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Heterologous metalloprotein biosynthesis in *Escherichia coli*: conditions for the overproduction of functional copper-containing nitrite reductase and azurin from *Alcaligenes xylosoxidans*

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This paper reports on the optimization of conditions for the overproduction and isolation of two recombinant copper metalloproteins, originally encoded on the chromosome of the dentrifying soil bacterium *Alcaligenes xylosoxidans*, in the heterologous host *Escherichia coli.* The trimeric enzyme nitrite reductase (NiR) contains both type-1 and type-2 Cu centres, whilst its putative redox partner, azurin I, is monomeric and has only a type-1 Cu centre. Both proteins were processed and exported to the periplasm of *E. coli*, which is consistent with their periplasmic location in their native host *A. xylosoxidans*. NiR could be readily purified from the periplasmic fraction of *E. coli* but the enzyme as isolated possessed only type-1 Cu centres. The type-2 Cu centre could be fully reconstituted by incubation of the periplasmic fraction with copper sulfate prior to enzyme purification. Azurin I could only be isolated with a fully occupied type-1 centre when isolated from the crude cell extract but not after isolation from the periplasmic fraction, suggesting loss of the copper due to proteolysis. Based on a number of criteria, including spectroscopic, mass spectrometric, biochemical and structural analyses, both recombinant proteins were found to be indistinguishable from their native counterparts isolated from *A. xylosoxidans*. The findings of this work have important implications for the overproduction of recombinant metalloproteins in heterologous hosts.

Keywords: copper proteins; heterologous protein production; denitrification; crystal structure; metal centres.

1. Introduction

The recent advent of large-scale genome sequencing and the concomitant development of functional and structural genomic programmes has become a major focus of current research. An important aspect of such advances is the development of suitable systems for the large-scale high-throughput production of recombinant proteins for structural and biochemical analyses. The traditional means of achieving this objective is by making use of appropriate heterologous host organisms, such as *E. coli*, for recombinant protein production. Recent bioinformatic studies (Degtyarenko, 2000) have revealed that it can be anticipated that a large proportion (at least 30%) of proteins encoded in known genomes are metalloproteins. Therefore, in addition to general concerns of protein folding, other considerations must be made, which include whether metal centres are accurately and completely incorporated into the recombinant protein within the heterologous host. In this paper we address this potential problem by examining the incorporation of copper atoms into two metalloproteins: the small monomeric cupredoxin azurin I and the trimeric nitrite reductase (NiR), both of which are encoded in the genome of the denitrifying bacterium *Alcaligenes xylosoxidans* (Abraham *et al.*, 1993; Dodd *et al.*, 1995; Prudêncio *et al.*, 1999).

Dissimilatory NiR is a key enzyme in the denitrification process in which nitrate undergoes stepwise reduction to the gaseous products nitrous oxide and dinitrogen (see Scheme; Zumft, 1997; Eady & Hasnain, 2003). The first committed step in this process involves the one-electron reduction of nitrite to nitric oxide, catalysed by NiR. Two distinct classes of periplasmic NiRs catalyse this reaction: one containing cd_1 heme as the prosthetic group and the other containing copper (Eady & Hasnain, 2003). The two Cu centres in NiR from

1) $NO_3^- \leftrightarrow NO_2^- \longrightarrow NO \longrightarrow N_2O \longrightarrow N_2$

2) $NO_2^- + e^- + 2H^+ \longrightarrow NO + H_2O$
(E_0' at pH 7.0 = +370 mV)

A. xylosoxidans comprise a type-1 centre, which imparts the blue colour to this enzyme, and a type-2 centre, which makes no contribution to the visible spectrum of the protein. The type-2 centre is the site of nitrite reduction and the electron for this reaction (see Scheme) is delivered from the type-1 centre. Reduced azurin I has a type-1 Cu centre, and donates the electron to the type-1 Cu centre of NiR (Dodd *et al.*, 1995), presumably through the formation of a protein–protein complex. The electron donor to oxidized azurin I has not been identified.

The blue NiR from *A. xylosoxidans* has been characterized extensively and its structure has been determined to atomic resolution (Ellis *et al.*, 2003). Native azurin I has also been isolated from *A. xylosoxidans* (Dodd *et al.*, 1995) and more recently the gene encoding this redox protein has been cloned (R. L. Harris, R. R. Eady, S. S. Hasnain and R. G. Sawers, unpublished observations). Overproduction of the recombinant NiR has been achieved in *E. coli* and it is exported to the periplasmic compartment in the heterologous host (Prudêncio *et al.*, 1999). In the present study we report on the isolation of recombinant forms of both NiR and azurin I from the heterologous host *E. coli*, and we highlight important considerations in producing active recombinant protein with a full complement of metal centres.

