Characterization of a molybdenum cofactor biosynthetic gene cluster in *Rhodobacter capsulatus* which is specific for the biogenesis of dimethylsulfoxide reductase

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The DMSO reductase of *Rhodobacter capsulatus* contains a pterin molybdenum cofactor (Moco) and is located in the periplasm. DNA sequence analysis identified four genes involved in the biosynthesis of the Moco (*moaA*, *moaD*, *moeB* and *moaC*) immediately downstream of the *dor* (DMSO respiratory) gene cluster. *Rhodobacter capsulatus* MoaA was expressed in *Escherichia coli* as a His₆-tagged protein. Although, the expressed protein formed inclusion bodies, EPR spectroscopy showed that MoaA contains a [3Fe-4S] cluster. A *moaA* mutant was constructed and its phenotype indicates that the Moco biosynthetic gene cluster downstream of the *dor* operon is specific for the biogenesis of DMSO reductase. Two forms of DMSO reductase were purified by immunoaffinity chromatography from the *moaA* mutant. A mature form of DMSO reductase was located in the periplasm and a precursor form was found in the cytoplasm.

Keywords: molybdenum cofactor, dimethylsulfoxide reductase, *Rhodobacter capsulatus*

INTRODUCTION

Apart from nitrogenase, all enzymes containing molybdenum form a coherent group known as the molybdenum oxotransferases and hydroxylases (Kisker *et al.*, 1997; Hille, 1996). The common feature of these oxomolybdenum enzymes is the presence of a pterin molybdenum cofactor (Moco). In oxomolybdenum enzymes from eukaryotes the organic component of Moco is a tricyclic pterin derivative known as molybdopterin (MPT) and its carbon side chain ends with a phosphate group. In almost all bacterial oxomolybdenum enzymes the MPT is in a dinucleotide form and this has been revealed in a spectacular fashion via the high resolution crystal structures of DMSO reductase (Schindelin *et al.*, 1996; Schneider *et al.*, 1996; McAlpine *et al.*, 1997), aldehyde oxidoreductase (Romeo *et al.*, 1995) and formate dehydrogenase (Boyington *et al.*, 1997).

Furthermore, it appears that in formate dehydrogenase and DMSO reductase there are two MPT guanine dinucleotide units per Mo atom. The sulfur atoms of the ene-dithiolate side chains of both MPT guanine dinucleotide moieties are ligands to the Mo centre.

Although it is now established that there is a diversity of nucleotide derivatives of MPT, there is a wealth of biochemical genetic evidence that there is a common pathway of synthesis of MPT (Johnson & Rajagopalan, 1987a, b). Much of this information has come from the analysis of pleiotropic mutants of *Escherichia coli* which are defective in various steps in Moco biosynthesis. These mutants, identified via the chlorate-resistant phenotype (Chl mutants), have been redesignated and at least five distinct loci (*moa*, *mob*, *mod*, *moe* and *mog*) are now recognized as genes or gene clusters encoding enzymes involved in synthesis of MPT and its dinucleotide forms. In *E. coli* the *moa* locus contains five genes that are involved in the early stages of the synthesis of MPT (Johnson & Rajagopalan, 1987a, b; Rivers *et al.*, 1993). *moeB* and *moaD* are thought to encode proteins involved in the addition of sulfur to these

Abbreviations: Moco, pterin molybdenum cofactor; MPT, molybdopterin.

The GenBank accession number for the sequence reported in this paper is AF012844.

moieties, since *moeB* and *moaD* mutants accumulate a sulfur-free molecule known as precursor Z, derived from the action of MoaABC (Pitterle & Rajagopalan, 1989). The enzymic step catalysed by MoaABC is still unclear. Although *E. coli* is an excellent system for the study of oxomolybdenum enzymes and Moco biosynthesis, other systems, notably the purple photosynthetic bacteria *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, are now well-developed. For example, *R. capsulatus* possesses a periplasmic DMSO reductase (McEwan *et al.*, 1991) whose structure is now defined at 1.8 Å resolution (McAlpine *et al.*, 1997), a cytoplasmic xanthine dehydrogenase (Leimkuhler *et al.*, 1998) and in some strains a periplasmic nitrate reductase (McEwan *et al.*, 1984).

