ELSEVIER

Biochimica et Biophysica Acta 1412 (1999) 47–55

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Cytochrome *c″* from the obligate methylotroph *Methylophilus methylotrophus*, an unexpected homolog of sphaeroides heme protein from the phototroph *Rhodobacter sphaeroides*

Klaus Klarskov [a], David Leys [a], Katrien Backers [a], Helena S. Costa [b], Helena Santos [b], Yves Guisez [a], Jozef J. Van Beeumen [a,*]

[a] *Department of Biochemistry, Physiology and Microbiology, Laboratory of Protein Biochemistry and Protein Engineering, State University of Gent, Ledeganckstraat 35, B-9000 Gent, Belgium*

[b] *Instituto de Tecnologia Quimica e Biologica, Universidade Nova de Lisboa, Apartado 127, 2780 Oeiras, Portugal*

Received 18 January 1999; received in revised form 16 March 1999; accepted 25 March 1999

Abstract

The complete primary structure of an unusual soluble cytochrome *c* isolated from the obligate methylotrophic bacterium *Methylophilus methylotrophus* has been determined to contain 124 amino acids and to have an average molecular mass of 14 293.0 Da. The sequence has two unusual features: firstly, the location of the heme-binding cysteines is far downstream from the N-terminus, namely at positions 49 and 52; secondly, an extra pair of cysteine residues is present near the C-terminus. In both respects, cytochrome *c″* is similar to the oxygen-binding heme protein SHP from the purple phototrophic bacterium *Rhodobacter sphaeroides*. In contrast to SHP, cytochrome *c″* changes from low-spin to high-spin upon reduction, due to dissociation of a sixth heme ligand histidine which is identified as His-95 by analogy to the class I cytochromes *c*. The distance of His-95 from the heme (41 residues) and the presence of certain consensus residues suggests that cytochrome *c″* is the second example of a variant class I cytochrome *c*.

Keywords: Cytochrome *c*; Primary structure; Methylotroph; Redox–Bohr effect

1. Introduction

Many different bacterial cytochromes have been discovered and, among these, the most unusual are those which change spin-state upon reduction [1]. Bacterial cytochrome *c* peroxidase is perhaps the best known hemoprotein for a change from low-spin to high-spin, presumably to poise the heme for binding peroxide [2–4]. A minor cytochrome from the purple sulfur bacterium *Chromatium vinosum* changes from high-spin to low-spin upon reduction, but the functional role for this change in spin-state is unknown [5].

A protein from the methylotrophic bacterium *Methylophilus methylotrophus*, designated cytochrome *c″*, was found, like peroxidase, to change from low-spin to high-spin upon reduction. The pro-

Abbreviations: ESMS, electrospray ionization mass spectrometry; LC-MS, liquid chromatography coupled to mass spectrometry; SHP, sphaeroides heme protein; MALDI-MS, matrix-assisted laser desorption mass spectrometry; mcd, magnetic circular dichroism

* Corresponding author. Fax: +32 (9) 264-5338; E-mail: jozef.vanbeeumen@rug.ac.be

0005-2728/99/$ – see front matter
PII: S0005-2728(99)00050-X

tein was given the trivial name of 'cytochrome *c* double-prime' because their discoverers observed similarities in the absorption spectrum to cytochrome *c′* (single prime) in the reduced form, but pronounced differences in the oxidized form [6]. Cytochrome *c″* has a mass of 15 kDa, it contains a single heme, and has a redox potential of −60 mV at pH 7.0. As shown by EPR, mcd and NMR studies, the cytochrome *c″* is a unique example of a monoheme *c* protein with a near-perpendicular orientation of the histidinyl ligands [7,8]. The structure of the heme-pocket has been extensively studied using NMR and ultraviolet/visible spectrophotometry [9]. Taking into account the strong pH dependence (redox–Bohr effect) of the midpoint redox potential of the cytochrome, it was concluded that the τ-NH of an axial histidine becomes detached in the reduced state and that the pK_a value changes from 8.1 in the latter to 2.4 in the oxidized state [9,10]. Further elucidation of the dynamics, by measuring the $^1H/^2H$ exchange rates, provided circumstantial evidence that it is the presumed sixth ligand, and not the fifth ligand histidine that detaches and protonates upon reduction. This is thus an example of a soluble cytochrome capable of transferring protons as well as electrons in a process that causes a major change in the structure of the heme-binding site. The N-terminal sequence was reported up to residue 62 and the heme-binding site was located at Cys-49 and -52 [11].

