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Crystallization and preliminary X-ray analysis of two pH-dependent forms of cytochrome c_2 from *Rhodopseudomonas palustris*

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Cytochrome c_2 from *Rhodopseudomonas palustris* has been crystallized in two different crystal forms: a monoclinic form I at pH 4.4 from both reduced and oxidized protein solution and a trigonal form II at pH 9.0 from reduced protein solution. Complete 1.7 and 1.4 Å resolution data sets were collected from the oxidized form I and from the form II, respectively. The preliminary structures show an important change in the iron coordination environment in the trigonal form obtained at basic pH arising from the substitution of the Met ligand by an ammonia molecule.

1. Introduction

Cytochromes c_2 (cyts c_2) are water-soluble electron carriers involved in the electron-transport chains of photosynthesis and respiration in most photosynthetic non-sulfur purple bacteria and some non-phototropic bacteria. They belong to the class I cytochromes and form a fairly homogeneous group of proteins with 30–40% homology in amino-acid sequence (Ambler *et al.*, 1979). The prokaryotic cyts c_2 have similar properties to those of mitochondrial cyts *c*, but have more variable reduction potentials. The reduction potential is about +260 mV in all eukaryotic cyts *c*, while it varies from +250 to +450 mV in the bacterial cyts c_2 (Meyer *et al.*, 1983). The cyts c_2 also show significant variations in their molecular sizes, which range from 12 kDa ('small type', resembling cyts *c*) to 13–14 kDa ('large type'; Ambler *et al.*, 1979). The strictly invariant primary sequences of the eukaryotic cyts *c* have a preponderance of positively charged amino acids compared with the negatively charged amino acids (net charge +6), while the prokaryotic cyts c_2 normally do not show this large disparity between positively and negatively charged residues (Salemme, Kraut *et al.*, 1973).

To date, X-ray structures of cyt c_2 isolated from *Rhospirillium rubrum* (Salemme, Freer *et al.*, 1973), *Rhodobacter capsulatus* (Benning *et al.*, 1991), *Paracoccus denitrificans* (Benning *et al.*, 1994), *Rhodobacter sphaeroides* (Axelrod *et al.*, 1994), *Rhodopseudomonas viridis* (Sogabe & Miki, 1995) and *Rhodopila globiliformis* (Benning *et al.*, 1996) have been determined. Five or six α-helices, arranged around the haem, characterize their highly conservative folding motif. Differences in local structural features, both within the cyts c_2 family and in comparison with the eukaryotic analogues, are of interest in the investigation of the factors controlling the redox potentials and electron-transfer rates in *c*-type cyts.

The protein conformation and the electronic distribution within the haem are sensitive to ionizing residues and at extreme pH values axial haem ligation is also markedly influenced (Moore & Pettigrew, 1990). Although pH-dependent forms and ligand binding in *c*-type cyts are not directly related to their physiological function, they may provide valuable information on protein stability and folding (Dumortier *et al.*, 1998).

Multiple pH-dependent states for a number of *c*-type cyts have been characterized (Moore & Pettigrew, 1990). For example, the pH-induced protein conformational transitions and changes in the ligation state of the haem iron in cyt c_2 from *R. palustris* have been monitored by electrochemical and spectroscopic measurements (Battistuzzi *et al.*, 1995; Bertini *et al.*, 1998). At pH values above 8, the appearance of the new voltammetric wave and of an additional NMR signal pattern was interpreted as the result of the formation of a new species with distinct structural and electronic properties of the haem group. This and previous work (Timkovich *et al.*, 1984; Banci *et al.*, 1998) show that the alkaline ionization is probably a consequence of the replacement of the methionine axially coordinated to the haem iron with another (most probably N-donor) ligand. It has been proposed that in oxidized *c*-type cyts the N^{ζ} of a Lys residue substitutes the S of the Met ligand in the iron coordination at high pH values (Moore & Pettigrew, 1990; Ubbink *et al.*, 1994).

