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PrrC from *Rhodobacter sphaeroides*, a homologue of eukaryotic Sco proteins, is a copper-binding protein and may have a thiol-disulfide oxidoreductase activity

Alastair G. McEwan[a,*], Allison Lewin[b], Sharon L. Davy[b], Ruth Boetzel[b], Andrew Leech[c], Daniel Walker[c], Tania Wood[a], Geoffrey R. Moore[b,*]

[a] *School of Molecular and Microbial Sciences, The University of Queensland, Brisbane, Qld 4072, Australia*
[b] *School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ, UK*
[c] *School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK*

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Abstract PrrC from *Rhodobacter sphaeroides* provides the signal input to a two-component signal transduction system that senses changes in oxygen tension and regulates expression of genes involved in photosynthesis (Eraso, J.M. and Kaplan, S. (2000) Biochemistry 39, 2052-2062; Oh, J.-I. and Kaplan, S. (2000) EMBO J. 19, 4237-4247). It is also a homologue of eukaryotic Sco proteins and each has a C-x-x-x-C-P sequence. In mitochondrial Sco proteins these cysteines appear to be essential for the biogenesis of the Cu_A centre of respiratory cytochrome oxidase. Overexpression and purification of a water-soluble and monomeric form of PrrC has provided sufficient material for a chemical and spectroscopic study of the properties of the four cysteine residues of PrrC, and its ability to bind divalent cations, including copper. PrrC expressed in the cytoplasm of *Escherichia coli* binds Ni^{2+} tightly and the data are consistent with a mononuclear metal site. Following removal of Ni^{2+} and formation of renatured metal-free rPrrC (apo-PrrC), Cu^{2+} could be loaded into the reduced form of PrrC to generate a protein with a distinctive UV-visible spectrum, having absorbance with a λ_{max} of 360 nm. The copper:PrrC ratio is consistent with the presence of a mononuclear metal centre. The cysteines of metal-free PrrC oxidise in the presence of air to form two intramolecular disulfide bonds, with one pair being extremely reactive. The cysteine thiols with extreme O_2 sensitivity are involved in copper binding in reduced PrrC since the same copper-loaded protein could not be generated using oxidised PrrC. Thus, it appears that PrrC, and probably Sco proteins in general, could have both a thiol-disulfide oxidoreductase function and a copper-binding role. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: PrrC; Sco; Cytochrome oxidase; Photosynthesis response regulation; Copper protein

1. Introduction

Bacteria that stain positive in the 'oxidase' test have a respiratory pathway which has an organisation similar to that of the mitochondrion, in which a terminal oxidase is downstream of a cytochrome bc_1 complex [1]. Some of these bacteria possess a cytochrome aa_3 that contains identical redox centres and core polypeptides that are homologous to those of the mitochondrial oxidase: subunit I contains haem *a* and the haem a_3-Cu_B binuclear centre, and subunit II contains the binuclear Cu_A centre [2,3]. The Cu_A centre faces the periplasm in Gram-negative bacteria and accepts electrons from a cytochrome *c* [4]. More commonly, a cytochrome cbb_3 is found in oxidase-positive bacteria in addition to, or instead of, cytochrome aa_3 [5]. Cytochrome cbb_3 is also a member of the cytochrome oxidase superfamily, and its subunits I and II are similar to those of cytochrome aa_3. The haem-containing redox centres of the two oxidases are also similar, except that haem *b* is present in place of haem *a* in cytochrome cbb_3. However, cytochrome cbb_3 lacks the Cu_A centre, whose function is taken by a dihaem *c*-type cytochrome.

Genome sequence analysis [6–8] has revealed that two species of bacteria in the genus *Rhodobacter* and several other oxidase-positive bacteria also possess a homologue of the mitochondrial Sco proteins. In yeast, Sco1 has been shown to be essential for the correct assembly of a functional cytochrome aa_3, probably through assisting with the acquisition of copper by cytochrome aa_3 [9–11]. Support for this view comes from the observation that overexpression of Sco1 can suppress the phenotype of a mutant which lacks the copper chaperone COX17, which is involved in transfer of copper from the cell cytosol to the mitochondrial intermembrane space [9]. Recently, it was shown that YpmQ, the Sco homologue of *Bacillus subtilis*, is essential for formation of cytochrome aa_3 [12] and it was suggested that it is required for provision of copper for the formation of the Cu_A centre of cytochrome aa_3. The situation in *Rhodobacter* species is less clear. Mutation of the *sco* homologue in *Rhodobacter capsulatus*, *senC*, resulted in a mutant that was oxidase-negative in the classical microbiological test [6] and since *R. capsulatus* only possesses cytochrome cbb_3, which lacks the Cu_A centre, this might have suggested that *senC* was required for formation of Cu_B. However, Eraso and Kaplan [8] have subsequently shown that in *Rhodobacter sphaeroides*, mutants lacking the Sco homologue

*Corresponding authors. Fax: (61)-7-3365 4620; (44)-1-603 592697.
E-mail addresses: mcewan@mailbox.uq.edu.au (A.G. McEwan), g.moore@uea.ac.uk (G.R. Moore).

