Two-iron rubredoxin of *Pseudomonas oleovorans*: production, stability and characterization of the individual iron-binding domains by optical, CD and NMR spectroscopies

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A minigene encoding the C-terminal domain of the 2Fe rubredoxin of *Pseudomonas oleovorans* was created from the parental *alk G* gene contained in the expression plasmid pKK223-3. The vector directed the high-level production of the C-terminal domain of this rubredoxin; a simple procedure was used to purify the recombinant domain in the 1Fe form. The 1Fe form of the C-terminal domain was readily converted into the apoprotein and cadmium forms after precipitation with trichloroacetic acid and resolubilization in the presence or absence of cadmium chloride respectively. In steady-state assays, the recombinant 1Fe C-terminal domain is redox-active and able to transfer electrons from reduced rubredoxin reductase to cytochrome *c*. The absorption spectrum and dichroic features of the CD spectrum for the iron- and cadmium-substituted C-terminal domain are similar to those reported for the iron- and cadmium-substituted *Desulfovibrio gigas* rubredoxin [Henehen, Pountney, Zerbe and Vasak (1993) *Protein Sci.* **2**, 1756–1764]. Difference absorption spectroscopy of the cadmium-substituted C-terminal

domain revealed the presence of four Gaussian-resolved maxima at 202, 225, 240 and 276 nm; from Jørgensen's electronegativity theory, the 240 nm band is attributable to a CysS-Cd(II) charge-transfer excitation. Attempts to express the N-terminal domain of the 2Fe rubredoxin directly from a minigene were unsuccessful. However, the N-terminal domain was isolated through cleavage of an engineered 2Fe rubredoxin in which a factor Xa proteolysis site had been introduced into the putative interdomain linker. The N-terminal domain is characterized by absorption spectra typical of the 1Fe rubredoxins. The domain is folded as determined by CD and NMR spectroscopies and is redox-active. However, the N-terminal domain is less stable than the isolated C-terminal domain, a finding consistent with the known properties of the full-length 2Fe and cadmium-substituted *Ps. oleovorans* rubredoxin.

Key words: alkaline hydroxylation, electron transfer, metalloprotein.

INTRODUCTION

The rubredoxin-reductase–rubredoxin electron transfer complex of *Pseudomonas oleovorans* is a physiological complex that donates electrons to a membrane-bound ω -hydroxylase. This redox chain is responsible for the hydroxylation of aliphatic hydrocarbons, functionalized hydrocarbons and various aromatic compounds [1–3]:

Rubredoxin reductase is a NADH-dependent flavoprotein that transfers reducing equivalents from NADH to a novel 2Fe rubredoxin [2]. The hydroxylase component receives electrons from reduced rubredoxin and is a member of the non-haem di-iron family of enzymes [4] that includes methane mono-oxygenase, toluene 4-mono-oxygenase, stearyl-acyl carrier protein Δ^9 desaturase, ruberythrin and ribonucleotide reductase.

The rubredoxins are non-haem iron proteins whose active sites comprise an iron atom tetrahedrally co-ordinated to four cysteine residues [5]. Rubredoxins serve as electron carriers and most have a molecular mass of approx. 6 kDa. Most rubredoxins are restricted to anaerobes; NADH-dependent rubredoxin reductases have been identified and partly purified from some of these organisms [6,7]. The first gene encoding rubredoxin to be isolated was that from *Clostridium pasteurianum* [8]. Recently a eukaryotic rubredoxin has been identified and characterized from the

unicellular alga *Guillardia theta* and found to be associated with photosystem II [9,10]. The metabolic roles of rubredoxin in general are poorly understood. However, roles as a cofactor of a terminal oxidase in *Desulfovibrio gigas* [11] and in hydrogen oxidation in *Azotobacter vinelandii* [12] have been described. Rubredoxin is also used as an electron donor in the superoxide reductase electron transfer chain in anaerobic organisms [13,14]. In aerobic bacteria, rubredoxin acts as an electron carrier in the alkane hydroxylation system [15,16]. Although rubredoxins are presumed to function as electron carriers, the rubredoxin of *Ps. oleovorans* is the only member of this family of proteins for which the electron transfer reactions have been delineated in detail [17]. Cloning and sequencing of the genes encoding proteins involved in the hydroxylation of alkanes established the role of rubredoxin in transferring electrons from a specific NADH-dependent rubredoxin reductase to the ω -hydroxylase [18,19]. *Ps. oleovorans* rubredoxin is unique in that it contains two binding sites for iron. Unlike other rubredoxins (molecular mass approx. 6 kDa), the rubredoxin of *Ps. oleovorans* is a 19 kDa protein and the gene encoding the protein (*alk G* [18]) is most probably the product of a gene duplication event. The 2Fe form of the rubredoxin is believed to be the physiological form but it is less stable than the readily isolated 1Fe form [20]. The 1Fe form is also active in its reaction with rubredoxin reductase [2,17]. Cleavage of the 2Fe rubredoxin at residue Met-50 with CNBr yields a C-terminal peptide (residues 51–173) that can be folded

Abbreviations used: 1D, one-dimensional; 2D, two-dimensional; HSQC, heteronuclear single-quantum coherence; IPTG, isopropyl β -D-thiogalactoside.

