Synthesis of Adenylated Molybdopterin
AN ESSENTIAL STEP FOR MOLYBDENUM INSERTION*

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The molybdenum cofactor (Moco) is part of the active site of all molybdenum (Mo)-dependent enzymes, except nitrogenase. Moco consists of molybdopterin (MPT), a phosphorylated pyranopterin with an endo-dithiolate coordinating Mo and it is synthesized by an evolutionary old multistep pathway. The plant protein Cnx1 from Arabidopsis thaliana catalyzes with its two domains (E and G) the terminal step of Moco biosynthesis, the insertion of Mo into MPT. Recently, the high-resolution MPT-bound structure of the Cnx1G domain (Cnx1G) has been determined (Kuper, J., Llamas, A., Hecht, H. J., Mendel, R. R., and Schwarz, G. (2004) Nature 430, 803–806). Besides defining the MPT-binding site a novel and unexpected modification of MPT has been identified, adenylated MPT. Here we demonstrate that it is Cnx1G that catalyzes the adenylation of MPT. In vitro synthesized MPT was quantitatively transferred from Escherichia coli MPT synthase to Cnx1G. The subsequent adenylation reaction by Cnx1G was Mg$^{2+}$ and ATP-dependent. Whereas Mn$^{2+}$ could partially replace Mg$^{2+}$, ATP was the only nucleotide accepted by Cnx1G. Consequently the formation of pyrophosphate was demonstrated, which was only nucleotide accepted by Cnx1G. Catalytically inactive Cnx1G mutant variants showed impaired MPT adenylation confirming that MPT-AMP is the reaction product of Cnx1G. Therefore Cnx1G is a Mo adenylyltransferase catalyzing the activation of MPT, a universal reaction in the Moco synthetic pathway because Cnx1G is able to reconstitute also bacterial and mammalian Moco biosynthesis.

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Molybdenum (Mo) plays important roles as the active center in all Mo enzymes (1), catalyzing key metabolic reactions necessary for sustaining the sulfur, nitrogen, and carbon cycles in organisms ranging from bacteria to human (2). With the exception of nitrogenase, Mo is activated and chelated by the molybdenum cofactor (Moco) (3). Moco consists of Mo covalently bound via the dithiolate moiety of a conserved tricyclic pterin referred to as molybdopterin (MPT), which is highly conserved in eukaryotes, eubacteria, and Archaea (4). Mo enzymes are essential for diverse metabolic processes like nitrate assimilation in autotrophs and phytohormone synthesis in plants (5) or sulfur detoxification and purine catabolism in mammals (6). Human Moco deficiency is a rare but severe hereditary disease characterized by the pleiotropic loss of sulfite oxidase, xanthine oxidase, and aldehyde oxidase activity (7). Affected patients die early because no therapy was available. Very recently we have described a first substitution therapy of Moco deficiency in an animal model (8).

The conservation of Moco is also reflected by a conserved biosynthetic pathway that can be divided into three steps (3, 4). At least six gene products catalyzing Moco biosynthesis have been identified in humans (9–11), plants (4), and bacteria (3). In the first step, a guanosine derivative is converted into the sulfur-free intermediate precursor Z, a tetrahydropyranopterin with a geminal diol and cyclic phosphate (12, 13). In the second step the heterotetrameric MPT synthase transfers two sulfur atoms to the C1’ and C2’ carbons of the precursor Z pyrano ring thus forming the MPT ene-dithiolate (14, 15). In the final and so far most enigmatic step of Moco biosynthesis, a Mo atom is attached to one (in pro- and eukaryotes) or two MPT dithiophosphates (in prokaryotes). Furthermore, in bacteria nucleotides are linked via a pyrophosphate bond to MPT forming the so-called MPT dinucleotide cofactors that are found either in mono-MPT (16) or bis-MPT containing enzymes (17). After completion of biosynthesis the mature cofactor has to be inserted into the Mo enzymes. In the green alga Chlamydomonas reinhardtii a Moco carrier protein that protects and transfers Moco to nitrate reductase has been identified (18).

Macromolecular structure.

