Communication

Molybdenum Cofactor Biosynthesis

THE PLANT PROTEIN Cnx1 BINDS MOYLBDOPTERIN WITH HIGH AFFINITY

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The molybdenum cofactor is an essential part of all eukaryotic molybdoenzymes. It is a molybdopterin (MPT) revealing the same core structure in all organisms. The plant protein Cnx1 from Arabidopsis thaliana is involved in the multi-step biosynthesis of molybdenum cofactor. Previous studies (Stallmeyer, B., Nerlich, A., Schiemann, J., Brinkmann, H., and Mendel, R. R. (1995) Plant J. 8, 751–762) suggested a function of Cnx1 in a late step of cofactor biosynthesis distal to the formation of MPT, i.e. conversion of MPT to molybdenum cofactor. Here we present the first biochemical evidences confirming this assumption. The protein Cnx1 consists of two domains (E and G) homologous to two distinct Escherichia coli proteins involved in cofactor synthesis. Binding studies with recombinantly expressed and purified Cnx1 and with its single domains revealed a high affinity of the G domain to MPT (kd = 0.1 μM) with equimolar binding. In contrast, the E domain of Cnx1 binds MPT with lower affinity (kd = 1.6 μM) and in a cooperative manner (nH = 1.5). The entire Cnx1 showed a tight and cooperative MPT binding. Based on these data providing a common link between both domains that matches the previous characterization of plant and bacterial Cnx1 homologous mutants, we present a model for the function of Cnx1.

The molybdenum cofactor (Moco) is an integral constituent of all molybdoenzymes (with the exception of nitrogenase) where it forms part of the catalytically active center (1). It consists of molybdopterin (MPT), a unique 6-alkylpterin containing one Mo atom via a dithiolene group to its four-carbon side chain, and reveals the same core structure in eukaryotes, eubacteria, and archaea (2). Because molybdoenzymes are essential for such diverse metabolic processes like nitrite assimilation in autotrophs, sulfur detoxification in mammals, and phytohormone synthesis in plants, the survival of the organisms depends on their ability to synthesize Moco. The biosynthesis of this unique cofactor is complex because it requires the multi-step synthesis of the MPT moiety and the subsequent incorporation of Mo into MPT. In all organisms studied so far at least six gene products are involved in the formation of active Moco; first models for the biosynthetic pathway of Moco in Escherichia coli (2) and plants (3) are proposed.

The first plant gene (cnx1) involved in Moco biosynthesis has been isolated by functional complementation of the Moco-deficient E. coli mutant mogA (4). The encoded protein Cnx1 consists of two domains where the N terminus (E domain) is homologous to E. coli MoeA and the C terminus (G domain) is homologous to E. coli MogA, both proteins being involved in Moco synthesis (see Fig. 1). In contrast to mogA, E. coli mutants in moeA cannot be complemented by the plant protein Cnx1 (4). Further, Cnx1 shows homologies to two eukaryotic proteins: Cinnamon (Moco protein from Drosophila (5)) and Gephyrin (neuroprotein from mammals (6)). Both homologous proteins show an inverted order of the conserved domains.

First insights into the putative function of Cnx1 came from the characterization of the E. coli Moco mutant mogA, which had a residual nitrate reductase activity of about 1% of wild type (7) and is able to synthesize MPT up to 15% of wild type level (8). Growing the mogA mutant under high molybdate concentrations (1–10 mM), the nitrate reductase activity is partially restored to 10–13% of wild type level (7) without changes in MPT levels (8), thus showing that mogA mutants are still able to synthesize MPT. Their defect should therefore reside in the last step of Moco formation, i.e. in the insertion of Mo into MPT. In contrast to MogA, nearly nothing is known about the E. coli protein MoeA homologous to the Cnx1 E domain. Mutants in moeA have no residual molybdoenzyme activities (9), are not Mo-repairable (7), but showed 3% of wild type MPT (9), which indicates that MoeA might be also involved in a step distal to MPT synthesis. The observed lack of moeA complementation by Cnx1 (4) indicates a significant divergence in the function of MoeA between prokaryotes and eukaryotes, which could go back to the final attachment of a nucleotide to the MPT moiety being only observed in prokaryotes (10).

The cnx1 gene from Arabidopsis thaliana was mapped to the chl-6 locus (4). Mutants in this locus are Moco-deficient but can be partially repaired by growth on high concentrations of molybdate (11). In Nicotiana plumbaginifolia, it was shown that mutants in the Mo-repairable cnxA locus are principally able to synthesize MPT; however, they are unable to produce active Moco (12). Thus it was suggested that Cnx1 should be involved in the last step of Moco synthesis, i.e. the insertion of Mo into MPT and/or intracellular Mo processing (3, 4, 13). For the other two eukaryotic Cnx1 homologs, Cinnamon and Gephyrin, no suggestion for a specific function in Moco synthesis has been reported yet.

