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Production and preliminary characterization of a recombinant triheme cytochrome c_7 from *Geobacter sulfurreducens* in *Escherichia coli* ☆

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Abstract

Multiheme cytochromes *c* have been found in a number of sulfate- and metal ion-reducing bacteria. *Geobacter sulfurreducens* is one of a family of microorganisms that oxidize organic compounds, with Fe(III) oxide as the terminal electron acceptor. A triheme 9.6 kDa cytochrome c_7 from *G. sulfurreducens* is a part of the metal ion reduction pathway. We cloned the gene for cytochrome c_7 and expressed it in *Escherichia coli* together with the cytochrome *c* maturation gene cluster, *ccmABCDEFGH*, on a separate plasmid. We designed two constructs, with and without an N-terminal His-tag. The untagged version provided a good yield (up to 6 mg/l of aerobic culture) of the fully matured protein, with all three hemes attached, while the N-terminal His-tag appeared to be detrimental for proper heme incorporation. The recombinant protein (untagged) is properly folded, it has the same molecular weight and displays the same absorption spectra, both in reduced and in oxidized forms, as the protein isolated from *G. sulfurreducens* and it is capable of reducing metal ions in vitro. The shape parameters for the recombinant cytochrome c_7 determined by small angle X-ray scattering are in good agreement with the ones calculated from a homologous cytochrome c_7 of known structure.

Keywords: Cytochrome *c*; Cytochrome c_7; Cytochrome *c* maturation proteins; Heterologous expression; *Geobacter sulfurreducens*; Multiheme cytochrome *c*; Small angle X-ray scattering

1. Introduction

Cytochromes *c* are electron transfer proteins involved in respiratory processes of almost all organisms. They have heme prosthetic groups covalently bound to the polypeptide chain via two thioether bonds. Cytochromes *c* display the typical CXXCH sequence pattern in which the histidine residue serves as one of the axial ligands to the iron of the heme. Multiheme cytochromes have been found in the periplasm of sulfate-reducing bacteria [1–4]. The best studied are tetraheme cytochromes (c_3 superfamily).

A triheme cytochrome, cytochrome c_7 from *Geobacter sulfurreducens* (also designated as 9.6 kDa cytochrome *c* or CytP) has been described earlier [5–7]. *G. sulfurreducens* is one of a family of microorganisms that can oxidize organic compounds with Fe(III) oxide or other metal oxides as terminal electron acceptors [8,9]. The cytochrome c_7 is located in the periplasm and is involved in electron transport. Current studies suggest that this cytochrome could be directly responsible for the reduction of soluble metal ion species such as U(VI) that can center the periplasm [6]. This cytochrome consists of 71 amino acids and contains three heme groups. It is homologous to the cytochrome c_7 purified from *Geobacter metallireducens* [10] and to the cytochrome c_7 from *Desulfuromonas acetoxidans*. The structure of the latter was determined by X-ray diffraction [11]. The triheme cytochromes c_7 can be considered members of the c_3 superfamily that lack heme 2 and the corresponding fragment of the polypeptide chain. The cytochromes c_7 appear to be the prevalent cytochromes in

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some organisms, e.g. a preliminary analysis of the *G. sulfurreducens* genome did not reveal any genes encoding c_3-like tetraheme cytochromes but did identify a plethora of genes for c_7-like cytochromes (Y.Y.L., M.S., unpublished data).

Multiheme cytochrome *c* proteins such as c_7 have been shown to exhibit a metal ion reductase activity, which makes them prospective biotechnological tools, including their possible application for bioremediation. To understand the function of cytochrome c_7, detailed structural, mutational and functional studies have to be carried out. These studies require large amounts of c_7 protein. For mutational studies, the gene has to be heterologously overexpressed; the yields of mutant proteins are usually lower than that of the wild-type proteins. Heterologous expression of multiheme proteins in *Escherichia coli*, the most common expression host, often fails because of low efficiency of post-translational maturation (covalent attachment of hemes). Only recently has a solution been found, which employs an *E. coli* gene cluster *ccmABCDEFGH* responsible for maturation of its own *c*-type cytochromes under anaerobic conditions [12–14]. These genes are a part of the so-called "aeg46.5" operon [15] and encode eight proteins, CcmABCDEFGH, involved in a number of different steps providing transport of heme groups through the inner membrane and their covalent attachment to the polypeptide chain [16–19]. Coexpression of genes coding for *c*-type cytochromes with genes from a separate plasmid, pEC86, which contains the maturation gene cluster, resulted in production of *c*-type cytochromes in *E. coli* but in relative low yields for multiheme cytochromes [20–28]. Here we describe an expression system in *E. coli* for overproduction of cytochrome c_7 from *G. sulfurreducens*. Using this system, we have obtained a good yield and based on initial physicochemical characterization, we show that the recombinant cytochrome c_7 is indistinguishable from the c_7 isolated from *G. sulfurreducens*.

2. Methods

2.1. Sequence data

Sequence data for *G. sulfurreducens* cytochrome c_7 and its gene were obtained from The Institute for Genomic Research Web site at http://www.tigr.org. GenBank accession number for the gene sequence is AF505790.

2.2. Bacterial strains and plasmids

E. coli strain XLl-Blue (Stratagene) was used for subcloning. BL21(DE3) (Novagen) was used for production of the His-tagged version of the cytochrome c_7, JM83 [29] and HM125 [20,30] were used for production of the wild-type protein without the His-tag. Cultures were routinely grown in 2 × YT medium or on 2 × YT agar plates [31]. Growth media were supplemented with carbenicillin, 100 μg/ml, and where appropriate, chloramphenicol, 20 μg/ml. Vectors pET-15b and pET-22b(+) were obtained from Novagen. Plasmid pkIVlen004, which is vector pASK40 containing an immunoglobulin light chain fragment [32], was provided by Dr. R. Raffen (Argonne National Laboratory). Plasmid pEC86, a derivative of pACYC184, containing the *ccm* gene cluster was a kind gift from Dr. L. Thöny-Meyer (Zürich, Switzerland).