Recently, we have completed the sequencing of the *dor* operon of *R. capsulatus*. This gene cluster encodes proteins involved in DMSO respiration (Shaw *et al.*, 1999). In this paper we describe the analysis of Moco biosynthetic genes which are clustered 3' to the *dor* genes of *R. capsulatus*. Analysis of the predicted amino acid sequence of the DMSO structural gene (*dorA*) (Shaw *et al.*, 1996) revealed that it possesses an unusually long signal peptide which contains the 'twin arginine' motif characteristic of exported proteins which bind complex prosthetic groups (Berks, 1996). It has been suggested that proteins of this type may be secreted via a secretory pathway that is distinct from, or is a modification of, the Sec pathway (Berks, 1996). In this paper the effect of a mutation in *moaA* on oxomolybdenum enzyme activity has been investigated and the cellular localization of the DMSO polypeptide in this mutant is described.

METHODS

Bacterial strains and plasmids. Strains and plasmids used during this study are described in Table 1. Typically, *R. capsulatus* strain 37b4 was grown phototrophically on RCV medium as described by Weaver *et al.* (1975). *E. coli* was grown routinely on LB medium with antibiotics added where required. For the growth of *R. capsulatus*, antibiotic concentrations were ($\mu\text{g ml}^{-1}$): gentamicin (Gm), 5; tetracycline (Tc), 1. Antibiotic concentrations for *E. coli* growth were ($\mu\text{g ml}^{-1}$): Gm, 5; Tc, 10; ampicillin (Ap), 100. Plasmid pFR400 harbouring the *R. sphaeroides nap* gene cluster (Reyes *et al.*, 1996) was a generous gift from Dr Conrado Moreno-Vivian (Universidad de Cordoba, Spain) and was transferred to *R. capsulatus* by conjugation with *E. coli* as described by Masepohl *et al.* (1988).

DNA sequencing and sequence analysis. The Moco biosynthetic gene cluster of the phagemid pALS1 (Shaw *et al.*, 1996) was sequenced using the dye terminator method using an Applied Biosystems 373A automated sequencer. DNA was sequenced in both directions using primers designed from new sequence information (Brendel & Trifonov, 1984). Sequence alignments and analysis was performed using programs in the Wisconsin GCG software package version 8.1-UNIX.

Expression of *R. capsulatus* MoaA in *E. coli*. *moaA*, located on pALS1, was amplified by PCR with the Expand High Fidelity PCR System (Boehringer Mannheim). The typical reaction

was made up as follows: 5 μl 10× PCR buffer (Boehringer Mannheim), 1.5 mM MgCl_2 , 0.2 mM each dNTP, 5% DMSO, 1 μM each primer, 1 unit *Taq/Pwo* DNA polymerase (New England Biolabs) and 50 ng template DNA, made up to 50 μl with sterile distilled water. Cycles were performed with an initial 1 min denaturation step at 95 °C followed by 30 cycles of 94 °C for 1 min, 42 °C for 1 min and 72 °C for 7 min. The product of the PCR amplification was purified using the QIAquick PCR purification kit (QIAGEN). The *moaA* PCR product was cloned into *EcoRI/BamHI* sites of pUC8 to create pJPS2. An *NdeI-BamHI* fragment of pJPS2 was then cloned into pET15b (Novagen) creating the MoaA expression vector, pJPS4.

For the production of MoaA, pJPS4 was transformed into strain BL21(DE3) and a 200 ml culture of these transformed cells was grown until the OD_{600} had reached 0.6. IPTG was then added to a concentration of 0.4 mM and the cells were allowed to grow for a further 4 h. The cells were harvested by centrifugation at 5000 g for 10 min at 4 °C before being washed twice in 50 mM Tris/HCl, pH 8.0. The cells were disrupted by two passages through a French pressure cell at approximately 11 000 p.s.i. Large cell fragments and unbroken cells were removed by centrifugation at 25 000 g for 15 min at 4 °C. The cell-free extract was stored at -70 °C until required.