Here we present the first biochemical evidences for the predicted function of Cnx1. We show a high affinity binding of MPT to the Cnx1 G domain and demonstrate that the Cnx1 E domain binds MPT as well, but with lower affinity and in a cooperative manner. The data suggest a functional link between both domains in eukaryotic organisms.
EXPERIMENTAL PROCEDURES

Materials—All chemicals used were from highest quality available. Xanthine oxidase (EC 1.1.3.22) from buttermilk grade I (0.69 units/mg) was obtained from Sigma (Munich, Germany). Prepacked gel filtration nick columns were used as recommended by the supplier (Pharmacia, Freiburg, Germany).

Plasmids, Strains, and Growing Conditions—For overexpression and purification of recombinant proteins the QAExpress kit from QIAgen (Hilden, Germany) for metal ion chelating chromatography of His-tagged proteins was used. The His tag (six amino acids) was localized at the C-terminal end of the protein (pQE-60 vector) separated by two additional amino acids. Prior to protein expression, the recombinant plasmids were transformed into the expression strain M15 [pREP4] provided by QIAgen. Cells carrying the expression constructs were grown under aerobic conditions in 250 ml of culture medium up to an A600 of 0.7–2.0, followed by induction with 1 ml isopropyl-β-D-thiogalactosidase. The expression culture was continued for 5–20 h at 20–37 °C and then harvested (5,000 × g, 10 min, 4 °C).

Purification of the His-tagged Proteins—Purification of the recombinantly expressed proteins was performed as recommended by the supplier under native conditions at 4 °C. Purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis on 7.5–12.5% polyacrylamide gels, and pure fractions were dialyzed against 100 mM Tris/HCl, pH 7.5, 1 mM EDTA, concentrated in ultrafiltration cells using YM10 membranes (Amicon), sterile filtered through 0.1-μm membranes and stored at 4 °C. Protein concentration was determined by UV absorption measurements using the calculated extinction coefficient (14) of the analyzed polypeptides (Cnx1, 37,380 M 2 cm; Cnx1 G domain, 16,680 M 2 cm).

Molecular Weight Determination and Isoelectric Focusing—For determination of the native molecular weight of the expressed proteins, fast protein liquid gel filtration chromatography was performed on a Superdex 200 column (Pharmacia) in 50 mM Tris/HCl, pH 7.5, 300 mM NaCl at 0.4 ml/min flow rate. The molecular weight was calculated using a calibration curve of standard proteins (LMW/HMW-Kit; Pharmacia). Isoelectric focusing was performed with Phast-Gels (pH 3–9) on the PhastSystem as recommended by the supplier (Pharmacia).

MPT Binding Experiments—MPT binding experiments were performed with protein-free MPT isolated from xanthine oxidase. Active MPT (in the reduced form) was used for binding experiments. Protein-bound and unbound MPT was analyzed separately after conversion to its stable oxidized fluorescent product Form A. For preparation of 250–350 pmol MPT, 200 μl of xanthine oxidase were gel filtrated under anaerobic conditions against MPT buffer (100 mM Tris/HCl, pH 7.2, 5 mM sodium malonate, 5 mM reduced glutathione) using nick columns and denatured by heat treatment for 100 s at 80 °C. After gel filtration (nick column) against anaerobic MPT buffer, the MPT-containing salt fraction was collected and used immediately for binding experiments. This fraction also contained the inactive air-oxidized product of MPT, which was subtracted from the protein-bound MPT. The gel filtration of the Cnx1 holoprotein did not give a proper resolution of peaks; only fast protein liquid gel filtration chromatography of the Cnx1 holoprotein revealed the correct translation start. Fast protein liquid gel filtration chromatography of the Cnx1 holoprotein did not give a proper resolution of peaks; only dispersed peaks occurred with shoulders between 200 and 600 kDa where Cnx1 eluted in all fractions, indicating a wide range of different oligomers. In contrast, the gel filtration of the Cnx1 G domain resulted in two distinct peaks of equal size eluting at 80.2 and 152.2 kDa, representing a trimer (calculated 77.8 kDa) and an hexamer (calculated 155.6 kDa), respectively. Subsequent gel filtration of the separated trimer and hexamer resulted in an elution of the corresponding fraction as a single peak, indicating stable forms of multimers. On gel filtration, the majority of Cnx1 E domain behaved as a dimer (calculated 97.2 kDa) eluting at 84 kDa (data not shown). The isoelectric points of Cnx1 and the E domain were found to be at pH 5.2, and the G domain focused at pH 6.6 (data not shown).