2.3. Construction of expression plasmids

DNA manipulations generally followed standard published procedures [31]. The genomic DNA of *G. sulfurreducens* was provided by Dr. D.R. Lovley (University of Massachusetts, Amherst). Oligonucleotides were synthesized by P. Gardner (University of Chicago). Pfu Turbo DNA-polymerase (Stratagene) was used for all PCR amplifications. QIAquick PCR purification kit or QIAquick Gel Extraction kit (Qiagen) was used for purification of the PCR product. All constructs were confirmed by DNA sequencing with the use of BigDye Terminator Sequencing kit (Applied Biosystems). The DNA fragment encoding the amino acid sequence of the mature protein was amplified from the genomic DNA by PCR, with primers CC1-F (5′-ctcgccc*catatg*gccgacgacatcgtc) and CC1-R (5′-ttt*ggatcc*tcattacttcttgtggcac), which introduced *Nde*I and *Bam*HI restriction sites, respectively (underlined). The resulting DNA fragment (240 bp) was digested with restriction enzymes *Nde*I and *Bam*HI, and cloned into vector pET-15b (Novagen). The construct obtained encoded the mature cytochrome fused to a cleavable N-terminal His-tag and was named pCC21 (Fig. 1). Next, pCC21 was digested with restriction enzymes *Nco*I and *Bam*HI and the resulting DNA fragment (290 bp) was cloned into vector pET-22b(+) (Novagen). The new construct, named pCH33, encoded a polypeptide consisting of the pelB leader sequence followed by a cleavable His-tag and the mature cytochrome sequence (Fig. 1).

In order to construct a plasmid for expression of the wild-type protein (untagged), the DNA fragment consisting of the sequence of the mature cytochrome and adjacent 3′ untranslated region including *Hin*dIII restriction site (361 bp) was amplified, with primers OLC (5′-*caggcagccgacg*acatcgtc) and T7TER (5′-gctagttattgctcagcgg) and pCH33 as a template. Then, the DNA fragment encoding the OmpA leader sequence and adjacent 5′ untranslated region including *Xba*I restriction site (118 bp) was amplified, with primers K4rev (5′-cacacaggaaacagctatgacc) and OLL (5′-*gtcggctgcctgag*caacggtag) and plasmid pkIVlen004 as a template. Primers OLC and OLL contained complementary sequences of 12 bases (underlined). Therefore, the two PCR fragments obtained overlapped and were recombined by overlap PCR [33]. The resulting PCR fragment was digested with restriction enzymes *Xba*I and *Hin*dIII and ligated into pkIVlen004 digested with the same enzymes (Fig. 2). This construct was named pCK32.

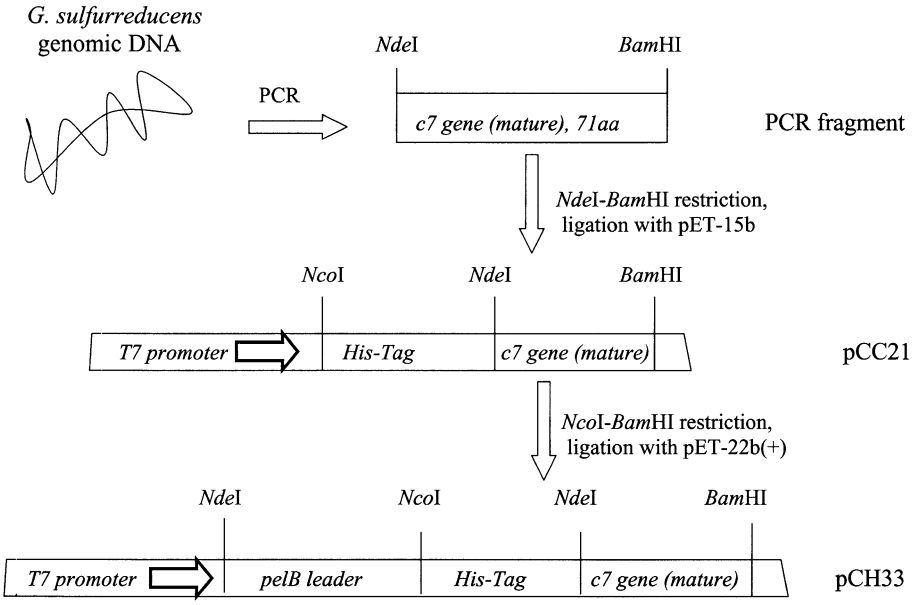

Fig. 1. Design of a recombinant cytochrome c_7 gene with His-affinity tag. The DNA fragment encoding the mature sequence of the cytochrome c_7 was amplified from the genomic DNA by PCR, it was digested with restriction enzymes *Nde*I and *Bam*HI, and was cloned into vector pET-15b. The construct obtained encoded the mature cytochrome fused to a cleavable N-terminal His-tag. Further, the construct was digested with *Nco*I and *Bam*HI and the resulting DNA fragment was cloned into the vector pET-22b(+).

2.4. Production and purification of the His-tagged protein

E. coli cultures were grown in two different ways: (1) cells were grown to mid-exponential phase at 37 °C at a shaking speed of 150–200 rpm and then induced with 1 mM IPTG and incubated overnight at 30 °C; (2) cells were grown at 30 °C at the same shaking speed for approximately 24 h with no induction at all (basal level of transcription was

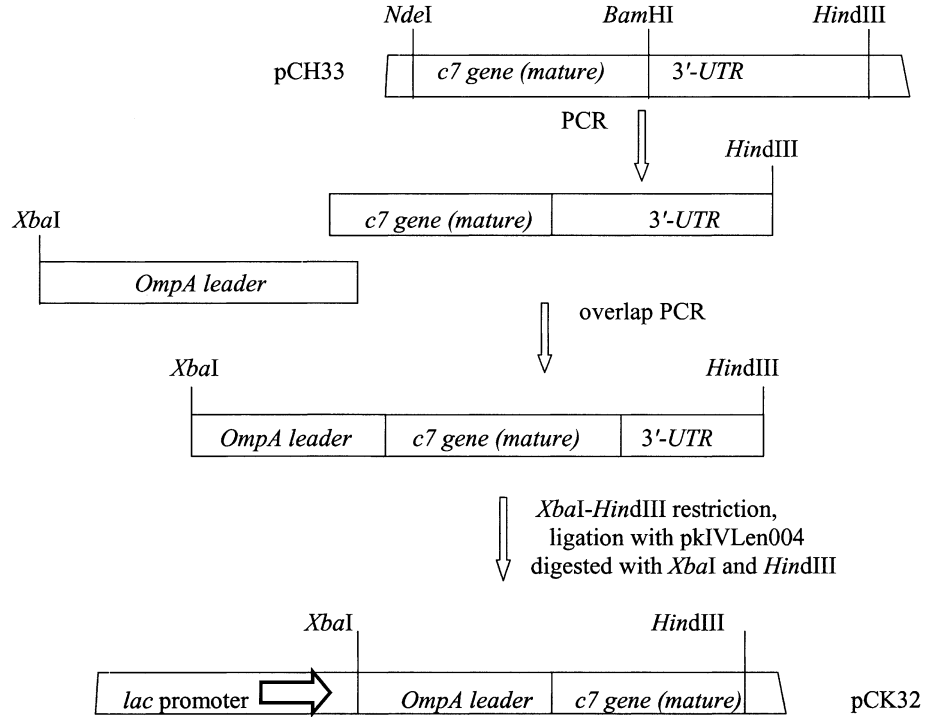

Fig. 2. Cloning of the recombinant cytochrome c_7 gene into pASK40 vector. A DNA fragment encoding the sequence of the mature protein and 3′-untranslated region (3′-UTR) was amplified from the pCH33 construct. The resulting fragment was recombined by overlap PCR with a DNA fragment encoding the OmpA leader sequence and the ribosome binding site amplified from pASK40. The product of the overlap PCR was digested with enzymes *Xba*I and *Hin*dIII and was ligated with pkIVlen004 digested with the same enzymes.

