Rhodobacter capsulatus XdhC Is Involved in Molybdenum Cofactor Binding and Insertion into Xanthine Dehydrogenase*

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Rhodobacter capsulatus xanthine dehydrogenase (XDH) is a cytoplasmic enzyme with an (αβ), heterodimeric structure that is highly identical to homodimeric eukaryotic xanthine oxidoreductases. The crystal structure revealed that the molybdenum cofactor (Moco) is deeply buried within the protein. A protein involved in Moco insertion and XDH maturation has been identified, which was designated XdhC. XdhC was shown to be essential for the production of active XDH but is not a subunit of the purified enzyme. Here we describe the purification of XdhC and the detailed characterization of its role for XDH maturation. We could show that XdhC binds Moco in stoichiometric amounts, which subsequently can be inserted into Moco-free apo-XDH. A specific interaction between XdhC and XdhB was identified. We show that XdhC is required for the stabilization of the sulfurated form of Moco present in enzymes of the xanthine oxidase family. Our findings imply that enzyme-specific proteins exist for the biogenesis of molybdenozymes, coordinating Moco binding and insertion into their respective target proteins. So far, the requirement of such proteins for molybdenzyme maturation has been described only for prokaryotes.

Xanthine oxidoreductase is a complex metalloflavoprotein that catalyzes the hydroxylation of hypoxanthine and xanthine, the last two steps in the formation of urate, using a water molecule as the ultimate source of oxygen incorporated into the product (1). The enzyme exists in two forms; the xanthine dehydrogenase form (XDH; EC 1.17.1.4)2 uses NAD+ as electron acceptor, whereas the xanthine oxidase (EC 1.17.3.2) form uses O2 as electron acceptor (2). Xanthine oxidoreductases are found both in eukaryotes and prokaryotes, with the enzymes isolated from bovine milk and Rhodobacter capsulatus being functionally and structurally the best characterized (3, 4). R. capsulatus XDH is a cytoplasmic enzyme with an (αβ), heterodimeric structure, with the two subunits encoded by the xdhA and xdhB genes, respectively (5). It was shown that XdhA binds the two [2Fe-2S] clusters and the FAD cofactor, whereas XdhB binds the molybdopterin (MPT) form of the molybdenum cofactor (Moco) (4, 5). DNA sequence analysis revealed that a third gene is co-transcribed with xdhAB, designated xdhC (6). It was shown by interposon mutagenesis that the xdhC gene product is required for XDH activity; however, XdhC was not identified as a subunit of active XDH (4, 6). Analysis of the Moco content of inactive XDH purified from an R. capsulatus xdhC− strain showed that in the absence of XdhC, Moco is not inserted into XDH, but XDH still consisted of an (αβ), heterodimer with a full complement of FeS clusters and FAD (6). An interaction of XdhC with apo-XDH was not analyzed; however, studies of the electrophoretic mobility revealed a different conformation of Moco-free XDH in xdhC− and Moco-deficient R. capsulatus mutant strains in comparison with mature XDH (6). It was assumed that during Moco biosynthesis, the Moco-free apo-XDH stays in a suitable “open” conformation for the insertion of Moco into XdhB. XdhC was proposed to act as an XDH-specific Moco carrier protein, a Moco insertase, or a chaperone involved in proper folding during or after the insertion of Moco into XDH. In previous work, we have reported a system for the heterologous expression of R. capsulatus XDH in Escherichia coli (7). Whereas preparations of bovine milk XDH/xanthine oxidase typically possess a large quantity of the desulfo form of the enzyme (8), R. capsulatus XDH can be purified from E. coli cells in a form with a full complement of the equatorial Mo=S ligand required for functionality (9). This shows that E. coli contains a sulfurtransferase capable of adding the terminal sulfur ligand to the molybdenum site of Moco; however, nothing is yet known about the nature of this protein in E. coli.