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1 The abbreviations used are: Mo, molybdenum; Moco, molybdopterin cofactor; MPT, molybdopterin; precursor Z, substrate of MPT synthase; HPLC, high performance liquid chromatography; Cnx1G, Cnx1 G domain.
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FIG. 1. Schematic representation of the proposed reaction catalyzed by Cnx1G. Cnx1G is proposed to catalyze the adenylation of MPT. Shown are MPT and MPT-AMP as well as the derived oxidation products FormA and FormA-AMP, respectively, that were used to quantify product formation by HPLC analysis. The dithiolene ligand of MPT and MPT-AMP is indicated by an “R” that can either be a proton or a copper atom as shown in the recent molydbopterin-bound Cnx1G structure (24).

Experimental Procedures

Materials—All chemicals used were of highest quality available. Inorganic E. coli pyrophosphatase (EC 3.6.1.1; 1.09 units/mg) was obtained from Sigma. Nickel-nitriotriacetic acid Superflow matrix was purchased from Pierce. Prepacked NICK columns were used as recommended by the manufacturer (Amersham Biosciences).

Plasmids, Expression, and Purification of Cnx1G Domain Variants—The previous described expression plasmids of Cnx1G domain (pQE60cnx1g828) and its variants were used as recommended by the manufacturer (22). Proteins were concentrated (Centricon, Amicon) to 5 mg/ml and stored in aliquots in liquid nitrogen. Cnx1E was purified as C-terminal His-tagged protein as described (20).

Purification of Precursor Z, E. coli MPT Synthase, and In Vitro Synthesis of MPT—Precursor Z was purified as described (12). E. coli thiohoxydylated MPT synthase was in vitro assembled from its subunits according to Gutzke et al. (14). Standard in vitro synthesis of MPT was performed in a 100-μl reaction volume (100 mM Tris/HCl, pH 7.2) containing 340 pmol of MPT synthase and 355 pmol of precursor Z. The reaction was anaerobically incubated for 2 min at room temperature until completion (12).

Size Exclusion Chromatography—Size exclusion chromatography was performed using the HR30/10 Superdex 200 column (Amersham Biosciences) equilibrated in 20 mM Tris/HCl, 50 mM NaCl, pH 8.0. Proteins (3.3–10 μmol) were loaded in a total volume of 100 μl and separated with 0.2 ml/min flow rate. Fractions of 500 μl were collected and the protein content was determined by HPLC FormA analysis. The molecular weight was determined using a calibration curve obtained from the retention times of standard proteins (Amersham Biosciences).

Adenylation of MPT—For quantitative transfer of MPT to Cnx1G the MPT synthase reaction mixture was incubated for 1 h with 1 nmol of Cnx1G. If not stated otherwise, the adenylation reaction was performed with 10 μl of MPT synthase reaction mixture containing 10 μM Cnx1G. The reaction was started immediately or after 1 h preincubation with MPT synthase by adding MgCl₂ or any other bivalent cation and ATP or any other nucleotide. The standard adenylation reaction was performed with 5 mM MgCl₂ and 1 mM ATP for 2 h. In some experiments the adenylation was performed with 25 μM Superdex 200-purified Cnx1G-MPT complex (with 10 μM ATP bound). In other cases 1–5 units of pyrophosphatase was added to the reaction. All reactions were stopped by the addition of 300 μl of 100 mM Tris/HCl, pH 7.2, and 50 μl of 1% I₂, 2% KI, 1 % HCl.

Determination of MPT and AMP-MPT—MPT was detected by HPLC FormA analysis as described (20) (see also Fig. 1). To separate MPT and MPT-AMP, oxidation was performed at room temperature overnight. By this procedure MPT-AMP was converted into FormA-AMP (Fig. 1) that was separated from FormA by QAE anion exchange chromatography and subsequently treated with pyrophosphatase and alkaline phosphatase (25) thus yielding FormA-dephospho that was quantified as described above (20).