Abbreviations: PrrC, the Sco homologue from *R. sphaeroides*; ht-PrrC, his-tagged recombinant PrrC; rPrrC, recombinant PrrC starting at residue 25 of PrrC and with a five amino acid extension at the N-terminus; apo-PrrC, metal-free rPrrC; ESI-MS, electrospray ionisation mass spectrometry; NTA, nitrilotriacetic acid; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol

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PrrC are not affected in cytochrome cbb_3 provided there is no polar affect on the expression of the transcriptional regulator PrrA that lies downstream of PrrC. Furthermore, it appears that biogenesis of the Cu_B centre requires a distinct assembly protein, Cox11p, in mitochondria and *R. sphaeroides* [13]. The presence of a Sco homologue in a bacterium that lacks an oxidase with a Cu_A centre is indicative of one or more functions for bacterial Sco proteins that are additional to copper acquisition, and these include the sensing of oxygen and control of the transcription of photosynthesis (PS) genes in purple phototrophic bacteria [6–8,14].

Genetic and physiological analyses of yeast and bacterial systems have identified the possible function of Sco proteins without providing any molecular details. A deeper understanding of the mechanism of action of proteins in this family would be greatly helped by biophysical studies of a Sco protein, since bioinformatic approaches in this context can only be indicative. Protein sequence analysis has identified a key region of sequence identity (C-x-x-x-C-P) that is conserved in all mitochondrial Sco proteins and their bacterial homologues. Initially, it was suggested that the C-x-x-x-C-P motif might be a part of a ferredoxin-like [Fe–S] centre [6]. Later it was suggested that this motif might form part of a copper centre similar to the binuclear Cu_A sites of cytochrome aa_3 [9], a proposal that would be consistent with a role for Sco proteins in copper metabolism. Most recently, secondary structure prediction for Sco proteins has revealed that they have similarities to peroxiredoxin/thiol-disulfide oxidoreductases, which led to the proposal that the C-x-x-x-C-P region is involved in thiol-disulfide oxidation reduction [15]. Mitochondrial and bacterial Sco proteins are known to be membrane-bound [2–4,8,12] and this can provide a significant restriction to biophysical studies. In this paper we describe the overexpression and purification of a water-soluble form of the Sco homologue from *R. sphaeroides*, PrrC, together with spectroscopic and chemical investigations of its ability to bind metal ions. We show that it does bind first-row transition metal ions, including copper.

2. Materials and methods

2.1. Expression and purification of PrrC

The cloning strategy for expression of a water-soluble form of PrrC involved amplification of the *prrC* gene from nucleotide 73 in the coding sequence. Oligonucleotide primers were designed with flanking restriction enzyme sites: the forward primer was 5′-GCGGG-ATCCTTCCTCGGCAGATCCGAA-3′ with a *Bam*HI restriction site and the reverse primer was 5′-GAAAAGCTTATCCTCAGC-CATGATTGT-3′ containing a *Hin*dIII restriction site. Approximately 1 ng of plasmid pJE1404, harbouring the *prr* gene cluster [7] was used as a template in a 50 μl reaction volume containing 200 μM of each dNTP, 445 ng each of forward and reverse primers, 2.4 units *Taq* polymerase and reaction buffer supplied by the manufacturer (New England Biolabs, Beverly, MA, USA) with 1.5 mM $MgCl_2$. PCR amplification was performed using an initial denaturation step of 2 min at 94°C followed by 30 cycles of 1 min at 42°C and 6 min at 72°C. Following amplification, samples were incubated at 72°C for 10 min and then cooled to 4°C. The 625 bp PCR product was purified using QIAquick PCR purification kit (Quiagen, Chatsworth, CA, USA). The truncated *prrC* gene was cloned into the *Bam*HI and *Hin*dIII sites of the expression plasmid pPROEX HT b (Gibco BRL, Grand Island, NY, USA) to generate plasmid pPRRC1 and transformed into *Escherichia coli* BL21. The resulting construct harbours a gene encoding a truncated PrrC protein, comprising of 212 of the 231 residues of intact PrrC, with an N-terminal six-histidine tag.