High Affinity Binding of MPT to Cnx1 G Domain—The presence of different mutants in Cnx1 and homologous proteins suggested a function of Cnx1 in the conversion of MPT to Moco (3, 4, 9, 13). To play this role, Cnx1 and particularly its G domain homologous to E. coli MogA should be able to interact/bind to MPT and/or Mo.

Because of the extreme sensitivity of protein-free MPT to

Fig. 1. Comparison of proteins and domains homologous to Cnx1 from A. thaliana. Note that MoeA and MogA are two distinct, separately expressed E. coli proteins.
oxidation, a method is needed where binding of MPT to proteins and subsequent separation of bound and unbound MPT can be performed rapidly and in small volumes. Here we used gel filtration and ultrafiltration followed by I2/KI oxidation of MPT to its fluorescent dephosphorylated product Form A (2, 15), subsequently quantified on HPLC and compared with a Form A standard (Fig. 2A).

After coincubation of 184 nM MPT with 88 nM Cnx1 G domain, 34 nM MPT could be detected in the excluded fraction of gel filtration (measured as Form A) represented by the single peak at 5 min of retention time (Fig. 2C). Further, no Form B (air oxidation product of MPT (15), which was formed during MPT preparation by heat treatment of xanthine oxidase) is coeluting with protein so that the binding of MPT is very specific, and neither Form B nor some of the other contaminations from the salt fraction bind to the Cnx1 G domain. Using 88 and 164 nM Cnx1 G domain for binding experiments with different MPT concentrations (2–300 nM), the plot of bound and unbound MPT showed a typical Michaelis-Menten kinetic (Fig. 3A). By regression of both binding curves, a dissociation constant \( k_D \) of 113 and 167 nM, respectively, was calculated (Fig. 3D), corresponding to a calculated number of binding sites of \( n = 64 \) nM (with 88 nM protein) and \( n = 110 \) nM (with 164 nM protein), respectively (Fig. 3D). Considering the errors indicated, a binding of 1 mol MPT to 1 mol Cnx1 G domain can be assumed, which confirms a nearly quantitative yield of Form A during MPT oxidation and purification on QAE chromatography and HPLC.

Ultrafiltration was used for separating bound and free MPT after coincubation with Cnx1 G domain to avoid the change in equilibrium between free and protein-bound MPT that occurs during gel filtration experiments. The binding of MPT could be verified. Analysis of the absolute amounts of Form A and Form B in both the retained protein fraction and flow-through salt fraction showed an increase of Form A (representing the bound MPT) in the protein fraction, whereas Form B, which did not bind to Cnx1 G domain, is decreased (Fig. 2D). Calculating the concentrations of Form A and Form B in both fractions showed no change in Form B concentration, whereas Form A (MPT) is highly increased in the protein fraction, which indicates MPT binding to the G domain. Using 142 nM protein for binding of different MPT concentrations (Fig. 3A), a \( k_D \) of 81 nM could be obtained from the fitted binding plot (Fig. 3D). Taking into account the result obtained by gel filtration, a slow dissociation of bound MPT from Cnx1 G domain can be suggested with an average \( k_D \) of 120 nM for MPT binding to Cnx1 G domain. Using ultrafiltration, also the predicted equimolar binding could be confirmed (\( n = 119 \) nM, Fig. 3D).

FAD was present in the MPT preparations used for the MPT binding assays. A typical preparation of 300 nM MPT yielded 20–30 \( \mu \)M FAD. Adding up to 300 \( \mu \)M FAD had no effect on the binding properties of Cnx1 G domain. Further, binding of FAD to Cnx1 G domain could not be detected in the presence or in the absence of MPT, which was expected because no putative binding site for FAD was found within Cnx1.

**Binding of MPT to Cnx1 and the E Domain—**Using the E domain of Cnx1 for MPT binding experiments, on gel filtration a coelution of MPT in the protein fraction was found, however in lower quantities as compared with Cnx1 G domain. Using bovine serum albumin as control no coelution of MPT was detectable (Fig. 2B). Analysis of protein-bound MPT (87 nM and 300 nM protein) at different MPT concentrations by plotting against unbound MPT resulted in a cooperative binding curve (Fig. 3B). Postulating binding at equal molarities we calculated by Hill plotting a \( k_D \) of 1.6 \( \mu \)M with a Hill coefficient of 1.50 (Fig. 3D).

