MoaA of *Arthrobacter nicotinovorans* pAO1 involved in Mo-Pterin cofactor synthesis is an Fe-S protein

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Abstract MoaA, involved in an early step in the biosynthesis of the molybdopterin cofactor (MoCo), has not yet been characterized biochemically and the reaction it catalyzes is unknown. We overexpressed MoaA from pAO1 of *Arthrobacter nicotinovorans* in *Escherichia coli* as a N-terminal fusion with either glutathione-S-transferase or a 6-histidine tag. The pAO1 encoded MoaA as well as the fusion proteins functionally complement *E. coli moaA* mutants. Here we show that purified MoaA contains approximately 4 μM Fe and approximately 3 μM acid-labile S/μM protein. EPR spectroscopy revealed a predominant signal at $g_{av} = 2.01$, indicative of a [3Fe-xS] cluster.

Key words: MoaA; Molybdopterin cofactor; *Arthrobacter nicotinovorans*; Fe–S cluster; Cys mutagenesis

1. Introduction

Synthesis of the molybdopterin-dinucleotide cofactor (MoCo) and its insertion into apoenzymes, a prerequisite for the catalytic activity of a large group of complex enzymes [1], requires the activity of several *mol* gene products (for a discussion of nomenclature see reference [2], for a review see [3]). The genes coding for these products have been identified in *Escherichia coli* by mutational analysis, but until now few proteins have been characterized biochemically. The product of *moaA* is involved in an early step in the metabolic pathway leading to MoCo synthesis; its biochemical function, however, is unknown [3]. A *moaA* copy which functionally complements in vivo *E. coli moaA* mutants has been identified on pAO1 of *Arthrobacter nicotinovorans* [4]. Comparison of the DNA-deduced amino acid sequence of MoaA from various organisms shows a high degree of similarity at two conserved cysteine clusters: cluster I and cluster II [4]. Site-directed mutagenesis of *moaA* carried on pAO1 revealed that the conserved cysteine residues of cluster I are essential for MoaA activity [4]. In the present report we demonstrate that the cysteine residues of cluster II are also essential and that MoaA of *A. nicotinovorans* is an Fe-S protein.

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Abbreviations: GTS-MoA, glutathione-S-transferase-MoaA; EPR, electron paramagnetic resonance; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

2. Material and methods

2.1. Chemicals and biochemicals

Isopropyl-β-thiogalactoside and ampicillin were from Sigma (Munich, Germany). All other chemicals were of highest quality available. Glutathione-S-transferase (GST) affinity chromatography material and chelating Sepharose were provided with the glutathione-S-transferase and 6-His tag purification kits of Pharmacia (Freiburg, Germany). Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany).

2.2. Bacterial strains, plasmids, site-directed mutagenesis, preparation of cell extracts and isolation of the fusion proteins

As a host for recombinant plasmids were used *E. coli* JM109 cells grown in LB medium [5]. The construction of a N-terminal fusion between the truncated GST and MoaA, the complementation assays and the site-directed mutagenesis of cysteine residues of cluster II of MoaA were performed essentially as described in [4]. For the replacement of Cys^{277} and Cys^{280} by serine we used the mutagenic primers 5′-$_{879}$GCCGTTCTCGAGTGATTGCCGC$_{900}$-3′ and 5′-$_{884}$TCTGCTCCGATTCTAGACGTACC$_{906}$-3′, for the 5′ end respectively, and the oligonucleotide 5′-$_{1296}$CCGCGCCAGGATCCGGGGG$_{1278}$-3′ as 3′ primer for both mutageneses. Replacement of Cys^{294} was performed with the 5′ primer 5′-$_{10}$ACTGTCCAGCTAGGCATACCGC$_{31}$-3′ and the mutagenic 3′ primer 5′-$_{957}$CTCCTCCCGGGAGAACAGTGAGCTCAT$_{930}$-3′. The Cys^{36}–Cys^{280} double mutant was obtained by reconstituting the *moaA* gene from the corresponding single mutants. Addition of a 6-His tag to the N-terminus of MoaA was performed according to [6]. Induction of cells carrying recombinant plasmids, preparation of cell extracts and the one-step affinity chromatography isolation of MoaA fusion proteins were performed as recommended by the supplier of the isolation kits (Pharmacia, Freiburg, Germany). The purified proteins were analysed by SDS- and native-PAGE on 10% polyacrylamide gels.