This report describes the detailed analysis for the requirement of XdhC to produce active XDH during heterologous expression in E. coli TP1000 cells. Analysis of XDH expressed under different aeration levels in the presence or absence of XdhC showed that, especially under aerobic conditions, XdhC is required to produce active XDH containing the terminal sulfur ligand of Moco. In addition, we purified and characterized XdhC after heterologous expression in E. coli. We could show that XdhC binds Moco/MPT in stoichiometric amounts and is able to insert bound Moco into Moco-free apo-XDH. In addition, a specific interaction between XdhC and XdhB was identified. We showed that XdhC acts as a Moco-binding protein, which protects the sulfurated form of Moco from oxidation. We propose that sulfurated Moco is inserted into apo-XDH by the aid of XdhC to produce active XDH. This is the first example of a system-specific protein involved in maturation of a molybdenozyme for which Moco binding could be shown.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Media, and Growth Conditions—E. coli ER2566 (DE3) cells and plasmid pTYB2 were obtained from New England Biolabs, and expression vector pET29b was purchased from Novagen. E. coli TP1000 cells (10) were used for expression of XDH from plasmid pSL207 (7) and for expression of the Moco domain of human sulfate oxidase (hSO) from pTG818 (11). Moco-free apo-XDH was obtained after expression in E. coli RK5200 (chlA::Mu cts [moaA−]) (12). Molybdenum-free hSO was obtained after expression in E. coli RK5202 (child202::Mu cts [moaC−]) (13). Expression of XDH from pSL181 was carried out in E. coli RK4353 (DE3) cells (14). His6-tagged CsdA was expressed in E. coli BL21 (DE3) cells (Novagen) from plasmid pSL215...
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and purified as described previously (15). Cell strains containing expression vectors were grown aerobically in LB medium at 30 °C in the presence of either 150 μg/ml ampicillin or 25 μg/ml kanamycin.

Construction of Expression Vectors—For expression of xdhB and xdhC, primers were designed to allow cloning into the Ndel and KpnI sites of expression vector pTBY2, resulting in plasmid pAK22 and pAK20, respectively. For construction of pSL181, the Ndel-HindIII xdhABC fragment from pSL207 was cloned into pET29b, resulting in a C-terminal His6 fusion to XdhC. pMS3 was obtained from pSL207 by deletion of a 500-bp Xhol fragment in the coding sequence of xdhC.

Expression and Purification of XdhB and XdhC—For the heterologous expression of R. capsulatus XdhC in E. coli, pAK22 and pAK20 were transformed into E. coli ER2566(DE3). The proteins were expressed and purified following the IMPACT instruction manual (New England Biolabs) as described previously (16). XdhB was further purified by an additional gel filtration step. Immediately before use, XdhC was transferred into the required buffer by gel filtration using PD10 columns (GE Healthcare).

Heterologous Expression of Xdh in the Absence and Presence of XdhC under Different Oxygen Supply—XDH was expressed in E. coli TP1000 or DH5α cells (17) with the expression vectors pSL207 (xdhABC) and pMS3 (xdhAB) under different oxygen supply by using shaking rates of 50, 130, and 210 rpm. After expression, XDH was purified by Ni2+-NTA chromatography (Qiagen) and Q-Sepharose ion exchange chromatography (GE Healthcare).

Co-purification of XDH with XdhC—For co-purification of XDH with XdhC, pSL181 was transformed using the E. coli RK4353(DE3) strain, and cells were grown in 1 liter of LB medium. For purification of the C-terminal His6-tagged XdhC, 0.5 ml of Ni2+-NTA matrix was used. The column was washed with 10 column volumes of 50 mM phosphate buffer, 100 mM NaCl, pH 8.0, containing 10 mM imidazole and 20 column volumes of the buffer containing 20 mM imidazole. The proteins were eluted with the same buffer containing 250 mM imidazole and immediately analyzed by SDS-PAGE. Moco was extracted from 1 ml of 390 μM hSO and added to the crude extract in conjunction with 80 μM Na2MoO4 prior to Ni2+-NTA chromatography. XDH showed no interactions with the Ni2+-NTA matrix under these conditions. Moco was quantified as Form A.

Sulfuration of Moco Bound to XdhC by CsdA—240 μM CsdA was persulfurated by incubation with 10 mM L-cysteine for 15 min at 4 °C, and excess cysteine was removed by passage through a PD10 column.

For in vitro sulfuration experiments, 3 μM XdhC was incubated in a total volume of 0.5 ml under anaerobic conditions with 32 μM Moco extracted from hSO, 60 μM CsdA, and 150 μM sodium dithionite for 30 min. CsdA was removed by Ni2+-NTA chromatography (0.5-ml bed volume) equilibrated in 50 mM phosphate buffer, 300 mM NaCl, pH 8.0, and a volume of 1 ml of the flow-through was collected and incubated with 0.75 μM apo-XDH for 2 h. Control experiments were carried out without inclusion of XdhC, CsdA, or sodium dithionite in the incubation mixtures. As positive controls, either 60 μM sodium sulfide was added to the incubation mixtures containing XdhC for chemical sulfuration of Moco or 32 μM Moco extracted from active XDH.

Enzyme Assays—XDH assays were performed at room temperature using a Shimadzu UV-2401PC spectrophotometer. 1 ml of mixture contained 500 μM xanthine and 1 mM NAD+ in 50 mM Tris, 1 mM EDTA, pH 7.5. The specific enzyme activity (units/mg) is defined as the reduction of 1 μmol of NAD+/min/mg of enzyme.