In the present work, we have determined the remaining part of the amino acid sequence of cytochrome *c″* using modern analytical techniques, including capillary LC-MS. The sequence is compared to the only homologous protein so far known, the oxygen-binding SHP of *Rhodobacter sphaeroides*.

2. Materials and methods

2.1. Materials

All enzymes used in this study were obtained from Boehringer (Mannheim, Germany). The capillary column (0.32×200 mm) was packed in the laboratory with ODS-AQ C18 (5 μm particle size, 120 Å pore size) (YMC, Shermbeck, Germany) according to Davis et al. [12]. The narrow-bore column ODS-AQ C18 (2.1×100 mm) was also purchased from YMC. HPLC solvents were obtained from Rathburn (Walkerburn, UK).

2.2. Removal of heme from holocytochrome c″

Cytochrome *c″* was isolated from *M. methylotrophus* as described previously [6]. Removal of heme was carried out for 6 h at room temperature, after dissolving the protein (32 μg) in 40 μl 50% aqueous acetonitrile containing 0.1% TFA and 1 μg/μl mercury chloride. Excess of reagents and heme were removed by gel filtration on a 0.8×10 cm column packed with Bio-Gel P-6 (Bio-Rad, Eke, Belgium) equilibrated and eluted with 5% aqueous acetonitrile containing 0.05% TFA.

2.3. Enzyme digestions

Holocytochrome *c″* (64 μg) was digested for 3 h with 2.6 μg endo-proteinase Lys-C in 30 μl 25 mM Tris-HCl buffer, pH 8.5. The apocytochrome (3.8 μg) was digested for 6 h with *Staphylococcus aureus* Glu-C proteinase (enzyme/substrate ratio 1:37, w/w) in 20 μl 100 mM sodium hydrogen phosphate buffer, pH 7.8. Subdigestion of peptide K8 (150 pmol) with subtilisin (1 μg) was performed in a 50 mM Tris-HCl buffer, pH 8.0, for 1 h. All digestions were carried out at room temperature.

2.4. Liquid chromatography

The narrow-bore/capillary LC-EMS interface employed during this work is comparable, with few modifications, to the set-up described elsewhere [13]. Peptides separated on the narrow-bore system were detected on a 1000S diode-array detector (Perkin Elmer, Applied Biosystems Division, Foster City, CA). The solvents used for the separations consisted of 5% (solvent A) and 80% (solvent B) aqueous acetonitrile containing 0.05% TFA. Peptides derived from Lys-C digestion of holocytochrome *c″* were separated using the following linear gradient conditions; 0–35% solvent B in 40 min followed by an increase to 100% in 10 min at a flow rate of 200 μl/min. Peptides obtained with *S. aureus* protease digestion of apocytochrome *c″* were separated on the

capillary column using a linear gradient from 5 to 45% solvent B in 45 min followed by an increase to 100% solvent B in 10 min at a constant flow rate of 4 μl/min.

2.5. Mass spectrometry

2.5.1. Pneumatically assisted electrospray ionization mass spectrometry

All analyses were performed on a Bio-Q quadrupole mass spectrometer upgraded to the Quatro II version (Micromass, Altrincham, UK). Analysis of the intact holocytochrome was carried out using the standard probe supplied with the instrument. The sample was dissolved to a concentration of 5–10 pmol/μl in 50% aqueous acetonitrile, containing 0.2% formic acid, and introduced at a flow rate of 6 μl/min. Spectra were acquired in the *m/z* range 600–1800 in 12 s.

For LC-ESMS, a modified commercial triaxial liquid sheath probe was used. The modification consisted in replacing the original capillaries with two metal capillaries of the following dimensions: for the inner tube, 25.4 μm (i.d.)×178 μm (o.d.)×31 cm, for the outer tube 178 μm (i.d.)×330 μm (o.d.)×25 cm. The probe was connected to the UV-detector outlet by 100 cm long peek tubing, 64 μm (i.d.)×508 μm (o.d.). During on-line LC-ESMS operation, spectra were acquired for 6 s in the *m/z* range 385–1800, and the total ion current recorded. Calibration of the instrument was carried out using either myoglobin or a mixture of polyethylene glycols (average masses 400, 1000 and 2000 Da) at a resolution sufficient to resolve the monoisotopic peaks from the latter at *m/z* 1840, 20% above baseline.