EPR spectroscopy of MoaA. Inclusion bodies containing MoaA were washed twice with 0.2 mg lysozyme ml^{-1} /1 mM EDTA/1 mg sodium deoxycholate ml^{-1} , followed by two washes of 50 mM Tris/HCl, pH 8.0. The insoluble MoaA was then resuspended in 300 μl 50 mM Tris/HCl, pH 8.0. EPR spectra were measured at X-band on a Bruker ESP300E continuous wave spectrometer loaded with version 3.02 of Bruker ESP300E software. Microwave frequency calibration was performed using an EIP 548B frequency counter, while the magnetic field was calibrated using a Bruker ER-035 M Gauss meter. The liquid helium temperatures (ca. 2 K) required for the measurement of EPR spectra from [Fe-S] clusters were achieved using an Oxford Instruments ESR910 liquid helium flow-through cryostat, in conjunction with an Oxford Instruments ITC-4 variable temperature controller. Computer simulation of EPR spectra were performed using the Sophe computer simulation software suite (Wang & Hanson, 1995, 1996).

Construction of a *moaA* mutant. *moaA* was disrupted by insertion of a Gm resistance cassette into a *BglII* site at position 489 in the coding region of *moaA*. The Gm cassette does not possess a transcription terminator and thus expression of genes downstream of the cassette can occur provided the Gm cassette is in the correct orientation. A *BamHI* DNA fragment from pR2021 containing the Gm cassette (Hirsch & Beringer, 1984) was cloned into the *BglII* site of *moaA* in pJPS2, creating pJPS7. To ensure the transcription of the genes downstream from *moaA* would occur the orientation of the Gm cassette was checked by restriction analysis. The mobilization genes required for successful bacterial conjugation were cloned into the *EcoRI* site of pJPS7 from pR459II to create pJPS8. pJPS8 was then transferred by conjugation from *E. coli* S17-1 to *R. capsulatus* as described by Masepohl *et al.* (1988), creating *R. capsulatus* 37b4 MAA.

Purification of DMSO reductase using a Protein A HiTrap column. A HiTrap Protein A column (Pharmacia) was equilibrated with 5 ml 10 mM Tris/HCl, pH 7.5. Anti-DMSO reductase antibody (2 mg) was loaded onto the column and washed again with 5 ml 10 mM Tris/HCl, pH 7.5. The cell-

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype and properties*	Reference or source
<i>R. capsulatus</i>		
37b4	Wild-type	Kaufmann <i>et al.</i> (1984)
37b4 MAA	<i>moaA::Gm</i>	This work
<i>E. coli</i>		
BL21 (DE3)	F ⁻ <i>ompT</i> [<i>lon</i>] <i>hsdS_B</i> (<i>r_b</i> ⁻ <i>m_b</i> ⁻); an <i>E. coli</i> B strain with DE3, a λ prophage carrying the T7 RNA polymerase gene; Ap ^r	Studier <i>et al.</i> (1990)
JM109	<i>endA1 recA1 syrA96 thi hsdR17</i> (<i>r_k</i> ⁻ <i>m_k</i> ⁻) <i>relA1 supE44 λ^- Δ(lac-proAB)</i> [F', <i>traD36 proAB lacI^q ΔM15</i>]	Yanisch-Perron <i>et al.</i> (1985)
S17-1	RP4-2 (Tc::Mu) (Km::Tn7); integrated into chromosome	Simon <i>et al.</i> (1983)
Plasmids		
pALS1	pBK-CMV phagemid vector containing <i>R. capsulatus dorA</i> ; Km ^r	Shaw <i>et al.</i> (1996)
pUC8	Ap ^r <i>lacZα</i>	Vieira & Messing (1982)
pJPS2	<i>R. capsulatus moaA</i> in pUC8	This work
pET15b	Expression vector	Novagen
pJPS4	<i>R. capsulatus moaA</i> in pET15b	This work
pR2021	Plasmid containing Gm cassette	Hirsch & Beringer (1984)
pJPS8	<i>moaA::Gm</i> in pUC8 <i>mob</i> (RP4); Tc ^r	This work
pFR400	6.8 kb <i>Pst</i> I fragment containing <i>R. sphaeroides nap</i> gene cluster in pPHU231 <i>Mob⁺</i> ; Tc ^r	Reyes <i>et al.</i> (1996)

* Km, kanamycin.

free extract of the strain being analysed was then applied to the column and washed with 5 ml 10 mM Tris/HCl, pH 7.5, containing 500 mM NaCl. DMSO reductase was eluted from the column using 100 mM glycine, pH 2.5, and 250 μ l fractions collected. Each fraction collection tube contained 250 μ l 1 M Tris/HCl, pH 8.0, to quickly adjust the pH of the protein from pH 2.5. Presence of DMSO reductase was analysed by following the absorbance of the fractions at 280 nm. The protein-containing fractions were analysed on SDS-PAGE (7.5% acrylamide; 30:0.8 acrylamide/bis-acrylamide) as described by Laemmli (1971).