The data show that both domains are able to bind MPT, but the binding types and affinities to MPT are different. Mixing both domains at equal ratios led to a superimposition of E and G domain binding plots, thus indicating no interference between the two separated domains (data not shown). Finally, as

![Fig. 2. HPLC analysis of protein-bound and free MPT after separation by gel filtration and ultrafiltration.](image)
presented here support this assumption, revealing that Cnx1 is not active Mo because E. coli mutants in the homologous MogA protein are molybdate-repairable (7). We could not, however, detect Mo bound to the G domain (data not shown). Perhaps there is a positive cooperativity (sequential binding) between Mo and Mg where Mo binds to the G domain only after the MPT-binding site is occupied by Mo. It is also possible that Mo binding is only of a transient nature during formation of Moco and cannot be readily detected in equilibrium binding experiments.

Although speculative, we assume the following hierarchy of MPT binding to the Cnx1 protein: the G domain with high affinity for MPT acts as the initial MPT-acceptor. With ongoing saturation of the G domain, the critical concentration of MPT reaches a threshold, thereby enabling the neighboring E domain with lower binding affinity to take over MPT. This latter step is further facilitated by the E domain-specific cooperative character of binding with lower affinity. The postulated interaction between both domains could also explain the fusion of the two predecessor proteins observed during evolution. The results presented in this paper are the first biochemical evidence for Cnx1 to be directly involved in the conversion of MPT into Moco.

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**FIG. 3. MPT binding plots of Cnx1 G domain, E domain, and the entire Cnx1.** A, 88 (●) and 142 nm (□) Cnx1 G domain were used for plotting and calculating the MPT binding kinetics obtained by gel filtration. In ultrafiltration experiments 142 nm (●) G domain was used. B, cooperative binding plot of 87 nm (○) versus 300 nm (□) Cnx1 G domain. Assuming an equimolar binding of MPT the curves were calculated by fixing (fix.) the number of maximal binding sites (n) to the used protein concentrations. C, MPT binding of 300 (○) and 408 nm (□) Cnx1 showing both Michaelis-Menten (dotted line) and cooperative regression (solid line). D, summary of the obtained binding parameters. nH, Hill coefficient.

**DISCUSSION**

The Cnx1 protein from A. thaliana consists of two domains that are homologous to two distinct E. coli proteins, both involved in Moco biosynthesis. This two-domain structure of Cnx1 formed the basis for our biochemical analysis of the separately expressed and purified domains of Cnx1 as compared with the entire Cnx1 protein. Not only the Cnx1 protein (4) but also its G domain alone (data not shown) is able to complement the E. coli mutant mogA, which shows that at least the G domain alone is functionally active. Characterization of Cnx1-related mutants from plants (11, 12, 13) and E. coli (9, 11) showed that (a) the defect of the mutation could be overcome by high concentrations of molybdate in the growth media and (b) the mutants are still able to synthesize MPT but not active Moco, so that it was suggested that Cnx1 is probably involved in a late step of Moco biosynthesis distal to the formation of MPT, i.e. insertion of Mo into MPT. Therefore Cnx1 should be able to interact with MPT and/or Mo. Our results presented here support this assumption, revealing that Cnx1 is able to bind MPT. The low kD of 0.1 μM for dissociation of bound MPT from the G domain of Cnx1 indicates a high affinity binding. Curve extrapolation shows an equimolar binding, which serves also as a good internal control for quantitation of MPT via Form A. Also the E domain of Cnx1 was found to bind MPT. This was surprising but confirms a functional link between the E domain and the G domain. The higher kD of 1.6 μM on the basis of the cooperative binding type (nH = 1.5) indicates a weaker interaction between MPT and the E domain. The binding of MPT to the entire Cnx1 protein is comparable with that of the G domain; however, the binding type fitted better with a weak cooperative regression similar to that of the E domain. Thus the Cnx1 holoprotein reflects binding characteristics (high affinity combined with cooperativity) seen in both domains. Taking into account that both of the Cnx1 domains are able to bind MPT, one could speculate about a common binding fold occurring in both domains. In contrast to the G domain, not all Cnx1 protein could be saturated with MPT (binding ratio, 1:8 to 1:15). We can rule out degradation of Cnx1 as a possible explanation because both freshly prepared Cnx1 as well as Cnx1 stored for prolonged times showed a similar low ratio for MPT binding. Other reasons could be that part of the Cnx1 protein is inactive or that MPT binds only to specific Cnx1 multimers. The difference in MPT binding of the mixed separated domains to that of the entire Cnx1 protein reveals an as yet unknown interaction between the domains within the Cnx1 holoprotein where both domains are tightly linked.

The Cnx1 G domain is a likely candidate for the binding of Mo because E. coli mutants in the homologous MogA protein are molybdate-repairable (7). We could not, however, detect Mo bound to the G domain (data not shown). Perhaps there is a positive cooperativity (sequential binding) between MPT and Mo where Mo binds to the G domain only after the MPT-binding site is occupied by MPT. It is also possible that Mo binding is only of a transient nature during formation of Moco and cannot be readily detected in equilibrium binding experiments.

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