phe42	β-Turn (type II)
Pro46–Glu53	α-Helix (B)
Thr59–Asp61	γ-Turn
Ile63–Ala68	α-Helix (C)
Pro70–Glu80	α-Helix (D)
Lys81–Lys83	γ-Turn
Ala84–Ala87	3 ₁₀ -Helix (E)
Glu100–Thr112	α-Helix (F)

random coil stretching from Val88 to Asn99. The Ala84–Asp85–Gln86–Ala87 residues form the unusual isolated 3₁₀-helix secondary structural element. These residues also belong to the rare six-residue insertion Lys83–Val88 preceding the heme-binding methionine. This uncommon insertion has been detected recently in the primary sequence of the *Rhodospirillum centenum* cytochrome *c*₂ (Samyn et al. 1998). The 3₁₀-helix presents three H-bonds, one between Lys83 O and Gln86 N, one between Ala84 O and Ala87 N, and one between Asp 85 O and Val 88 N.

All non-Gly residues fall within the allowed regions of the Ramachandran plot (Fig. 1A) except Met23 ($\phi = -147$;

$\psi = -125$) and Asp69 ($\phi = -167$; $\psi = 91$). The Asp69 residue links the C and D helices. The Met23 residue takes part in a Met23–Gly25 γ -turn (Milner-White et al. 1988), which is directly in contact with the D heme edge on the histidine ligand side (Fig. 3). This γ -turn is stabilized by two H-bonds, the first involving Met23 N and Gly25 O and the second between Gly25 N and Cys16 O. A further stabilization comes from the H-bonds of the Wat3 molecule. Several cytochrome *c*₂ structures show a residue exposed to the solvent with a similar backbone conformation approximately in the same position below the C heme edge on the histidine side (Table 2). This position is occupied frequently by a Lys residue. It has been proposed that the additional hydrogen bond made by lysine N ϵ gains the stability of this strained folding (Sogabe and Miki 1995; Benning et al. 1996). In cytochrome *c*₂ from *Rps. capsulatus*, this position is occupied by a Gly residue that shows similar main chain torsion angles ($\phi = -141$ and $\psi = -145$; Benning et al. 1991). The Gly residue, which is essentially lacking in a side chain, can confer a high degree of local flexibility to the polypeptide chain and can allow this conformation. This local strain was observed also in several mitochondrial cytochromes *c*, in chloroplast cytochromes *c*₆, and in other bacterial *c*-type cytochromes (Table 2). In cytochrome *c*₂ from *Rps. palustris*, a methionine residue (Met23) occupies this position. The side chain of methionine has a relatively strong steric hindrance and is unable to form strong H-bonds. Furthermore, the side chain of Met23 assumes two different orientations in Mol B. The presence of a residue near the exposed edge of the heme, with the main-chain torsion angles ϕ and ψ of approximately -140° and -130° seems a typical feature of *c*-type cytochromes (Table 2).

The two crystallographically independent cocrystallized sulphate ions are located near Mol A and Mol C and interact with the Arg18 side chains (Fig. 3). Of the 653 water molecules located in the oxidized structure, and of the 613 in the reduced structure, 328 are related by noncrystallographic symmetry in all of the four subunits. Three of these water molecules are buried within the interior of each globular protein molecule. The Wat1 molecule forms three H-bonds with Asn48 ND2, Tyr66 OH, and Thr91 OG1. The Wat2 molecule is located near the propionate group bonded to the heme pyrrolic ring A and forms four H-bonds with propionate O2A, Arg34 NE, Lys35 O, and Thr38 N. The Wat3 molecule is located close to the Met23–Gly25 γ -turn and forms three H-bonds with Arg18 N, Lys21 O, and Pro25 O.