2.5.2. Matrix-assisted laser desorption mass spectrometry

Maldi was performed on a VG TofSpec SE (Micromass, Wythenshawe, UK) using the reflectron mode. Data-acquisition parameters and further processing was carried out applying the OPUS software. Sample preparation was performed using the following procedure. The matrix (α-cyano-4-hydroxycinnamic acid) was dissolved in acetonitrile/ethanol (1:1 v/v) to a concentration of 9 μg/μl. The sample was diluted in 20% aqueous acetonitrile containing 0.1% TFA, to about 1 pmol/μl, followed by addition of an equal volume of the matrix solution. Typically 0.5–1 μl was air dried on the target.

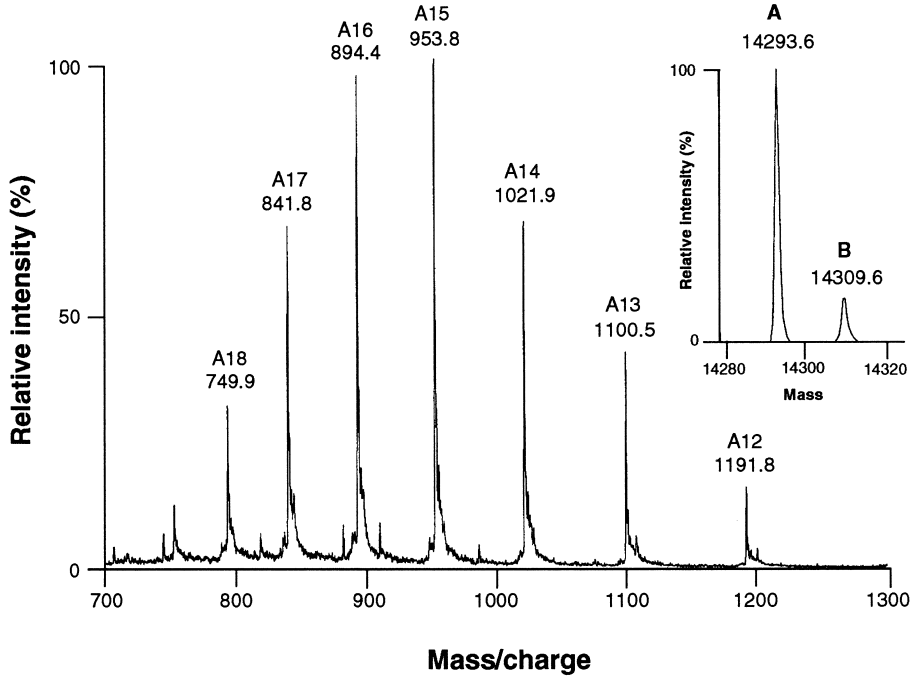

Fig. 1. Electrospray ionization mass spectrum of holocytochrome *c*″. The insert shows the molecular mass obtained after application of the maximum entropy algorithm (part of the Masslynx software version 2.1).

2.6. Sequence analysis

Edman degradation was performed on an 476A pulsed liquid phase sequenator equipped with an on-line 120A PTH amino acid analyzer (Perkin Elmer, Applied Biosystems Division, Foster City, CA).

3. Results

3.1. Molecular weight determination of cytochrome c″

ESMS analysis of native cytochrome *c*″ revealed the presence of two components of masses 14 293.6 and 14 309.9 Da, respectively (Fig. 1). The mass difference of 16.3 Da indicated that the less abundant species could be due to partial oxidation. To verify the number of hemes, the cytochrome was incubated in an acidic aqueous acetonitrile solution containing mercury chloride. Traditionally, the reaction is performed in acidified buffers containing an excess of urea or guanidine-HCl. These salts unfortunately hamper the mass spectrometric analyses. We therefore carried out the heme release, and also obtained complete reaction, using aqueous acetonitrile containing TFA and mercury chloride in appropriate concentrations (see Section 2.2). The mass of the apoprotein determined by MALDI-MS was 13 874.2 Da. The mass difference of 419.4 Da corresponds to the removal of a single heme (calculated M_r 615.7) and the incorporation of one mercury ion (calculated M_r 200.7).