high enough to produce significant amounts of apo-protein). The cells were harvested, resuspended in Extraction/Wash buffer (Clontech; 50 mM sodium phosphate, pH 7.0, 300 mM NaCl) with 50 mM imidazole and then disrupted by sonication. The supernatant after centrifugation (20–25 min, 25 000 × *g*, 4 °C) was loaded onto TALON resin (Clontech). Electrophoretically pure protein was eluted with the same buffer containing 100 mM imidazole.

2.5. Production and purification of the wild-type (untagged) protein

E. coli cultures were grown to mid-exponential phase at 30 °C at a shaking speed of 200 rpm, and then induced with 10 μM to 1 mM IPTG. After overnight incubation under the same conditions, the cells were harvested and the periplasmic fraction was isolated by osmotic shock [32]. The cell pellet was gently resuspended in ice-cold TES buffer (0.5 M sucrose, 0.2 M Tris–HCl, pH 8.0, 0.5 mM EDTA), 50 ml/l of culture. Then ice-cold water was added (0.5–1 × initial volume) and the suspension was incubated on ice with gentle shaking for 1–2 h and then centrifuged at 12 000 × *g* for 20 min at 4 °C. The supernatant contained the periplasmic fraction. It was concentrated either by ultrafiltration in an Amicon stirred ultrafiltration cell (YM3 membrane) or by loading it onto 2 × 5 ml Econo-Pac High S cartridges (Bio-Rad) followed by elution with a high salt buffer (10 mM Tris–HCl, pH 8.5, 1 M NaCl). The buffer was subsequently exchanged to 10 mM Tris–HCl, pH 8.5 by dialysis or using Econo-Pac 10DG desalting columns (Bio-Rad) and the sample was applied to 2 × 5 ml Econo-Pac High S cartridges equilibrated with the same buffer. Protein was eluted with 150 ml 10 mM Tris–HCl buffer pH 8.5 that contained a gradient of 0–300 mM NaCl.

2.6. Production and purification of the apo-protein

Production and purification of the apo-protein was performed essentially as that of the wild-type, but the plasmid pCK32 encoding the apo-protein was transformed into strain JM83 that did not contain the cytochrome *c* maturation plasmid pEC86.

2.7. Mass-spectrometry and N-terminal sequencing

Mass-spectrometry and N-terminal sequencing were performed in the HHMI Biopolymer Laboratory and W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

2.8. Biochemical methods

Spectrophotometric measurements were performed on a Shimadzu UV160U spectrophotometer in a cell with a path length of 1 cm. Cytochrome c_7 concentration was determined by two different methods: (1) Bradford method, Coomassie Plus Protein Assay Reagent (Pierce) with BSA as a standard; (2) by absorbance of the reduced *c*-type cytochrome α-band at 552 nm using an extinction coefficient of 32.5 mM^{-1} cm^{-1} of the wild-type protein isolated from the *G. sulfurreducens* bacteria [5]. To determine heme concentration, we assumed that each heme contributed equally to the extinction coefficient of the mature protein and therefore the absorption at 552 nm could be used as a measure of the heme content. Cytochrome samples were reduced by addition of a few crystals of solid sodium dithionite (Sigma). Gel electrophoresis was carried out in 4–20% gradient gels (BioWhittaker Molecular Applications) or 8–25% gradient Phast gels (Pharmacia). The gels were stained with Coomassie blue.

2.9. X-ray scattering measurements

X-ray scattering experiments were performed at the BESSRC beam line 12-ID of the Advanced Photon Source (APS) at Argonne National Laboratory [34]. The X-ray wavelength was set at 1.0 Å by double-crystal Si(111) monochromators, and scattering was measured with a two-dimensional mosaic CCD detector. Scattering patterns were collected with the use of a flow cell; X-ray damage was minimized by flowing the samples. The 2-D scattering images were first corrected for spatial distortion and sensitivity of the detector, and then were radially averaged to produce a 1-D map of scattered intensity versus q. Sample scattering profiles, $I(q)$, were obtained from the difference of the 1-D measurements of scattered intensities measured for the cytochrome c_7 solutions and the solution background.

3. Results

3.1. Design and properties of a recombinant cytochrome with a His-tag

A partial amino acid sequence of the mature protein was obtained from Dr. D.R. Lovley and Dr. J. Lloyd (University of Massachusetts, now at University of Manchester, UK). It was used to find the gene with the BLAST software [35] from the preliminary genome sequence of *G. sulfurreducens* available on the TIGR Web site (http://www.tigr.org). Analysis of the deduced amino acid sequence using the SignalP software (http://www.cbs.dtu.dk/services/SignalP, [36]) predicted a signal sequence cleavage site between residues Ala20 and Ala21, which was consistent with the data of Lloyd et al. [7]. The gene sequence of the cytochrome c_7 was recently deposited into the GenBank by Dr. Lloyd, accession number is AF505790.

The DNA fragment encoding the amino acid sequence of the mature protein was amplified from the genomic DNA by PCR methods and was cloned into vector pET-15b (Novagen). The construct obtained, designated pCC21, encoded

the mature cytochrome fused to a cleavable N-terminal His-tag (Fig. 1). The His-affinity tag was intended to facilitate the purification procedure. The *N*col–*Bam*HI restriction fragment of pCC21 was cloned into vector pET-22b(+). The vector pET-22b(+) includes the pelB leader sequence that directs the secretion of the newly synthesized polypeptide chain into the periplasm necessary for proper post-translational processing [26,37]. The new construct, named pCH33, provided for production of a polypeptide consisting of the pelB leader sequence followed by a cleavable His-tag and the mature cytochrome sequence under control of the T7*lac* promoter (Fig. 1).

Both pCC21 and pCH33 constructs were expressed in strain BL21(DE3) cotransformed with plasmid pEC86 providing for the constitutive expression of the *ccm* genes. Only in the case of pCH33 construct that contained the leader sequence did we obtain a pink-colored cell pellet after induction (the cell pellet obtained by centrifugation of culture had pink color), which is a sign of heme incorporation [26]. In the absence of pEC86, both constructs failed to develop color after induction.