32 ml of an overnight culture of *E. coli* BL21 (pPRRC1) was used to inoculate 5 l LB medium (8×625 ml in 2.5 l flasks) containing 100 μg/ml ampicillin. The cultures were shaken at 37°C until they reached an OD_{550} of 0.8 then expression of *prrC* was induced by addition of IPTG to 1 mM. Growth of the cultures continued for a further 2.5 h, and then cells were harvested, washed once in 50 mM Na_2PO_4 (pH 8.0) containing 300 mM NaCl and frozen as pellets at −20°C. Frozen cells were resuspended in lysis buffer (50 mM Na_2PO_4 (pH 8.0) containing 300 mM NaCl) containing 5 mM imidazole to a volume of 70 ml, and cells broken by sonication. Membranes and unbroken cells were removed by centrifugation. The his-tagged PrrC (ht-PrrC) was purified by affinity chromatography using a BIOCAD-Sprint system. The sample was charged onto a Ni-nitrilotriacetic acid (NTA) column, washed with lysis buffer containing 50 mM imidazole, and the ht-PrrC was eluted with 400 mM imidazole in the same buffer. Chromatography fractions were analysed by SDS–PAGE and SELDI-MS to identify those containing ht-PrrC, and these fractions were pooled and dialysed against 50 mM Tris–HCl (pH 8.0). The histidine tag of ht-PrrC was cleaved using rTEV protease (Gibco BRL) according to the manufacturer's instructions except that a ratio of approximately 100 units of protease per 5 mg ht-PrrC protein was used. The cleavage reaction was carried out at 4°C for 40 h. Cleavage of the ht-PrrC was confirmed by SELDI-MS analysis. Cleaved recombinant PrrC (rPrrC) was resolved from ht-PrrC by chromatography on a Ni-NTA column as described above; fractions containing rPrrC did not bind to the column but were eluted in the charging and washing step with lysis buffer containing 5 mM imidazole. The final purification step for rPrrC was dialysis against 50 mM Tris–HCl (pH 8.0).

2.2. Protein and metal analysis

Purity of PrrC preparations was determined by SDS–PAGE [16] and by mass spectrometry. Electrospray ionisation mass spectra (ESI-MS) were collected on a Micromass platform I mass spectrometer calibrated with horse heart myoglobin. The solvent used was 1:1 acetonitrile and water containing 0.1% formic acid, and samples were run at a flow rate of 20 μl min^{-1}. Native gels were run with a discontinuous buffer system [17]. The N-terminal amino acid sequence and the amino acid composition of metal-free rPrrC (apo-PrrC) were determined by AltaBioscience (University of Birmingham, Birmingham, UK). Analytical ultracentrifugation was performed on a Beckman-Coulter Optima XL/I using 12 mm path length double sector charcoal filled Epon cells, monitoring protein absorbance at 280 nm. Three loading concentrations were used and two or more rotor speeds in each experiment. Data were analysed using software supplied by Beckman. UV-visible electronic absorption spectra were collected with Perkin Elmer Lambda 900 and Hitachi U2000 spectrophotometers, using a 1 cm pathlength. X-band EPR spectra were measured at the University of Essex (Colchester, UK), using a Bruker EMX spectrometer equipped with a flow cryostat and spherical resonator. Circular dichroism spectra were collected with an Applied Photophysics π* spectrometer and NMR spectra with a Varian Unity-Inova 500 or 600 MHz spectrometer. Metal ion analyses were carried out with Thermal Jarrell Ash Polyscan 61e and Varian Vista Pro axial ICP-OES spectrometers. The former was equipped with a radial torch and employed a polychromator system with photomultiplier tube detector, and the latter was equipped with an axial torch and CCD detector for multi-wavelength measurements.

2.3. Reactivity of cysteines

Determination of the free thiol content of metal-free PrrC was carried out using Ellmans' reagent, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) [18]. 0.9 ml of 14 μM PrrC in 50 mM Tris–HCl buffer (pH 8.0) and 0.9 ml of the same buffer was used in the reference cuvette. A freshly prepared solution of DTNB was added to both cuvettes to give a final concentration of 1.5 mM DTNB. After mixing, the absorbance at 412 nm was recorded as a function of time until no further change was seen. The concentration of free thiol was calculated by assuming one molecule of 2-nitro-5-thiobenzoate anion $(TNB)^-$ is released for every free thiol in the protein, with an extinction coefficient (ε_{412}) of 14 000 M^{-1} cm^{-1} [18].