Redox-coupled structural changes

The native form structures of *Rps. palustris* cytochrome *c*₂ obtained in both redox states confirm that the conformational differences between the two redox states are small. The root mean square (RMS) difference of C α positions is 0.14 Å (0.11 Å for molecule A, 0.22 Å for molecule B, 0.13

Fig. 2. Cartoon representation of the polypeptide chain backbone of the native *Rps. palustris* cytochrome *c*₂ structure with heme and iron ligands in stick. The elements of secondary structure of the protein are five α helices (A, B, C, D, F), one 3₁₀ helix (E), six β turns, and three γ turns.

Fig. 3. Stereo view of the heme edge exposed to the solvent of the oxidized native form of *Rps. palustris*. The external Met23 residue involved in a strained folding near the exposed edge of the heme, important nearby residues, buried water molecules, and the cocrystallized sulphate ion are shown.

Å for molecule C, and 0.11 Å for molecule D). A significant structural change occurs only in the Mol B in which the Val88-Gly89 main-chain peptide bond flip shifts the backbone of the Asp85-Val90 region (Fig. 1B). Val88 is the last residue of the 3_{10} helix secondary structure. The movement of the C α position in this backbone region is 0.64 Å. The heme iron displacement, the geometry of the heme group, and the conformation of the ligands appear to be scarcely influenced by the oxidation state of the cytochrome c_2 . In agreement with the previous crystallographic works on c-type cytochromes, no large variation of the iron axial coordination distances is observed. The mean value of the iron-nitrogen bond length to His17 is 2.02 Å in the oxidized state and 1.99 Å in the reduced state, having a standard error of the mean of 0.04 Å; whereas the mean values of the iron-sulphur distances to Met93 are 2.33 and 2.39 Å, with a standard error of the mean of 0.02 and 0.03 Å in the oxidized and reduced states, respectively. The correlation of the positional change of a highly conserved water molecule located in the heme binding pocket with the change of the iron oxidation state has been widely discussed for the eukaryotic cytochromes c (Takano and Dickerson 1981a,b; Louie and Brayer 1990; Bushnell et al. 1990; Moore and Pettigrew 1990; Berghuis et al. 1994a,b; Lett et al. 1996). A similar positional change of a conserved water molecule has been observed in the bacterial *Rps. viridis* cytochrome c_2 (Sogabe and Miki 1995), which presents a reduction potential of 285 mV, close to that of the eukaryotic cytochromes c , i.e., 260 mV (Pettigrew et al. 1978). On the contrary, the present cytochrome c_2 has a higher redox potential (350

mV) and the corresponding water molecule (Wat1) is detected in a similar position in both redox states.

Molecular structure of the ammonia adduct

One polypeptide chain, one ammonia molecule, two sulphate ions, and 238.5 water molecules are present in the asymmetric unit of this ammonia adduct. The overall refined architecture of this form is similar to that of the native form except for the 3_{10} helix length and the conformation of the loop involving Met93. The C α atoms of the native and the ammonia complex superimpose with a RMSD of 0.7 Å. With respect to the native form, in the ammonia adduct, the Lys92-Met93 main-chain peptide bond is flipped, and this forces the loop to turn away from the core of the protein (Fig. 4A). The sulphur atom of Met93 does not coordinate the heme iron atom and is located ~10.5 Å away from it. The main-chain torsion angles of the region involved in the conformational transition between the two forms of *Rps. palustris* cytochrome c_2 are shown in the Ramachandran plot of Figure 5. Despite the dramatic change in the chemical environment of the prosthetic group (Fig. 6), the residues mainly involved in this conformational transition are only three: Lys92-Met93-Thr94. In particular, Met93 changes its position in the Ramachandran plot (Fig. 5). In the native form, it lies in the β -sheet region, whereas in the ammonia adduct, it lies in the left-handed helix region. In the Ramachandran plot (Fig. 5), Lys92 and Thr94 lie in the right-handed α -helix region in the native structure, whereas