Furthermore, it is known that pyridine, imidazole and ammonia are able to displace the Met ligand (Shao *et al.*, 1993, 1995; Banci *et al.*, 1998; Dumortier *et al.*, 1998). The crystal structure of cytochrome c_2 from *Rhodobacter*

Table 1
Data-collection statistics.

Values in parentheses refer to the outer resolution shell. Outer-shell data is in the resolution range 1.79–1.70 Å for form I and 1.47–1.40 Å for form II.

Form	I (pH 4.4)	II (pH 9.0)
Space group	$P2_1$	$P3_221$
Temperature (K)	100	100
Resolution range (Å)	50.0–1.7	50.0–1.4
Unit-cell parameters (Å, °)	a = 50.31	$a = b$ = 64.67
	b = 71.53	c = 68.25
	c = 66.66	γ = 120.0
	β = 93.52	
V (Å^3)	239400	247200
Z	8	6
Number of observations	194662	394132
Unique reflections	51959	32980
$R_{\rm merge}$† (%)	0.087 (0.358)	0.096 (0.507)
Completeness (%)	99.9 (99.7)	99.8 (99.8)
$I/\sigma(I)$	11 (3.6)	20.5 (5.9)

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum\langle I\rangle$.

sphaeroides, where an exogenous imidazole molecule binds the haem Fe ion, is the only example so far known among *c*-type cyts with a replaced axial Met (Axelrod *et al.*, 1994).

The primary sequence of the 'large-type' cyt c_2 from *R. palustris* (Ambler *et al.*, 1979) shows a unusual six-residue insertion which precedes the haem-binding Met (Gly82–Ala87) and a large disparity between positively (15) and negatively (10) charged amino acids not present in any other resolved structure of cyts c_2. We report here the crystallization of the cyt c_2 from *R. palustris* at pH 4.4 and pH 9.0, together with data collection and preliminary structural analyses to 1.7 and 1.4 Å resolution, respectively.

2. Materials and methods

2.1. Synthesis and purification

Cyt c_2 obtained from *R. palustris* strain 42 OL of the culture collection of the Research Center on Autotrophic Microorganisms (Florence) was purified as described previously (Bertini *et al.*, 1998). The purity of the protein was checked by measuring the $\varepsilon_{280}/\varepsilon_{415}$ ratio of the reduced form, which was obtained by addition of β-mercaptoethanol.

2.2. Crystallization

Crystallization experiments of cyt c_2 from *R. palustris* were performed using the hanging-drop vapour-diffusion method at 291 K. Stock protein solution was 0.67 m*M* (9.0 mg ml^{-1}) in cyt c_2 buffered to pH 6.0 in 200 m*M* phosphate. The oxidized and reduced forms of cyt c_2 were obtained by adding an excess of ferricyanide and sodium dithionite, respectively. After centrifugation, 2 µl of supernatant protein solution was mixed with the same volume of the reservoir solution and equilibrated against 1 ml of the reservoir solution.

Table 2
Roto-translation solutions obtained by molecular replacement for the four independent molecules of form I.

	α	β	χ	T_x	T_y	T_z	Corr	R
Solution 1	192.09	87.88	304.88	0.4423	0.000	0.0000	26.8	60.5
Solution 2	192.09	87.88	304.88	0.9425	0.3439	0.4999	49.1	55.5
Solution 3	168.09	91.92	124.58	0.9412	0.0412	0.0000	45.7	53.7
Solution 4	168.09	91.92	124.58	0.4415	0.3853	0.4992	53.0	54.0

Crystals grew from 42–44% saturated ammonium sulfate and 0.1 *M* citrate buffer pH 4.4, with both oxidized and reduced protein solutions. Optimized crystallization trials of cyt c_2 typically produced needle-shaped red crystals in 3–7 d (form I) having maximum dimensions of about 1.0 × 0.2 × 0.02 mm. The resulting crystals were extremely fragile and sensitive to slight changes in temperature and mother liquor and decomposed quickly even upon gentle manipulation. Adding 20% glycerol as co-precipitant, we were able to obtain better separated and larger crystals. Under these conditions, crystals require at least 10 d to grow.