Enzyme assays. DMSO reductase was assayed as described by McEwan *et al.* (1985). Protein determination was performed using the bichinonic acid reagent with bovine serum albumin as a standard (Smith *et al.*, 1985). Nitrate reductase was assayed as for DMSO reductase except that nitrate was used in place of DMSO as the substrate. Xanthine dehydrogenase activity was measured as described by Leimkuhler *et al.* (1998).

RESULTS

A Moco biosynthetic gene cluster is located 3' to the *dor* gene cluster

The cloning of *dorA* from a λ library of *R. capsulatus* genomic DNA and its nucleotide sequence has been reported (Shaw *et al.*, 1996). Further sequence analysis 5' to *dorA* has identified genes encoding electron transfer proteins of the DMSO respiratory chain (Shaw *et al.*,

Fig. 1. Organization of the Moco biosynthetic gene cluster identified downstream of *dorB* of *R. capsulatus*.

1999). A putative transcription terminator 3' to *dorA* has been identified immediately beyond the *dorA* stop codon (Fig. 1). Beyond the transcription terminator five ORFs were identified. The first ORF has been named *dorB*; it encodes a putative protein of about 11 kDa and its role in the biogenesis of DMSO reductase is discussed in the accompanying paper (Shaw *et al.*, 1999). A 215 bp intergenic region separates the stop codon of *dorB* from four ORFs (Fig. 1). These ORFs were closely linked; the end of the coding sequence of each ORF was only 1 base proximal to the putative translation start site of the next ORF.

DNA sequence analysis of the three additional ORFs showed that they have high sequence similarity to MPT biosynthetic genes. The first ORF was identified as *moaA*. One base after the stop codon of *moaA* is the potential start site of another ORF that shows extensive

Fig. 2. Sequence alignment of MoaA from *R. capsulatus* (rcapsMoaA), *E. coli* MoaA (ecoliMoaA), *A. nicotinovorans* MoaA (arthroMoaA) and NarA from *B. subtilis* (bsubNarA). Conserved residues are highlighted with an asterisk and similar residues are marked with a dot. The numbers on the top line indicate the amino acid numbering of arthroMoaA.

sequence similarity to *moaD*. The next ORF begins one base ahead of the stop codon of *moaD* and appears to be another Moco biosynthetic gene, *moeB*. Beyond *moeB*, a small region of DNA (150 bases) was sequenced to the end of pALS1 and this exhibits extensive sequence similarity to the MPT biosynthetic gene *moaC*. No transcription terminators were identified in this MPT biosynthetic gene cluster.

Analysis of the predicted MoaA amino acid sequence

The ORF identified as *moaA* encodes a protein of 328 aa with a calculated molecular mass of 35 820 Da and pI of 8.2. MoaA is involved in the formation of an early intermediate in MPT biosynthesis known as precursor Z (Johnson & Rajagopalan, 1987a, b). *R. capsulatus* MoaA shows a high level of sequence similarity to MoaA from *E. coli* (32% identity) and *Arthrobacter nicotinovorans* (37% identity) as well as to NarA from *Bacillus subtilis* (34%). Of particular interest is the conservation of the cysteine-rich regions at both the N

and C termini (Fig. 2), typical of MoaA proteins identified thus far. These cysteine regions are thought to be involved in the formation of an [Fe-S] cluster (Menendez *et al.*, 1995).

Analysis of the predicted MoaD and MoeB amino acid sequence

The second ORF in the MPT biosynthetic gene cluster encodes MoadD. This protein is part of the converting factor, a complex involved in sulfur addition to precursor Z (Pitterle & Rajagopalan, 1989; Pitterle *et al.*, 1993). The calculated molecular mass and pI for MoadD are 8895 Da and 4.4, respectively. The MoadD sequence from *R. capsulatus* was found to be 27% identical to *E. coli* MoadD. No significant sequence similarity was observed between MoadD from *R. capsulatus* and the α subunit of nitrate reductase A from *E. coli* as observed for MoadD from *E. coli* (Rivers *et al.*, 1993).