Table 2. Comparison of the main chain torsion angles, Φ and Ψ (deg), of the Met23 residue in *Rps. palustris* cytochrome c_2 with those found in several X-ray structures of bacterial cytochromes c_2 , mitochondrial cytochromes c , chloroplast cytochromes c_6 , and other bacterial c -type cytochromes for the analogous residue exposed to the solvent in the approximately same position below the C heme edge on the histidine side

Cytochrome	c-type	PDB code	Res. (Å)	Residue	Φ	Ψ
<i>Rps. palustris</i>	c_2	1fj0	1.7	Met23	-147	-125
	c_2	li8o	2.0	Met23	-153	-134
	c_2	li8p	1.2	Met23	-141	-131
<i>Rps. viridis</i>	c_2	lco6	1.6	Lys26	-126	-132
<i>Rb. capsulatus</i>	c_2	lc2r	2.5	Gly30	-141	-145
<i>R. globiformis</i>	c_2	lhro	2.2	Lys31	-141	-134
<i>P. denitrificans</i>	c_2	lcot	1.7	Lys34	-117	8
<i>Rb. sphaeroides</i>	c_2	lca	2.2	Lys35	-110	-77
	c_2	lcxb	2.0	Lys35	-112	-99
	c_2	lcxc	1.6	Lys35	-123	18
<i>Rs. rubrum</i>	c_2	3c2c	1.7	Lys27	-66	-69
	c_2	2c2c	2.0	Lys27	-73	-63
Yeast iso-1	c	lycc	1.2	Lys27	-124	-128
Rice	c	lccr	1.5	Lys35	-132	-143
Tuna	c	3cyt	1.8	Lys27	-105	-132
	c	5cyt	1.5	Lys27	-71	-59
Horse	c	lhrc	1.9	Lys27	-105	-136
<i>Bacil. pasteurii</i>	c_6	lb7c	1.0	Ala22	-146	-112
<i>Mon. brunii</i>	c_6	lctj	1.1	Gly23	-91	-160
<i>Scen. obliquis</i>	c_6	lc6r	1.9	Asn23	-99	-154
<i>Chlam. reinhardtii</i>	c_6	lcyi	1.9	Asn23	-137	99
	c_6	lcyj	1.9	Asn23	-151	101
<i>P. denitrificans</i>	c_{552}	lq13	1.4	Gly25	-145	-125
	c_{552}	lq14	1.5	Gly25	-145	-121
<i>P. aeruginosa</i>	c_{551}	351c	1.6	Met22	-113	-101
	c_{551}	451c	1.6	Met22	-112	-98

The PDB code of the structures and the relative resolution limit are also reported.

they lie in the β -sheet region in the present structure. A similar methionine displacement was observed in the structure of cytochrome c_2 from *R. sphaeroides*, in which an exogenous imidazole molecule binds the heme iron atom (Fig. 4B) (Axelrod et al. 1994). This structure was determined at 2.2 Å resolution with crystals grown in ammonium sulphate using imidazole as a buffer solution at pH 7.0. It is apparent that the conformational change observed in the imidazole derivative of *R. sphaeroides* cytochrome c_2 is quite different from that in the *Rps. palustris* cytochrome c_2 . The conformation change starts at the Met ligand, but the peptide chain of the imidazole complex gradually reverts to the folding of the native backbone after 5–6 residues. Furthermore, the side chain of the methionine ligand shows a relatively small shift when compared to that in the ammonia adduct. The *trans* conformation of the Met side chain, observed in both of the native forms of these cytochromes, is maintained in the imidazole adduct of *R. sphaeroides* cytochrome c_2 , whereas in the ammonia complex of *Rps. palustris* cytochrome c_2 , it is changed to the *gauche+* conformation. An opposite behavior is observed for the side-chain conformation of the neighbor Phe95, the invariant residue

located in the surface region of c -type cytochromes, which is an integral component in the maintenance of the hydrophobic heme pocket (Louie and Brayer 1989; Moore and Pettigrew 1990). In both of the native forms of these cytochromes and in the ammonia adduct, it assumes a *trans* conformation, whereas in the imidazole complex, it has a *gauche+* conformation. As a consequence, in the imidazole complex of *R. sphaeroides* cytochrome c_2 , the hydrophobic interaction between the phenylalanine side chain and heme moiety is lost, whereas it is maintained in the ammonia complex of *Rps. palustris* cytochrome c_2 .