The free thiol contents of native and unfolded PrrC were determined as follows. A 60 μM solution of PrrC in 50 mM Tris–HCl (pH 8.0) was treated overnight with 20 mM dithiothreitol (DTT), then the reducing agent was removed by passing the sample (0.5 ml) down a 20 ml Sephadex G15 desalting column equilibrated with

50 mM Tris–HCl (pH 8.0). The eluted protein was tested for free thiols as above. The treatment with DTT was repeated with 6 M guanidinium hydrochloride in all buffers in order to unfold the PrrC, and the thiol determination carried out as for PrrC treated with DTT alone, with a value of 13 700 M^{-1} cm^{-1} for ε_{412} [18]. Alkylated PrrC was obtained by reacting the DTT-reduced protein in 50 mM Tris–HCl (pH 8.0) with a 10-fold excess of iodoacetamide, followed by gel filtration on a PD-10 column (Pharmacia) to remove excess reagent and DTT.

3. Results

3.1. Expression and purification of a water-soluble form of PrrC

Analysis of the secondary structure of PrrC predicts that it has a single membrane-spanning helix at the amino terminus [7]. The remainder of the protein is predicted to be mainly α-helical and not to be hydrophobic in character. Recent topological studies using PrrC::PhoA protein fusions are consistent with this prediction, and they indicate that PrrC is anchored to the cytoplasmic membrane via its amino terminus while the remainder of the protein protrudes into the periplasm [8]. The topology of PrrC suggested that it should be possible to produce a water-soluble form of this protein if it was expressed in the absence of its hydrophobic amino terminus. Thus, a fragment of the *prrC* gene lacking the first 72 nucleotides was cloned into an expression vector. Preliminary experiments showed that the ht-PrrC could be expressed at high levels in *E. coli* and confirmed that the truncated recombinant protein was water-soluble and located in the cytoplasm (data not shown). ht-PrrC was purified by affinity chromatography as described in Section 2 and the six-histidine tag was then removed by proteolysis. rPrrC was separated from intact ht-PrrC by a second round of affinity chromatography. SDS–PAGE analysis showed that ht-PrrC had a higher molecular mass than rPrrC (Fig. 1) and this was consistent with SELDI mass spectrometric analysis of both proteins (data not shown). The preparation of rPrrC was homogeneous, as indicated by SDS–PAGE (Fig. 1) and N-terminal sequencing of the protein in solution, which identified a single amino acid sequence. The sequence of the N-terminus was Gly-Ala-Met-Gly-Ser-Phe. This corresponds to the sequence of PrrC beginning at residue Phe25 with a five amino acid N-terminal appendage remaining from the rTEV protease cleavage of the ht-PrrC. Electrospray mass spectrometry of rPrrC gave a mass that was 1 Da more than the expected mass as calculated from the predicted sequence of oxidised rPrrC (23 417 Da).

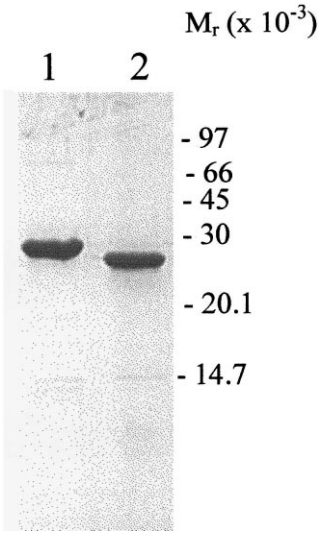

Fig. 1. Coomassie-stained SDS–PAGE gel of purified PrrC. Lane 1, 5 μg ht-PrrC; lane 2, 5 μg rPrrC.

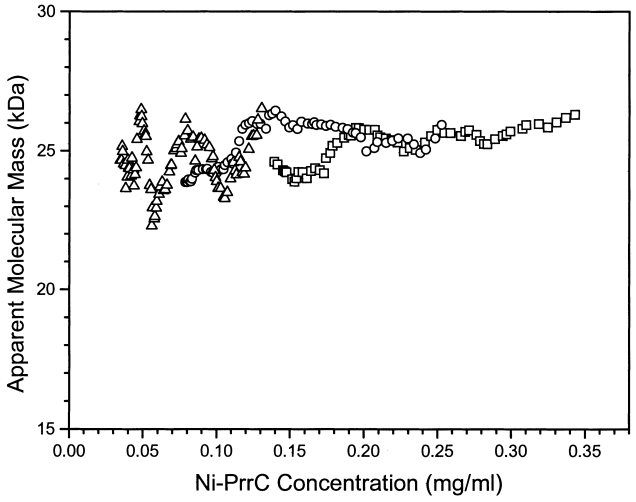

Fig. 2. Sedimentation equilibrium analytical ultracentrifugation analysis of PrrC. Equilibrium distributions from three different loading concentrations (triangle, 0.12 mg/ml; circle, 0.23 mg/ml; and square, 0.47 mg/ml) plotted as apparent weight-average molecular mass distributions.