3.2. Sequence analysis of the cytochrome c″

The complete sequence of the cytochrome *c*″ (Fig. 2) was obtained as follows. The first 61 amino acid residues have previously been established [11]. Ami-

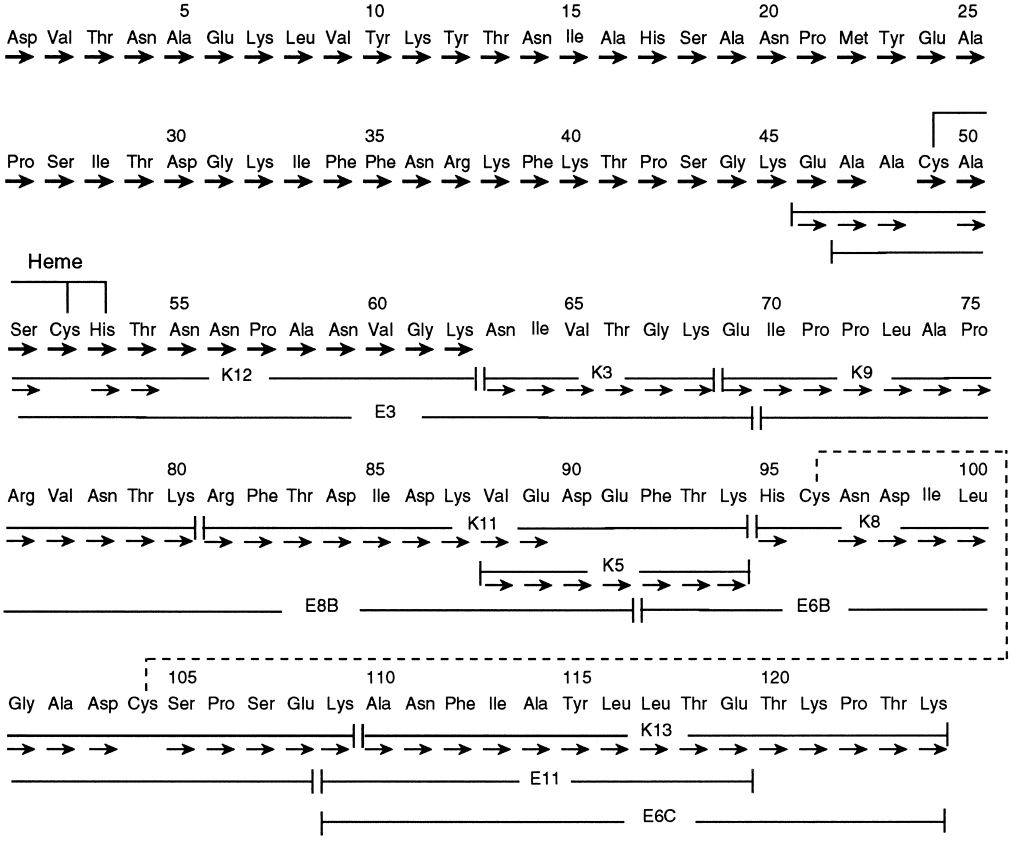

Fig. 2. Amino acid sequence of cytochrome *c*″ from *M. methylotrophus*. Bold arrows indicate residues as determined by Costa et al. [11]. Edman degradation of peptides obtained after digestion of native cytochrome *c*″ with Lys-C endoproteinase (prefix K) are presented by plain arrows. Peptides generated by Glu-C digestion of apocytochrome (prefix E), are also included in the figure confirming, through mass analysis, the connection between different Lys-C peptides.