Determination of Moco—In vitro conversion of MPT into Moco was determined by the nit-1 reconstitution assay using freshly desalted nit-1 extract that was passed twice through NICK columns equilibrated with nit-1 buffer (19). In vitro Moco synthesis was performed in 20 μl containing 10 μl of nit-1 protein extract, 1 mM reduced glutathione, 0.3 mg/ml of MPT, and 5 μM of in vitro synthesized MPT according to the protocol of the assay, 1 μM Cnx1G, and when required 1 μM Cnx1E. Other additives are indicated. The reaction was incubated for 1 h at room temperature followed by the determination of reconstituted NADPH-nitrate reductase activity. Nitrite formed in a final volume of 125 μl was quantified by the absorbance at 540 nm using a 96-well plate reader (Versa max, Molecular Devices). One unit of Moco activity was defined as reconstituted nit-1 nitrate reductase activity sufficient to produce an increase of 1.0 absorbance unit at 540 nm per 25-min reaction time and nmo1 of Cnx1G.

Determination of Pyrophosphate (PP)—PP, released during the adenylation reaction of MPT was detected by the PFK20 Pyrophosphate Assay Kit (Molecular Probes). PP was detected in 50 μl of adenylation reaction mixtures containing 25 μM Cnx1G with or without 10 mM MgCl₂, 1 mM ATP, and 10 μM MPT. The reaction mixtures were incubated for 2 h at 37 °C in a microtiter plate, afterward the absorbance at 565 nm was measured using a microtiter plate reader.

Results

Cnx1G Sequesters MPT from MPT Synthase—It has been demonstrated previously that Cnx1G binds MPT with high affinity (20). To investigate the function of Cnx1G in a defined in vitro system we have synthesized MPT using in vitro assembled thiohoxydylated E. coli MPT synthase (14) and purified precursor Z (12). Co-incubation of 340 pmol of MPT synthase with 335 pmol of precursor Z revealed its quantitative conversion into MPT (data not shown). Size exclusion chromatography of this reaction mixture (Fig. 2A, thin line) showed a co-elution of 85 pmol of MPT (detected as FormA, Fig. 2B, black bars) with the heterotetrameric MPT synthase. This finding indicates that upon completion of the MPT synthase reaction the product MPT remains partially bound to the enzyme. After co-incubation of similar amounts of MPT synthase and precursor Z with 1000 pmol of Cnx1G, a total of 300 pmol of MPT was recovered in the Cnx1G-containing fraction indicating the tight binding of MPT to Cnx1G. The fact that (i) MPT elution was shifted from MPT synthase-containing fractions to Cnx1G-containing fractions and (ii) that the total recovery of MPT was dramatically higher with Cnx1G than with MPT synthase demonstrates that MPT binding to Cnx1G is stronger than to the MPT synthase. The recovery of 300 pmol of MPT indicates the quantitative conversion of 335 pmol of precursor Z. These experiments were performed with full-length Cnx1G domain (residues 460–670) (21) to separate the Cnx1G trimer (72 kDa) from the MPT synthase tetramer (54 kDa). Because of the efficient transfer of MPT to Cnx1G all following experiments, if not stated otherwise, were performed without the separation of both proteins by using the truncated version of Cnx1G (residues 460–628) (22).

The Adenylation of MPT by Cnx1G—In the high-resolution
molybdopterin-bound structure of the Cnx1G S583A variant MPT-AMP has been identified (24). Whereas Cnx1G also showed MPT-AMP, but somewhat less than S583A, the catalytically inactive variant D515H contained only MPT and no MPT-AMP. This finding suggested a function of Cnx1G in the adenylation of MPT. After probing the quantitative transfer of MPT from MPT synthase to Cnx1G we investigated the in vitro formation of MPT-AMP. In the presence of MPT synthase, precursor Z, and Cnx1G the in vitro formation of MPT-AMP was detected when MgCl2 (10 mM) and ATP (1 mM) were present (Fig. 3B). The de novo synthesis of MPT-AMP was accompanied by a depletion of free (non-modified) MPT (Fig. 3). The remaining FormA peak in Fig. 3A is probably because of a partial hydrolysis of MPT-AMP during the oxidation procedure resulting in FormA-dephospho. Our result shows that Cnx1G is indeed able to catalyze the adenylation of MPT, a reaction that is magnesium- and ATP-dependent.