3.2. rPrrC as isolated contains a bound metal ion

Preparations of rPrrC were red-pink and it was assumed that this colour must arise from the absorption of a metal-containing centre. Metal ion analyses revealed that the purified protein contained a considerable amount of bound metal ions, especially Ni and Fe (data not shown). Adventitious metal ions were removed by dialysis as described in Section 2 with the resulting protein retaining its red-pink colour. The UV-visible electronic absorption spectrum of rPrrC after dialysis exhibited absorption bands at 385 nm with a very weak absorbance band at 550 nm (Fig. 3). Metal analysis showed that this form of PrrC, henceforth known as Ni-PrrC, contained 0.8 g atom of nickel per mol of protein. The metal ions did not fly with the rPrrC in the mass spectrometer. Sedimentation equilibrium experiments revealed that the Ni-rPrrC was monomeric and gave no evidence for oligomerisation (Fig. 2). This indicates that ligands to the bound nickel are provided by a single polypeptide.

Ni-rPrrC did not show an EPR signal with a conventional X-band EPR spectrometer at 10 K, indicating that the nickel is not mononuclear Ni^{+} or Ni^{3+}. [^{1}H]NMR spectra of Ni-PrrC did not show any hyperfine-shifted resonances arising from ligands to paramagnetic Ni^{2+} (data not shown). In general, such signals are readily detected [19,20] and their absence suggests the nickel is present in a diamagnetic state. This could come about from mononuclear Ni^{2+} in a square-planar environment [21] or arise from magnetic coupling between two or more Ni^{2+} ions. However, the stoichiometry of ~0.8 Ni^{2+} per rPrrC and the monomeric state of rPrrC rules out magnetically coupled Ni^{2+} ions. Square-planar Ni^{2+} complexes are diamagnetic and generally red to yellow in colour, in contrast to the green/blue colour of octahedral and tetra-

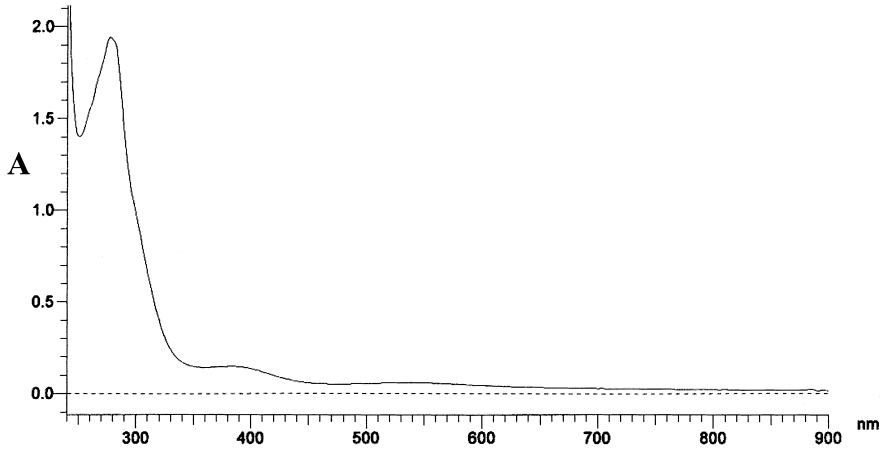

Fig. 3. UV-visible spectrum of Ni-rPrrC (110 μM) in 50 mM Tris–HCl pH 8.0.

hedral Ni^{2+} complexes, as a result of d–d transitions in the visible region of the spectrum [21]. Thus, the present spectroscopic data for Ni-PrrC suggest a planar environment for the metal ion.

3.3. Formation of apo-PrrC

The nickel present in rPrrC is a result of the use of the Ni^{2+} affinity column. It is unlikely to be a physiologically relevant metal ion as far as PrrC is concerned, and if there is such an ion it is likely to be copper for the reasons given in Section 1. Incubation of rPrrC in 6 M Gdn–HCl, 2 mM EDTA and 2 mM DTT overnight at 25°C removed all visible absorption arising from bound metal ions. The protein was renatured by dialysis against 2 M urea, 2 mM EDTA and 2 mM DTT, 50 mM Tris–HCl (pH 8.0) and finally against 50 mM Tris–HCl (pH 8.0), 2 mM DTT. The absence of absorbance at wavelengths greater than 300 nm suggested that nickel had been lost from this preparation. This form of the protein is henceforth known as apo-PrrC. The absence of transition metal ions in apo-PrrC was confirmed by metal analysis. One-dimensional [^{1}H]NMR spectra (Fig. 4) and two-dimensional NMR spectra (data not shown) of metal-free PrrC and Ni-PrrC confirmed that renaturation resulted in apo-PrrC becoming fully refolded. CD spectroscopy of apo-PrrC also confirmed that it had refolded (data not shown). The molecular mass of apo-PrrC determined by ESI-MS was 23 420 Da, 3 Da more than the predicted value. Resolved resonances in the [^{1}H]NMR spectra of Ni-PrrC and apo-PrrC had similar linewidths (Fig. 4), suggesting that apo-PrrC is like Ni-PrrC in being monomeric.