In the ammonia complex, the movement of the methionine ligand provokes an extended change in the H-bond network around the heme group. In particular, the Thr91 side chain shows a different rotamer conformation in the two forms (χ_1 is 180° in the native form and 64° in the ammonia adduct). The three hydrogen bonds formed by OH of Thr91 with the highly conserved internal water molecule Wat1, the oxygen OD2 of the heme propionate group, and the N of Lys92, observed in the native form, are replaced by three new hydrogen bonds with O Asn71, OH Thr94, and an internal water molecule, Wat5. The backbone conformation

Fig. 4. Superimposition of the local environment around the heme group in the native form (light sketch) and ammonia complex (dark sketch) of the cytochrome c_2 from *Rps. palustris* (A), and around the heme group in the native form (light sketch) and imidazole complex (dark sketch) of the cytochrome c_2 from *R. sphaeroides* (B).

of the Lys92 residue is stabilized in the ammonia complex by the O Lys92–Wat5 hydrogen bond. The Lys92 side chain shows the same gauche+ rotamer conformation in all three structures of this cytochrome. The backbone of the other residues involved in the conformational transition is stabilized further by two hydrogen bonds (O Met93–Wat4 and N Phe95–Wat4) formed by a water molecule located in the heme pocket.

Another interesting feature of the ammonia adduct structure is that the isolated 3_{10} helix is one residue shorter than that found in the native form. The Val88 residue is the amino acid that causes this shortening. In the native structure, this residue is the last residue of the 3_{10} helix secondary structure. In the cytochrome c_2 –ammonia complex, the Val88–Gly89 main-chain peptide bond is flipped with respect to the native form. This change of backbone direction breaks the Asp85–Val88 H-bond and results in a shortening of the 3_{10} helix one residue earlier than the native form.

From inspection of the first electron density map of the cytochrome c_2 crystallized at basic pH, it appeared that the Met93 ligand was displaced by a single nonhydrogen atom, with respect to that obtained at acid pH (Garau et al. 2000). The high affinity of the iron atom for nitrogen ligands suggested that the sixth axial ligand position is occupied by an ammonia molecule produced by the precipitating agent $(\text{NH}_4)_2\text{SO}_4$ at basic pH. This assignment is now supported by the thermal factor analysis, the coordination distances,

Fig. 5. Ramachandran plot of the main-chain torsion angles (ϕ , ψ) of the region involved in the conformational transition between the two forms of *Rps. palustris* cytochrome c_2 . The residues of the native form (full circles) are joined by straight lines with the same residue of the ammonia complex (triangles).

and the H-bond scheme of this high-resolution structure. In fact, the final refinement gave a B factor of $6.0 \text{ \AA}^2$ for the axial nitrogen atom. This value is close to those of the iron atom ($6.3 \text{ \AA}^2$), the *trans* NE2 His ($6.5 \text{ \AA}^2$), and the equatorial pyrrole nitrogen atoms (NA $6.3 \text{ \AA}^2$, NB $6.2 \text{ \AA}^2$, NC $6.6 \text{ \AA}^2$, and ND $6.7 \text{ \AA}^2$). An oxygen atom refined in this site gives a significantly higher thermal factor ($8.5 \text{ \AA}^2$). Such a thermal factor is unlikely for an exogenous axial ligand also

Fig. 6. Electron density map around the prosthetic group of *Rps. palustris* cytochrome c_2 ammonia complex. The 2Fo–Fc map (contour levels at 3σ) shows the NH_3 exogenous ligand coordinated to the heme iron.