Next we performed MPT-AMP synthesis experiments with different MgCl2 (0–10 mM) and ATP (0–10 mM) concentrations (Fig. 4, A and C) demonstrating maximal activity with 10 mM MgCl2 and 1 mM ATP. Already with 1 mM MgCl2 and 1 mM ATP or 10 mM MgCl2 and 100 μM ATP 75% of maximal activity was obtained. Interestingly, 10 mM ATP resulted in less MPT adenylation than with 1 mM, indicating an inhibition of Cnx1G function. ATP inhibition might be either because of (i) unspecific and competitive binding of the nucleotide to the MPT binding site of Cnx1G or (ii) because of pyrophosphate (PPi) that might be formed by spontaneous ATP hydrolysis and would be therefore increased at higher ATP concentrations. Therefore we investigated both substrate specificity and the effect of PPi, on the Cnx1G-mediated MPT adenylation.

Substrate Specificity of MPT Adenylation Reaction—Nucleotide hydrolysis is dependent on divalent metal ions with Mg2+ as the most prominent co-substrate. We investigated other metal ions such as Mn2+, Ca2+, Li2+, Ni2+, Zn2+, and Cu2+ (each 10 mM) for their ability to promote MPT adenylation in the presence of 1 mM ATP and MPT (Fig. 4B). With Mg2+ the highest activity was obtained suggesting this ion as native co-substrate for MPT-AMP synthesis. The only other metal that was able to promote MPT adenylation was Mn2+ with about 50% of the magnesium-dependent MPT-AMP production under experimental conditions.

Regarding the specificity for the nucleotide that is transferred to MPT by Cnx1G we investigated the acceptance of either ADP and AMP or other nucleotides such as GTP, CTP, and UTP (Fig. 4D). The results show that ATP is the only acceptable substrate of Cnx1G suggesting a structural preference to bind the adenosine in the negatively charged surface depression of Cnx1G (24). Furthermore, the presence of excess AMP (10 mM) had no effect on the ATP-dependent MPT adenylation.
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FIG. 5. The formation of pyrophosphate and inhibition of MPT adenylation. A, formation of pyrophosphate upon MPT adenylation. Purified Cnx1G-MPT complex was prepared as shown in Fig. 2A and concentrated to 25 μM yielding 10 μM bound MPT that corresponded to 500 pmol of MPT in the reaction mixture. Pyrophosphate formation was detected as described under “Experimental Procedures.” The reaction was performed in the absence or presence of 10 mM MgCl₂ and 0.1 mM ATP. Note that 0.1 mM ATP already contained substantial amounts of pyrophosphate. As negative control MPT-free Cnx1G was used. B, pyrophosphate inhibition of Cnx1G-dependent MPT adenylation. In vitro synthesis of MPT-AMP was performed under standard conditions (see Fig. 4) in the presence of 10 mM MgCl₂, 1 mM ATP, and 0–10 mM inorganic pyrophosphate PPᵢ. The observed inhibition by 1 mM pyrophosphate was efficiently suppressed by 1 and 5 units of pyrophosphatase (PPase). MPT-AMP formation was detected and quantified as described in the legend to Fig. 4.

PPᵢ, glutamate, and ATP (data not shown), which excludes an unspecific binding of the adenine.

Formation of Pyrophosphate during MPT Adenylation and Pyrophosphate Inhibition—So far we have demonstrated the in vitro synthesis of MPT-AMP. However, during ATP hydrolysis a second product, inorganic PPᵢ, should be released. To detect PPᵢ, formation we first purified the Cnx1G-MPT complex as shown in Fig. 2. In total, 1250 pmol of Cnx1G containing 500 pmol of MPT (detected as FormA) were used for each adenylation experiment. MPT-AMP synthesis was performed in the presence or absence of 10 mM MgCl₂ and 100 μM ATP (5000 pmol in total). Reactions that contained ATP either without Mg²⁺ or without MPT (negative control) showed 200 pmol of pyrophosphate, which supports our previous assumption that PPᵢ is present in the ATP batch derived from spontaneous hydrolysis (Fig. 5A). However, the highest level (500 pmol) of PPᵢ production was detected when MPT, MgCl₂, and ATP were simultaneously present in the reaction mixture demonstrating the MPT- and Mg-dependent formation of 300 pmol of PPᵢ. This result further demonstrates that MPT binding to Cnx1G is a prerequisite for ATP hydrolysis.