3.4. Reactivity of cysteine residues in apo-PrrC

Initial attempts to bind Cu^{2+} to apo-PrrC did not give a product with any notable UV-visible spectrum. These reconstitution experiments were carried out using apo-PrrC that had been reduced with DTT but had been exposed to O_2 in solutions after removal of excess DTT. Therefore, we investigated the redox state of cysteine residues in apo-PrrC by determining whether they were able to react with DTNB [18]. It was found that the cysteines in the protein could be considered fully oxidised, with less than 0.5 thiol per protein free to react. Given that the same protein is a monomer, it was assumed that the cysteines were forming intramolecular disulfides. An attempt was made to reduce the cysteines with

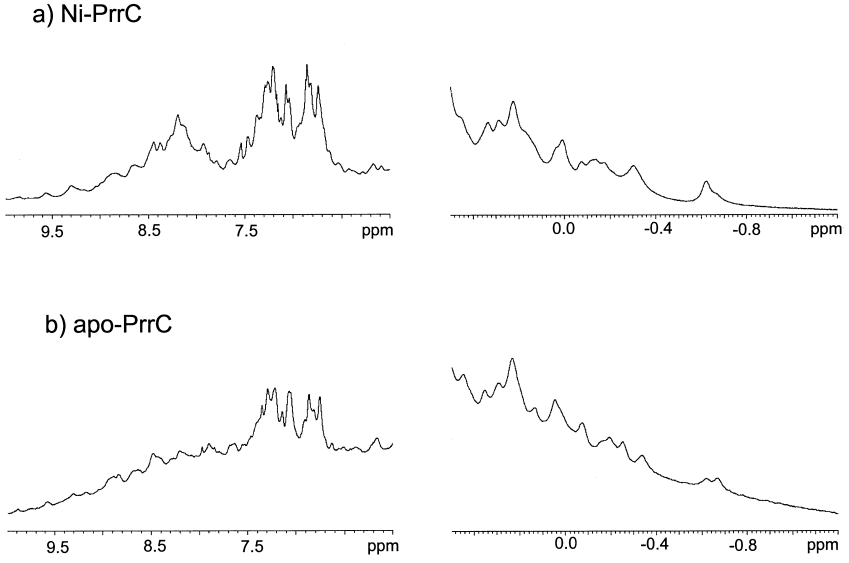

Fig. 4. 600 MHz [^{1}H]NMR spectra of (a) Ni-PrrC and (b) apo-PrrC in 50 mM Tris (pH 7.0) and at 25°C.

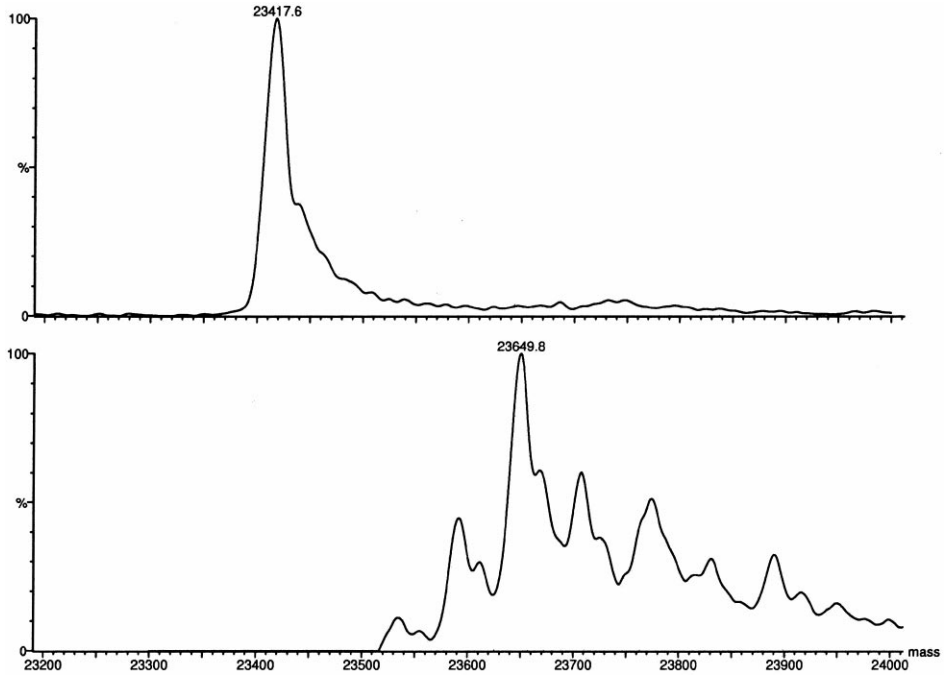

Fig. 5. Electrospray mass spectrometry of native and iodoacetamide modified PrrC. Top: Native PrrC as prepared. Bottom: The major component of the iodoacetamide modified protein has a mass of 23 649.8 Da corresponding to modification of all four cysteine residues. The higher molecular weight peaks with lower intensity arise from species with more than four alkylated groups, presumably reflecting the reactivity of iodoacetamide with functional groups other than thiols.