held by three strong H-bonds (Fig. 7). Furthermore, the axial distance of 2.11 Å found for the exogenous ligand coordination is consistent with an Fe-NH₃ bond length. In fact, a statistical analysis of available small-molecule crystal structures reveals that the mean value of the Fe-NH₃ bond length calculated from nine iron-ammonia complexes is 2.10(3) Å, whereas the corresponding mean value for the Fe-OH bond obtained from 13 iron-hydroxo complexes is 1.91(2) Å. However, no X-ray structural determination of an ammonia adduct of iron porphyrins has been so far reported. The structurally most-related complex characterized is Fe(DBPh₂)₂(NH₃)(Py), in which (DBPh₂)₂ is the macrocyclic equatorial ligand bis(dimethylglyoximate-diphenylborylated) (Vernik and Stynes 1996). In this complex, the Fe-NH₃ axial distance is 2.035(5) Å. Mononuclear hydroxo-iron(III) porphyrins are normally unstable (Buchler et al. 1982), and the only two examples characterized structurally are pentacoordinated complexes, the hydroxo-iron(III) $\alpha\gamma$ bis(tert-butyl)-octaethyl- $\alpha\gamma$ dihydroporphyrin (Fe-OH = 1.89(1) Å) (Buchler et al. 1982) and the hydroxo-iron(III) meso-tetraphenylporphyrin (Haryono et al. 1998). Nevertheless, unambiguous assignment of the sixth axial ligand derives from the H-bond network around the heme group of the present high-resolution crystal structure. In fact, the H-bond scheme (Fig. 7) reveals that the exogenous ligand is a three-proton donor in the H-bonds that it forms with Tyr66, Wat4, and Wat6.

The coordination distance of the His ligand in this low-spin cytochrome c₂ complex is 2.01 Å, close to that found in the native form. Furthermore, the heme group is not completely planar but is distorted into a saddle shape. This equatorial ligand distortion is very similar, within experimental error, to that of the native form. This suggests that the axial methionine ligand has no role in the deformation of porphyrin moiety.

Fig. 7. H-bond scheme around the NH₃ exogenous ligand of *Rps. palustris* cytochrome c₂ ammonia complex.

There are eight buried water molecules in the ammonia complex structure (Wat1–Wat8), whereas only three are present in the native-form structures. Three of these (Wat1–Wat3) are located in similar positions to those of Wat1, Wat2, and Wat3 molecules found in the native-form structures. Wat4, Wat5, and Wat6 are located in the space cleared by the Met93 side chain, whereas Wat7 and Wat8 are located near the A propionate group.

Conclusions

The complete primary structure of the *Rps. palustris* cytochrome c₂ protein, expressed from the strain 42OL, was obtained by X-ray analysis and checked by mass spectrometry. It confirms that sequence differences between independent strains of the same bacteria are as large as those between mitochondrial cytochrome c of mammals (Ambler et al. 1979). The X-ray structures of the *Rps. palustris* cytochrome c₂ show an unusual isolated 3₁₀-helix formed by the rare six-residue insertion (Gly82–Ala87), which precedes the heme-binding Met93. The structural comparison of the present cytochrome c₂ with other c-type cytochromes has revealed that the presence of an external residue involved in a strained folding near the exposed edge of the heme is a typical feature of this family of proteins. The differences in the native structure between the two redox states are small. Different from the structural features observed in eukaryotic cytochromes c and in the prokaryotic *Rps. viridis* cytochrome c₂, the water molecule (Wat1), located in the heme-binding pocket (Fig. 3) and conserved among c-type cytochromes, is detected in the same position in both redox states. Recently, structural determinations of the cytochrome c₆ from *Scenedesmus obliquus* in both redox states revealed no redox-dependent movement of the internal water molecule (Schnackenberg et al. 1999). As *Rps. palustris* cytochrome c₂, the midpoint redox potential of cytochromes c₆ is usually ~100 mV higher than that of the eukaryotic cytochromes (Kerfeld 1997) and than that of the *Rps. viridis* cytochrome c₂.