2.3. Determination of Fe and acid-labile S content of MoaA

The Fe content of GST-MoaA was determined by atomic mass spectrometry with the aid of a Perking-Elmer ICP/6500 XR spectrophotometer on 8 ml of a 0.76 mg/ml protein sample at the Mineralogic Institute, University Freiburg. Acid-labile S was determined on 4 ml of 2 mg/ml protein samples as described in [7] and [8] with omission of the extraction step.

2.4. EPR spectroscopy

EPR spectroscopy was performed on 10 mg/ml protein samples at 70 K with a Varian X-Band Spectrometer set at a microwave frequency of 8.978 GHz, an incident microwave power of 2 mW, a modulation frequency of 100 kHz and a field modulation amplitude of 2 mT.

3. Results and discussions

An analysis of the brownish colored, affinity-purified MoaA by SDS-PAGE is presented in Fig. 1. Presence of the reducing agent β-mercaptoethanol in the protein sample did not affect the migration behaviour of MoaA, consistent with the absence of a disulfide bridges from the molecule. The GST-MoaA fusion protein migrated with a molecular mass of 67 kDa as expected from the sum of the molecular masses of the trun-

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cated GST (26 kDa) and 39 kDa of MoaA; the 6-His-MoaA migrated on SDS-PAGE as an approximately 40 kDa protein. Analysis of the migration behaviour of GST-MoaA and 6-His-MoaA on native PAGE with bovine albumine (68 kDa), 6-His-6-hydroxy-D-nicotine oxidase (49 kDa) and rat liver dimethylglycine dehydrogenase (96 kDa) as molecular mass standards indicated that the MoaA proteins migrated as monomers (result not shown). UV-visible spectrophotometry of the protein revealed no significant features (not shown).

Complementation assays of *E. coli moaA* mutants with plasmids carrying pAO1 *moaA* with the cysteine residues of cluster II mutated one at the time into serine were negative. Apparently these residues are essential for MoaA function.

Determination of the Fe and acid-labile S content of MoaA resulted in approximately 4 μM Fe/μM GST-MoaA and 3 μM acid-labile S/μM GST-MoaA or 6-His-MoaA (relative protein concentration). Typical EPR spectra of wild-type and Cys-mutant MoaA proteins are presented in Fig. 2. Both fusion proteins exhibited the same EPR features (Fig. 2, compare spectra 1 and 2). The predominant signal was at g_{av} of 2.01, which is characteristic for [3Fe-xS] clusters [9]. The S content of MoaA would be consistent with a [3Fe-3S] cluster. However, due to limitations in the accuracy of the acid-labile S determination, a [3Fe-4S] cluster may not be excluded. As expected for this type of Fe-S clusters [9] addition of the reducing agent sodium dithionite decreased the intensity of the $g = 2.01$ signal (Fig. 2, spectrum 3). Surprisingly, the Cys double mutant with the cysteine residue in the middle of each of the two clusters replaced by serine exhibited the same EPR signals as the wild-type MoaA protein (Fig. 2, spectrum 4). The single cysteine mutant with the first cysteine of the cluster I replaced by serine, however, exhibited a reduced iron content (1 μM Fe/μM relative protein concentration) and a reduced EPR signal (Fig. 2, spectrum 5).

The results presented demonstrate that the MoaA protein encoded by *moaA* of pAO1 is an Fe-S protein. Given the high degree of similarity among the deduced amino acid sequences of known *moaA* genes we propose that this represents a general feature of MoaA proteins. As probable ligands for the Fe-S cluster we assume the cysteine residues of the two cysteine clusters of MoaA. The amino acid residue spacing on

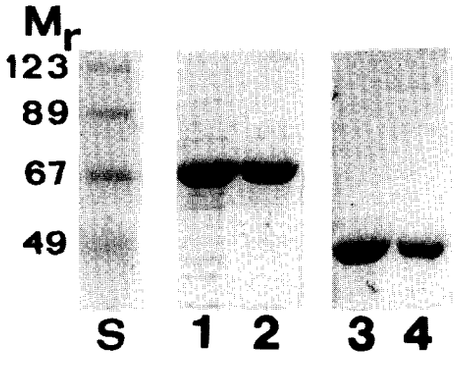

Fig. 1. SDS-PAGE analysis of purified MoaA fusion proteins. Lanes 1 and 3, 5 μM of a 2 mg/ml GST-MoaA or 6-His-MoaA solution (relative protein concentration), respectively, were diluted in 2 vol. SDS-PAGE sample buffer with mercaptoethanol, incubated 5 min at 95°C and applied to the 10% polyacrylamide gel. Lanes 2 and 4, identical samples to those of lanes 1 and 3, but in the absence of mercaptoethanol.