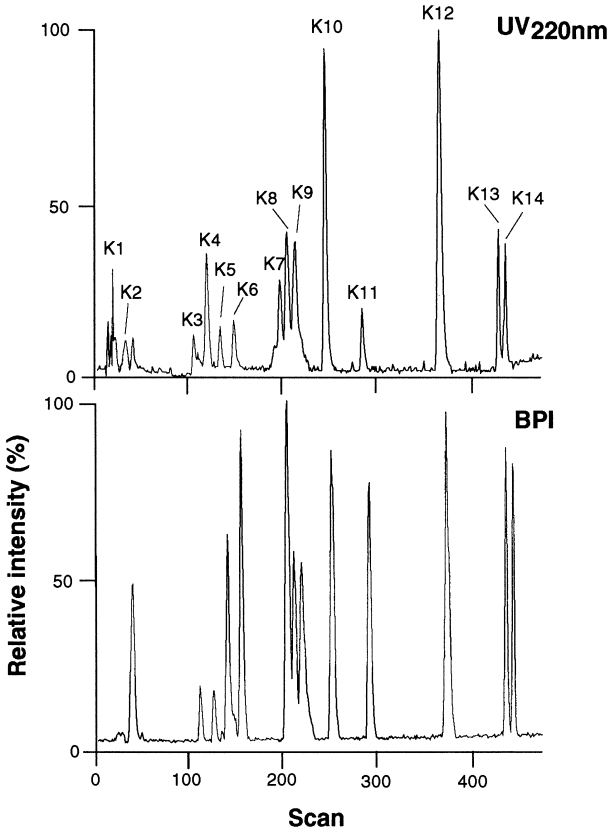

Fig. 3. On-line LC-ESMS analysis of peptides obtained after Lys-C digestion of holocytochrome *c*″. An amount of 1.2 nmol was injected. The *y*-axis represents the UV-absorbance (0.2 absorbance units full-scale) and the relative base peak intensity (BPI), respectively. The *x*-axis shows the number of mass spectra acquired during the analysis (one scan corresponds to 6 s).

no acid analysis of the holoprotein [9], having revealed the presence of 16 lysine residues, indicated Lys-C endoproteinase to be a suitable enzyme for proteolysis. To determine the optimal conditions for this cleavage, the digestion was monitored in a time-course experiment by capillary LC-ESMS (data not shown). The results showed that nearly complete hydrolysis was achieved after 3 h, allowing the identification of all the unknown peptides which, together with the known N-terminal sequence, revealed that the complete sequence of the cytochrome was covered. Preparative on-line LC-ESMS analysis yielded 15 peaks (Fig. 3) of which the mass of 14 peptides could be determined (Table 1). The molecular weight information and the specificity of the protease used allowed unambiguously assignment of peptides K1, K2, K4, K7 and K10 within the N-terminal sequence. Detection of the peptides at 370 nm (results not shown) indicated that fragment K12 (Table 1) was the candidate heme-binding peptide. The difference between the measured and the expected molecular mass of 71.3 Da suggested the presence of one alanine residue, not mentioned when the N-terminal sequence was reported [11]. Edman degradation of the first four residues confirmed this assumption (Fig. 2), whereas all the remaining peptides except one could be entirely sequenced (Fig. 2). Edman degradation of peptide K8 revealed two blank cycles. The difference between the calculated and measured molecular weight indicated the presence of two cysteine residues forming a disulphide bridge. This was further confirmed by MALDI-MS analysis of the peptide before and after proteolysis with subtilisin (Fig. 4). An increase of 18 Da in the molecular mass was observed for the digested peptide which is consistent with a cleavage between the two cysteines and the addition of one water molecule.

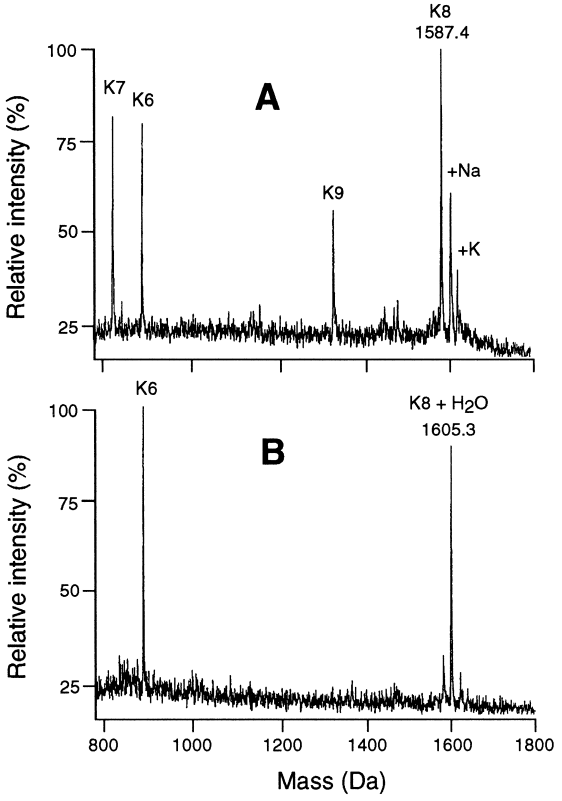

Fig. 4. Laser desorption ionization mass spectrum of fraction K8 (Fig. 2 and Table 1) before (A) and after (B) subdigestion with subtilisin.