Metal and Moco/MPT Analysis—The molybdenum and iron contents of the purified proteins were quantified by inductively-coupled plasma optical emission spectrometry (ICP-OES) analysis with a PerkinElmer Life Sciences Optima 2100 DV ICP-OES. 500 μl of a 10 μM solution was mixed with 500 μl 65% nitric acid and incubated overnight at 100 °C before the addition of 4 ml of water. As reference, the multielement standard solution XVI (Merck) was used.

Moco/MPT was quantified by conversion to Form A as described earlier (16, 18). Moco/MPT was extracted by the addition of 50 μl of acidic iodine. Following incubation at room temperature for 1 h, excess iodine was removed by the addition of 55 μl of 1% ascorbic acid, and the sample was adjusted with 1 ml Tris to pH 8.3. The phosphate monoester of Form A was cleaved by the addition of 40 mM MgCl2 and 1 unit of calf intestinal alkaline phosphatase. After the addition of 10 μl of acetic acid, Form A was identified and quantified by HPLC analysis with a C18 reversed phase HPLC column (4.6 × 250-mm ODS Hypersil; particle size 5 μm) with 5 mM ammonium acetate, 15% methanol at an isocratic flow rate of 1 ml/min. In-line fluorescence was monitored by an Agilent 1100 series detector with an excitation at 383 nm and emission at 450 nm. For quantification of Form A, the corresponding fractions were collected, and the concentration was determined as described earlier (18).

Quantification of the Cyanolyzable Sulfur—250 μl of a 13 μM XDH in 100 mM Tris acetate, pH 8.6, was incubated with 27.5 μl of 1 mM KCN overnight at 4 °C as originally described by Massey and Edmondson (19). The protein was separated with a 3000 molecular weight cut-off Centricon concentrator (Millipore), and 250 μl of the SCN−-containing flow-through was mixed with 250 μl of ferric nitrate reagent (10 g of Fe(NO3)3 × 9H2O and 20 ml of 65% HNO3 per 150 ml). The FeSCN2− complex was quantified at 460 nm using a standard curve from 1–100 μM KSCN.

Generation of Free Moco/MPT—The Moco domain of hSO was expressed from pTGG18 in E. coli TP1000 and purified as described by Temple et al. (11). To 200 μl of hSO (220 μM in 100 mM Tris, pH 7.2), 50 μl of 1 mM Na2MoO4 was added prior to heat treatment at 95 °C for 5 min. After centrifugation at maximum speed for 5 min, the supernatant was used as a Moco source.

For purification of molybdenum-free MPT, hSO was expressed from pTGG18 in E. coli RK5202 (13) without the addition of 1 mM Na2MoO4, and Na2MoO4 was omitted from the incubation mixtures. The sulfurated form of Moco was obtained from 80% active XDH using the same procedure.

Reconstitution of Apo-XDH with Moco—To obtain Moco-free XDH, pSL207 was expressed in E. coli RK5202 (mocA−) cells, and apo-XDH was purified immediately before use by Ni2+-NTA affinity chromatography. Varying amounts of XdhC (0–15 μM) were incubated with an excess of purified Moco from hSO at 4 °C. Unbound Moco was removed from the incubation mixtures by gel filtration using Nick columns (GE Healthcare). The amount of Moco bound to XdhC was quantified by conversion to Form A, and the Form A concentration was determined using a Form A standard curve. 300 μl of Moco-XdhC was incubated with 100 μl of 4 μM apo-XDH for 90 min at 4 °C. After incubation, XDH was separated from XdhC by Ni2+-NTA chromatography. As control, different concentrations of free Moco (0–35 μM) were incubated with apo-XDH. The amount of Moco inserted into XDH was quantified by conversion to Form A. The same procedure was used to insert the sulfurated form of Moco (obtained from heat-treated XDH) into apo-XDH in the presence or absence of XdhC. 2 μM XdhC were preincubated with Moco for 5, 30, or 60 min at 4 °C before the addition of 0.8 μM apo-XDH. In parallel, extracted Moco was incubated without any additions before the addition of apo-XDH at the same time points. After an incubation time of 2 h at 4 °C, XDH activity was tested.
Binding of Moco/MPT to XdhC — 6 μM XdhC (in 100 mM Tris, pH 7.2) was incubated with free Moco/MPT (0–35 μM) for 15 min at 4 °C. The samples were transferred to Microcon concentrators (molecular weight cut-off 10,000; Millipore) and centrifuged at 14,000 × g for 5 min. As a control, free Moco/MPT was used in the absence of XdhC. The flow-through containing unbound Moco/MPT was converted to the stable oxidized fluorescent degradation product Form A, as described under “Experimental Procedures.” 100% was set to a fully sulfurated XdhC after heterologous expression in E. coli. Moco was injected at 25 °C for 4.5 min at a flow rate of 20–40 μl/min. As a control, free Moco/MPT was used in the absence of XdhC. Binding curves were normalized by subtraction of buffer injection curves for all flow cells. The concentration of the terminal sulfur ligand of Moco was determined spectrophotometrically as an iron-thiocyanate complex at 420 nm as described under “Experimental Procedures.” 100% was set to a fully sulfurated control XdhC.