The strain BL21 (DE3) transformed with both pCH33 and pEC86 was used for the production of preparative amounts of the His-tagged protein. The protein was purified in a single step by immobilized metal affinity chromatography on cobalt-containing TALON resin. The purity of the cytochrome with His-tag is shown by SDS-PAGE gel electrophoresis (Fig. 3, lane 1). The protein has a total of 21 additional residues, therefore, it is larger than the wild-type cytochrome c_7. Both oxidized and reduced forms of the cytochrome had spectra (Fig. 4B) essentially the same as those of the native protein (Fig. 4A). The yield of the pure protein was about 50 mg/l of culture as determined by the Bradford method. However, the heme concentration calculated from absorbance at 552 nm was 5- to 10-fold lower than expected based on the protein concentration, which indicated that a large portion of the protein lacked hemes.

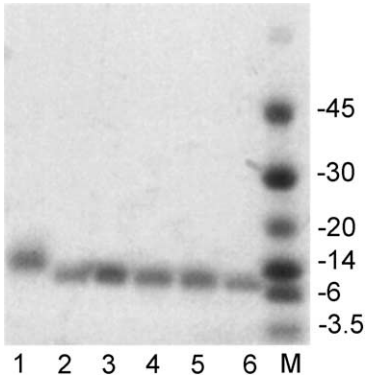

Fig. 3. SDS-PAGE (4–20% gradient gel) of different samples of the cytochrome c_7. Lane 1, the His-tagged protein; lane two, protein isolated from *G. sulfurreducens*; lanes 3–6, fractions 47, 51, 58, and 67 of recombinant cytochrome c_7 eluted from an ion-exchange column (see Fig. 5); M, molecular weight markers (Bio-Rad). The gels were stained with Coomassie blue.

To slow down the protein synthesis, we also expressed the pCH33 construct without induction with IPTG. The level of "constitutive" expression due to "leakage" of T7*lac* promoter in pET-22b(+) was high enough to provide a significant amount of the protein during overnight growth of the host cells at 30 °C. For the "constitutively" produced cytochrome c_7 the heme to protein ratio was slightly better than for the cytochrome obtained in the case of IPTG-induced expression. The heme to protein ratio was 3- to 4-fold lower than the expected value for a triheme cytochrome.

3.2. Design and properties of a recombinant cytochrome with the wild-type sequence (untagged)

Another construct was designed based on vector pASK40, a pUC derivative [38]. This vector features the *lac* promoter and OmpA leader sequences and has been used in our lab for expression of antibody light chain fragments [32,39]. The DNA fragments encoding the sequence of the mature cytochrome and the OmpA leader sequence were amplified from plasmids pCH33 described above and pkIVlen004, respectively, and then recombined by overlap PCR. The resulting PCR fragment was cloned into pkIVlen004 (Fig. 2). The plasmid pkIVlen004 is the pASK40 vector that contains an antibody light chain fragment [32]. This construct named pCK32 provided expression of a polypeptide consisting of the OmpA leader sequence and the mature cytochrome sequence.

The protein produced in strain HM125 was purified in two major steps: first, the periplasmic fraction was isolated by osmotic shock. The protein is basic, therefore, for purification it was applied to High S cation exchange resin at pH 8.5 and eluted with a NaCl gradient. The elution profile is shown in Fig. 5. Since the protein does not contain Trp or Tyr residues, the absorbance at 280 nm was not a reliable indicator of protein concentration. Therefore, we monitored the protein concentration at 210 nm, where the peptide bonds have high extinction coefficients, and at 408 nm where the heme of oxidized cytochrome absorbs. Based on the elution profile, four heme-containing peaks were identified: fractions 46–48, 51–52, 58–60, and 66–68. The SDS PAGE patterns of fractions 47, 51, 58, and 67 are shown in Fig. 3 together with the c_7 isolated from *G. sulfurreducens* provided by Dr. J. Lloyd and the His-tagged version of the protein. Each sample, with the possible exception of fraction 67 runs as a single band of ~10 kDa molecular weight. The His-tag protein (lane 1) clearly has a higher molecular weight, while fraction 67 (lane 6) has a lower molecular weight than the wild-type protein (lane 2). The absorption spectra of the oxidized and reduced proteins are shown in Fig. 4. The protein concentration for each fraction determined by the Bradford method, is compared with the heme concentration measured by absorbance of the reduced protein at 552 nm in Table 1.