2. Experimental

2.1. Bacterial growth

The *Escherichia coli* strain used in this study was BL21(DE3) F^- *ompT gal [dcm] [lon] hsdS*$_B$ ($r_B^-m_B^-$; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene (Studier & Moffat, 1986). The plasmids used were pEnirsp-1 (Kan^R) (Prudêncio *et al.*, 1999) and pTAZU-1 (R. L. Harris, R. R. Eady, S. S. Hasnain and R. G. Sawers, unpublished observations), which is a pT7-7 (Ap^R) derivative (Novagen). Antibiotics were used at a final concentration of 50 μg ml^{-1}.

E. coli strains were grown routinely in Luria Broth (LB) [1% *w*/*v*, bactotryptone (Difco), 0.5% *w*/*v* yeast extract (Difco), 1% *w*/*v* NaCl] at 310 K. Small-scale cultures (up to 10 ml) for testing protein overproduction were grown aerobically in sterile conical flasks filled to approximately 10% of their volume with growth medium. For large-scale isolation of NiR and azurin I, 1 l fermentor cultures were grown in HYE 50 medium [per liter; 3 g KH_2PO_4, 6 g Na_2HPO_4, 0.5 g NaCl, 2 g casein hydrolysate, 10 g $(NH_4)_2SO_4$, 35 g glycerol, 20 g yeast extract, 0.5 g $MgSO_4{\cdot}7H_2O$, 40 mg $FeSO_4{\cdot}2H_2O$, 20 mg citric acid, 30 mg $CaCl{\cdot}2H_2O$, 8 mg thiamin hydrochloride and 0.5 ml trace-element solution]. Media were also supplemented with $CuSO_4$ (0.1 m*M*). Overproduction of NiR was performed at 303 K and achieved by adding 0.25 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested approximately 4 h after induction had been initiated. For overproduction of azurin I the fermentor was run at 310 K and with a constant stirring speed of 300 r.p.m. until the cultures had attained an $OD_{600\,nm}$ of 10 when the stirring speed was increased to 1000 r.p.m. The pH of the cultures was maintained at 7.1. Induction of high-level protein production was initiated when the cultures had reached a turbidity ($OD_{600\,nm}$) of 25, through the addition of 0.5 m*M* IPTG. The temperature of the culture was reduced to 301.5 K. The cells were cultured for a further 3 h (final $OD_{600\,nm}$ of 40) prior to harvesting by centrifugation. Generally, 40–50 g wet weight of cells were derived from each fermentation. Antibiotics were purchased from Sigma and stock solutions were prepared in water and filter-sterilized through sterile 0.2 μm syringe filters (Sartorius). Cells were used immediately for the isolation of recombinant protein or were stored frozen at 193 K until required.

2.2. Protein purification

Native NiR and native azurin I were purified from *A. xylosoxidans* as described previously (Abraham *et al.*, 1993; Dodd *et al.*, 1995). Recombinant NiR was purified from the periplasmic fraction of *E. coli* as described (Prudêncio *et al.*, 1999) and recombinant azurin I was purified from the crude extract of recombinant *E. coli* cells using a single carboxymethyl cellulose step in a similar procedure to that described for NiR. Briefly, after cell disruption by French press (16000 p.s.i.) and centrifugation, the crude extract was passed over a DEAE cellulose column equilibrated with 100 m*M* tris-HCl pH 7.4 buffer. Under these conditions azurin I did not bind to the column and the flow-through was collected and precipitated with solid ammonium sulfate, which was added to a final saturation of 75%. After collection of the ammonium sulfate precipitate by centrifugation, the material was resuspended in 50 ml of water and dialyzed for 48 h against water. After clarification of the dialysate by centrifugation, the suspension was loaded onto a CM cellulose column (10 cm × 5 cm) that had been equilibrated with 500 m*M* MES [2-(*N*-morpholino)ethanesulfonic acid], pH 6.0, followed by washing with two column volumes of water. The overproduced azurin I has a pronounced absorbance band at 620 nm, which is characteristic of oxidized type-1 Cu sites. Recombinant azurin I bound as a tight blue band to the top of the column. After washing the column extensively with water, azurin was eluted with 200 m*M* tris-Cl pH 8 containing 200 m*M* NaCl. The pure azurin I preparation was then dialyzed against 50 m*M* MES buffer, pH 6. Generally, approximately 0.5 mg of pure NiR and 2 mg of pure azurin I were obtained per g wet weight of cell paste.