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around iron [2] and cobalt [21]; the folded peptide can act as an electron carrier during steady-state hydroxylation reactions [2,22]. The corresponding N-terminal fragment (residues 1–50) can be transiently folded around iron but it is highly labile [2].

Rubredoxins have been studied extensively from the structural viewpoint. Three-dimensional structures with resolutions smaller than 1.5 Å have been reported [23–27], and NMR studies have been used to investigate metal ion ligation [28–30], contributions made by protein structure to the overall thermostability of rubredoxins [31] and solution structure [32,33]. However, despite this detailed structural knowledge, little is known about the function of rubredoxins in general. With this deficiency in mind, we have chosen to study the rubredoxin from *Ps. oleovorans*, for which there is a well-defined biochemical role. A structural determination of the 2Fe rubredoxin, rubredoxin reductase and ultimately the productive electron transfer complex formed by the two proteins will provide valuable information on electron transfer mechanisms in this physiological redox system. However, a major question arising from a study of this system is the role of the labile N-terminal domain in the recognition of its physiological redox partners and electron transfer. Clearly, detailed functional and structural characterization of the component domains of the 2Fe rubredoxin is required. Here we report the genetic excision and purification of the N-terminal and C-terminal domains of *Ps. oleovorans* rubredoxin and studies of the spectroscopic and functional properties of the two domains.

EXPERIMENTAL

Chemicals and enzymes

Complex bacteriological media were from Unipath Ltd. Ethidium bromide, horse cytochrome *c*, cadmium chloride and ferrous ammonium sulphate were from Sigma; ¹¹³cadmium chloride was from Promochem Ltd. Restriction enzymes, phenyl-Sepharose, the metal-chelating column and the expression vector pKK223-3 were from Pharmacia. Pre-swollen DEAE-cellulose (DE-52) anion-exchange medium was from Whatman Biosystems Ltd. Sephadex G25 columns were supplied by Bio-Rad. Rubredoxin reductase was purified from a recombinant host as described previously [17], and the concentration of purified enzyme was determined at 450 nm by using a molar absorption coefficient of 11100 M⁻¹·cm⁻¹ [2]. The 1Fe di-domain rubredoxin was purified as described [20].

Recombinant DNA methods

Bacteria were cultured in double-strength YT medium that was supplemented, where appropriate, with ampicillin. Plasmid DNA was prepared with Wizard Maxi and Mini Preps (Promega). The expression construct (pKR10 [20]) for di-domain rubredoxin containing the full-length *alk* G gene has been described previously. The primers 5'-GCT GAA GCA GTG GTT TAA CCG ACA AGC CTT AGA GAA ATT G-3' and 5'-GCA ATT TCT CTA AGC TTG TCG GTT AAA CCA CTG CTT CAG C-3' were used in conjunction with the Quickchange mutagenesis kit (Stratagene) to engineer a translation termination codon at the location of the codon for Ala-86 in the wild-type gene, producing an expression construct (designated pKR11) for the N-terminal iron-binding domain of the 2Fe rubredoxin. To create an expression plasmid for the isolated C-terminal iron-binding domain, an *Eco*RI site was first engineered at the location of two codons encoding Ala-83 and Val-84 of the wild-type gene, and the codon for Val-85 was converted into one encoding methionine. These changes were introduced simultaneously by

using the oligonucleotides 5'-CAA GTT TAA CTG CTG AAG AAT TCA TGG CGC CGA CAA GCT TAG AGA AAT TGC C-3' and 5'-GGC AAT TTC TCT AAG CTT GTC GGC GCC ATG AAT TCT TCA GCA GTT AAA CTG G-3'. After mutagenesis, a 252 bp DNA fragment encoding the entire N-terminal iron-binding domain and half the putative interdomain linker was excised by digestion with *Eco*RI and removed by gel electrophoresis. Re-ligation of the resultant plasmid brought Met-85 (introduced in the preceding mutagenesis reaction) to the same location as Met-1 in the wild-type rubredoxin expression plasmid (pKR10) to produce a plasmid construct (designated pKR12) capable of expressing the C-terminal portion of the *alk* G gene.

A version of the *alk* G gene encoding recombinant rubredoxin containing an additional six histidine residues at the C-terminus was engineered by using the primers 5'-GCT CTA CGA GGA AAA GCA CCA TCA CCA CCA TCA CTG ACC CGG GGA TCC GTC GAC C-3' and 5'-GGT CGA CGG ATC CCC GGG TCA GTG ATG GTG GTG ATG GTG CTT TTC CTC GTA GAG C-3'. The resultant construct was designated pKR13. A factor Xa proteolysis site (Ile-Glu-Gly-Arg) was then introduced into this gene by mutagenesis of the codons corresponding to residues Ala-96, Asp-97, Val-98 and Lys-99 by using the primers 5'-GCT TAG AGA AAT TGC CTA GTA TTG AGG GGC GCG GCC AAG ATC TAT ATA AAA CTC AAC C-3' and 5'-GGT TGA GTT TTA TAT AGA TCT TGG CCG CGC CCC TCA ATA CTA GGC AAT TTC TCT AAG C-3'. The resultant construct was designated pKR14. All modified genes contained in the new plasmid constructs were completely resequenced to check that spurious changes had not arisen during mutagenesis.