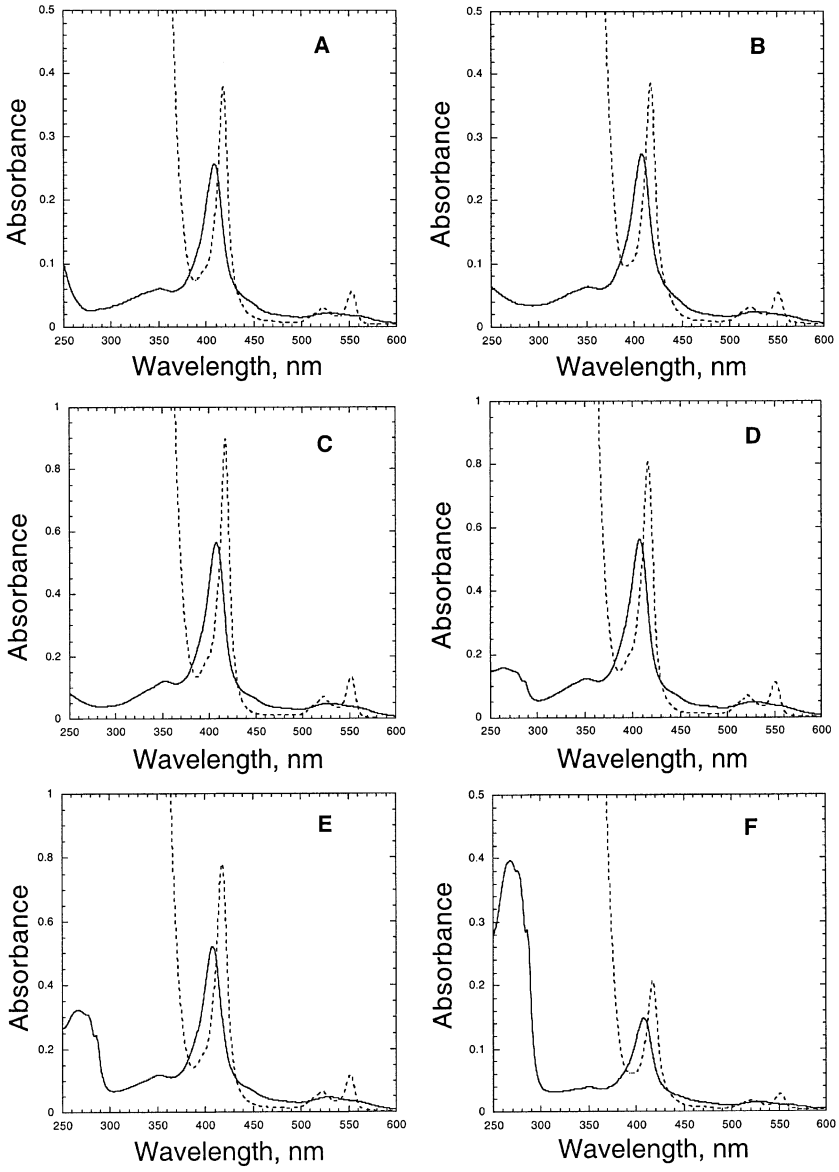

Fig. 4. Absorption spectra of oxidized (solid line) and reduced (dashed line) forms of the cytochrome c_7 preparations described in the text. (A) Protein purified from *G. sulfurreducens*, concentration 0.016 mg/ml; (B) the His-tagged protein, concentration 0.06 mg/ml; (C–F) fractions 47 (0.04 mg/ml), 51 (0.05 mg/ml), 58 (0.09 mg/ml), and 67 (0.05 mg/ml) of the recombinant cytochrome c_7 eluted from an ion-exchange column (see Fig. 5). All samples have the same peak absorption wavelengths both in the reduced (418, 522, 552 nm) and in the oxidized forms (408, 530 nm).

The major peak fractions 46–48 (Fig. 5) were eluted at ~ 120 mM NaCl concentration. The relative protein and heme concentration shown in Table 1 indicated that this fraction had the proper heme content for a fully mature protein, three heme groups per molecule. Mass-spectrometry data confirmed this, showing a molecular mass of 9573 Da, which is in excellent agreement with that reported earlier (9.57 kDa [5]) and with the value calculated from the amino acid sequence (the calculated weight of the polypeptide part is 7725 Da and the weight of a heme group is 616 Da [13]; $7725 + 3 \times 616 = 9573$). N-terminal sequencing showed that the eight N-terminal residues were ADDIVLKA, which indicates the correct processing of the signal sequence. Spectra of both oxidized and reduced forms (Fig. 4C) were similar to those of the native protein (Fig. 4A). The function of the recombinant c_7 was compared with the one isolated from *G. sulfurreducens*. Their capability to reduce Fe(III) citrate in vitro [6] was indistinguishable (Dr. D.R. Lovley, private communication).

Based on the data in Table 1, we assumed that the second (fractions 51–52) and third peaks (fractions 58–60) corresponded to 2 hemes/molecule and 1 heme/molecule species, respectively. The mass-spectrometry results confirmed a molecular mass of 8956 Da for the second peak, which is in good agreement with the calculated value for a two-heme species ($7725 + 2 \times 616 = 8957$). However, for the third peak, the same molecular mass of 8955 Da was measured as for the second peak. Three different two-heme/molecule

Table 1
Determination of heme incorporation in the peak fractions shown in Fig. 5[a]

Fraction number	C_p, protein concentration determined by Bradford method (μM)	C_h, heme concentration determined by absorbance at 552 nm (μM)	C_h/C_p
46	172	524	3.04
47	225	718	3.20
48	131	442	3.38
51	52	103	1.98
52	45	82	1.81
58	129	150	1.17
59	147	129	0.87
60	114	69	0.61
66	71	38	0.53
67	89	38	0.42
68	88	28	0.32

[a] To determine heme concentration, we assumed that each heme contributed equally to the extinction coefficient of the mature protein and therefore the absorption at 552 nm could be used as a measure of the heme content.

species of cytochrome $c_7 - s$ are possible, they are expected to have different folds which could lead to different surface charge distribution and consequently different ion-exchange properties. The reasons for the increased absorption of the third peak at ~270 nm (Fig. 4E), and at 210 nm (Fig. 5) are not clear. The last peak (fractions 66–68), according to the mass-spectrometry data contains the apo-protein as the major species, 7721 Da, and two species of 8337 and 8954 Da, both at the amounts of <10% of the major one, that have mass values close to the one- and two-heme species. The calculated values for apo, one- and two-heme proteins are 7725, 8341, and 8957 Da, respectively.

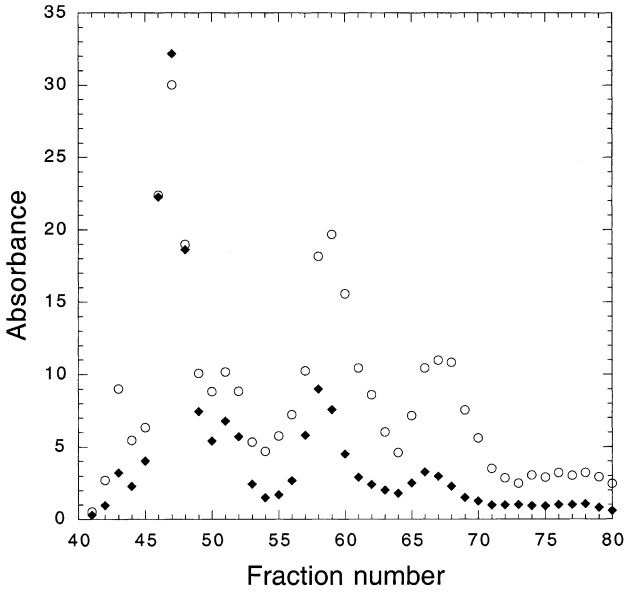

Fig. 5. The elution profile from ion exchange chromatography of the recombinant cytochrome c_7. The absorbance of fractions eluted between 100 and 300 mM NaCl are shown. The volume of each fraction is 2.5 ml. The absorbance of the peptide bonds at A_{210} is shown in open circles and the absorbance of the heme of the oxidized cytochrome at A_{408} is shown in filled diamonds.

To get an increased yield of the c_7, we expressed the pCK32 construct containing the gene for the wild-type cytochrome, both in *E. coli* strains JM83 [29] and HM125, both were harboring pEC86 plasmid. Strain HM125 lacks periplasmic protease DegP [20,30]. No significant difference in the yields from the two strains were observed. The yield of the mature protein after purification was 3 mg/l of culture. To improve the yield, we also tested different IPTG concentrations for induction; the range was from 10 μM to 1 mM. We found that decreasing the amount of IPTG led to higher yields of the fully matured species, together with the decrease of the partially modified species. The yield of the recombinant c_7 from *G. sulfurreducens* reached 6 mg/l when 10 μM IPTG was used for induction.