2.3. Metal determination

The copper and zinc content of samples of NiR and azurin I were measured on wet-ashed samples (Abraham *et al.*, 1993),

using inductively coupled plasma emission spectroscopy by Southern Analytical (Sussex, UK).

2.4. Spectroscopic methods

Electron paramagnetic resonance (EPR) data were collected using a Bruker ER ER200 D-SRC spectrometer fitted with an ER042 MRH microwave bridge with an ER033C field frequency lock and an Oxford Instruments Ite^{503} temperature controller. EPR spectra were simulated using the program *WINEPR* (SimFonia, Bruker). Spin quantizations were made according to the method of Åasa & Vänngård (1975), by comparing the area of the experimental curve under non-saturating conditions to that obtained, under the same conditions, for a sample of Cu(II)-EDTA.

2.5. Other methods

Molecular weight determinations were undertaken using matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry and by quadrupole/time-of-flight (Q-ToF) mass spectrometry.

MALDI-ToF mass spectrometry was performed using a Reflex III MALDI-ToF mass spectrometer (Bruker UK, Coventry, UK). A 10 mg ml^{-1} solution of 3,5-dimethoxy-4-hydroxycinnamic acid (Fluka, Dorset, UK) in acetone was prepared. This was mixed with the protein sample and spotted directly onto a stainless steel target plate. Mass spectra were acquired in positive-ion reflection mode. Accurate calibration was achieved with reference to the singly and doubly charged molecular ions of horse heart myoglobin (Sigma), spotted adjacent to the sample.

Q-ToF mass spectrometry was performed in the following way: protein was diluted in 50/50 water/acetonitrile + 0.1% formic acid to a concentration of 5 pmol µl^{-1}, loaded into coated borosilicate glass capillaries (Micromass, Manchester, UK) and sprayed into the nano-electrospray ion-source of a Q-ToF 2 mass spectrometer (Micromass, Manchester, UK) in positive-ion mode. Charge-states from the resulting spectra were deconvoluted using the *MaxEnt* algorithm (Micromass, Manchester, UK) to enable determination of exact molecular weights.

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS-PAGE) was used routinely to check the purity of protein samples throughout the purification. SDS-PAGE was performed according to Laemmli (1970). Typically, 12.5% (*w*/*v*) or 15% (*w*/*v*) acrylamide gels were run. For whole cell extracts, $OD_{600\,nm}$ was measured and a volume of $1.2/OD_{600\,nm}$ ml of cell suspension was centrifuged and the cell pellet was resuspended in 80 µl of SDS sample buffer.

3. Results and discussion

3.1. Heterologous synthesis of NiR in *E. coli*

The *nirA* gene could be overexpressed in exponentially growing *E. coli* BL21(DE3) cells containing vector pEnirsp-1 by inducing the T7 RNA polymerase-directed transcription of this gene with IPTG. Overproduction of NiR was optimized on a small scale (5–10 ml cultures) with regard to growth temperature, IPTG concentration and duration of induction. Growth of the cultures at 303 K and inducing enzyme production by adding 0.25 m*M* IPTG when the cells had attained an optical density of ∼0.4–0.5, as determined by measuring the absorbance at 600 nm, were found to be optimal conditions (Prudêncio *et al.*, 1999; and data not shown). The extent of cell growth subsequent to induction is also an important parameter and this was assayed in a 1 l batch-fed fermentor. Cells were grown at 303 K and induction with 0.25 m*M* IPTG was carried out when the $OD_{600\,nm}$ was 0.5. Samples were then collected at 30 min intervals for up to 5 h and the production of NiR was analysed by SDS-PAGE (Fig. 1*a*). The overproduced protein is present in two different forms, which correspond to an unprocessed form of the protein, of higher apparent molecular mass (∼43 kDa), and a mature form of the protein, of lower apparent molecular mass (∼35 kDa). Overproduction of NiR worked well for a number of conditions analysed. However, incubating the cultures at 303 K for 3–4 h subsequent to induction yielded the highest ratio of mature:unprocessed forms of the protein, together with the highest levels of soluble mature enzyme (Fig. 1*a*).