RESULTS

Heterologous Expression and Purification of R. capsulatus XdhC in E. coli — For purification of XdhC, a fusion protein was generated containing both a C-terminal intein tag and a chitin-binding domain for affinity purification. After binding to chitin beads, cleavage with dithiothreitol resulted in purified XdhC in a soluble form with a yield of 0.2 mg of protein/liter of E. coli culture (Fig. 1). The purified protein displayed a single band on Coomassie Brilliant Blue-stained SDS-polyacrylamide gels with a monomeric mass of 33 kDa, which is in close correspondence to the calculated monomeric mass of 33.4 kDa. The observed elution position of native XdhC from a size exclusion chromatography suggested that XdhC exists as a dimer in solution (data not shown).

Characterization of the Role of XdhC for the Maturation of R. capsulatus XDH in E. coli — As reported previously, XdhC was shown to be essential for the insertion of Moco into XDH when expressed in R. capsulatus under anaerobic conditions, but not for XdhC heterologously expressed under aerobic conditions in E. coli TP1000 cells, containing a deletion in the mobAB genes (6, 7). However, in both cases, XDH was found to be inactive. To determine whether different aeration levels during expression influence the levels of Moco insertion into XDH, R. capsulatus XDH was expressed in E. coli TP1000 cells in the presence or absence of XdhC under variation of shaking velocities (50, 130, and 210 rpm). XDH was purified (see “Experimental Procedures”), and XDH activity was determined, in addition to the content of molybdenum, iron, cyanolyzable sulfur, and MPT in the enzyme (Table 1). As shown in Table 1, XdhC was not required for the insertion of MPT into XDH; however, increasing aeration resulted in lower levels of MPT in the purified protein. Analysis of the molybdenum content of the proteins corresponded well with the MPT content, showing that mainly all MPT bound to the protein is present as Moco. In addition, all enzyme preparations contained the appropriate levels of FeS and FAD cofactors, as judged by their respective visible absorption spectra (data not shown) and by determination of the iron content (Table 1). However, XDH activity was drastically decreased in the absence of XdhC with increasing levels of culture aeration (Table 1). Whereas XDH expressed in the absence of XdhC at 50 rpm under semiaerobic conditions retained 30% of its activity, the protein expressed at 210 rpm showed that loss of enzyme activity correlated well with a low concentration of the cyanolyzable sulfur present in Moco. Thus, during heterologous expression in E. coli TP1000, XdhC is not required for Moco

TABLE 1

Effect of XdhC on activity and cofactor content of R. capsulatus XDH after heterologous expression in E. coli TP1000 cells under different culture aeration

| rpm Values, expression of genes on plasmid used | Molybdenum | Iron | Cyanolyzable sulfur | Activity | MPT/Moco |
|-----------------------------------------------|------------|------|---------------------|----------|----------|
| 50 rpm, xdhABC (pSL207)                        | 71.5 ± 6.7 | 105.2 ± 5.0 | 63.5 ± 0.6 | 37.8 ± 0.7 | 72.7 ± 5.9 |
| 50 rpm, xdhAB (pMS3)                           | 71.0 ± 5.7 | 101.7 ± 1.4 | 25.5 ± 2.1 | 11.7 ± 0.6 | 67.7 ± 2.4 |
| 130 rpm, xdhABC (pSL207)                       | 71.5 ± 0.3 | 97.6 ± 3.1 | 66.9 ± 1.8 | 45.3 ± 0.7 | 71.8 ± 4.1 |
| 130 rpm, xdhAB (pMS3)                          | 71.0 ± 0.7 | 93.6 ± 0.4 | 2.5 ± 0.9  | 0.95 ± 0.03 | 65.2 ± 4.9 |
| 210 rpm, xdhABC (pSL207)                       | 31.3 ± 1.1 | 106.0 ± 4.8 | 31.1 ± 0.6 | 35.1 ± 0.3 | 50.8 ± 4.4 |
| 210 rpm, xdhAB (pMS3)                          | 32.6 ± 3.3 | 101.1 ± 3.9 | 0.85 ± 0.9 | 0.19 ± 0.02 | 49.4 ± 2.8 |

a Molybdenum (μg molybdenum/μg XDH) and iron (μg [2Fe2S]/μg XDH) were determined by ICP-OES (see “Experimental Procedures”) using a multielement standard.

b Determined spectrophotometrically as an iron-thiocyanate complex at 420 nm as described under “Experimental Procedures.” 100% was set to a fully sulfured control XDH.

c Specific enzyme activity (units/mg) is defined as the reduction of μmol of NAD/min/mg of enzyme under assay conditions.

d MPT/Moco was quantified after conversion to the stable oxidized fluorescent degradation product Form A, as described under “Experimental Procedures.” 100% was set to a control XDH with a full complement of Moco.