Purification of the individual domains

The C-terminal domain was purified with the use of the protocol developed for full-length recombinant 2Fe rubredoxin [20]. The N-terminal domain was isolated initially as a fusion protein with the C-terminal domain (expressed from plasmid pKR14) containing an additional six histidine residues at the C-terminus. *Escherichia coli* strain TG1 transformed with plasmid pKR14 was grown in double-strength YT medium at 37 °C in the presence of 100 µg/ml ampicillin. Harvested cells were resuspended in buffer A (0.1 M potassium phosphate buffer, pH 7.0) and disrupted in a French press at 4 °C at a pressure of 140 MPa. The extract was clarified by centrifugation at 15000 *g* for 90 min and fractionated with solid (NH₄)₂SO₄. The precipitate obtained with 40–60 % satd (NH₄)₂SO₄ was dissolved in buffer A and dialysed against two changes of buffer A (10 litres each). The dialysed solution was then applied to a column (5 cm × 12 cm) of ion-exchange resin (DE-52) equilibrated with buffer A. After being washed, the column was developed with a gradient of KCl (0–0.5 M) in buffer A. Mutant di-domain rubredoxin was eluted at approx. 0.2 M KCl. Fractions were pooled and applied to a metal-chelating column (5 ml) (HiTrap; Pharmacia) charged with 50 mM nickel sulphate and equilibrated with binding buffer [50 mM potassium phosphate (pH 7.5)/1.0 M NaCl/5 mM imidazole]. The column was washed with 100 ml of binding buffer and 20 ml of wash buffer [50 mM potassium phosphate buffer (pH 7.5)/1.0 M NaCl/60 mM imidazole], and the protein was then eluted with elution buffer [50 mM potassium phosphate buffer (pH 7.0)/1.0 M NaCl/1 M imidazole]. Release of the N-terminal domain from the purified mutant di-domain rubredoxin was achieved by the addition of factor Xa (50 µg of mutant di-domain per unit of factor Xa) for 24 h at room temperature. The proteolytic digest was desalted to remove

Figure 1 Sequence alignments of rubredoxins from different organisms

The top six sequences are from rubredoxins whose structures have been solved by X-ray crystallography. The sources of the data are *Clostridium pasteurianum* [39], *Pyrococcus furiosus* [37], *Desulfovibrio vulgaris* (strain Hildenborough) [40], *Desulfovibrio vulgaris* (strain Miyazaki) [41], *Desulfovibrio gigas* [42], *Desulfovibrio desulfuricans* [43], *Butyrivibrio methylotrophicum* [44], *Heliobacillus mobilis* [45], *Megasphaera elsdenii* [46], *Peptococcus aerogenes* [47], *Clostridium thermosaccharolyticum* [48], *Acinetobacter calcoaceticus* [15], *Clostridium perfringens* [49] and *Guillardia theta* [9]; PoNt and PoCt are the N-terminal and C-terminal domain regions of the *Ps. oleovorans* rubredoxin respectively [50]. The numbering is as follows: the top numbers are for typical rubredoxins, the bottom two numberings are for PoNt and PoCt. Alignments were performed with ClustalW with the 'blossum' weight matrix, a gap penalty of 25 and a gap extension penalty of 0.05.

imidazole and then reapplied to the HiTrap column (buffer conditions as above) to separate the released N-terminal domain from the C-terminal domain. Fractions (1 ml) were collected as the column was washed with binding buffer.

Isolation of cadmium-substituted rubredoxin domains

Cadmium-substituted rubredoxin domains were isolated after precipitation of the purified proteins with trichloroacetic acid and incubation with cadmium chloride under anaerobic conditions as described previously for the di-domain rubredoxin [20].

CD and NMR spectroscopies

CD spectroscopy was performed with a Jasco J-715 spectropolarimeter with a 1 mm pathlength. Ten scans, over the range 190–260 nm at 20 °C, were averaged. The integration time was 50 nm/min. No smoothing algorithms were employed. The sample concentration was 0.2 mg/ml.