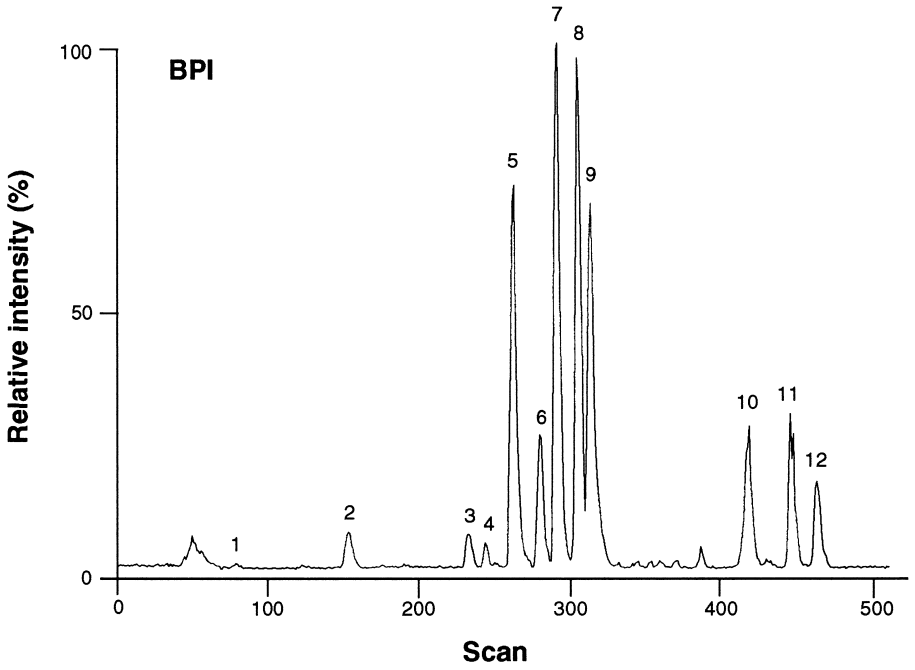

Fig. 5. Capillary LC-ESMS analysis of peptides generated by Glu-C endoproteinase digestion of the apocytochrome *c″*. About 10 pmol was injected.

The sequence order of the Lys C-peptides K3, K8, K9 and two partially digested peptides, K11 and K13, which at this point remained to be established, was obtained using the molecular weight information from a capillary LC-ESMS analysis of the apocytochrome after limited V8 protease digestion (Fig. 5, Table 2).

Table 1
LC-ESMS analysis of peptides derived from endoproteinase Lys-C digestion of native cytochrome *c″*

HPLC fraction	Measured[a] M_r (Da)	Calculated[b] M_r (Da)	Sequence position
K1	488.5	488.5	41–45
K2	775.9	775.8	1–7
K3[c]	630.6	630.7	63–68
K4	521.6	521.7	8–11
K5[c]	867.0	866.9	88–94
K6	894.1	894.0	81–87
K7	824.2	824.0	33–38
K8[c,d]	1 587.0	1 586.7	95–109
K9[c]	1 335.1	1 334.6	69–80
K10	2 280.6	2 280.5	12–32
K11[c]	1 743.3	1 742.9	81–94
K12[c,e]	2 302.8	2 303.3	46–62
K13[c]	1 710.4	1 710.0	110–124
K14	1 383.7	1 383.6	110–121

[a]The measured masses are obtained from the LC-ESMS analysis shown in Fig. 2.
[b]The average M_r weights were calculated using Biolynx (Micromass).
[c]Peptides submitted to Edman degradation.
[d]Peptide containing intra-sulfide bridge at positions 96 and 104.
[e]Heme-containing peptide.

Table 2
Mass data from the LC-ESMS analysis of peptides obtained after Glu-C endoproteinase digestion of $HgCl_2$-treated apocytochrome *c″*

HPLC fraction	Measured[a] M_r (Da)	Calculated M_r (Da)	Sequence position
E1	647.5	647.6	1–6
E2	602.4	602.3[b]	25–30
E3	2498.0	2498.2[c]	47–69
E5	1884.6	1884.2	31–46
E6			
A	602.4	602.7	85–89
B	1834.9	1835.0[d]	92–108
C	1837.6	1838.2	109–124
E7	2142.8	2145.5	7–24
E8			
A	2469.4	2468.8	25–46
B	2554.2	2553.9	70–91
E9	5034.6	5034.1[c]	47–91
E10	13877.0	13876.1[c,d]	1–124
E11	1282.5	1282.5	109–119
E12	3099.6	3099.5[d]	92–119

[a]The molecular masses are derived from the LC-ESMS analysis shown in Fig. 3.
[b]The sequence was confirmed from a separate LC-ESMS/MS analysis (data not shown).
[c]One Hg atom (M_r 200.6) is included in the calculated molecular weight.
[d]Peptide containing intra-sulfide bridge at positions 96 and 104.