Fig. 3. (a) SDS-PAGE analysis of the expression of *R. capsulatus* MoaA in *E. coli*. Lanes: 1, molecular mass standards; 2, soluble fraction; 3, pellet after low speed centrifugation. (b) SDS-PAGE analysis of MoaA used for EPR spectroscopy. Lanes: 1, molecular mass standards; 2, washed inclusion bodies containing MoaA.

Sequence analysis of the ORF immediately downstream from *moaD* identified it as *moeB*. MoeB is responsible for supplying sulfur to the converting factor in the conversion of precursor Z to MPT (Pitterle & Rajagopalan, 1989; Pitterle *et al.*, 1993). MoeB comprises 253 aa with a calculated molecular mass of 26.6 kDa. As expected, the amino acid sequences of MoeB from *R. capsulatus* and *E. coli* are highly similar (41% identity). *R. capsulatus* MoeB was also highly similar to ThiF from *E. coli* (41% sequence similarity). ThiF is required for the synthesis of thiazole in the thiamine biosynthetic pathway (Vander Horn *et al.*, 1993).

Expression of *R. capsulatus moaA* in *E. coli* and spectroscopic characterization of MoaA

The presence of the conserved cysteine residues in MoaA and the sequence similarity of this protein to NifB has led to the suggestion that this protein contains an [Fe-S] cluster (Rivers *et al.*, 1993; Menendez *et al.*, 1995). This has been confirmed in the case of the *Arthrobacter* MoaA where an [Fe-S] cluster was identified spectroscopically (Menendez *et al.*, 1995). *R. capsulatus* His₆-MoaA was expressed in *E. coli* as described in Methods. Analysis of cell extracts by SDS-PAGE showed that the recombinant plasmid pJPS4, containing *moaA*, directed the synthesis of a 38 kDa polypeptide. No His₆-MoaA appeared water-soluble but was present as inclusion bodies (Fig. 3a) which formed a pale brown pellet after centrifugation. Varying cell growth conditions, including decreasing the IPTG concentration used for induction of *moaA* expression and growing cells at 25 °C, were tried in an attempt to obtain soluble MoaA. However, no soluble MoaA was obtained. The inclusion bodies were washed to remove contaminating

material and Fig. 3(b) shows the polypeptide composition of the MoaA-containing sample after these washing steps. This sample was used for EPR spectroscopy.

An X-band EPR spectrum of resting MoaA at 2 K is shown in Fig. 4 (spectrum A). Resonances around $g = 2.01$ were observed, typical of a species containing a single unpaired electron. These resonances were not observed above 20 K and no other resonances were observed over a wide range of magnetic fields (0–1.5 T). Reduction of the sample with sodium dithionite destroyed the $g = 2.01$ EPR signal, indicating that it is the resting (oxidized) form of MoaA which contains a paramagnetic centre. Computer simulation of spectrum A in Fig. 4 with an $S = 1/2$ spin Hamiltonian ($H = \beta B \cdot g \cdot S$) and the orthorhombic g -matrix ($g_1 = 2.0040$, $g_2 = 2.0154$, $g_3 = 2.0232$) yielded the spectrum shown in Fig. 4 (spectrum b). The g -value anisotropy, line shape and redox characteristics of this spectrum are consistent with a $[3Fe-4S]^{1+0}$ cluster being present in the sample of purified MoaA.

The effect of a *moaA* mutation on the activity of oxomolybdenum enzymes in *R. capsulatus*