After showing the formation of PPᵢ we tested the effect of externally added PPᵢ (0–10 mM) onto the synthesis of MPT-AMP (Fig. 5B). In the presence of 1–10 mM PPᵢ an almost complete inhibition of the Cnx1G-mediated MPT adenylation was observed (Fig. 5B, black bars), which was completely overcome by the addition of inorganic pyrophosphatase (Fig. 5B, white bars). These data demonstrate that increasing concentrations of PPᵢ inhibit the formation of MPT-AMP, probably because of a shift in the reaction equilibrium from ATP hydrolysis toward ATP synthesis as it was found in several enzymes involved in adenylation processes (26). Accordingly, the observed inhibition with a high ATP concentration (10 mM) was completely reverted by pyrophosphatase treatment (data not shown) confirming the presence of PPᵢ.

Reaction Rates and Kinetic Parameters of MPT Adenylation—We have shown that Cnx1G catalyzes the adenylation of MPT, a reaction that is dependent on Mg²⁺, ATP, and release PPᵢ. Next we investigated the reaction rates under “standard conditions” using 10 mM MgCl₂ and 1 mM ATP. When precursor Z conversion and MPT-AMP synthesis were started simultaneously MPT adenylation reached saturation after a 1-h reaction time (Fig. 6A) corresponding to a reaction rate of 0.4 pmol of MPT-AMP/min. However, when Cnx1G was first preincubated for 1 h with MPT synthase and precursor Z, allowing the efficient transfer of synthesized MPT to Cnx1G, the subsequent MPT adenylation was significantly faster (Fig. 6B) with 0.8 pmol of MPT-AMP/min. Under those conditions saturation started after 30 min. Based on this observation we propose a relatively slow transfer of MPT from MPT synthase as one rate-limiting factor. Conclusively, when using purified Cnx1G-MPT complex (see Fig. 2A) an MPT-AMP synthesis rate (Fig. 6B, 1.2 pmol of MPT-AMP/min) was observed similar to that seen with preincubation (Fig. 6A). When reaching saturation all free (non-adenylated) MPT was converted into MPT-AMP (data not shown, compare also Fig. 3).

We have shown that PPᵢ is released during MPT adenylation and that increasing amounts of PPᵢ inhibit Cnx1G. As PPᵢ is formed during the synthesis it should also inhibit the reaction
resulting in a decreased reaction rate with time. Therefore adenylation was performed in the presence of inorganic pyrophosphatase resulting in a significantly increased MPT adenylation rate (Fig. 6B, 2.5 pmol of MPT-AMP/min). Almost complete saturation of MPT-AMP synthesis was reached after 10–15 min.

To obtain catalytic parameters MPT adenylation was performed with MPT-bound Cnx1G at different ATP concentrations (50–1000 μM). A clear ATP-dependent reaction rate was observed as indicated by the resulting double-reciprocal plot (Fig. 6C). The calculated kcat of 0.0012 s−1 indicates that the adenylation reaction is relatively slow. The Km value for ATP of 62 μM is almost 1000-fold higher than the Kd value of MPT (100 nM) (20). This finding supports our previous observation that first MPT binds to Cnx1G before ATP can be hydrolyzed.

The Active Site of Cnx1G—Previous studies have described different Cnx1G variants with modifications in highly conserved surface residues (23). Some of those residues are located in the MPT-binding site (Thr-542, Ser-573) and others are part of or in close proximity to the adenosine-binding site (Ser-476, Asp-486, Asp-515, Arg-547) (24). The latter are able to bind MPT but are catalytically inactive (21, 23). Here we have analyzed these Cnx1G variants for their ability to catalyze MPT adenylation. In the absence of pyrophosphatase (Table I) all variants with a complete loss of MPT binding (S573A, T542D, T542A) showed no detectable MPT-AMP synthesis, confirming our finding that MPT binding is essential for nucleotide transfer. Two other variants with partially impaired MPT binding (T542S, T542A) were able to synthesize MPT-AMP but with significantly reduced rates corresponding well to their reduced levels in MPT binding (23). Cnx1G variants impaired in catalysis (D515H, S476A, D486A) showed also no MPT adenylation. One variant (R547E) with wild type-like MPT binding and slightly reduced activity (23) showed a clear reduction in MPT adenylation. Finally, we investigated the fully active SS83A variant, which was even more active than Cnx1G. This increased adenylation rate explains why MPT-AMP was trapped in the crystal structure (24).