DTT. Following removal of the DTT by a desalting column and immediate reaction with DTNB, two free thiols per protein were detected. Alkylation with iodoacetamide followed by ESI-MS also showed only two thiols were modified. The reduction with DTT was repeated, using 6 M Gdn–HCl to denature the apo-PrrC, and again the protein reacted with DTNB immediately following the removal of the DTT. In this case 2.0 free thiols per protein were observed. The unreactivity of the other two cysteine residues to DTNB and iodoacetamide could be due to DTT not being able to reduce the disulfide, or to extreme O_2 sensitivity of the corresponding thiols. Since the DTNB assay cannot be used in the presence of excess DTT we employed an alkylation procedure to investigate the oxidation state of the cysteines. ESI-MS of denatured rPrrC modified with iodoacetamide in the presence of DTT gave a major peak with a mass of 23 650 Da, consistent with the modification of all four cysteines in PrrC (Fig. 5). In summary, the data concerning the reactivity of the four cysteines of rPrrC indicate that they oxidise in the presence of air to form two intramolecular disulfide bonds. One pair of thiols has an extreme sensitivity to air oxidation and within minutes after the removal of excess reductant is oxidised.

3.5. Formation of Cu-rPrrC

Copper-containing DTT-reduced PrrC (Cu-rPrrC) was formed by passage of DTT-reduced apo-PrrC through a 20 ml Sephadex G15 gel filtration column, made with deoxygenated buffers and run under N_2. 100 mM $CuCl_2$ in 50 mM Tris–HCl (pH 8.0) was applied to the column and after it had passed through so that the top 25% of the column was essentially copper-free, DTT-reduced apo-PrrC was applied to the column and the protein eluted through it with 50 mM Tris–HCl (pH 8.0). The anaerobic gel filtration column removed DTT from the reduced apo-PrrC prior to it passing through the $CuCl_2$ band. Metal ion analysis showed that the eluted protein contained $\sim$0.7 Cu^{2+} per rPrrC. Cu-rPrrC ran on native polyacrylamide gels with the same mobility as the metal-free rPrrC (data not shown), consistent with it too being monomeric.

Cu-rPrrC has a distinctive UV-visible absorption spectrum with an absorbance maximum at 360 nm (Fig. 6). The addition of $CuCl_2$ to DTT-reduced apo-PrrC immediately after separation of the rPrrC from excess DTT by an aerobic gel filtration column, under conditions when reactivity toward DTNB revealed the presence of only two thiols per PrrC molecule, failed to generate the UV-visible absorption spectrum of Cu-rPrrC. Thus it appears that the cysteine thiols with extreme O_2 sensitivity are involved in copper binding.

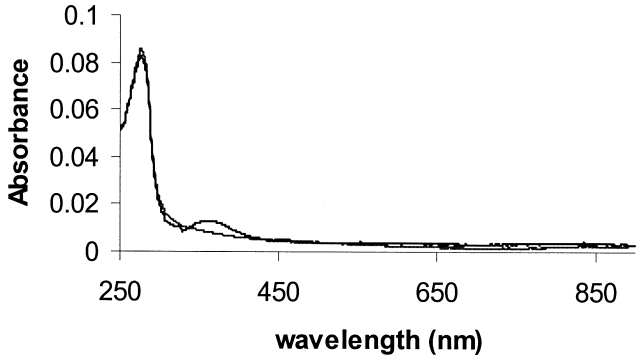

Fig. 6. The UV-visible spectrum of 5 μM apo-PrrC (broken line) and 5 μM Cu-PrrC (solid line) in 50 mM Tris–HCl (pH 8.0).