Purification of the mature NiR polypeptide was from the periplasmic fraction of *E. coli* (Prudêncio *et al.*, 1999). The N-terminal amino acid sequence of the mature polypeptide was determined to be Q-D-A-D-K-L, which is in perfect agreement with that predicted for the mature NiR protein lacking the signal sequence and is identical to that determined for native NiR isolated from *A. xylosoxidans* (Vandenberghe *et al.*, 1998). Taken together, these data provide clear evidence that the native NiR signal peptide is recognized and efficiently and specifically cleaved by the *E. coli* export apparatus.

Recombinant NiR purified from various growths of *E. coli* cells was characterized in terms of its catalytic, spectroscopic and biochemical properties. These were compared with those of the wild-type NiR (wt-NiR) from *A. xylosoxidans* and the two proteins were shown to be indistinguishable with regard to the parameters studied (see Prudêncio *et al.*, 1999, 2001).

The final confirmation that the recombinant NiR protein is indeed indistinguishable from the native enzyme isolated from *A. xylosoxidans* was confirmed by the determination of the X-ray crystal structures of both enzymes (Ellis *et al.*, 2001, 2003).

3.2. Correlation between Cu content and activity of NiR

Purified concentrated recombinant NiR is dark blue in colour. The colour is due to absorbance maxima at 595 nm and 470 nm and is an indication of the occupancy of the type-1 Cu centre (Abraham *et al.*, 1993). Measurement of the ratio of the absorbance at 280 nm compared with that at 595 nm gives a good indication of the type-1 centre occupancy. Type-1 centres fully loaded with Cu have an absorbance ratio of between 12 and 16. Higher ratios indicate incomplete occupancy. NiR that was purified without treatment has a ratio of ∼20. The EPR spectrum of this protein shows the typical features of type-1