ND, not detectable.
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insertion into XDH, but in particular under aerobic conditions XdhC is essential for the stabilization of the sulfurated form of Moco. We also investigated whether the presence of Moco in XDH in the absence of XdhC is due to the usage of E. coli TP1000 cells for expression, which accumulate Moco to unphysiological high levels due to an impaired conversion of Moco into bis-MGD. Our analyses showed that expression of xdhAB in DH5α cells under high culture aeration resulted in an inactive, Moco-free XDH (data not shown), consistent with the results obtained for XDH purified from R. capsulatus xdhC− cells. Thus, under physiological concentrations of Moco, XdhC is essential for Moco insertion into XDH both in R. capsulatus and E. coli.

Analysis of Moco/MPT Binding to XdhC—From the previous results, the question arose whether XdhC is able to bind Moco and might act as a Moco insertase for XDH, inserting the sulfurated form of Moco and protecting it from oxidation.

Since hSO contains the MPT form of Moco and can be purified in large quantities (11), recombinant, heat-denatured hSO was chosen as a source of Moco/MPT for binding studies. To compare differences in binding of MPT or Moco to XdhC, Moco was obtained from the Moco domain of hSO expressed in TP1000 cells in the presence of molybdate, whereas molybdenum-free MPT was obtained from inactive hSO expressed in RK5202(modC−) cells in the absence of molybdate. To determine the dissociation constants of Moco/MPT to XdhC, purified XdhC was incubated with extracted Moco/MPT for 15 min before unbound Moco/MPT were separated by ultrafiltration using a membrane with a molecular weight cut-off of 10,000. The Moco/MPT concentration in the flow-through was quantified after conversion to the stable, fluorescent oxidation product Form A (see “Experimental Procedures”).

Quantification of Moco/MPT after ultrafiltration in the presence and absence of XdhC allowed the determination of $K_d$ values of Moco/MPT binding to XdhC (Fig. 2). The amount of Moco/MPT bound to XdhC and free Moco concentrations were calculated according to the determined free and total Moco concentrations and the given total XdhC concentration. Fitting revealed a function according to the law of mass action for a Moco/MPT-XdhC-ratio of 1:1, with a Moco saturation of 0.97 ± 0.06 (Fig. 2A) and a MPT saturation of 0.95 ± 0.11 (Fig. 2B). A $K_d$ value of 3.6 ± 0.1 μM was obtained for Moco binding, and a $K_d$ value of 3.5 ± 0.3 μM was obtained for MPT binding to XdhC at 4 °C, showing that XdhC is able to bind both molecules with the same efficiency in stoichiometric amounts.

Analysis of the Ability of XdhC to Insert Moco into Apo-XDH—Further, it was of interest to determine whether XdhC is also able to transfer the bound Moco into purified Moco-free apo-XDH. Moco-containing XdhC was incubated with freshly purified apo-XDH for 90 min at 4 °C, before XdhC was removed from the incubation mixture by affinity chromatography (see “Experimental Procedures”). Since the Moco used for this experiment was obtained from hSO containing the dioxo form of Moco lacking the terminal sulfur ligand required for XDH activity, the reconstitution of XDH with Moco could not be determined by regaining XDH activity. Thus, to determine the amount of Moco present in reconstituted XDH, the protein was subjected to iodine oxidation to produce the fluorescent Form A derivative of the cofactor, which can be quantified by HPLC analysis.

As shown in Fig. 3A, XdhC was able to insert bound Moco into Moco-free apo-XDH. The control experiment using free Moco showed the same level of Moco insertion into XDH. A half-maximal Moco insertion at 4 °C was observed at a Moco/XDH ratio of about 2:1. The achievable saturation level of XDH with Moco depended on the preparation of XDH and, as shown in Fig. 3A, was in the range of about 15%. A control experiment using bovine serum albumin instead of XDH showed that Moco insertion was specific to apo-XDH (data not shown). This reconstitution efficiency is less than the in vitro insertion of Moco described for hSO, where about 50% could be achieved (20). However, since XDH displays a 2.5 times higher molecular mass (275 kDa) than hSO (~110 kDa), the lower levels of in vitro Moco reconstitution might be due to the complexity of the XDH structure and to a less stable conformation of the Moco-free apo-XDH.