The ^1H and ^{113}Cd NMR spectra of the C-terminal domain were recorded with a Bruker AMX-600 spectrometer. The NMR sample (2–5 mM protein in 20 mM potassium phosphate buffer, pH 7.6) was made up in either $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (9:1, v/v) or $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (1:99, v/v). Spectra were recorded at 298 and 313 K. The chemical shifts of ^{113}Cd were referenced to 0.1 M $^{113}\text{Cd}(\text{ClO}_4)_2$ at 0 p.p.m. Proton chemical shifts were referenced to 2,2-dimethylsilapentane-5-sulphonic acid at 0 p.p.m. Homonuclear two-dimensional double-quantum-filtered COSY, TOCSY and NOESY data were acquired by using standard pulse sequences; where appropriate, ^{113}Cd -decoupling, with a GARP (globally optimized alternating-phase rectangular pulses) decoupling sequence, was incorporated during the evolution and acquisition periods. For all experiments, solvent suppression was achieved by a low-power pre-irradiation. To obtain ^{113}Cd - ^1H connectivities, a standard heteronuclear single-quantum coherence (HSQC) pulse sequence was used with polarization transfer delays optimized for coupling constants of either 40 or 17 Hz. All heteronuclear experiments were performed with ^{113}Cd -decoupling during acquisition. All spectra were measured in phase-sensitive modes. COSY data matrices were 4096×512 , NOESY data matrices were 2048×512 and ^{113}Cd - ^1H two-dimensional (2D) HSQC matrices were 4096×128 real-time domain data-points.

Electrospray MS

Mass measurements were obtained with a MicroMass Platform single-quadropole electrospray mass spectrometer. The cone potential was 30–32 V. Data analysis was performed with Mass-Lynx software from MicroMass. Samples were diluted in 50% (v/v) acetonitrile/0.3% (v/v) formic acid at a concentration of 50 pmol/ μl .

Kinetic measurements

Steady-state kinetic measurements were performed with a 1 cm light path in a final volume of 1 ml. The desired concentrations of horse heart cytochrome *c* (83 μM), rubredoxin (5–100 nM), rubredoxin reductase (2 μM) and NADH (0.3 mM) were obtained by making microlitre additions from stock solutions to the assay mix. Assays were performed in 50 mM Tris/HCl buffer, pH 7.8. Reactions were started by the addition of NADH, and the increase in A_{550} due to the reduction of cytochrome *c* (ϵ 21000 $\text{M}^{-1}\cdot\text{cm}^{-1}$) was measured with a Hewlett Packard 8452a single-beam diode-array spectrophotometer. All data were collected at 30 °C.

Limited proteolysis

Rubredoxin (200 μg) was incubated with trypsin (10 μg) at 37 °C. Samples (10 μl) were removed at intervals and mixed with an equal volume of SDS/PAGE loading buffer and boiled for 5 min. Samples were analysed by SDS/PAGE.

RESULTS AND DISCUSSION

Overexpression and purification of the N-terminal and C-terminal rubredoxin domains

Sequence alignment of *Ps. oleovorans* rubredoxin with all 1Fe rubredoxins sequenced so far reveals that the former comprises two domains separated by a large interdomain linker of 65 residues (Figure 1). The presence of two 'structured' domains is substantiated further by the finding that the 2Fe rubredoxin can be cleaved into individual iron-binding domains by treatment with CNBr at a unique methionine residue (Met-51) within the linker region [2]. However, the asymmetric cleavage of rubredoxin might contribute to the poor stability of the N-terminal domain

Figure 2 SDS/PAGE analysis of the expression, purification and proteolysis of the N-terminal and C-terminal domains of *Ps. oleovorans* rubredoxin

(A) Expression of the C-terminal and N-terminal domains in *E. coli* strain TG1. Lane 1, molecular mass markers (83, 63, 45, 29 and 14 kDa); lane 2, purified full-length rubredoxin from *Ps. oleovorans*; lane 3, expression of *Ps. oleovorans* 2Fe rubredoxin in *E. coli* strain TG1 from plasmid pKR10; lane 4, as lane 3 but with induction by IPTG (1 mM); lane 5, expression of the C-terminal domain from plasmid pKR12 in *E. coli* strain TG1; lane 6, as lane 5 but with induction by IPTG (1 mM); lane 7, total cell analysis of *E. coli* strain TG1 transformed with plasmid pKR11 (encoding the N-terminal domain); lane 8, as lane 7, but with induction by IPTG (1 mM). The N-terminal domain is expected to migrate at the same apparent molecular mass as the C-terminal domain (lanes 5 and 6). (B) Samples at various stages of the purification of the C-terminal domain. Lane 1, *E. coli* strain TG1 transformed with plasmid pKR12; lane 2, after $(\text{NH}_4)_2\text{SO}_4$; lane 3, pooled fractions after ion-exchange chromatography; lane 4, pooled fractions after hydrophobic interaction chromatography. The purified C-terminal domain migrates as multiple bands in SDS/PAGE owing to the facile nature by which disulphide cross-links are formed in the absence of the metal ion [20]. (C) Limited proteolysis of the purified C-terminal domain. Lane 1, purified C-terminal domain; multiple bands (indicated by the label CR) are due to the facile formation of disulphide bonds with this domain even under reducing conditions. Lanes 2–8, samples analysed at various time points (0.5, 1, 2, 5, 10, 20 and 40 min respectively) throughout the limited proteolysis. The band labelled 'tryp' has been identified as trypsin. Band x is the major digestion product referred to in the text. (D) Isolation of the N-terminal domain by proteolysis of the rubredoxin fusion protein with factor Xa. Lane 1, purified C-terminal domain; lane 2, purified wild-type di-domain rubredoxin; lane 3, purified mutant di-domain rubredoxin containing the His-tag and factor Xa cleavage site; lane 4, as lane 3 but after digestion with factor Xa for 24 h; lane 5, protein (His-tagged C-terminal domain) retained on metal-chelating column after digestion of mutant di-domain rubredoxin with factor Xa; lane 6, protein (released N-terminal domain after digestion of mutant di-domain with factor Xa) eluted with binding buffer from the metal-chelating column. (E) Amino acid sequence of the C-terminal rubredoxin domain. T1 to T8 indicate the locations of potential trypsin cleavage sites. T3 is the cleavage site giving rise to band x in (C). The alanine residue after the T5 cleavage site denotes the predicted start of the 'structured' domain.