4. Discussion

Cytochrome *c″* contains 124 residues and has a calculated average mass of 14293.0 Da which is in excellent agreement with the mass determined by ESMS (14292.9 ± 1.2 Da).

The primary structure of the cytochrome *c″* from *M. methylotrophus* has many remarkable features. Firstly, although it contains only one 'classical' heme-binding site (Cys X X Cys His) and has a polypeptide chain length of around 120 amino acids, which is typical for some class I cytochromes occurring in prokaryotes [14], the position of this heme-binding site is preceded by 48 residues instead of the 'prototypic' N-terminal stretch of 15–17 residues. Secondly, cytochrome *c″* contains a pair of cysteines in addition to the two cysteines involved in the heme-binding site; they are located at positions 96 and 104. The occurrence of two additional cysteine residues, linked by a disulphide bridge, is an extremely rare phenomenon in monoheme cytochromes and is in fact only documented in the cytochromes c_5 from

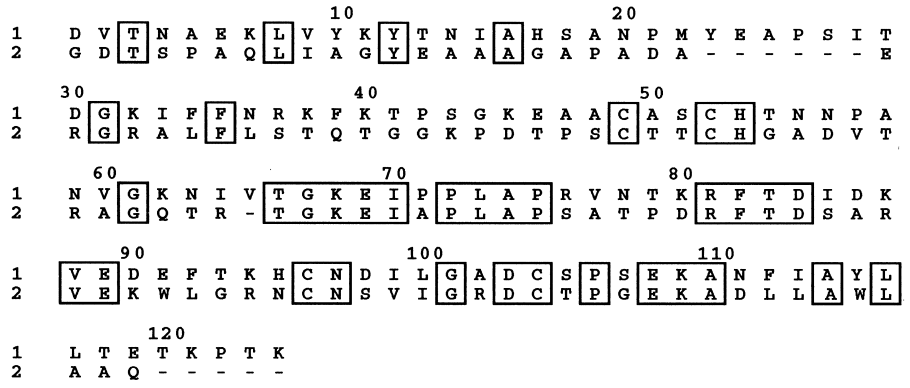

Fig. 6. Alignment of *Methylothrophus methylophilus* cytochrome *c″* (1) with the oxygen-binding heme protein (SHP) from *Rhodobacter sphaeroides* (2).

Azotobacter and *Pseudomonas* species. In the latter case, however, there are only two residues that separate the cysteines [15]. Thirdly, there is no methionine in the C-terminal part of the polypeptide chain which can take the role of the sixth heme ligand. A methionine does occur in the low-spin cytochromes of class I where it is separated from the fifth heme ligand histidine by about 40 residues (as in the cytochromes c_5).

Although these structural features are unusual, they do not appear to be unique to cytochrome *c*″. We recently determined the covalent structure of an oxygen-binding heme protein (SHP) from the purple phototrophic bacterium *Rhodobacter sphaeroides* in which the heme-binding site is also located near the center of the protein [13]. Although the physiological role of SHP is still unknown, it has been demonstrated that this cytochrome transiently binds oxygen at physiological pH during autoxidation [16].

A BLAST search [17] found weak homology between them, but failed to identify other possible homologs. As shown in the alignment of Fig. 6, we have postulated a single 6-residue insertion in cytochrome *c*″ near the N-terminus. Only a single-residue insertion is necessary between the heme-binding site and the disulfide. Nevertheless, there is only 32% overall identity between both proteins. There are nine identical residues in the midsection which increases the likelihood that these two proteins are, in fact, homologous.

A case for homology with class I cytochromes was previously made for SHP [13]. If SHP and cytochrome *c*″ are related to this class of redox proteins, then the sixth heme ligand to cytochrome *c*″ should be in the vicinity of the disulfide. There is a His at position 95 in cytochrome *c*″ immediately in front of the first cysteine of the disulfide, but the analogous residue in SHP is an Asn. This difference may be expected because the sphaeroides protein is always high-spin and should have a weak-field ligand or no ligand at all. Thus, His-95 in cytochrome *c*″ has a p*K* of 2.4 in the oxidized protein and of 8.1 in the reduced protein consistent with its role as the heme sixth ligand only in the oxidized state [9,10].