The clustering of Moco biosynthetic genes and *dor* genes raised the question of whether the former are specific for expression of the DMSO reductase or for all oxomolybdenum enzymes in *R. capsulatus*. To address this question a *moaA* mutant was constructed by marker exchange and this resulted in the insertion of a Gm interposon into the genomic copy of *moaA*. This Gm interposon was inserted in an orientation such that there would be transcriptional read-through from the Gm interposon into the Moco biosynthetic genes that lie downstream of *moaA*. The specific activity of xanthine dehydrogenase was 0.31 mU mg⁻¹ in wild-type *R. capsulatus* and 0.28 mU mg⁻¹ in the *moaA* mutant, indicating that the mutation in *moaA* had little effect on xanthine dehydrogenase activity. In contrast, the activity of DMSO reductase in the *moaA* mutant (0.07 mU mg⁻¹) was only about 3% of that found in wild-type cells (2.65 mU mg⁻¹). However, Western blotting of cell-free extracts showed that the level of DMSO reductase polypeptide was essentially the same in wild-type cells and the *moaA* mutant (data not shown). These data suggested that the *moaA* gene that had been mutated was specifically required for the biogenesis of holo-DMSO reductase but not for other oxomolybdenum enzymes. *R. capsulatus* strain 37b4 does not possess a nitrate reductase but can express the periplasmic nitrate reductase (encoded by the *nap* operon) of *R. sphaeroides* (Solomon, 1998). The level of nitrate reductase activity was similar in wild-type *R. capsulatus* (44.8 mU mg⁻¹) and the *moaA* mutant (46.2 mU mg⁻¹) when the *nap* genes of *R. sphaeroides* were expressed from a plasmid. These results are consistent with the view that the copy of *moaA* that lies downstream of the *dor* gene cluster must be specific for expression of DMSO reductase.

Fig. 4. X-band EPR spectrum of resting His₆-MoaA. (A) Experimental spectrum. (B) Computer simulation. Microwave frequency = 9.4452 GHz, $T = 2$ K.

Fig. 5. SDS-PAGE analysis of DMSO reductase purified from cell fractions of the *moaA* mutant by immunoaffinity chromatography. (a) Lanes: 1, molecular mass standards; 2, purified DMSO reductase standard; 3, eluant containing DMSO reductase from cell-free extracts. (b) Lanes: 1, molecular mass standards; 2, purified DMSO reductase standard; 3, eluant containing DMSO reductase from a periplasmic fraction. (c) Lanes: 1, molecular mass standards; 2, purified DMSO reductase standard; 3, eluant containing DMSO reductase from a cytoplasmic fraction.

Synthesis and secretion of the DMSO reductase polypeptide in a *moaA* mutant

Previous studies of the secretion of DMSO reductase from *R. sphaeroides* (Yoshida *et al.*, 1991) have shown that molybdate deficiency or a mutation in the Moco biosynthetic pathway leads to accumulation of the DMSO reductase polypeptide inside the cell. The *moaA* mutant of *R. capsulatus* generated in this study allowed us to analyse the secretion of the DMSO reductase polypeptide in a defined genetic background. A Western blot of cell fractions of the *moaA* mutant was probed with anti-DMSO reductase antibody. DMSO reductase polypeptide was found in both the cytoplasmic and periplasmic fractions of the cell with no detectable protein in the membrane (data not shown). The results indicated that the *moaA* mutation was affecting the secretion of DMSO reductase and this led us to analyse the DMSO reductase polypeptides more closely. Anti-DMSO reductase antibody was bound to a Hi-Trap Protein A affinity column and this was used to purify

DMSO reductase polypeptides from the *moaA* mutant and wild-type cells. Fig. 5 shows a SDS-PAGE analysis of polypeptides eluted from the antibody affinity column at pH 2.5 during the chromatography of cell-free extracts and cell fractions. Apart from polypeptides corresponding to IgG light and heavy chains, two polypeptides in the molecular mass range of DMSO reductase were purified from the cell extracts of the *moaA* mutant (Fig. 5a, lane 3). The smaller polypeptide had an identical mobility to the polypeptide purified from strain 37b4 (Fig. 5a, lane 2) while the larger polypeptide had a molecular mass of about 90 kDa. Analysis of a periplasmic fraction using the same immunoaffinity chromatography system showed that the smaller polypeptide could be purified from this cellular fraction (Fig. 5b, lane 3) while the large polypeptide could be purified from the cytoplasmic fraction (Fig. 5c, lane 3). In wild-type cells only the smaller form of DMSO reductase was detected and it was located in the periplasm (data not shown).

DISCUSSION

In this paper we have described the analysis of part of a Moco biosynthetic gene cluster in *R. capsulatus* which appears to be specific for the biogenesis of DMSO reductase. The observation that the *moaA* mutant generated in this study continued to synthesize active nitrate reductase and xanthine dehydrogenase strongly suggests that there is a second *moaA* gene in *R. capsulatus*. It seems likely that the other components of the Moco biosynthetic gene cluster that have been sequenced will also be specific for the biogenesis of DMSO reductase. The question of whether all components of the Moco biosynthetic pathway are duplicated was not addressed in this study. However, copies of *moaC*, *moaD* and *moeB* that are not linked to the *dor* operon have been identified in *R. capsulatus* (S. Leimkuhler & W. Klipp, personal communication).