released by cleavage with CNBr, because the cleavage site is located within the N-terminal domain. Sequence alignments provide only a guide to the location of domain boundaries. Genetic excision of the two domains was achieved by cleaving the linker region at a central location. In this way, the inclusion

of a portion of the putative linker region with each domain provided a safeguard against disruption of the structured domain by inappropriate cleavage close to the domain–linker boundaries.

SDS/PAGE of *E. coli* strain TG1 transformed with the N-terminal and C-terminal domain expression plasmids revealed

that only the C-terminal domain was expressed at high levels within the recombinant host (Figure 2A). There was little evidence for either the isopropyl β -D-thiogalactoside (IPTG)-induced or uninduced expression of the N-terminal domain in *E. coli* strain TG1 (Figure 2A) or in the protease-deficient strain of *E. coli* (strain BL21 DE3) (results not shown). Lack of expression of the N-terminal domain probably reflects the poor structural stability of this domain, as inferred from metal binding NMR studies in the full-length di-domain rubredoxin [20] and the poor stability of the N-terminal fragment generated by the cleavage of rubredoxin by CNBr [2]. The C-terminal domain is expressed as a soluble protein; purification of the domain was achieved by using the protocol described previously for the di-domain rubredoxin [20] (Figure 2B). In SDS/PAGE, the purified C-terminal domain migrates as multiple bands. A similar observation was made with the di-domain rubredoxin; this is attributed to the formation of intramolecular disulphides on removal of the iron [20]. Approximately 30 mg of purified C-terminal domain was isolated from a 1 litre culture of the recombinant host. The N-terminal sequence (Pro, Thr, Ser, Leu, Glu; determined by gas-phase methods) and the mass of the domain [9690 ± 1 Da; determined by electrospray MS (expected mass 9691.6 Da)] are both consistent with those expected for the isolated C-terminal domain.

Limited proteolysis was performed on the C-terminal domain to ascertain whether the N-terminal region (putative linker sequence) is predominantly unstructured, as predicted from the sequence alignments (Figure 1). Digestion with trypsin released several bands that were stable for up to 10 min (Figure 2C). The red coloration of the solution, attributed to the ligation of iron by the C-terminal domain, was retained within the first 20 min of the digestion with trypsin but was lost thereafter. Electrophoretic analysis by SDS/PAGE revealed that beyond 20 min of digestion, the major high-molecular-mass band (band X) corresponding to the truncated and structured C-terminal domain is degraded. Analysis of the N-terminal sequences of the major digestion products released during the early stages of proteolysis revealed that cleavage occurs within the conjectured linker peptide region of the C-terminal domain (Figures 2C and 2E). The N-terminus of the major digestion product (band X; Figure 2C) begins at residue Thr-106 (i.e. cleavage at site T3; Figure 2E). A sequence alignment with other rubredoxin sequences suggests that the N-terminal region of the structured C-terminal domain is located at Ala-118. This predicted location for the boundary between the linker region and the structured domain might account for the apparent resistance to cleavage by trypsin between 0 and 10 min at sites T4 and T5 (Figure 2E), because these sites are probably afforded some protection by the structured C-terminal domain. The loss of red coloration of the reaction mixture between 20 and 40 min suggests a loss of structural stability, leading to the release of iron from the domain. The loss of iron beyond 20 min might result from cleavage at T4 and T5 or an exposed trypsin site in the C-terminal domain, leading to a loss of structural integrity and rapid digestion of the domain [rapid digestion of the domain after 20 min would explain why further stable bands of smaller apparent molecular mass than band X (i.e. cleavage at T3) are not observed during SDS/PAGE]. Alternatively, the linker region might be needed to maintain the stability of the C-terminal domain; early removal of the linker by proteolysis might lead to structural instability and further rapid proteolysis after collapse of the C-terminal domain. To distinguish between these two possibilities, digestion with trypsin was arrested after 10 min of incubation by the addition of tosyl-lysylchloromethane ('TLCK'). After addition of the inhibitor, the red coloration of

the proteolysed C-terminal domain (cleaved at site T3) was stable for several hours, indicating that the absence of the linker region does not impart instability on the structured C-terminal domain.