4. Discussion

Genetic and biochemical analysis, mainly in yeast, has established that the uptake of copper and its metabolism within eukaryotic cells is tightly regulated [22–24]. The avidity of Cu^+ and Cu^{2+} for nitrogen and sulfur donors and its activity as an oxidising catalyst has led to specific metallo-chaperones evolving to protect the cell against free copper and target this cation to its subcellular location for insertion into specific enzymes. All of the available evidence indicates that mitochondrial Sco1 has a central role in the provision of copper for the Cu_A centre of cytochrome aa_3 oxidase [9–11], and yeast two-hybrid screening assays show [25] that Sco proteins interact with the specific cytochrome aa_3 oxidase metallochaperone, COX17 [26,27]. Direct evidence that mitochondrial Sco proteins bind copper has only been reported since submission of this manuscript: Nittis et al. [28] have shown that a water-soluble domain of yeast Sco1 binds a single Cu^+ ion. The results presented in this paper, showing that a recombinant bacterial Sco homologue, PrrC, binds copper with the formation of a mononuclear metal centre, suggest this is a common feature of Sco proteins.

There are a number of potential metal-binding amino acids conserved in all mitochondrial Sco proteins and their bacterial homologues [8]. These include the two cysteines in the Sco C-x-x-x-C-P motif and a conserved histidine, two tyrosines and two aspartates. Cu^{2+} binds to many proteins via cysteine ligands, often becoming reduced to Cu^+ in the process. Examples include the copper chaperone Atx1 [29] and the metal-binding domains from the Menkes copper-transporting ATPase [30], both of which supply at least two cysteine thiols that bind Cu^+. Recent spectroscopic and site-directed mutagenesis studies of Sco1 are consistent with three ligands to the Cu centre being provided by the C-x-x-x-C-P motif and a conserved histidine [28]. This central role for the Sco motif cysteines in Cu binding is in agreement with previous studies that have shown that the cysteines are critical for biogenesis of the Cu_A centre of cytochrome aa_3 [9–12]. It has been suggested that Sco itself contains a Cu_A-like centre [9] but Cu-rPrrC is monomeric and only contains one copper ion. Furthermore, the UV-visible electronic absorption spectrum of Cu-rPrrC (Fig. 6) is quite distinct from that of the Cu_A centre [31].

In a theoretical study based on protein sequence analysis Chinenov [15] has drawn attention to the possibility that Sco proteins act as thiol-disulfide oxidoreductases, rather than as copper-binding proteins. A key part of the rationale for this suggestion is that secondary structure predictions for Sco proteins suggest that their tertiary structures resemble those of characterised peroxiredoxins. An important general characteristic of peroxiredoxins is that their redox active thiol-disulfide cysteines are extremely sensitive to O_2 oxidation [32], as is at least one of the pairs of cysteines of apo-PrrC. Therefore, our data are consistent with PrrC having the kind of thiol-disulfide oxidoreductase function Chinenov envisages. However, there is another possibility: PrrC, and other Sco proteins, could have both a thiol-disulfide oxidoreductase function and a copper-binding role. Similar, though not identical, bifunctional behaviour is shown by the Hsp33 chaperone protein of *E. coli* [33]. This binds Zn^{2+} at a site created by four cysteines. On oxidation of the cysteine thiols to form disulfide bonds, the Zn^{2+} is expelled from the protein. The analogy with copper binding to PrrC is not exact however. Unlike Cu-rPrrC, Zn^{2+}-containing Hsp33 is dimeric [33–36]; oxidation of the cysteines leading to the formation of two intermolecular disulfide bonds. Nevertheless, the correlation of structure and function for Hsp33 does suggest a model for the function of Sco proteins in cytochrome aa_3 oxidase assembly. On oxidation of Cu-containing Sco, one or more intramolecular disulfide bonds are formed with copper being expelled from the C-x-x-x-C-P site, either moving to a weak binding site that could be formed from conserved amino acids in Sco proteins or transferred directly to cytochrome oxidase or an appropriate assembly factor. O_2 would not be the oxidant in this scheme because the UV-visible spectrum of Cu-PrrC (Fig. 6) is stable in air.

Although there is clear evidence for a role for bacterial Sco homologues in the biogenesis of cytochrome aa_3 oxidase in *B. subtilis*, it is also apparent that the bacterial Sco protein must have additional function(s) since it is present in bacteria such as *R. capsulatus* that lack a cytochrome oxidase with a Cu_A centre. Kaplan and co-workers have presented strong evidence that PrrC in *R. sphaeroides* is the signal input into a two-component signal transduction [37] system that senses changes in oxygen tension [8,14]. They propose that the CcoQ protein interacts with PrrC and relays information regarding the rate of electron transfer via the cytochrome cbb_3 oxidase. In this way the cell is able to monitor oxygen availability by monitoring the activity of this cytochrome oxidase. They have also proposed that PrrC transmits a signal to the sensor kinase PrrB, thereby stimulating a phosphatase activity or inhibiting its kinase activity [8]. Whichever activity of PrrC is affected, it acts to dampen expression via the PrrA response regulator, a global regulator of PS gene expression. The presence of a copper centre in PrrC and the reactivity of its thiol groups suggests that this signal transduction process may involve oxidation-reduction reactions.

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