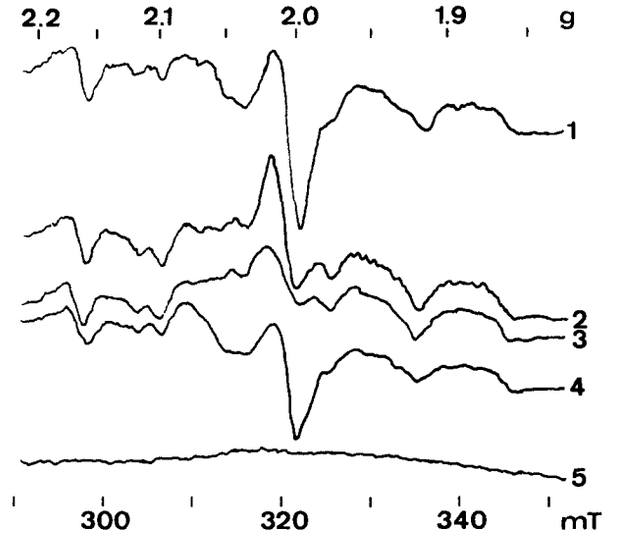

Fig. 2. EPR spectroscopy of wild-type and Cys-mutant MoaA fusion proteins. Spectra of (1) 6-His-MoaA; (2) GST-MoaA; (3) GST-MoaA in the presence of 10 mM sodium dithionite; (4) GST-MoaA Cys^{36}-Cys^{280} double mutant; (5) GST-MoaA Cys^{32} mutant. *G* values are indicated on the top of the figure; the magnetic field strength is indicated in millitesla (mT).

these clusters is $Cys^{32}xxxCys^{36}xxCys^{39}\cdots Cys^{277}xxCys^{280}$ $13xCys^{294}$. Site-directed mutagenesis revealed that each cysteine of the clusters is essential for MoaA function. Since the double mutant is still able to assemble an Fe-S cluster but the single mutant is not, as shown by the EPR spectroscopy, we speculate that the cysteine residues involved in coordination of the Fe-S cluster are Cys^{32}, Cys^{39}, Cys^{277} and Cys^{294}. The cysteine residues Cys^{36} and Cys^{280} may be involved in the modulation of the redox state of the Fe-S cluster required for the activity of MoaA or may serve another essential role. From our results one may assume that MoaA represents an oxido-reductase. Alternatively MoaA may be involved in the S donation to the converting factor in the assembly of the Mo-cofactor. Such an assumption may be supported by the reported amino acid similarity between MoaA and NifB [4], which is known to have this function in the assembly of the NifB cofactor [10].

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References

[1] Kretzer, A., Fruntzke, K. and Andreesen, J.R. (1993) J. Gen. Microbiol. 139, 2763–2772.
[2] Shanmugam, K.T., Stewart, V., Gunsalus, R.P., Boxer, D.H., Cole, J.A., Chippaux, M., DeMoss, J.A., Giordano, G., Lin, E.C.C. and Rajagopolan, K.V. (1992) Mol. Microbiol. 6, 3452–3454.
[3] Rajagopolan, K.V. and Johnson, J.L. (1992) J. Biol. Chem. 267, 10199–10202.
[4] Menéndez, C., Igloi, G., Henninger, H.-P. and Brandsch, R. (1995) Arch. Microbiol. 164, 142–151.
[5] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[6] Berthold, H., Scanarini, M., Abney, C.C., Frorath, B. and Northemann, W. (1992) Prot. Exp. Pur. 3, 50–56.

[7] Sakurai, H., Lien, S. and SanPietro, A. (1982) Anal. Biochem. 119, 372–377.
[8] Beinert, H., Emptage, M.H., Dreyer, J.-L., Scott, R.A., Hahn, J.E., Hodgson, K.O. and Thomson, A.J. (1983) Proc. Natl. Acad. Sci. USA 80, 393–396.
[9] Cammack, R., Patil, D.S. and Fernandez, V.M. (1985) Biochem. Soc. Transac. 13, 572–578.
[10] Shah, V.K., Jeffrey, R.A., Spangler, N.J. and Ludden, P.W. (1944) J. Biol. Chem. 269, 1154–1158.