SDS/PAGE of *E. coli* strain TG1 transformed with the mutant di-domain expression construct (pKR14) encoding rubredoxin with the internal factor Xa cleavage site and the histidine tag indicated that this protein is expressed at high levels. The full-length protein was purified to homogeneity with the procedure described above (Figure 2D). The protein migrated as a single band of approx. 20 kDa during SDS/PAGE. N-terminal sequence analysis of the purified domain revealed that the first six residues are Ala, Ser, Tyr, Lys, Cys and Pro, which is consistent with the predicted sequence of the mutant *alk G* gene. Moreover, analysis by electrospray MS indicated the mass of the protein to be 19640 ± 4 Da (expected mass 19632.5 Da). Cleavage of the purified protein with factor Xa, followed by separation of the N-terminal and C-terminal fragments by metal-ion affinity chromatography, enabled the isolation of the N-terminal domain from the full-length mutant rubredoxin (Figure 2D). Digestion resulted in the appearance of a diffuse band migrating with apparent mass 10 kDa and the loss of the parental 20 kDa band (Figure 2). Analysis by electrospray MS revealed that the diffuse band comprised two components of masses 10497 ± 1 Da (the N-terminal domain, expected mass 10502.6 Da) and 9145 ± 2 Da (the His-tagged C-terminal domain; expected mass 9147.9 Da), with a combined mass of 19642 ± 3 Da. Removal of the His-tagged C-terminal domain was achieved by metal-ion affinity chromatography. The isolated N-terminal domain migrated as a single band during SDS/PAGE (Figure 2D). The domain has the N-terminal sequence expected for the released N-terminal domain (Ala, Ser, Tyr, Lys, Cys, Pro) and has a mass of 10500 ± 1 Da (as determined by electrospray MS) as expected for the released N-terminal domain.

Spectroscopic and kinetic properties of the isolated domains

The electronic absorption spectrum of the isolated C-terminal domain is characteristic of the simple 1Fe rubredoxins (Figure 3). Absorption maxima are located at 498, 380 and 280 nm, and A_{280}/A_{498} is 4.0. The spectrum is similar to that observed for the chemically cleaved C-terminal domain, for which A_{280}/A_{498} is 4.3–4.5 [2]. The larger ratio reflects the larger size of the chemically cleaved domain, which has a mass of approx. 13 kDa. The electronic spectrum of the genetically excised C-terminal domain was stable for 25 h at 20 °C.

In contrast with the C-terminal domain, the N-terminal domain was colourless 'as purified' and lacked a signature in the visible region characteristic of rubredoxins. However, the apo-domain was readily converted into the iron-bound form by precipitation with trichloroacetic acid with the reconstitution method described previously for the di-domain rubredoxin [20]. The spectrum of the reconstituted domain had absorption maxima at 448 and 280 nm; although it had a large shoulder centred on 350–380 nm, it lacked the well-defined maxima at 380 nm of other rubredoxins (Figure 3). The poor spectral definition in this region probably reflects the structural instability of the N-terminal domain and/or the harsh procedures used to reconstitute the domain with iron. A_{280}/A_{488} is higher (5.0) than the corresponding value for the transiently stable, reconstituted N-terminal domain obtained by chemical cleavage with CNBr (ratio 4.3–4.5 [2]), again reflecting the additional sequence derived from the linker region present in the genetically constructed N-terminal domain. The smaller N-terminal domain generated by cleavage with CNBr was reported to be very unstable (half-life 30 min at room temperature) [2]. By contrast, the proteolytically

Figure 3 UV-visible spectra of the purified C-terminal (broken line) and N-terminal (solid line) domains

Protein samples were contained in 50 mM potassium phosphate buffer, pH 7.0.

Figure 4 Reduction of cytochrome *c* by rubredoxin reductase mediated by the N-terminal and C-terminal domains of rubredoxin and the 1Fe di-domain rubredoxin

Symbols: ●, 1Fe di-domain rubredoxin; ○, N-terminal domain; △, C-terminal domain. Rubredoxin concentrations were determined from iron content. Linear correlation coefficients obtained in data fitting are: 0.973 (C-terminal domains), 0.981 (N-terminal domain) and 0.996 (1Fe di-domain).

generated N-terminal domain generated in the study described here was considerably more stable, with a half-life of approx. 5 h at 20 °C (as determined by iron loss at 490 nm).

Both the isolated N-terminal and C-terminal domains of *Ps. oleovorans* rubredoxin are functional in transferring electrons from rubredoxin reductase to horse cytochrome *c* (Figure 4). The results clearly indicate that both the N-terminal and C-terminal domains can form productive electron transfer complexes with rubredoxin reductase and accept electrons from the enzyme-bound FAD. The results suggest that electron transfer from rubredoxin reductase to the 1Fe di-domain rubredoxin is more efficient than to either of the N-terminal and C-terminal domains, although it should be recognized that the interaction of cytochrome *c* with the di-domain and its component domains might also differ.

