Role of the C-Terminal Gly-Gly Motif of *Escherichia Coli* MoaD, a Molybdenum Cofactor Biosynthesis Protein with a Ubiquitin Fold†

Jennifer Schmitz,‡ Margot M. Wuebbens,§ K. V. Rajagopalan,§ and Silke Leimkühler*,‡

Institute of Biochemistry and Biology, University of Potsdam, 14476 Potsdam, Germany, and Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received September 27, 2006; Revised Manuscript Received November 16, 2006

ABSTRACT: In *Escherichia coli*, the MoaD protein plays a central role in the conversion of precursor Z to molybdopterin (MPT) during molybdenum cofactor biosynthesis. MoaD has a fold similar to that of ubiquitin and contains a highly conserved C-terminal Gly-Gly motif, which in its active form contains a transferrable sulfur in the form of a thiocarboxylate group. During MPT biosynthesis, MoaD cycles between two different heterotetrameric complexes, one with MoaE to form MPT synthase and the other with MoeB, a protein similar to E1 in the ubiquitin pathway, to regenerate its transferrable sulfur. To determine the specific roles of each of the two terminal Gly residues with regard to the MoaD cycle, variants at the penultimate (Gly80) or terminal (Gly81) residues of both MoaD and thiocarboxylated MoaD were created. These variants were analyzed to determine their effects on complex formation with MoaE and MoeB, formation of the MoaD−acyl-adenylate complex, transfer of sulfur to precursor Z to form MPT, and total cofactor biosynthesis. The combined results show that while conservative substitutions at Gly80 had little effect on any of the processes that were examined, the terminal MoaD residue (Gly81) is important for transfer of sulfur to precursor Z and essential for formation of the MoaD−AMP complex. These results further our understanding of the mechanistic similarities of the MoaD−MoeB reaction to that of the ubiquitin−E1 system.

In *Escherichia coli*, molybdenum cofactor (Moco)[1] biosynthesis comprises four steps: (1) conversion of GTP to precursor Z, (2) transformation of precursor Z to molybdopterin (MPT), (3) insertion of molybdenum onto MPT to form Moco, and (4) the attachment of GMP to form MPT-guanine dinucleotide. MPT synthase is the protein responsible for conversion of precursor Z to MPT. The synthase is a heterotetrameric complex of two MoaE (∼16850 Da) and two MoaD (∼8750 Da) subunits (*1*, *2*). The high-resolution crystal structure of MPT synthase revealed that within the complex, the two MoaE subunits form a central dimer, and the MoaD subunits are located at opposite ends of the dimer (*3*). MoaD contains 81 amino acid residues, and homologous proteins have been identified in a number of organisms, including bacteria, archea, and eukaryotes. In its active form, MoaD contains a C-terminal thiocarboxylate (MoaD-SH) that acts as the sulfur donor for the dithiolene group formed during conversion of sulfur-free precursor Z to MPT (*2*, *4*, *5*). Structural studies revealed that MoaD shares a fold with ubiquitin (Ub) despite the absence of detectable sequence homology between the two proteins other than a C-terminal Gly-Gly motif (*3*). This motif is shared by all members of the family of ubiquitin-like (Ubl) proteins with the exception of *Aspergillus nidulans* CnxG which contains a serine in place of the glycine at the penultimate position (*6*).

For MPT synthase to act catalytically, it is necessary to regenerate its transferrable sulfur after each round of MPT synthesis. Both ATP and the MoeB protein are required for this regeneration, with MoaD and MoeB forming a tetrameric complex (*7*). *E. coli* MoeB is significantly similar in sequence to two segments of the E1 enzyme for Ub (Uba1). Ub and most Ubl proteins are activated in an ATP-dependent process that involves the initial formation of an acyl-adenylate between the C-terminus of the Ubl protein and AMP (*8*). The acyl-adenylate is nucleophilically attacked by a conserved active site cysteine residue in the activating enzyme (E1), leading to the formation of a thioester bond between the cysteine and the C-terminal Gly-Gly motif of Ub (*9*, *10*). Subsequently, Ub is further transferred to a conjugating enzyme (E2) and to Ub ligases (E3 enzymes) in transthioesterification reactions (*11*). Biochemical and structural analysis of the MoeB−MoaD complex revealed that the role of MoeB is solely to activate the C-terminus of MoaD via formation of an acyl-adenylate and that no thioester intermediate is formed between MoaD and MoeB. Therefore, the interaction of MoeB with MoaD resembles only the first step of Ub activation by E1 (*3*, *12*, *13*). The formation of MoaD-SH from the acyl-adenylate requires the action of a persulfide-containing protein the identity of which has not been

†This work was supported by National Institutes of Health Grant GM00091 (to K.V.R.) and Deutsche Forschungsgemeinschaft Grant LE1171/5-1 (to S.L.) as well as the Fonds der Chemischen Industrie.