3.3. Small angle X-ray scattering

We performed small angle X-ray scattering study of different species eluted during the ion-exchange chromatography (fraction 47, mixture of fractions 51–52 and mixture of fractions 58–60) as well as the apo-cytochrome (that was produced separately in the absence of the Ccm proteins). Fig. 6 shows the experimental small angle scattering curves and the calculated scattering curve based on the *D. acetoxidans* cytochrome c_7 structure. The lack of secondary peak at $q = 0.4$ reflects the lack of defined secondary structures in

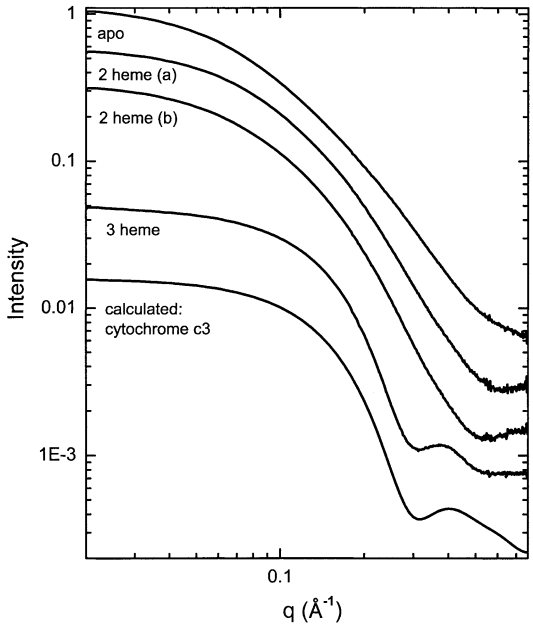

Fig. 6. The small angle X-ray scattering patterns of the apo-form; two versions of the two-heme forms: a (fractions 58–60) and b (fractions 51–52); and the three-heme form, fraction 47 (see Fig. 5) are compared with the calculated curve for the homologous protein from *D. acetoxidans* [11]. The absence of secondary peak at $q = 0.4$ of the apo- and two-heme forms suggests the lack of secondary structure for these incompletely assembled species. The logarithm of the intensity is plotted against the logarithm of the scattering vector q, where $q = 4\pi\sin\theta/\lambda$ and λ is the X-ray wavelength and 2θ is the scattering angle.

the incompletely assembled species [40]. The calculated scattering curve [41] was derived from the coordinates of the *D. acetoxidans* cytochrome c_7 (PDB code: 1HH5; [11]) that has 46% sequence identity with *G. sulfurreducens* c_7. The radii of gyration (Rg) values measured are 12.3, 20.6, 19.3, and 22.0 Å for the first, second and third peaks in Fig. 5, and the apo-cytochrome, respectively. The calculated Rg for c_7 derived from *D. acetoxidans* is 11.7 Å, which is in good agreement with the value measured for the three-heme species showing that they have similar three-dimensional structures. The higher measured value of the radius of gyration for *G. sulfurreducens* c_7 might reflect the increased lysine content of this protein.

4. Discussion

Numerous attempts to express genes of various *c*-type cytochromes in *E. coli* have met with different degrees of success. In some cases, recombinant cytochromes either were exported to the periplasm but remained in the form of apo-cytochromes, e.g. cytochrome c_3 from *Desulfovibrio vulgaris* (Hildenborough) [42] or accumulated as precursors in the cytoplasmic membrane, e.g. *c*-type cytochromes from *Rhodopseudomonas viridis* [43]. Correctly matured *c*-type cytochromes were obtained but with low yields, for cytochrome c_6 from cyanobacteria *Synechocystis* [44] and from *Anabaena* [45], for cytochrome c_{550} from *Bacillus subtilis* [46], and from *Thiobacillus versutus* [47], and also for cytochrome c_2 from *Rhodobacter sphaeroides* [48].

The development of the expression system for *c*-type cytochromes employing coexpression of *ccm* genes from a separate plasmid in *E. coli* [20] considerably increased both yields and reliability of cytochrome *c* expression: e.g., of c_3 tetraheme cytochromes from *Desulfovibrio desulfuricans* [22] and from *D. vulgaris* Hildenborough [24]; and a diheme cytochrome from *Haemophilus influenzae* [27]. In our experiments, however, we obtained a little or no holoprotein in the case of the His-tagged version and significant amounts of partially modified species for the wild-type cytochrome c_7 in addition to the fully mature protein when expressed in the above system.

It has been suggested for cytochromes *c* that coordination by non-native histidine ligands could lead to misfolded species, resulting in a kinetic trap that allows the misfolded proteins to accumulate [49,50]. We speculate that partially matured two-heme and one-heme species in the present study are unproductive intermediates where incorrect histidine residues coordinate to a heme group already attached and prevent further heme incorporation due to structural or spatial constraints. The small angle X-ray scattering results suggest that these partially matured proteins are not well ordered. The coordination of hemes by non-native residues might also be the reason why the His-tagged protein fails to assemble properly. The N-terminal chain segment that contains the His-tag has seven histidine residues and two methionine residues. These potential ligands could coordinate to a heme, thereby distorting the structure and interfering with proper heme attachment. Lower yields for His-tagged cytochromes compared to the untagged versions have also been reported by other investigators [27,51].

A possible reason for failure to modify all heme attachment sites is a relatively high rate of protein synthesis, therefore the amounts of the Ccm proteins present might be insufficient to provide hemes for *all* heme attachment sites in *all* newly synthesized polypeptide chains. To correct this problem, we decreased the rate of protein synthesis by two methods: (a) by the use of an alternate promoter and (b) by lowering the IPTG concentration. (a) The rate of protein synthesis provided by the *lac* promoter in pASK40-based construct we used is lower than the protein synthesis facilitated by the T7 promoter. In a T7-based system described earlier for a monoheme cytochrome [26], only 15–30% of the molecules contained hemes. (b) We found that by decreasing IPTG concentration from 1 mM to 10 μM doubled the yield of the fully matured form. This observation is in agreement with what was seen for the expression of the cytochrome *c* domain of cytochrome cd_1 from *Paracoccus pantotrophus* where additional induction of the *tac* promoter with IPTG resulted in lower yields, and media richer than LB (2 × TY and TB) not only did not increase the yield but also caused a large degree of cell lysis [28].

The expression system reported here provides for up to 6 mg/l of culture of correctly folded recombinant triheme c_7 cytochrome from *G. sulfurreducens*. To the best of our knowledge, the yields obtained in *E. coli* for other recombinant multiheme cytochromes published up until now [20,22,24,27] have been significantly lower, 1 mg/l or less. Using *Shewanella oneidensis* as an expression host, Ozawa et al. [52] were able to obtain yields of ~2 mg/l for a tetraheme cytochrome c_3.

In summary, we described the overproduction of the recombinant cytochrome c_7 from *G. sulfurreducens* in *E. coli*. The coexpression of the *E. coli* Ccm maturation proteins with the cytochrome c_7 in a pASK40-based construct resulted in good yield of the fully matured protein. This system will be useful for the large-scale production of wild-type and mutant cytochromes for NMR and X-ray diffraction studies.