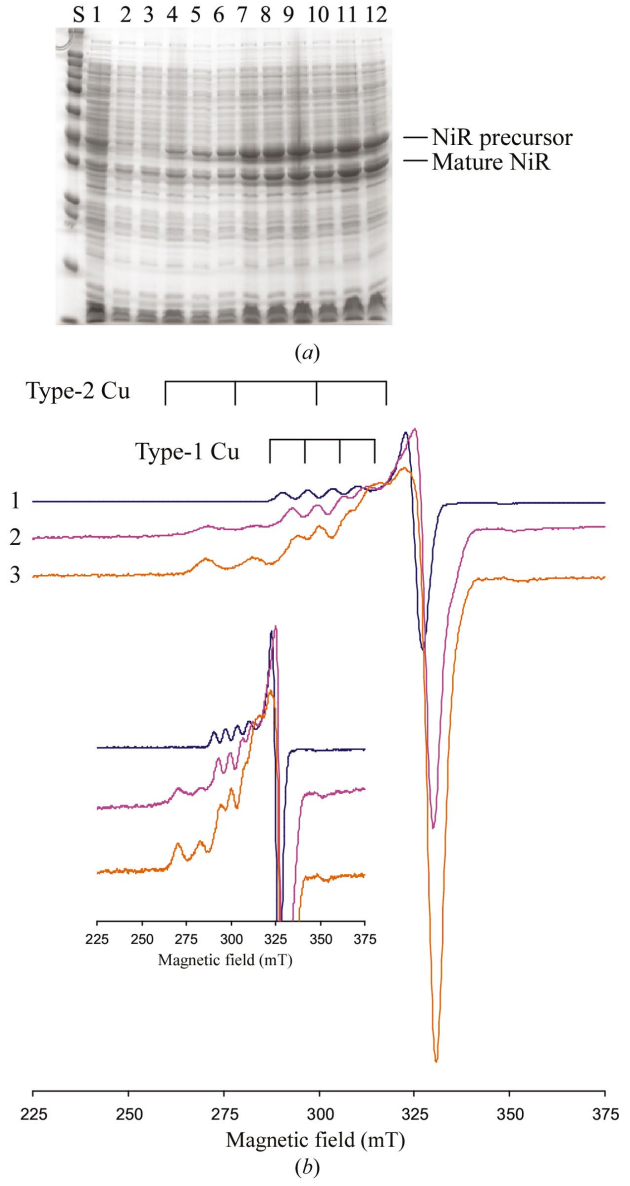

Figure 1
Analysis of recombinant NiR. (*a*) SDS-PAGE containing 12.5% (*w*/*v*) acrylamide of whole cell extracts of BL21(DE3) containing pEnirsp1 grown at 303 K in a 1 l fermenter. Induction at $OD_{600\,nm} \simeq 0.5$: lane S, molecular mass markers; lane 1, negative control, BL21(DE3); lane 2, sample taken immediately after addition of 0.25 m*M* IPTG; lanes 3 to 12, samples taken at 30 min intervals after induction. (*b*) EPR spectra of recombinant NiR in 20 m*M* MES buffer, pH 6.0. Spectrum 1. As-purified protein (T2D form) at 9.4 mg ml^{-1} (255 µ*M* monomer). Spectrum 2. Protein incubated with 1 m*M* $CuSO_4$ after purification (partially reconstituted form) at 6.1 mg ml^{-1} (165 µ*M* monomer). Spectrum 3. Protein purified after incubation of the periplasmic fraction with $CuSO_4$ (fully reconstituted form) at 4.8 mg ml^{-1} (135 µ*M* monomer). The spectra were recorded at 60 K at a microwave frequency of 9.312 GHz and normalized for ease of comparison. The type-1 and type-2 features are indicated.

Cu and a complete absence of a type-2 Cu EPR signal (Fig. 1*b*). Metal analysis indicated that this protein contains ~2.1 Cu atoms per trimer and the specific activity of this enzyme measured with the MV/dithionite assay (Abraham *et al.*, 1993) is only 11 units per mg of protein (Table 1), where one unit is defined as 1 µmol of nitrite reduced per minute. This shows that this form of the enzyme is deficient in the type-2 Cu centre (T2D) and also does not have a full type-1 Cu complement.

Table 1
Relationship between occupancy of the Cu centres and enzyme activity of NiR.

	Specific activity (µmoles NO_2^- reduced)	Metal content (mol Cu/mol trimer)	Type-1 Cu	Type-2 Cu
Enzyme preparation				
T2D	11.0	2.1	Partial	0
Pure + Cu†	47.0	3.8	Full	Partial
Periplasm + Cu	168.0	6.3	Full	Full

† Method of Cu reconstitution involved incubation of pure enzyme or periplasmic fraction including overproduced NiR with 1 m*M* $CuSO_4$.

Upon incubation of the purified protein with 1 m*M* $CuSO_4$, type-2 Cu EPR features appear in the spectrum of NiR (Fig. 1*b*, trace 2). The intensity of these features is lower than that of the type-1 signal, the ratio $Abs_{280\,nm}/Abs_{595\,nm}$ is reduced to ~16 and the Cu content is increased to ~3.8 Cu atoms per trimer, based on metal analysis (Table 1). This indicates that the type-2 Cu sites in the protein have been only partially reconstituted, whilst there is a full complement of type-1 Cu. Again, this result is consistent with the approximate fourfold increase in activity observed for this form of the enzyme.

It is possible to isolate recombinant NiR that has a full complement of type-1 and type-2 Cu after incubation of the periplasmic fraction with 1 m*M* $CuSO_4$ (Fig. 1*b*, trace 3). The $Abs_{280\,nm}/Abs_{595\,nm}$ ratio of this enzyme was ~13 and the protein was shown to have ~6.3 Cu atoms per trimer and a maximal activity of 168 per mg of protein (Table 1). The correlation between the presence of type-2 Cu centres and the activity of NiR is consistent with the notion that these centres are the sites of catalysis (Libby & Averill, 1992; Howes *et al.*, 1994; Abraham *et al.*, 1997; Suzuki *et al.*, 1997; Dodd *et al.*, 1998; Farver *et al.*, 1998).

Owing to its location at the subunit boundary, it is likely that the type-2 Cu is readily removed from the enzyme during purification. The results above suggest that the incorporation of Cu into the type-2 centres of NiR *in vivo* is facilitated by the activity of a periplasmic enzyme. The fact that when the purified enzyme is incubated with $CuSO_4$ only partial reconstitution of the type-2 Cu sites occurs is in accord with previous observations that suggested the presence of a metal chaperone in *A. xylosoxidans*, involved in the insertion of type-2 Cu into NiR (R. Eady, unpublished data). The fact that activation of the enzyme is more effective on a concentrated periplasmic protein extract than on a diluted periplasmic fraction is in accord with the presence of such an activity in the periplasm of *E. coli* cells and suggests that, *in vivo*, recombinant NiR is fully loaded with Cu. The role of metal chaperones in the assembly of the CuZn superoxide dismutases is well documented (Lamb *et al.*, 2001; Bartnikas & Gitlin, 2003).