MPT-AMP synthesis in the presence of pyrophosphatase showed an overall increase in activity for all variants examined before (Table I). Furthermore, a detectable activity for the MPT binding-deficient SS73A variant was found. When comparing the rates of reaction with and without pyrophosphatase an 2.2-fold increase has been observed for Cnx1G in the presence of pyrophosphates. Except for T542A all other detectable mutant activities were less sensitive to pyrophosphatase indicating no major reduction in the pyrophosphate release. However, in the case of T542A the 2.8-fold increased reaction rate might indicate a functional role of this residue in product release, which is not surprising as it is located in close proximity to the pyrophosphate bond in MPT-AMP.

**In Vitro Moco Synthesis from Precursor Z—**So far we have demonstrated that in vitro synthesized MPT is converted by Cnx1G into MPT-AMP. Finally we could demonstrate that in vitro synthesized MPT-AMP can be further converted into active Moco, which was detected by reconstitution of the apoprotein reductase in desalted *Neurospora crassa nit-1* protein extract. Moco synthesis was dependent on Mg2+ and ATP in a similar way as the synthesis of MPT-AMP (data not shown) demonstrating that the amount of in vitro synthesized Moco is directly related to the rate of MPT-AMP synthesis.

**DISCUSSION**

Moco biosynthesis is highly conserved between eukaryotes and prokaryotes, a fact that is illustrated by the efficient reconstitution of *E. coli* Moco synthesis by the plant protein domain Cnx1G (21). Therefore, we have chosen Cnx1 as the representative protein to investigate the last step of Moco biosynthesis. Although MPT binding to Cnx1 has been shown (20) the mechanism of Mo insertion remained enigmatic. For Cnx1G, besides its MPT binding, a second function in Mo insertion was shown and both properties were mapped onto the structure of Cnx1G (22) thus defining its active site (23). Recently, we reported the MPT-bound structure of Cnx1G that confirmed the proposed MPT binding site and revealed a novel mechanistically important intermediate, MPT-AMP (24). Therefore we investigated in this study the synthesis of MPT-AMP.

First we transferred de novo synthesized MPT from MPT synthase to Cnx1G providing the basis for a fully defined in vitro system to investigate MPT adenylation. In previous studies Cnx1G-MPT complexes were obtained by co-purification from *E. coli* (21, 23). Cnx1G showed Mg2+ and ATP-dependent in vitro synthesis of MPT-AMP. This reaction was highly specific as Mg2+ and ATP were the preferred substrates. Only Mn2+ was able to replace Mg2+ partially, which is seen in many reactions that are dependent on ATP hydrolysis (26). Upon MPT-AMP synthesis an almost equimolar release of PPi has been demonstrated. Because of the equilibrium nature of ATP hydrolysis/synthesis, increasing amounts of PPi resulted in the inhibition of MPT-AMP synthesis that could be completely suppressed by pyrophosphatase. As PPi is continuously formed during MPT adenylation the addition of pyrophosphatase showed a significant increase in the reaction rate. Furthermore, PPi release is dependent on the presence of MPT supporting the hypothesis that MPT binding is essential for ATP binding and subsequent hydrolysis. Large conformational changes in Cnx1G might be excluded as no differences were seen between the apo- and MPT-bound structure of Cnx1G (24). However, MPT binding was one rate-limiting factor of adenylation as the reaction rates were increased upon reincubation of Cnx1G with MPT synthase. The fact that MPT binding is a prerequisite to ATP hydrolysis is also supported by the large discrepancy between the Kd of MPT (0.1 μM) and the Km of ATP (62 μM).