Figure 5 Optical spectra and difference spectra of the cadmium-substituted forms of the purified N-terminal and C-terminal domains

Upper panel: optical spectra for cadmium-substituted N-terminal domain (solid line) and the apo-(N-terminal) domain (broken line). Inset, difference absorption spectrum [cadmium-substituted N-terminal domain minus apo-(N-terminal) domain]. Lower panel: optical spectra for cadmium-substituted C-terminal domain (solid line) and apo-(C-terminal) domain (broken line). Inset, difference absorption spectrum [cadmium-substituted C-terminal domain minus apo-(C-terminal) domain] and Gaussian analysis of the difference spectrum. Broken lines, individual Gaussian components; solid line, difference spectrum; ●, sum of Gaussian components.

Substitution of the individual domains with cadmium

Iron was replaced by cadmium in the isolated N-terminal and C-terminal domains by using the published method for the di-domain rubredoxin [20]. The electronic spectrum of the cadmium-substituted C-terminal domain revealed the presence of a broad absorption between 200 and 300 nm superimposed on the spectrum of the apo-protein (Figure 5), from which the difference spectrum was calculated. The difference spectrum could be resolved into four Gaussian components centred on 202, 224, 239 and 276 nm. The ability to deconvolve the difference spectrum into four components mirrors previous work on the parental di-domain rubredoxin from *Ps. oleovorans* [20] and also similar studies on the 1Fe rubredoxin from *D. gigas* [29]. Jorgensen's electronegativity theory [34] attributes the 239 nm band to a cadmium-thiolate charge-transfer transition. The precise assignment of the bands at 202 and 224 nm cannot yet be made but

Figure 6 Far-UV CD spectra of the iron-containing and cadmium-substituted N-terminal and C-terminal domains of *Ps. oleovorans* rubredoxin

Spectra were recorded for samples contained in 50 mM potassium phosphate buffer, pH 7.0. Upper panel, N-terminal domain; lower panel, C-terminal domain.

they are clearly charge-transfer in character. As discussed for the di-domain protein, the absorption band at 276 nm probably reflects a sharpening of tryptophan absorption by the presence of the cadmium ion [20]. A tryptophan residue is adjacent in sequence to one of the cysteinyl ligands of the metal in the isolated C-terminal domain (Figure 1). As with the electronic spectrum of the iron-containing C-terminal domain, the corresponding spectrum of the cadmium-substituted C-terminal domain was stable for more than 25 h at 25 °C.

The electronic absorption spectra and difference spectra for the cadmium-substituted N-terminal domain were less characteristic of conventional 1Fe rubredoxins. A broad absorption was apparent between 200 and 300 nm superimposed on the spectrum of the apoprotein. The difference spectrum was significantly different from that reported above for the C-terminal domain, and more than four Gaussian components were required to satisfy the spectrum. However, a sharpening of the 280 nm absorption is evident, again suggesting the enhancement of tryptophan absorption by the presence of the metal ion. The spectrum of the cadmium-substituted form was stable for 25 h at 20 °C, indicating that the cadmium form is more stable than the iron form. The unusual features of the spectra for both the cadmium-substituted and iron-substituted N-terminal domains might be related to the rapid exchange of metal ions between the

Figure 7 1D ^1H NMR spectra for the iron-containing and cadmium-substituted N-terminal and C-terminal domains of *Ps. oleovorans* rubredoxin

Spectra were recorded at 25 °C and pH 7.0. (a) N-terminal domain; (b) C-terminal domain.

protein and bulk solution. This rapid exchange has been seen in NMR studies of the N-terminal domain in the context of full-length di-domain rubredoxin in the cadmium-substituted form [20].

CD studies of the isolated N-terminal and C-terminal domains

CD spectroscopy was used to investigate the overall structure of the domains and the effects of replacing iron with cadmium. The far-UV spectrum of the iron-containing C-terminal domain has a large negative band at 204 nm, a smaller negative band at 225 nm and positive transitions at 210–220 nm and 190–200 nm (Figure 6). The spectrum is very similar to that reported for the 1Fe rubredoxins of *D. gigas* [29], *P. furiosus* [35] and *C. tepidum* [36], suggesting overall structural similarity to these proteins. Also, like the *D. gigas* rubredoxin, the C-terminal domain of *Ps. oleovorans* rubredoxin retains structure on removal of the iron (Figure 6). The binding of Cd(II) ions to the apo-(C-terminal) domain of *Ps. oleovorans* rubredoxin introduces metal-dependent changes in the CD spectrum. The major changes are an intensification of the negative bands seen at 202 and 225 nm; similar changes have been seen on introducing Cd(II) ions into the apo-form of the di-domain rubredoxin of *Ps. oleovorans* [20]. CD, NMR and Fourier-transform IR spectroscopies have been used previously to demonstrate that cadmium-substituted *D. gigas* rubredoxin is isostructural with the native iron-containing rub-

Figure 8 2D COSY spectra for the cadmium-substituted C-terminal domain rubredoxin and the cadmium-substituted di-domain rubredoxin

Spectra were recorded at 25 °C and pH 7.0. (a) Di-domain rubredoxin; (b) di-domain rubredoxin C-terminal domain.

redoxin [30]. The similarity of the electronic absorption and CD spectra of the iron and cadmium forms of the C-terminal domain of *Ps. oleovorans* rubredoxin and the corresponding forms of the *D. gigas* protein indicates that metal-ion substitution in the former is isostructural. The CD spectrum of the apo-(C-terminal) domain is also shown in Figure 6 and indicates that structural features in the domain are retained on removal of the metal.