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References

[1] F.S. Mathews, The structure, function and evolution of cytochromes, Prog. Biophys. Mol. Biol. 45 (1985) 1–56.
[2] M. Bruschi, Cytochrome c_3 (M(r) 26,000) isolated from sulfate-reducing bacteria and its relationships to other polyhemic cytochromes from *Desulfovibrio*, Methods Enzymol. 243 (1994) 140–155.
[3] I.B. Coutinho, A.V. Xavier, Tetraheme cytochromes, Methods Enzymol. 243 (1994) 119–140.
[4] S. Nørager, P. Legrand, L. Pieulle, C. Hatchikian, M. Roth, Crystal structure of the oxidized and reduced acidic cytochrome c_3 from *Desulfovibrio africanus*, J. Mol. Biol. 290 (1999) 881–902.
[5] S. Seeliger, R. Cord-Ruwisch, B. Schink, A periplasmic and extracellular *c*-type cytochrome of *Geobacter sulfurreducens* acts as a ferric iron reductase and as an electron carrier to other acceptors or to partner bacteria, J. Bacteriol. 180 (1998) 3686–3691.
[6] J.R. Lloyd, E.L. Blunt-Harris, D.R. Lovley, The periplasmic 9.6-kilodalton *c*-type cytochrome of *Geobacter sulfurreducens* is not an electron shuttle to Fe(III), J. Bacteriol. 181 (1999) 7647–7649.
[7] J.R. Lloyd, C. Leang, A.L. Hodges Myerson, M. Coppi, S. Cuifo, B. Methe, S.J. Sandler, D.R. Lovley, Biochemical and genetic characterization of CytP, a small soluble cytochrome of *Geobacter sulfurreducens*. Biochem. J. (submitted for publication).
[8] F. Caccavo Jr., D.J. Lonergan, D.R. Lovley, M. Davis, J.F. Stolz, M.J. McInerney, *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism, Appl. Environ. Microbiol. 60 (1994) 3752–3759.
[9] D.R. Lovley, M.J. Baedecker, D.J. Lonergan, I.M. Cozzarelli, E.J. Phillips, D.I. Siegel, Oxidation of aromatic contaminants coupled to microbial reduction, Nature 339 (1989) 297–299.
[10] E. Afkar, Y. Fukumori, Purification and characterization of triheme cytochrome c_7 from the metal-reducing bacterium, *Geobacter metallireducens*, FEMS Microbiol. Lett. 175 (1999) 205–210.
[11] M. Czjzek, P. Arnoux, R. Haser, W. Shepard, Structure of cytochrome c_7 from *Desulfuromonas acetoxidans* at 1.9 Å resolution, Acta Crystallogr., D 57 (2001) 670–678.
[12] L. Thöny-Meyer, F. Fischer, P. Kunzler, D. Ritz, H. Hennecke, *Escherichia coli* genes required for cytochrome *c* maturation, J. Bacteriol. 177 (1995) 4321–4326.
[13] L. Thöny-Meyer, Biogenesis of respiratory cytochromes in bacteria, Microbiol. Mol. Biol. Rev. 61 (1997) 337–376.
[14] M.D. Page, Y. Sambongi, S.J. Ferguson, Contrasting routes of *c*-type cytochrome assembly in mitochondria, chloroplasts and bacteria, Trends Biochem. Sci. 23 (1998) 103–108.
[15] J. Grove, S. Tanapongpipat, G. Thomas, L. Griffiths, H. Crooke, J. Cole, *Escherichia coli* K-12 genes essential for the synthesis of *c*-type cytochromes and a third nitrate reductase located in the periplasm, Mol. Microbiol. 19 (1996) 467–481.
[16] H. Schulz, R.A. Fabianek, E.C. Pellicioli, H. Hennecke, L. Thöny-Meyer, Heme transfer to the heme chaperone CcmE during cytochrome *c* maturation requires the CcmC protein, which may function independently of the ABC-transporter CcmAB, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 6462–6467.
[17] R.A. Fabianek, T. Hofer, L. Thöny-Meyer, Characterization of the *Escherichia coli* CcmH protein reveals new insights into the redox pathway required for cytochrome *c* maturation, Arch. Microbiol. 171 (1999) 92–100.
[18] H. Schulz, E.C. Pellicioli, L. Thöny-Meyer, New insights into the role of CcmC, CcmD and CcmE in the haem delivery pathway during cytochrome *c* maturation by a complete mutational analysis of the conserved tryptophan-rich motif of CcmC, Mol. Microbiol. 37 (2000) 1379–1388.
[19] L. Thöny-Meyer, Haem-polypeptide interactions during cytochrome *c* maturation, Biochim. Biophys. Acta 1459 (2000) 316–324.
[20] E. Arslan, H. Schulz, R. Zufferey, P. Kunzler, L. Thöny-Meyer, Overproduction of the *Bradyrhizobium japonicum c*-type cytochrome subunits of the cbb_3 oxidase in *Escherichia coli*, Biochem. Biophys. Res. Commun. 251 (1998) 744–747.
[21] B. Reincke, L. Thöny-Meyer, C. Dannehl, A. Odenwald, M. Aidim, H. Witt, H. Ruterjans, B. Ludwig, Heterologous expression of soluble fragments of cytochrome c_{552} acting as electron donor to the *Paracoccus denitrificans* cytochrome *c* oxidase, Biochim. Biophys. Acta 1411 (1999) 114–120.
[22] P.N. da Costa, C. Conte, L.M. Saraiva, Expression of a *Desulfovibrio* tetraheme cytochrome *c* in *Escherichia coli*, Biochem. Biophys. Res. Commun. 268 (2000) 688–691.
[23] J.A. Fee, Y. Chen, T.R. Todaro, K.L. Bren, K.M. Patel, M.G. Hill, E. Gomez-Moran, T.M. Loehr, J. Ai, L. Thöny-Meyer, P.A. Williams, F. Stura, V. Sridhar, D.E. McRee, Integrity of *Thermus thermophilus* cytochrome c_{552} synthesized by *Escherichia coli* cells expressing the host-specific cytochrome *c* maturation genes, *ccmABCDEFGH*: biochemical, spectral, and structural characterization of the recombinant protein, Protein Sci. 9 (2000) 2074–2084.
[24] M.L. Herbaud, C. Aubert, M.C. Durand, F. Guerlesquin, L. Thöny-Meyer, A. Dolla, *Escherichia coli* is able to produce heterologous tetraheme cytochrome c_3 when the *ccm* genes are co-expressed, Biochim. Biophys. Acta 1481 (2000) 18–24.
[25] N.J. Prince, L. Brennan, T.Q. Faria, E. Vijgenboom, G.W. Canters, D.L. Turner, H. Santos, High yield of *Methylophilus methylotrophus* cytochrome *c″* by coexpression with cytochrome *c* maturation gene cluster from *Escherichia coli*, Protein Expr. Purif. 20 (2000) 444–450.
[26] C. Sanders, H. Lill, Expression of prokaryotic and eukaryotic cytochromes *c* in *Escherichia coli*, Biochim. Biophys. Acta 1459 (2000) 131–138.
[27] A. Brigé, J.A. Cole, W.R. Hagen, Y. Guisez, J.J. Van Beeumen, Overproduction, purification and novel redox properties of the dihaem cytochrome *c* NapB, from *Haemophilus influenzae*, Biochem. J. 356 (2001) 851–858.
[28] E.H. Gordon, E. Steensma, S.J. Ferguson, The cytochrome *c* domain of dimeric cytochrome cd_1 of *Paracoccus pantotrophus* can be produced at high levels as a monomeric holoprotein using an improved *c*-type cytochrome expression system in *Escherichia coli*, Biochem. Biophys. Res. Commun. 281 (2001) 788–794.
[29] J. Vieira, J. Messing, The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers, Gene 19 (1982) 259–268.
[30] H.J. Meerman, G. Georgiou, Construction and characterization of a set of *E. coli* strains deficient in all known loci affecting the proteolytic stability of secreted recombinant proteins, Biotechnology (New York) 12 (1994) 1107–1110.
[31] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: a Laboratory Manual, , 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
[32] P. Wilkins Stevens, R. Raffen, D.K. Hanson, Y.L. Deng, M. Berrios-Hammond, F.A. Westholm, C. Murphy, M. Eulitz, R. Wetzel, A. Solomon, M. Schiffer, F.J. Stevens, Recombinant immunoglobulin variable domains generated from synthetic genes provide a system for in vitro characterization of light-chain amyloid proteins, Protein Sci. 4 (1995) 421–432.