To analyze whether XdhC is also capable of inserting the Mo=S form of Moco into XDH, the sulfurated form of Moco was obtained after heat treatment of active XDH. The Mo=S form of Moco was bound to XdhC as described above, and after the addition of XDH at different time points, XDH activity was analyzed. In a control experiment, the isolated Moco was incubated without XdhC, and XDH activity was only regained when mono-oxo Moco was bound to XdhC immediately after extraction. In the presence of XdhC, XDH activity decreased only slightly with increasing incubation times, clearly showing that XdhC is able to stabilize the sulfurated form of Moco.

![FIGURE 2. Binding of Moco and MPT to XdhC.](Image)
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Table 2

| Incubation mixtures prior to apo-XDH addition | Specific activity * | units/mg |
|---------------------------------------------|--------------------|---------|
| XdhC + CsdA + dithionite                   | 1.57 ± 0.09        |         |
| XdhC + dithionite                         | 0.21 ± 0.02        |         |
| XdhC + CsdA                               | ND                 |         |
| CsdA + dithionite                         | ND                 |         |
| XdhC + sulfide + dithionite               | 0.73 ± 0.13        |         |
| XdhC + Moco from XDH                      | 0.41 ± 0.05        |         |

* Specific enzyme activity (units/mg) is defined as the reduction of μmol of NADH/min/mg of enzyme under assay conditions.

and protects the Mo=S ligand from oxidation. The low values of XDH activity obtained are explained by the fact that the majority of the Mo=S ligand of Moco was lost during the extraction procedure from XDH, as previously shown by Wahl et al. (21) in experiments using radioactive labeled sulfur.

Sulfuration of XdhC-bound Moco by an l-Cysteine Desulfurase—In eukaryotes, like Drosophila melanogaster, Aspergillus nidulans, and Arabidopsis thaliana, two-domain proteins designated Mal-1, HxB, and Aba3, respectively, have been implicated in the specific sulfuration of the Moco-free form of XDH (30). Exchange of CsdA by sulfide as direct sulfur donor resulted in a 50% lower reconstitution of XDH activity, showing that Moco sulfuration by CsdA is more efficient. In addition, usage of sulfated Moco extracted from active XDH resulted in 75% lower XDH activity, underlining the findings shown above that Moco loses the sulfur ligand during the extraction procedure. The results point toward a general mechanism of Moco sulfuration by an l-cysteine desulfurase with the involvement of XdhC, and the specific protein involved in this reaction has to be identified in future studies.

Co-purification of XdhC and XDH—In order to determine whether XdhC can be co-purified with XDH, a plasmid was constructed (pSL181) expressing XdhABC as a C-terminal His6 fusion to XdhC (see “Experimental Procedures”). After expression in E. coli (pK4354DE3) cells, proteins were purified by Ni2+-NTA chromatography. SDS-PAGE analysis of the eluted fractions showed three distinct bands of 85, 60, and 55 kDa, which were identified to correspond to XdhB, XdhA, and XdhC-His6, respectively (Fig. 4A). Characterization of co-purified XDH revealed that the protein was mainly inactive due to the absence of Moco (Fig. 4B). The addition of Moco to the crude extract prior to Ni2+-NTA chromatography resulted in a reduced level of co-purified XDH (Fig. 4B). This shows that XdhC only forms a tight complex with XdhC, and XdhC is released from the complex after Moco insertion into XDH. No Moco was identified in the protein fraction to which Moco was added prior to Ni2+-NTA chromatography. Therefore, the Mo=S ligand from oxidation. The low values of XDH activity obtained are explained by the fact that the majority of the Mo=S ligand of Moco was lost during the extraction procedure from XDH, as previously shown by Wahl et al. (21) in experiments using radioactive labeled sulfur.