To expand upon the theme that cytochrome *c*″ and SHP may be variant class I cytochromes, we performed secondary structure calculations according to Chou and Fasman [18]. Four regions of cytochrome *c*″ are predicted to be helical, the latter two flanking the His-95 presumed heme ligand and the disulfide. Class I cytochromes all contain helices in these locations. In the case of strong structural relationship, the C-terminal helix should also contain some consensus residues for class I cytochromes, i.e. five hydrophobic residues in a row including a penultimate aromatic residue. Cytochrome *c*″ contains a FIAYL sequence (112–116) whereas SHP contains a LLAWL sequence, which matches the consensus very nicely. Although Chou–Fasman analysis does not predict an N-terminal helix in both proteins, such a helix is present in all class I cytochromes *c* and it should contain the consensus pattern of a Gly being separated from a aromatic residue by three residues occurring downstream. This signature is present in both proteins but it is separated from the heme-binding site by 13 instead of the usual three residues. This suggests that cytochrome *c*′ and SHP, relative to class I cytochromes *c*, contain a 10-residue insertion between the presumed N-terminal helix and the heme. We conclude from all of the above analyses that cytochrome *c*″ and SHP are very likely variant class I cytochromes, more exactly that they may be variants of cytochrome c_5. Three-dimensional structure analysis is needed to confirm this proposal.

Acknowledgements

The corresponding author is indebted to the FWO-Vlaanderen (Fund for Scientific Research-Flanders) for research grant G005497. David Leys is a Research Assistant of the same institution.

References

[1] G.R. Moore, G.W. Pettigrew, Cytochromes c – Evolutionary, Structural Physiochemical Aspects, Springer-Verlag, Berlin, 1990, ISBN 3-540-50852-X.
[2] V. Fülöp, C.J. Ridout, C. Greenwood, J. Hajdu, Structure 3 (1995) 1225–1233.
[3] G.W. Pettigrew, Biochim. Biophys. Acta 1058 (1991) 25–27.
[4] W. Hu, G. Van Driessche, B. Devreese, C.F. Goodhew, D.F. McGinnity, N. Saunders, V. Fulop, G.W. Pettigrew, J.J. Van Beeumen, Biochemistry 36 (1997) 7958–7966.

[5] D.F. Gaul, G.O. Gray, D.B. Knaff, Biochim. Biophys. Acta 723 (1983) 333–339.
[6] H. Santos, D.L. Turner, Biochim. Biophys. Acta 954 (1988) 277–286.
[7] M.J. Berry, S.J. George, A.J. Thomson, H. Santos, D.L. Turner, Biochem. J. 270 (1990) 413–417.
[8] H.S. Costa, H. Santos, D.L. Turner, Eur. Biophys. J. 25 (1996) 19–24.
[9] H.S. Costa, H. Santos, D.L. Turner, A.V. Xavier, Eur. J. Biochem. 208 (1992) 427–433.
[10] M. Coletta, H. Costa, G. De Sanctis, F. Neri, G. Smulevich, D.L. Turner, H.J. Santos, Biol. Chem. 272 (1997) 24800–24804.
[11] H.S. Costa, H. Santos, D.L. Turner, Eur. J. Biochem. 215 (1993) 817–824.
[12] M.T. Davis, T.D. Leu, Protein Sci. 1 (1992) 935–944.
[13] K. Klarskov, K. Backers, C. Dumortier, G. Van Driessche, T.E. Meyer, G. Tollin, M.A. Cusanovich, J.J. Van Beeumen, Biochemistry 37 (1998) 5995–6002.
[14] R.P. Ambler, Biochim. Biophys. Acta 1058 (1991) 42–47.
[15] R.P. Ambler, J. Hermoso, T.E. Meyer, R.G. Bartsch, M.D. Kamen, Nature 278 (1979) 659–660.
[16] T.E. Meyer, M.A. Cusanovich, Biochim. Biophys. Acta 807 (1985) 308–319.
[17] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, J. Mol. Biol. 215 (1990) 403–410.
[18] P.Y. Chou, G.D. Fasman, Biochemistry 13 (1974) 222–245.