The presence of two copies of *moaA* in *A. nicotinovorans* is established; one copy of *moaA* is located on a plasmid along with the structural gene for the oxomolybdenum enzyme nicotine dehydrogenase (*ndh*). This plasmid-borne copy of *moaA* is essential for formation of nicotine dehydrogenase but not for other oxomolybdenum enzymes in this bacterium (Menendez *et al.*, 1995). In *Methanobacterium formicicum* a putative *moaA* gene is located upstream of the formate dehydrogenase operon (White & Ferry, 1992). A *moaA* homologue in *B. subtilis*, known as *narA*, is immediately downstream of *narQ* (Glaser *et al.*, 1995). Thus, the linkage of *moaA* to the *dor* operon in *R. capsulatus* is not unusual, although it has not been observed for oxomolybdenum enzymes in *E. coli*. At this stage we cannot rule out the possibility that transcription of the other Moco biosynthetic genes can initiate downstream of *moaA*, but no obvious sequence corresponding to a promoter has been identified. The available data suggest that at least *moaA*, *moaD*, *moeB* and *moaC*, which lie downstream of the *dor* gene cluster, are specific for the biogenesis of DMSO reductase. In the absence of their expression a low level of DMSO reductase activity was observed, consistent with the presence of a second complement of Moco biosynthetic genes which have a role in synthesis of Moco for insertion into all oxomolybdenum enzymes. The reason why DMSO reductase requires a specific set of Moco biosynthetic genes is not clear but this enzyme is a major respiratory protein in the periplasm of *R. capsulatus*. The above observations raise the question of whether the *dor* genes and *moaA*, *moaD* and *moeB* form an operon? Although we have not yet performed detailed transcriptional studies, experiments with transcriptional *lac* fusions suggest that there might be transcriptional coupling of the *dor* genes and the Moco biosynthetic genes (P. S. Solomon & A. G. McEwan, unpublished observations).

MoeB and MoaD are thought to have a role in the incorporation of sulfur atoms into the MPT precursor, thereby producing the ene-dithiolate moiety that binds Mo. In *moeB* and *moaD* mutants an MPT precursor which lacks sulfur atoms, known as precursor Z, has

been identified (Pitterle & Rajagopalan, 1989; Pitterle *et al.*, 1993). Precursor Z cannot be detected in a *moaA* mutant and this has led to the view that MoaA is involved in one of the earliest steps in MPT synthesis (Johnson & Rajagopalan, 1987a, b). Recently, the early steps in MPT synthesis have been investigated in *E. coli* and it has been concluded that GTP is the precursor of MPT and that the pathway proceeds via steps that are distinct from those known for pteridine synthesis (Wuebbens & Rajagopalan, 1995).

The sequence of MoaA exhibits two highly conserved cysteine-rich segments towards the N and C termini of the protein. Site-directed mutagenesis studies of MoaA from *A. nicotinovorans* have shown that all cysteines are essential for MoaA function with two from each segment suggested to be required for the formation of an [Fe-S] cluster in *A. nicotinovorans* MoaA (Menendez *et al.*, 1995). The EPR spectroscopy of the His₆-MoaA (*R. capsulatus*) produced in *E. coli* confirms the presence of this [Fe-S] cluster. The EPR spectrum of oxidized MoaA can be assigned to a [3Fe-4S]¹⁺ cluster on the basis of the observed g-value anisotropy, temperature dependence and the loss of the spectrum upon reduction with sodium dithionite (Kent *et al.*, 1980; Kutcha *et al.*, 1986). The [Fe-S] cluster within oxidized MoaA cannot be [2Fe-2S]^{2+,1+} or [4Fe-4S]^{2+,1+} as the oxidized forms of these clusters are EPR-silent. The g-value anisotropy and temperature dependence is inconsistent with an oxidized [4Fe-4S]^{3+,2+} cluster (Pilbrow & Hanson, 1993).