Corresponding studies were also conducted on the various forms of the N-terminal domain. The CD spectrum of the iron-containing form has two negative bands at 202 and 227 nm (Figure 6). The spectrum is similar to that of the C-terminal domain except that the positive transitions are less pronounced in the N-terminal domain. Again, the apo-protein retains spectral signature, indicating that this form of the domain is folded. The introduction of cadmium induces small changes, particularly in the region 220–240 nm; similar changes (although more pronounced) are also seen on replacing iron with cadmium in the C-terminal domain.

Figure 9 NMR spectra of ^{113}Cd -substituted C-terminal domain of *Ps. oleovorans* rubredoxin

1D ^{113}Cd spectrum (A) and 2D [^{113}Cd - ^1H] HSQC spectrum (B) of *Ps. oleovorans* recorded at 25 °C and pH 7.0. The 2D spectrum was acquired with a polarization transfer delay of 6 ms.

NMR spectroscopy of the individual domains

The one-dimensional (1D) ^1H NMR spectra acquired for the cadmium-substituted C-terminal domain suggest the presence of a stable tertiary structure, including β -sheet secondary structure as evident by the large dispersion of amide resonance (approx. 7.0–10.0 p.p.m.) and the presence of H^α signals downfield of the water signal (approx. 4.7–5.5 p.p.m.) (Figure 7). This dispersion of resonances and the up-field shift of methyl resonances is similar that seen for the di-domain protein (L.-Y. Lian, A. Perry and N. S. Scrutton, unpublished work) and the zinc-substituted form of *P. furiosus* [37]. The spectrum for the iron-containing C-terminal domain demonstrates the effect of Fe(III) on the quality of the spectrum (see Figure 7b). There is significant line broadening and loss of signal in the spectrum of the iron-containing C-terminal domain, arising from the paramagnetic effect, compared with the spectrum of the cadmium-substituted C-terminal. This is similar to effects observed by Blake and co-workers [28,37] for Fe(II)-containing rubredoxin from *P. furiosus*.

The 1D ^1H NMR spectra of the cadmium-substituted N-terminal domain indicate that the protein is folded, as shown by the presence of methyl peaks shifted up-field (see Figure 7a). The poor recovery of recombinant N-terminal samples did not yield sufficient protein for a qualitative investigation of secondary structure elements, as was performed for the C-terminal domain. The spectra of the cadmium-substituted and iron-containing N-terminal domain show line broadening and shifting of peak positions, arising from paramagnetic effects (see Figure 7a). 2D NMR spectra of the C-terminal domain rubredoxin show an almost identical overlap with the respective peaks from the di-domain spectra (Figure 8). This is evidence that the structure of the C-terminal domain adopts the same conformation both when it exists as an independent domain and when present in the di-domain rubredoxin. It also suggests that there is not a major structural interaction between the two domains, because such an interaction would perturb the identical shifts, decreasing the amount of overlap observed.

The 1D ^{113}Cd NMR spectrum of the C-terminal domain exhibits a single narrow resonance at 732.3 p.p.m. (Figure 9A). This is in the expected range of 610–750 p.p.m. observed in other tetrahedral tetrathiolate cadmium sites [38]. The di-domain

rubredoxin contained two ^{113}Cd resonances at 732.2 and 730 p.p.m., with the peak at 730 p.p.m. being broader than that at 732.2 p.p.m. because of chemical exchange of the cadmium [20]. Our data for the C-terminal domain demonstrate that the ^{113}Cd resonances observed for the di-domain rubredoxin, at 732.2 and 730 p.p.m., are from ^{113}Cd bound to the C-terminal and N-terminal domains respectively, confirming the findings of Lee et al. that the N-terminal domain binds metal less strongly than the C-terminal domain [20]. The position of the H^β chemical shifts for the cysteine residues liganded to the $\text{Cd}(\text{II})$ are identified from the 2D (^1H – ^{113}Cd) HSQC experiment (Figure 9B).

Conclusions

Here we have demonstrated the feasibility of expressing functional domains of the 2Fe rubredoxin of *Ps. oleovorans*. Both domains are active in transferring electrons from rubredoxin reductase to horse cytochrome *c*; solution studies indicate that each domain is folded and assembled with the cofactor iron as expected for the simpler 1Fe rubredoxins of other species. Our studies reveal that the N-terminal domain is less stable than the C-terminal domain and that this is reflected in the rapid exchange of metal ions in the N-terminal domain. The ability to exchange the iron for cadmium in the individual domains will now open up detailed solution structural studies by NMR spectroscopy.

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