* To whom correspondence should be addressed: Institute of Biochemistry and Biology, University of Potsdam, 14476 Potsdam, Germany. Telephone: +49-331-977-5603. Fax: +49-331-977-5419. E-mail: sleim@uni-potsdam.de.

‡ University of Potsdam.

§ Duke University Medical Center.

[1] Abbreviations: Moco, molybdenum cofactor; MPT, molybdopterin; Ub, ubiquitin; Ubl, ubiquitin-like protein; MoaD-SH, thiocarboxylated MoaD; ITC, isothermal titration calorimetry; HPLC, high-pressure liquid chromatography; NR, nitrate reductase.

10.1021/bi062011w CCC: $37.00
Published on Web 12/21/2006

definitively established in *E. coli* (*14*). After reassociation of MoaD-SH with MoaE to form active MPT synthase, the thiocarboxylate sulfurs are transferred to the side chain of precursor Z to form MPT (*15*).

Recently, the crystal structure of the *E. coli* ThiS−ThiF complex involved in thiamine biosynthesis was determined (*16*). ThiS is a 7.2 kDa Ubl protein that is structurally similar to Ub and MoaD and also possesses the C-terminal Gly-Gly motif. ThiF shares sequence and structural similarities with MoeB and other Ubl-activating enzymes (*16*). In a manner similar to that observed for the MoaD−MoeB complex, an acyl-disulfide formation for the thiocarboxylation of ThiS was described rather than the formation of a thioester. The phylogenetic distribution of the enzymes involved in Moco and thiamine biosynthesis strongly suggests that both systems have evolved from a common ancestor and that the eukaryotic two-component Ubl−E1 systems are derived from these simpler systems.

Since the C-terminal Gly-Gly motif is the one common sequence feature of the Ubl proteins involved in both acyl-adenylate formation and thioester or acyl-disulfide formation, the roles of these two residues in the MPT biosynthesis pathway were examined with the hopes of shedding light on their relative importance in all similar protein systems. To this end, the motif in both MoaD and MoaD-SH was modified through site-directed mutagenesis. The modifications included a number of amino acid exchanges [G81A, G80A, G80E, and G80S (the variant found in *A. nidulans* MoaD)] as well as both a truncation (G81Δ) and extension (+82G) of MoaD and MoaD-SH. Each variant was assessed for complex formation with MoaE and MoeB, formation of the MoaD acyl-adenylate group, transfer of sulfur to precursor Z to form MPT, and total Moco biosynthesis. The ability of an alternative method of MoaD-SH formation that does not involve the normal in vivo MoeB activation route facilitated these experiments (*4*, *17*). Our results indicate that while conservative substitutions at Gly80 generally had little effect on any of the processes that were examined, the terminal MoaD residue (Gly81) is essential for formation of the MoaD−AMP complex. Interaction with MoeB and transfer of sulfur to precursor Z were also affected by amino acid exchanges at the last glycine, but not as drastically. These results provide insight into the role of the Gly-Gly motif in MPT formation and further our understanding of the mechanistic similarities of the MoaD−MoeB reaction and the Ubl−E1 systems.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis, Protein Expression, and Purification. MoaD was expressed from vector pMW15aD, and MoaD-SH was expressed from pMWTYBaD-SH in *E. coli* BL21(DE3) or *moaD*$^-$(DE3) cells. To synthesize MoaD-SH, the gene sequence of MoaD was cloned into the *E. coli* expression vector, pTYB1, resulting in a fusion protein containing both a C-terminal intein tag and a chitin-binding domain for affinity purification. Taking advantage of the intein-catalyzed self-cleavage reaction and the resulting transesterification, cleavage of the product with ammonium sulfide results in the thiocarboxylated form of MoaD (*4*, *5*). Single amino acid substitutions at the C-termini of the two proteins were created using the plasmids described above and the Transformer Kit from BD Biosciences. MoaE was expressed in *E. coli* BL21(DE3) cells from plasmid pMWaE15 as described by Wuebbens et al. (*5*). MoeB was expressed in *E. coli* BL21(DE3) cells from plasmid pMW15eB and purified as described by Leimkühler et al. (*12*).