At basic pH, crystals grew in the presence of 61% saturated ammonium sulfate and 0.1 *M* Tris–HCl buffer pH 9.0 from reduced protein solution. Single well shaped bipyramidal crystals (form II) appeared in about three weeks. No crystals were obtained starting from oxidized protein solution.

2.3. X-ray data collection and processing

X-ray diffraction experiments were carried out at the Elettra Synchrotron, Trieste, Italy. Data were collected using monochromatic radiation with wavelength 1.000 Å and a MAR Research 345 mm imaging-plate detector. Crystals were harvested with a small loop of rayon fibre into mother liquor containing 20% glycerol as cryoprotectant and were flash-frozen in a stream of N_2.

The reflections of form I crystals were indexed using a monoclinic unit cell (unit-cell parameters a = 50.31, b = 71.53, c = 66.66 Å, β = 93.5° for crystals obtained from oxidized protein solution; a = 49.90, b = 71.28, c = 67.07 Å, β = 93.7° for crystals obtained from reduced protein solution) with systematic absences in agreement with the $P2_1$ space group. Analysis of the diffraction pattern from form II crystals and structure solution reveal that they belong to the space group $P3_221$, with unit-cell parameters a = 64.67, c = 68.25 Å. The calculated Matthews coefficient $V_{\rm M}$ (Matthews, 1968) is 2.45 Å^3 Da^{-1} for form I, assuming eight molecules in the unit cell and a molecular weight of 12.2 kDa for the polypeptide chain (Ambler *et al.*, 1979). The $V_{\rm M}$ is 3.38 Å^3 Da^{-1} for form II, assuming six molecules in the unit cell. Two complete data sets from a monoclinic ferricytochrome c_2 crystal and from a trigonal crystal were collected to 1.7 and 1.4 Å resolution, respectively. Table 1 reports a summary of data-collection procedures and crystallographic statistics. The determination of unit-cell parameters, integration of reflection intensities and data scaling were performed using *MOSFLM* and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994).

2.4. Preliminary structure determination

The structure of form I was solved by molecular replacement using *AMoRe* (Navaza, 1994) as implemented in the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) and by using the information obtained from the Patterson map.

The starting model was the refined 2.0 Å structure of *Rhodospirillum rubrum* (Salemme, Freer *et al.*, 1973; PDB code 2c2c), whose amino-acid sequence shows a 42% identity with that of ATCC 17006/2.1.37 strain of *R. palustris* cyt c_2 (Swiss-Prot code CY22; Ambler *et al.*, 1979). All residues in *Rhodospirillum rubrum* cyt c_2 that differ from those of *R. palustris* cyt c_2 were mutated and the insertion and deletion regions were removed and joined, respectively.

Using data in the resolution range 8–2 Å, only two almost equivalent rotational solutions, related by a twofold rotor parallel to the *a* axis, were obtained. This suggests that the four independent molecules form two pairs with the same orientation. The presence of a very intense single peak (65% of the origin peak) in the Patterson map indicated that these pairs are related by the same translation vector (0.5, 0.34, 0.5). The rigid-body refinement of the four polypeptide chains obtained from the *AMoRe* solution (Table 2) improved the correlation coefficient and R factor to 55.9 and 51.7%, respectively. A random subset of data (5%) was omitted from all refinement calculations

to provide an assessment of the progress of refinement. Restrained positional and thermal factor refinement with non-crystallographic symmetry restraints improved the R and free R factors to 39.1 and 42.0%, respectively. Rebuilding of the model and location of solvent atoms were based on the analysis of $(2F_o - F_c)$ and $(F_o - F_c)$ maps and were performed on Silicon Graphics workstations using the program *O* (Jones *et al.*, 1991). The present R and free R factors are 18.8 and 22.9%, respectively. Further location of water molecules and final refinement using *REFMAC* (Collaborative Computational Project, Number 4, 1994) are still in progress.