3.3. Isolation of recombinant azurin I with a fully occupied type-1 Cu centre

The cloning and characterization of the gene encoding azurin I will be described elsewhere (R. L. Harris, R. R. Eady, S. S. Hasnain and R. G. Sawers, unpublished observations). For overexpression purposes the *azu-1* gene was cloned into the expression vector pT7-7 to create plasmid pTAZU-1, which is under the control of the phage T7 $\phi 10$ promoter. Optimization of azurin I overproduction was achieved on a small scale in the same manner as for NiR. Induction was achieved by adding 0.5 m*M* IPTG, with growth of the culture at 303 K.

Although azurin I could be shown to be exported to the periplasm of *E. coli* BL21, when the protein was purified from the periplasmic fraction it was colourless and contained no type-1 Cu (data not shown). However, when the enzyme was purified from the crude cell extract (Fig. 2) the protein was a deep blue colour and had an absorption maximum at 620 nm, characteristic of type-1 cupredoxin (Abraham *et al.*, 1997). The $A_{280\,nm}$:$A_{620\,nm}$ ratio was 2.6, which was a value indistinguishable from that of the native protein (Dodd *et al.*, 1995). The N-terminal sequence of recombinant azurin I was determined to be AEXSVD. If the residue X is assumed to be due to oxidation of a cysteinyl residue, the sequence matches exactly the first six amino acids of native azurin I (Inoue *et al.*, 1994; Dodd *et al.*, 1995).

3.4. Mass spectrometry of recombinant and native azurin I

MALDI-ToF and Q-Tof mass spectrometry are powerful techniques that allow the determination of the precise masses of proteins. Aliquots (50 pmol) of recombinant and native azurin I were analysed initially by MALDI-ToF, which revealed the presence of several different species (Fig. 3*a*). These species had a mass difference of either 38 or 22 Da. It is likely that the 38 Da species is due to bound potassium and that the 22 Da species is due to sodium binding. It is clear from the profiles, however, that azurin I isolated from *E. coli* and azurin I isolated from *A. xylosoxidans* are indistinguishable. The predicted mass of both azurins as determined by MALDI-ToF was 13960 Da. This is in accord with the monomeric nature of these electron transfer proteins.

Q-ToF analysis of recombinant azurin I showed additional features not identified by the MALDI-ToF analysis. Four pairs of repeating peaks were identified (Fig. 3*b*). The mass difference between the two peaks within each doublet was 63 Da, consistent with the loss or retention of copper. Each set of doublets was separated by a mass of 194–196 Da. MES buffer has a MW of 195.03, and these patterns suggest that up to three MES molecules were bound to the protein. Q-ToF calculated the mass of RecAz to be 14015 Da.

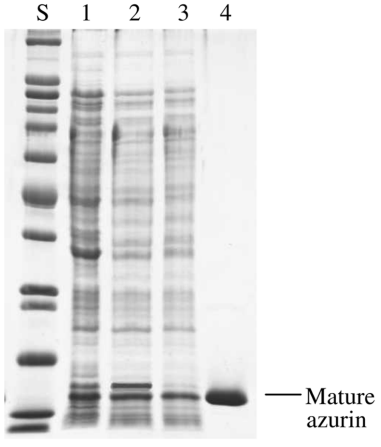

Figure 2
Purification profile of azurin I. SDS-PAGE (15% *w*/*v* polyacrylamide) analysis of various stages of protein purification is shown. Lane S, molecular mass standards; lane 1, whole cell lysate (50 µg protein); lane 2, crude cell extract (50 µg protein); lane 3, pool of DEAE flow-through (50 µg protein); lane 4, purified azurin I after CM-cellulose chromatography (5 µg protein).