The most striking feature of the structure obtained at alkaline pH is the modification of the coordination sphere of the iron center. The sulphur atom of the Met93 axial ligand does not coordinate the heme iron atom but is replaced by an exogenous ammonia molecule. This is the only X-ray structure so far reported of a heme coordinated by an ammonia molecule. The detachment of the methionine ligand is helped by a very localized change in the backbone conformation with maintenance of the overall protein folding. It is interesting to note that the crystal structure of *Rps. viridis* cytochrome c₂, obtained from crystals grown in similar conditions, does not show the Met ligand displacement (Sogabe and Miki 1995). Most likely, the 3₁₀-helix formed by the six-residues insertion Gly82–Ala87, present in the protein from *Rps. palustris*, increases Met loop flexibility and, con-

sequently, Met-iron bond lability. In addition, comparison between the structures of the native forms of the *Rps. palustris* and *Rps. viridis* cytochromes c_2 shows a difference in the hydrogen bond distance between the Tyr66 OH and the coordinated SD of the Met93 ligand. In the cytochrome c_2 from *Rps. palustris*, this distance is 3.34 Å (mean value with standard error of the mean of 0.02 Å), significantly longer than that found in the *Rps. viridis* cytochrome c_2 (3.12 Å). The H-bond formed by the coordinated Met ligand seems to be very important for the stabilization of the native form of c-type cytochromes. Also, in the native form of *R. sphaeroides* cytochrome c_2 , a very weak H-bond is present (3.48 Å). Probably the crystal formation of the *R. sphaeroides* cytochrome c_2 imidazole complex is also favored by the presence of this weak stabilization of the Met ligand through the Tyr H-bond.

Materials and methods

Synthesis and purification

Cytochrome c_2 obtained from a *Rps. palustris* strain 420L of the culture collection of the Research Center on Autotrophic Micro-organism (Florence, Italy) was purified as described previously (Bertini et al. 1998). The purity of the protein was checked from the ratio of its absorbance at 280 nm to that at 412 nm.

Mass spectrometry

Mass spectra were recorded with an SCIEX API I single-quadrupole mass spectrometer equipped with an ion-spray source. Calibration was performed with a PPG (poly-propylene glycol) calibration solution. Cytochrome c (Horse Heart type IV, Sigma) was used as the protein standard. Mass spectrometric standard deviations in the determination of cytochrome c molecular weight were ± 1 dalton. Proteins (~ 0.1 $\mu\text{g}/\mu\text{L}$) were dissolved in water and diluted with CH_3CN and HCOOH up to 50% and 0.1% v/v, respectively, before flow injection into the spectrometer (flow rate of 1.6 $\mu\text{L}/\text{min}$, positive mode, dwell time of 0.5 msec, step size 0.1 amu, 5–10 scans accumulated). Mass data were analyzed with the software supplied with the instrument.

Crystallizations and X-ray data collections

Crystallization experiments of the cytochrome c_2 from *Rps. palustris* were performed with the hanging-drop vapour diffusion method at 291K. Protein solution (9.0 mg/mL buffered to pH 6.0 in 20 mM phosphate) was either oxidized or reduced with an excess of ferricyanide or sodium dithionite, respectively. The oxidation state of the protein was monitored by absorbance spectrometry. Mixed together were 2 μL of protein solution and the same volume of the reservoir solution and equilibrated against 1 mL of the reservoir solution. At acid pH, crystals grew in 3–7 d from 42%–44% saturated ammonium sulphate and 0.1 M citrate buffer at pH 4.4 with oxidized and reduced protein solutions. At basic pH, the crystal used for data collection grew from reduced protein solution in about 3 wk in the presence of 61% saturated ammonium sulphate and 0.1 M Tris-HCl buffer at pH 8.5. Data collections were performed at the Elettra Synchrotron (Trieste, Italy)