Menendez *et al.* (1996) suggested that MoaA may be an oxidoreductase or that it is involved in sulfur donation to the converting factor (MoaD and MoeB). The idea of MoaA participating in the donation of sulfur to the converting factor seems unlikely. A MoaA mutant is unable to produce precursor Z, strongly indicating that MoaA is involved in the synthesis of precursor Z from GTP (Johnson & Rajagopalan, 1987a, b; Pitterle & Rajagopalan, 1989). Also, it has been clearly established with conclusive experiments by Rajagopalan and co-workers that MoeB (activating factor) is responsible for the donation of sulfur to the converting factor (Pitterle & Rajagopalan, 1989). Recently, it has become clear that [Fe-S] clusters have a multiplicity of functions (Beinert *et al.*, 1997). Although we have identified a [3Fe-4S] cluster in MoaA, it is possible that, like aconitase, the active form of MoaA may contain a [4Fe-4S] cluster. One possibility is that an Fe atom in the [4Fe-4S] cluster of MoaA acts as a Lewis acid and promotes a hydration or dehydration reaction which is proposed to occur during the conversion of a phosphorylated guanosine precursor to precursor Z (Wuebbens & Rajagopalan, 1995).

The involvement of MoeB in the addition of sulfur to precursor Z in *E. coli* was elucidated over 5 years ago (Pitterle *et al.*, 1993). As mentioned in the results section, MoeB from *R. capsulatus* not only exhibited high similarity to MoeB from *E. coli*, but also to the thiamine biosynthetic protein, ThiF, from *E. coli*. Vander Horn *et al.* (1993) determined that ThiF was involved in syn-

thesizing a precursor to thiamine, 4-methyl-5-(β -hydroxyethyl)thiazole (THZ) from 1-deoxy-D-threo-2-pentulose in a reaction also requiring tyrosine and cysteine. Interestingly, among other reactions, the synthesis of THZ involves the addition of a sulfur with the source of that sulfur identified as cysteine (DeMoll & Shive, 1985). Recently, it has been shown that ThiF catalyses the adenylation of another protein by ATP, ThiS, thereby activating this protein for sulfur transfer (Taylor *et al.*, 1998).

The results presented in Fig. 5 show that in the absence of *moaA* downstream of *dorA*, a precursor form of DMSO reductase appears to accumulate in the cytoplasm. The activity measurements in this study revealed only a very low level of active DMSO reductase in the periplasm. However, it appeared that about half of the DMSO reductase polypeptide produced was secreted. In contrast, in *E. coli* a mutation in *moaA* results in a complete absence of mature periplasmic TorA with only the precursor being present in the cytoplasm (Santini *et al.*, 1998). It is suggested that in the *R. capsulatus* *MoaA* mutant some secretion of holo-DMSO reductase can occur because, unlike the *MoaA* mutant of *E. coli*, Moco biosynthesis can occur at a low level in this mutant. We have observed that the precursor form of DMSO reductase is unstable (P. S. Solomon & A. G. McEwan, unpublished observations) and hence the level of cytoplasmic precursor may be underestimated in our experiments. Pulse-chase experiments would be required to confirm this view.

The traditionally held view of secretion is that periplasmic proteins are exported in an unfolded state via the Sec system (Pugsley, 1993; Driessen, 1994). However, Berks (1996) suggested that the export of periplasmic proteins containing a complex prosthetic group such as Moco would require a novel secretory pathway and support for this view has come from the observation that the export of TMAO reductase in *E. coli* is Sec-independent (Santini *et al.*, 1998). Recently, a system for the export of folded proteins containing a 'twin arginine' motif has been identified in *E. coli* (Sargent *et al.*, 1998; Weiner *et al.*, 1998). This system is encoded by the *tat* operon (also known as the *mtt* operon) and it is homologous to the Sec-independent protein translocation system (HCF106) found in chloroplasts (Settles *et al.*, 1997). It has already been noted that the homologues of HCF106 have been found in a number of bacterial genomes and thus it seems likely that the secretion of DMSO reductase in *R. capsulatus* will occur via this Sec-independent system.

ACKNOWLEDGEMENTS

This work was supported by grants from the Australian Research Council and the University of Queensland (A.G.M. and G.R.H.) and by an Australian Postgraduate Award (A.L.S.).

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Received 5 January 1999; revised 1 February 1999; accepted 23 February 1999.