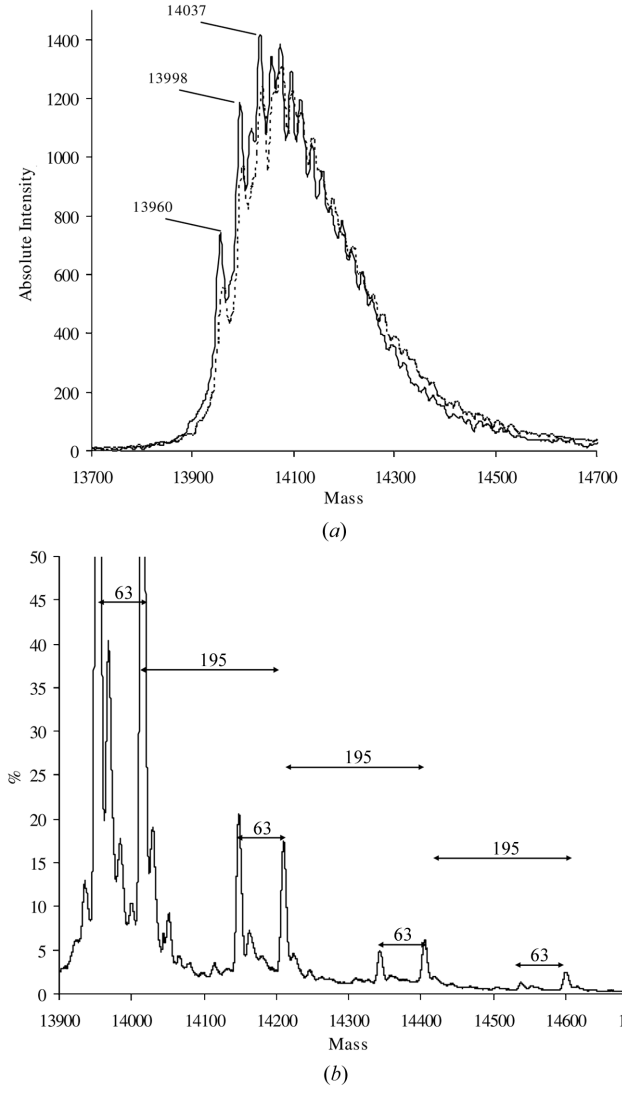

Figure 3
Analysis of recombinant azurin I. (*a*) The MALDI-ToF profiles of recombinant azurin (full line) and native azurin I (dotted line) are shown (see *Experimental* for details). The three most prominent peaks are highlighted. (*b*) Q-ToF of RecAz showing the four sets of doublets. The doublets are the Cu-containing and apo-forms of the protein. The four sets are consistent with up to three MES molecules binding to the protein.

For comparison, the mass of azurin was computationally determined using ExPASy's ProtParam tool (http://ca.expasy.org/cgi-bin/protparam). The program calculated the mass of the protein based on the amino acid sequence to be 13840 Da. This program did not take into account any cofactors, therefore Cu (63 Da) had to be added to give a final mass of 13903 Da. This is significantly different to the MALDI-ToF (13960) and the Q-ToF (14015) results. The reason for the differences between the deduced molecular mass and that derived from MALDI-ToF might result from the minimal ionic species in the MALDI-ToF experiment having one bound potassium plus one bound sodium ion. The discrepancy in size between the MALDI-ToF and Q-ToF data could signify some post-translational modification or tight binding of a non-specific unidentified species.

4. Conclusion

With the advent of structural genomics, the use of organisms such as *E. coli* as vehicles for heterologous overproduction of proteins has taken on a new significance. It is crucial that we are aware of the potential pitfalls in the overproduction of heterologous proteins, and this is particularly relevant for metalloproteins with complex cofactors. Only when we have optimized conditions and performed suitable analytical analyses can we be sure that what we see at a structural level is an accurate representation of the native protein in its homologous host. Two copper proteins from the Gram-negative denitrifying bacterium *A. xylosoxidans* with differing degrees of complexities with regard to their metal centres and their tertiary structures have been presented. Both proteins were successfully overproduced in *E. coli* BL21(DE3) and both proteins underwent export into the periplasmic space in the heterologous host, just as they do in *A. xylosoxidans*. However, different problems arose with optimizing overproduction of each protein. In the case of azurin I this was a problem of proteolysis and with NiR it was the necessity to reconstitute one of the metal centres. Nevertheless, it was possible to obtain products indistinguishable from the native proteins. These findings have broad and important implications for overproduction of heterologous proteins. For example, owing to the complex nature of metal-cofactor insertion in many proteins, where a large number of ancillary factors are often required for metal-centre maturation, this becomes a potentially significant problem in the choice of heterologous host. This is of particular relevance when analysing the genomes of organisms where the gene products have not been characterized and where it is unclear whether a metal centre is present in the protein.

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