using monochromatic radiation with wavelength of 1 Å and a MAR Research 345-mm imaging plate as detector. Before freezing, some crystals of ferricyanide (for the oxidized state) or sodium dithionite (for the reduced state) were dissolved in the drop containing the cytochrome c_2 crystals to provide a fresh oxidation and reduction environment. After 15 min, the protein crystals were passed quickly through a reservoir solution containing 20% glycerol as cryoprotectant and flash-frozen in a stream of N_2 at 100K. Table 3 reports a summary of data collection and crystallographic statistics. The determination of unit-cell parameters, integration of reflection intensities, and data scaling were performed using MOS-FLM and SCALA programs from the CCP4 suite (CCP4 1994).

Structure determinations and refinements

The structure determinations by molecular replacement have been described previously (Garau et al. 2000). The crystallographic refinement of the ammonia adduct was initiated by the partially refined structural model at 1.4 Å resolution with an R factor of 29.1% (Garau et al. 2000). Rebuilding of the model, amino acid substitutions, and location of solvent atoms were performed on Silicon Graphics workstations using the O program (Jones et al. 1991) and the enhanced $2F_o - F_c$ and $F_o - F_c$ maps as a guide. Refinement of the structure was performed using SHELXL program (Sheldrick 1997). Polypeptide atoms were restrained to the 1,2- and 1,3-target distances based on the study of Engh and Huber (1991), whereas the positional refinement of heme atoms and solvent molecules were not restrained. The positional refinement improved the R and R_{free} factors (Brünger 1992) to 18.1% and 19.9%, respectively; making all atoms anisotropic resulted in a drop of the R and R_{free} factors to 15.0% and 17.3%, respectively. Anisotropic thermal parameters of the water molecules were restrained with an effective standard deviation of 0.1 so that their U_{ij} components approximated the isotropic behavior, whereas protein atoms were restrained to make the U_{ij} values of neighboring atoms similar (Sheldrick 1997). Finally, the hydrogen atoms were added at calculated positions, and further minor adjustments were performed. Refinement statistics of the last cycle are reported in Table 3. To confirm the presence of the ammonia molecule as axial ligand, the nitrogen atom was replaced by an oxygen atom during parallel last-refinement cycles. Although no change in R factor values occurred, the temperature factor of the oxygen axial ligand increased from 6.0 to 8.5 Å².

With respect to the preliminary structural determination (Garau et al. 2000), the atomic model of the oxidized native form was further refined using the primary sequence obtained from the structure of ammonia adduct, and additional water molecules were introduced. The model was refined using the REFMAC program (Murshudov et al. 1997) with restrained positional and thermal factor refinement and noncrystallographic symmetry (NCS) restraints. The NCS restraint value of 0.05 Å was used for all four crystallographically independent protein molecules and for the water molecules related by pseudosymmetry. The NCS restraints for specific amino acids (side chain and backbone for Thr94 and Phe95 and only side chain for Asp2, Lys4, Lys11, Arg18, Glu53, Lys76, Lys96, and Lys114) were released when large differences in the conformation among the four independent units were detected in the Fourier maps. Refinement statistics of the last cycle are reported in Table 3.

The refinement of the reduced cytochrome c_2 structure was performed starting with the coordinates of the oxidized native form. The initial rigid body refinement using REFMAC gave an R factor of 31.1% for 34,721 unique reflections. After rebuilding the model and 50 cycles of positional refinement using REFMAC, the

Table 3. Data processing and refinement statistics for the native structures of *Rps. palustris* cytochrome c_2 (oxidized and reduced state) and for the ammonia adduct structure of this cytochrome