Sulfuration of XdhC-bound Moco by an l-Cysteine Desulfurase—In eukaryotes, like Drosophila melanogaster, Aspergillus nidulans, and Arabidopsis thaliana, two-domain proteins designated Mal-1, HxB, and Aba3, respectively, have been implicated in the specific sulfuration of the Moco-free form of XDH (22, 23). It was shown that this reaction is catalyzed by a protein domain expressing l-cysteine desulfurase activity (24). R. capsulatus and E. coli contain several l-cysteine desulfurases that could act as a sulfur donor for this reaction. In E. coli, three proteins were identified expressing l-cysteine desulfurase activity, which were designated lscS, CsdA, and CsdB (SufS) (25). Whereas all proteins act as sulfur-delivering proteins for the biosynthesis of iron-sulfur clusters (26–28), lscS is additionally involved in the biosynthesis of thiamine and thioridine (29). The specific protein for the sulfuration of Moco has not been identified in E. coli to date. To analyze whether an E. coli l-cysteine desulfurase is able to sulfurate Moco while bound to XdhC, we purified E. coli CsdA as a N-terminal His6 fusion (15). CsdA was chosen, since it expresses high l-cysteine desulfurase activity and acted as the best sulfur delivery protein for the generation of the dithiolene group of Moco in vitro (15). For the sulfuration experiments, the persulfurated form of CsdA was used (see “Experimental Procedures”). As shown in Table 2, XdhC incubated with an excess of dioxo Moco extracted from hSO3, persulfurated CsdA, and sodium dithionite prior to insertion into apo-XDH gave rise to the highest XDH activity. Since CsdA was removed from the incubation mixture by Ni2+-NTA chromatography before the addition of apo-XDH, the sulfuration of Moco occurs by CsdA, whereas Moco is bound to XdhC. Control experiments showed that the addition of dithionite is required for this reaction, which has been reported before for the resulfuration of dioxo Moco in vitro (30). Exchange of CsdA by sulfide as direct sulfur donor resulted in a 50% lower reconstitution of XDH activity, showing that Moco sulfuration by CsdA is more efficient. In addition, usage of sulfated Moco extracted from active XDH resulted in 75% lower XDH activity, underlining the findings shown above that Moco loses the sulfur ligand during the extraction procedure. The results point toward a general mechanism of Moco sulfuration by an l-cysteine desulfurase with the involvement of XdhC, and the specific protein involved in this reaction has to be identified in future studies.

Analysis of Binding between XdhC, Apo-XDH, and XdhB—In order to verify the binding between Moco-free apo-XDH and XdhC, Biocore instrumentation was used for real time detection of the spe-
specific interactions. All proteins were immobilized on the chip by amine coupling. The sensograms shown in Fig. 5A give the sum of the specific binding and the bulk effect. Compared with sensograms obtained with bovine serum albumin (data not shown), XdhC appeared to specifically interact with apo-XDH. In order to identify whether the specific binding site of XdhC is located on XdhB, the separate XdhB subunit was purified as described under “Experimental Procedures.” The single XdhB subunit existed as a monomer in solution and was shown to be Moco-free (data not shown). Using immobilized XdhB, the binding constants obtained with global analysis for increasing XdhC concentrations were approximately the same as determined for apo-XDH (Fig. 5B), showing that XdhC specifically interacts with the Moco-free form of the XdhB subunit.

**DISCUSSION**

The crystal structures of several molybdoenzymes revealed that Moco is deeply buried in the proteins, at the end of a funnel-shaped passage giving access only to the substrate (3, 4, 31, 32). This implied the requirement for specific chaperones to facilitate the insertion of Moco into the specific molybdoenzymes. In prokaryotes, specific chaperones were identified for a number of molybdoenzymes, like TorD for *E. coli* trimethylamine N-oxide reductase (TorA) or NarJ for *E. coli* nitrate reductase A (NarGHI) (33–35). TorD was shown to be a specific chaperone for TorA maturation, which interacts with apo-TorA and generates a form that is able to receive bis-MGD (36). A second role was attributed during the translocation process of TorA by the Tat (twin arginine transporter) translocon to prevent export of immature TorA (37, 38). The formation of active membrane-bound NarGHI in *E. coli* requires the presence of NarJ, which is not part of the active nitrate reductase complex (33). In the absence of NarJ, bis-MGD is not detectable in the unstable, inactive NarGHI complex. NarJ is involved in membrane anchoring of NarGHI to NarL after a NarJ-assisted assembly and bis-MGD insertion into NarGHI (39). It has also been suggested that NarJ plays a role in allowing interactions between NarG and the Moco biosynthesis proteins MogA, MoeA, MobA, and MobB (40). However, binding of Moco to neither TorD nor NarJ has been reported to date.

The role of *R. capsulatus* XdhC for the maturation of XDH seems to be different from the chaperone role of NarJ and TorD. We were able to...
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FIGURE 6. Model for the role of XdhC in XDH and Moco maturation. Moco is produced by molybdenum insertion into MPT catalyzed by MogA and MoeA and is further converted to bis-MGD by the MobA protein. Our results showed that XdhC binds Moco, and we suggest that the equatorial Mo=S is inserted into Moco while bound to XdhC. XdhC protects the mono-oxo form of Moco from oxidation and interacts with XdhB. After Moco insertion, XdhC dissociates from the complex. Nothing is known about the nature of the specific l-cysteine desulfurase involved in Moco sulfuration in prokaryotes to date.