[33] R.M. Horton, L.R. Pease, in: M.J. McPherson (Ed.), Directed Mutagenesis, Oxford Univ. Press, New York, NY, 1991, pp. 217–247.
[34] S. Seifert, R.E. Winans, D.M. Tiede, P. Thiyagarajan, Design and performance of a ASAXS instrument at the Advanced Photon Source, J. Appl. Crystallogr. 33 (2000) 782–784.
[35] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[36] H. Nielsan, J. Engelbrecht, S. Brunak, G. von Heijne, Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites, Protein Eng. 10 (1997) 1–6.
[37] L. Thöny-Meyer, P. Kunzler, H. Hennecke, Requirements for maturation of *Bradyrhizobium japonicum* cytochrome c_{550} in *Escherichia coli*, Eur. J. Biochem. 235 (1996) 754–761.
[38] A. Skerra, I. Pfitzinger, A. Pluckthun, The functional expression of antibody Fv fragments in *Escherichia coli*: improved vectors and a generally applicable purification technique, Biotechnology (New York) 9 (1991) 273–278.
[39] R. Raffen, P. Wilkins Stevens, C. Boogaard, M. Schiffer, F.J. Stevens, Reengineering immunoglobulin domain interactions by introduction of charged residues, Protein Eng. 11 (1998) 303–309.
[40] S. Arai, M. Hirai, Reversibility and hierarchy of thermal transition of hen egg-white lysozyme studied by small-angle X-ray scattering, Biophys. J. 76 (1999) 2192–2197.
[41] R. Zhang, P. Thiyagarajan, D.M. Tiede, Probing protein fine structures by wide angle solution X-ray scattering, J. Appl. Crystallogr. 33 (2000) 565–568.
[42] W.B. Pollock, P.J. Chemerika, M.E. Forrest, J.T. Beatty, G. Voordouw, Expression of the gene encoding cytochrome c_3 from *Desulfovibrio vulgaris* (Hildenborough) in *Escherichia coli*: export and processing of the apoprotein, J. Gen. Microbiol. 135 (1989) 2319–2328.
[43] R. Grisshammer, C. Oeckl, H. Michel, Expression in *Escherichia coli* of *c*-type cytochrome genes from *Rhodopseudomonas viridis*, Biochim. Biophys. Acta 1088 (1991) 183–190.
[44] A. Diaz, F. Navarro, M. Hervas, J.A. Navarro, S. Chavez, F.J. Florencio, M.A. De la Rosa, Cloning and correct expression in *E. coli* of the *petJ* gene encoding cytochrome c_6 from *Synechocystis* 6803, FEBS Lett. 347 (1994) 173–177.
[45] F.P. Molina-Heredia, M. Hervas, J.A. Navarro, M.A. De la Rosa, Cloning and correct expression in *Escherichia coli* of the petE and petJ genes respectively encoding plastocyanin and cytochrome c_6 from the cyanobacterium *Anabaena* sp. PCC 7119, Biochem. Biophys. Res. Commun. 243 (1998) 302–306.
[46] C. von Wachenfeldt, L. Hederstedt, *Bacillus subtilis* holo-cytochrome *c*-550 can be synthesised in aerobic *Escherichia coli*, FEBS Lett. 270 (1990) 147–151.
[47] M. Ubbink, J. Van Beeumen, G.W. Canters, Cytochrome c_{550} from *Thiobacillus versutus*: cloning, expression in *Escherichia coli*, and purification of the heterologous holoprotein, J. Bacteriol. 174 (1992) 3707–3714.
[48] A.G. McEwan, S. Kaplan, T.J. Donohue, Synthesis of *Rhodobacter sphaeroides* cytochrome c_2 in *Escherichia coli*, FEMS Microbiol. Lett. 50 (1989) 253–258.
[49] G.A. Elöve, A.K. Bhuyan, H. Roder, Kinetic mechanism of cytochrome *c* folding: involvement of the heme and its ligands, Biochemistry 33 (1994) 6925–6935.
[50] T.R. Sosnick, L. Mayne, R. Hiller, S.W. Englander, The barriers in protein folding, Nat. Struct. Biol. 1 (1994) 149–156.
[51] P.S. David, M.R. Morrison, S.L. Wong, B.C. Hill, Expression, purification, and characterization of recombinant forms of membrane-bound cytochrome *c*-550 nm from *Bacillus subtilis*, Protein Expr. Purif. 15 (1999) 69–76.
[52] K. Ozawa, A.I. Tsapin, K.H. Nealson, M.A. Cusanovich, H. Akutsu, Expression of a tetraheme protein, *Desulfubrio vulgaris* Miyazaki F cytochrome c_3, in *Shewanella oneidensis* MR-1, Appl. Environ. Microbiol. 66 (2000) 4168–4171.