Structure	Oxidized c_2	Reduced c_2	Ammonia c_2 adduct
Data collection and processing			
Space group	P2 ₁	P2 ₁	P3 ₂ 21
Unit-cell parameters (Å, °)	a = 50.31 b = 71.53 c = 66.66 β = 93.5	a = 50.12 b = 71.71 c = 67.36 β = 93.7	a = 64.83 b = 64.83 c = 68.55 γ = 120.0
No. of measurements	194662	106868	364867
No. of unique reflections	51959	34702	57362
R_{merge} (%)	8.7 (35.8)	17.5 (59.9)	5.4 (41.2)
$\langle I/\sigma(I) \rangle$	11.0 (3.6)	6.4 (2.0)	19.0 (4.1)
Multiplicity	3.7	3.1	6.3
Completeness (%)	99.9 (99.7)	99.7 (100)	99.3 (96.7)
Refinement statistics			
Resolution range (Å)	20–1.70	20–1.95	20–1.15
No. of reflection used	49259	32939	55707
R -factor ^a (%)	17.8	19.2	13.9
R_{free} -factor ^b (%)	21.8	23.7	16.7
No. of protein atoms	3420	3420	855
No. of heme atoms	172	172	43
No. of water molecules	653	613	238.5
Total number of atoms	4264	4205	1147.5
B-factor statistics			
Average B main chain (Å ²)	11.9	13.1	9.8
Average B side chain (Å ²)	16.3	16.5	13.4
Average B heme atoms (Å ²)	8.7	9.4	7.0
Average B waters (Å ²)	30.7	32.0	28.3
Overall B (Å ²)	16.4	17.4	11.6
RMSD from ideal geometry			
Bond length (Å)	0.010	0.028	0.010
Bond angle distances (°)	2.0	2.2	1.6

Values in parentheses refer to the outer resolution shell (1.79–1.70 Å oxidized state; 2.06–1.95 Å reduced state; 1.21–1.15 Å ammonia adduct).

^a $R_{\text{fact}} = \sum |F_{\text{obs}} - F_{\text{calc}}| / \sum F_{\text{obs}}$.

^b R_{free} calculated for 5% of the observed reflections.

R and R_{free} factors dropped to 19.3% and 24.6%, respectively. To remove the bias of the starting model further, 20 cycles were performed using the SHELXL program with default restraints (Sheldrick 1997). Using these weaker restraints, R decreased to 18.8% and R_{free} increased to 25.6%. The last 20 cycles of refinement, using REFMAC with NCS restraints (the same used for the oxidized protein with the further release of side chain and backbone of Ala87, Val88, and Gly89), gave the final R and R_{free} factors reported in Table 3. The coordinates and structural factors have been deposited in the Protein Data Bank under the accession codes 1fjo, 1i8p, and 1i8o.

Analysis of structures and structural comparisons

Model quality was checked with PROCHECK (Laskowski et al. 1993). The secondary structure of the protein was analyzed using the DSSP program (Kabsch and Sander 1983). Structural comparison between the native protein and its ammonia adduct was performed by RMS superposition of the protein Cα atoms of the subunit A of the oxidized native form using the program COMPARE (CCP4 1994). The superimposition of the peptide segments involved in the conformational transition between the native

form and N-ligand cytochrome c_2 complex of *Rps. palustris* (Fig. 4A) and of *R. sphaeroides* (Fig. 4B) was performed by RMS superposition of heme moiety of the native and N-adduct form using the lsq commands of the O program (Jones et al. 1991). Atomic coordinates for the imidazole adduct and native form of *R. sphaeroides* cytochrome c_2 were obtained from the Brookhaven Protein Data Bank (Bernstein et al. 1977) using the entries 1cxa and 1cxc, respectively. Statistical analysis of Fe–NH₃ and Fe–OH bond lengths in small-molecule crystal structures was performed on data from the Cambridge Structural Database (Allen et al. 1979; CSDS 5.20 Version, October 2000 with 224,400 entries).

Electronic supplemental material

Kinemages are available showing the elements of secondary structure and the conformational transition by superimposition of the ammonia complex and the native form of cytochrome c_2 from *Rps. palustris*.

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