demonstrate a first example for a system-specific protein required for the maturation of XDH, which binds Moco in stoichiometric amounts and is involved in protecting the sulfurated form of Moco from oxidation. It was shown that the dissociation constants for Moco and MPT binding to XdhC are the same. A tight interaction of XdhC with apo-XDH was shown, underlining the specific role of XdhC in XDH maturation. Unfortunately, it was not possible to purify XdhC from E. coli cells in a Moco-bound form, since during the process of purification, XdhC lost its bound Moco (data not shown). Thus, we were not able to give direct proof that the sulfurated form of Moco is bound to XdhC in the cell, which has to be verified in future studies. XdhC was shown to be essential for Moco insertion in R. capsulatus (6). We could show that expression of XDH in E. coli TP1000 cells in the absence of XdhC resulted in an unspecific incorporation of Moco. The TP1000 strain contains a deletion of the mobAB genes involved in bis-MGD formation (10). Thus, these cells accumulate MPT to unphysiological high concentrations, since so far, only one molybdoenzyme has been identified in E. coli binding the MPT form of Moco (41, 42). Our *in vitro* investigations showed that free Moco is inserted to the same level into XDH as XdhC-bound Moco. Thus, in the absence of XdhC, XDH might be able to incorporate free Moco when present in high concentrations in the cell. However, oxygen seems to be a major factor of free Moco inactivation, which makes it likely that under physiological conditions, Moco exists only as a protein-bound form in the cell. However, some differences seem to exist for Moco insertion between *R. capsulatus* and *E. coli*. Whereas in *E. coli* it is possible to incorporate MPT into molybdoenzymes in the absence of molybdate, a previous report showed that in *R. capsulatus*, Moco insertion into target enzymes occurs only after molybdenum chelation to the cofactor (43). It was also shown that in *R. capsulatus*, Moco-free apo-XDH has a different conformation in comparison with mature XDH, which could be visualized by a different electrophoretic mobility of the proteins (6, 43). However, a direct interaction of XdhC and apo-XDH has not been shown for XDH isolated from *R. capsulatus* to date. On the other hand, our results showed that XDH and XdhC form a tight complex, which dissociates after the insertion of Moco into XDH. Thus, it cannot be excluded that in addition to the role of protecting the mono-oxo Moco from oxidation, XdhC might serve *in vivo* as a chaperone that holds apo-XDH in a proper conformation until Moco is inserted.

The only other characterization of an XdhC-like protein was reported by Ivanov et al. (44), who described the functional expression of *Comamonas acidovorans* XDH in *E. coli*. Expression of *C. acidovorans* xdhAB yielded in a 10% active enzyme. However, when *Pseudomonas aeruginosa* xdhC was co-expressed (an XdhC homologue has not been identified in *C. acidovorans* to date), the enzyme was almost fully functional, showing that *P. aeruginosa* XdhC is able to participate in assembly of *C. acidovorans* XdhAB in *E. coli*. It was shown that in the absence of XdhC, molybdenum incorporation into the enzyme was not complete, a reaction that was dependent on the oxygen concentration of the culture medium. Their results implied that the presence of XdhC during expression increases the incorporation of sulfurated Moco into XDH; however, a direct proof of this hypothesis was not given in their report. In contrast, our results clearly show that XdhC stabilizes the sulfurated form of Moco and protects it from oxidation to the dioxo form.

In summary, we present a model for the role of XdhC for Moco and XDH maturation (Fig. 6). We suggest that XdhC binds Moco, which is produced by MoeA/MogA in the cell (45). XdhC especially protects the sulfurated form of Moco from oxidation, which is most likely generated by the activity of an l-cysteine desulfurase in the cell. The specific protein for the sulfuration of Moco has not been identified in either *R. capsulatus* or *E. coli* to date. However, our results showed that the *E. coli* CsdA protein is able to sulfurate Moco while bound to XdhC, pointing toward a general mechanism of Moco sulfuration by an l-cysteine desulfurase. For Moco insertion into XDH, XdhC binds specific to the XdhB subunit and inserts the matured Moco. We believe that under physiological Moco concentrations in the cell, XdhC ensures that only the mono-oxo form of Moco is incorporated into XDH. The unsulfurated form of Moco can be inserted nonspecifically, when high concentrations of free Moco are present. In conclusion, it seems likely that each prokaryotic molybdoenzyme has its own system-specific protein that plays a special role in Moco insertion and target protein folding. Whereas TorD and NarJ are more involved in correct assembly of their
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respective target proteins for bis-MGD cofactor insertion and the subsequent translocation of the mature protein, the specific binding of Moco to XdhC seems to be especially required for the protection of the mono-oxo form of Moco from oxidation. Surprisingly, the presence of these system-specific proteins for Moco insertion and molybdoenzyme maturation seems to be restricted to prokaryotes. Heterologous expression of hSO in E. coli gave rise to a high yield of active enzyme, showing that no protein for the insertion of the dioxygen form of Moco is required (11). Thus, so far no protein with a role similar to that of XdhC has been described for eukaryotic molybdoenzymes.

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