The structure solution of form II was searched in both space groups $P3_121$ and $P3_221$ by molecular replacement, using the atomic coordinates of form I as starting model. The initial R value for the molecular-replacement solution obtained in the $P3_221$ space group was 45.1 in the 8–2 Å resolution range. The restrained positional and thermal factor refinement improved the R and free R factors to 32.3 and 36.9%, respectively. The first $2F_o - F_c$ map clearly shows a modification to the coordination environment of the iron site (Fig. 1). In particular, a displacement of the Met93 ligand by a single non-H atom and a different folding in the Lys92-Met93-Thr94-Phe95 backbone with respect to the form I structure were observed. We are currently fitting the side chains of the model.

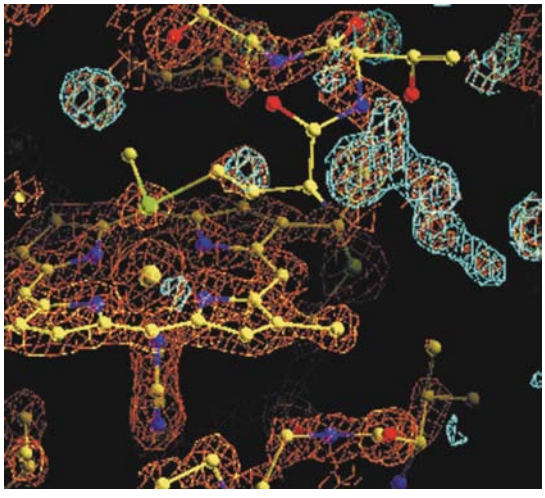

Figure 1
Electron-density maps around the haem moiety of form II obtained by the first refinement of the form I starting model. The $2F_o - F_c$ map at 2σ (brown) and the $F_o - F_c$ map at 3σ (cyan) clearly show the displacement of the Met93 ligand by a single non-H atom in the cyt c_2 crystallized at basic pH (form II) with respect to that obtained at acid pH (form I).

3. Discussion

We have obtained two pH-dependent crystal forms of *R. palustris* cyt c_2: the monoclinic form I at pH 4.4 both from reduced and oxidized protein solution and the trigonal form II at pH 9.0 from reduced protein solution only. Complete 1.7 and 1.4 Å resolution data sets using a synchrotron-radiation light source were collected from the oxidized form I and from form II, respectively.

Preliminary structure determination shows a conformational modification near the prosthetic haem group between the two pH-dependent forms. In particular, the axial Met ligand is displaced by a single heavy atom in the basic form II (Fig. 1). The high affinity of the Fe atom for nitrogen ligands suggests that this site is probably occupied by an ammonia molecule produced by the precipitant $(NH_4)_2SO_4$ at pH 9.0. This hypothesis is in agreement with spectroscopic measurements of the NH_3 adduct of the oxidized horse heart cyts *c* (Banci *et al.*, 1998).

The time required to obtain crystals from the reduced protein solution and the resistance to Met replacement of reduced cyts *c* (Pearce *et al.*, 1989; Banci *et al.*, 1998) and c_2 (Battistuzzi *et al.*, 1995) at high pH suggest that growth of form II crystals probably occurs with the oxidation of the iron, as observed in the cyt c_2 from *Rhodobacter sphaeroides* (Axelrod *et al.*, 1994).

It is interesting to note that the crystal structure of *R. viridis* cyt c_2 obtained from crystals grown in similar conditions to those of form II (0.1 *M* Tris–HCl buffer pH 8.5, 70% saturated ammonium sulfate at 277 K) does not show the Met-ligand displacement (Sogabe & Miki, 1995).

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