The adenylation catalyzed by Cnx1G is relatively slow. However, the kcat for ATP is within the physiological range and similar to other enzymes that catalyze AMP transfer reactions like the malonyl-CoA synthetase (27). Under optimal conditions adenylation rates were not comparable with MPT synthase, which converts stoichiometric amounts of precursor Z into MPT within 1 min (12) corresponding to a reaction rate of about 100 pmol of MPT/min. However, the rate-limiting step of MPT synthesis seems to be the regeneration of the thiocarboxylated small subunit MoaD (14), which is also dependent on the

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**Table 1**

| Pyrophosphatase* | Absent | Present | Increase† |
|-----------------|--------|---------|-----------|
|                 | Rate   | Rate    |           |
|                 | pmol/min | pmol/min |           |
| Cnx1G           | 0.824 | 1.819   | 2.21      |
| S476A           | ND   | ND      | ND        |
| D486A           | ND   | ND      | ND        |
| D515H           | ND   | ND      | ND        |
| T542S           | 0.082 | 0.127   | 1.55      |
| T542A           | 0.041 | 0.115   | 2.80      |
| T542D           | ND   | ND      | ND        |
| R547E           | 0.136 | 0.241   | 1.77      |
| S573A           | ND   | 0.056   | 0.96      |
| S583A           | 1.573 | 2.080   | 1.37      |

*5 units in each reaction.
†X-fold increased reaction rate in the presence of pyrophosphatase.
ND, not detectable.
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adenylation of MoaD, a stoichiometric reaction that was completed within 30 min (28).

Catalytic conversion of MPT into MPT-AMP was not observed (data not shown), which might be because of the tight binding of MPT-AMP to Cnx1G (24) and/or the missing E domain as the second domain of holo-Cnx1. In terms of overall reaction rates of Moco synthesis our and other data (28) indicate a relatively slow production of Moco. At least in eukaryotes only low-level induction of Moco synthesis has been observed, a fact that might indicate a functional importance of a Moco carrier (18).

After demonstrating that Cnx1G synthesizes MPT-AMP the question remained whether or not this activity is impaired in those Cnx1G variants that are unable to promote Mo insertion (21, 23). Indeed, all mutants either deficient in MPT binding or catalysis were defective in the adenylation of MPT-AMP. These results, based on the structural comparison of the MPT-AMP-bound SS83A structure (24) with the homologous fold of the magnesium-bound MoeA structure (29), both Asp-515 and Asp-548 might participate in the coordination of the catalytic Mg\(^{2+}\) ion. S476D and D486A, also impaired in catalysis (23), are probably essential rather for adenosine binding than being directly involved in catalysis. Finally, Thr-542 is in direct contact to the MPT phosphate (24) and was shown to be essential for MPT binding (23). As T542A and T542S show different activities in the absence of pyrophosphatase but similar adenylation rates in its presence one might conclude that Thr-542 is directly involved in catalysis. Finally, Thr-542 is in direct conformation of Asp-515 and Asp-548 essential for MPT adenylation. Based on the structural comparison of the MPT-AMP-bound SS83A structure (24) with the homologous fold of the magnesium-bound MoeA structure (29), both Asp-515 and Asp-548 might participate in the coordination of the catalytic Mg\(^{2+}\) ion. S476D and D486A, also impaired in catalysis (23), are probably essential rather for adenosine binding than being directly involved in catalysis. Finally, Thr-542 is in direct contact to the MPT phosphate (24) and was shown to be essential for MPT binding (23). As T542A and T542S show different activities in the absence of pyrophosphatase but similar adenylation rates in its presence one might conclude that Thr-542 is directly involved in pyrophosphate-bond formation and/or PP\(^{i}\) release. The results presented here show that Cnx1G is a MPT adenylyltransferase. As Cnx1E is needed for the subsequent magnesium-dependent Mo insertion reaction (data not shown) (24) this activity might be called Mo insertase. We propose that Cnx1G and Cnx1E act sequentially during Moco synthesis to promote efficient product/substrate channeling, which explains the evolutionary pressure for the domain fusion in eukaryotes. The reaction product MPT-AMP might be transferred to or directly processed by Cnx1E. Both cases would explain the weak but detectable binding of MPT to Cnx1E (20) as well as the structural similarity observed between both domains (30). As Mo insertion is dependent on MPT-AMP cleavage, one might conclude that the absence of molybdate would result in the accumulation of MPT-AMP. This hypothesis is supported by the observation in Hansenuela polymorpha where Mo-depletion resulted in the loss of MPT cytosine dinucleotide formation, which is the essential co-factor of carbon monoxide dehydrogenase (31). Under these conditions Moco synthesis would be completely arrested in the MPT-AMP state.

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2 G. Schwarz, unpublished data.