Detection of the MoaD−Acyl-Adenylate Complex. Individual MoaD variants and MoeB (each at 20 μM) were mixed with 50 μM ATP, 50 μM $MgCl_2$, 1 unit of pyrophosphatase (Sigma), and 1 μCi of [α-^{32}P]ATP or [γ-^{32}P]ATP (GE Healthcare) in a total volume of 200 μL of 100 mM Tris (pH 7.2). After 5 min, the sample was loaded onto a PD10 column (GE Healthcare) equilibrated with 100 mM Tris (pH 7.2) to separate protein-bound AMP from free ATP and inorganic phosphate. After the first 3 mL eluted from the column, the subsequent 0.5 mL containing the MoeB−MoaD−AMP complex was collected and the amount of radioactivity present was measured with a Wallac 1409 liquid scintillation counter using a counting time of 2 min. Protein-free mixtures containing [α-^{32}P]ATP or [γ-^{32}P]ATP were treated in the same manner, and the values obtained were substracted from the incubation mixtures.

Isothermal Titration Calorimetry (ITC). For the ITC experiments, all protein samples were dialyzed against 1 L of 100 mM NaCl, 50 mM Tris-HCl, and 1 mM EDTA (pH 7.5) at 4 °C. Purified MoaD variants were titrated as ligand into a 1.44 mL sample cell from a VP-ITC device (MicroCal), containing either MoaE or MoeB. Protein concentrations for MoaE and MoeB varied from 15 to 20 μM. MoaD or MoaD-SH variants (at concentrations of 150 or 200 μM) were titrated in 10 μL injections (30 injections total) to reach a final molar ratio of MoaD to MoaE or MoeB of 2:1 at the end of the experiment. All experiments were performed at 25 °C, and blank injections of titrant into buffer containing 10 or 20 μM BSA showed heat effects of injection, mixing, and dilution of less than 2%. For every injection, the binding enthalpy was calculated by integration of the peak area using ORIGIN. The association constant (K_a) and additional binding parameters (binding stoichiometry, enthalpy, and entropy) were obtained through curve fitting with ORIGIN.

Size Exclusion Chromatography of the MoaD−MoeB Complex. Size exclusion chromatography was performed at 8 °C using a Superdex 200 column (GE Healthcare) with a bed volume of 24 mL equilibrated in 100 mM Tris and 100 mM NaCl (pH 7.5) on an Äkta Basic system (GE Healthcare). Mixtures containing 13−14 μM MoaD, 30−33 μM MoeB, 10 mM ATP, 10 mM $MgCl_2$, and 5 units of pyrophosphatase were incubated for 15 min at 25 °C prior to gel filtration (flow rate of 0.5 mL/min).

MPT Synthase Reactions. Precursor Z was purified as described previously (*18*). Concentrated mixtures of MoaD-SH and MoaE were preincubated for 15 min and then transferred to multiple reaction tubes to yield a final concentration of 2.5 μM for each protein in 400 μL of 100 mM Tris (pH 7.2). Reactions were performed at room temperature, and the formation of MPT was initiated by the addition of 1.4 μM precursor Z. At specified time points, reactions were terminated by the addition of 50 μL of acidic iodine. This treatment also converts MPT to its fluorescent derivative form A, which can be quantified as previously described (*19*). Following incubation at room temperature for 14 h, excess iodine was reduced by the addition of 55 μL of 1% ascorbic acid, and each sample was adjusted to

Table 1: Activities of MoaD and Variants Assayed for Acyl-Adenylate Formation, Assembly with MoaE or MoeB, Conversion of Precursor Z to MPT, and NR Activity

	% adenylation[a]	formation of a complex with MoaE	formation of a complex with MoeB	% MPT formation (after 40 min)[a]	% NR activity[a]
wild type	100	yes	yes	100	100
+82G	—[b]	yes	yes	37	—[b]
G81Δ	—[b]	yes	no[b]	—[b]	—[b]
G81A	—[b]	yes	yes	40	7
G80S	128	yes	yes	98	87
G80A	154	yes	yes	96	94
G80E	126	no[b]	yes[c]	—[b]	—[b]

[a] Compared to the wild-type value. [b] None detected (<2%). [c] Only in the presence of ATP.

pH 8.3 with 1 M Tris. The phosphate monoester of form A was cleaved by the addition of 40 mM $MgCl_2$ and 1 unit of calf intestine alkaline phosphatase (Fermentas). After the addition of 20 μL of 50% acetic acid, form A was identified and quantified by HPLC analysis on a C18 reversed phase HPLC column (4.6 mm × 250 mm ODS Hypersil, particle size of 5 μm) equilibrated with 5 mM ammonium acetate and 15% methanol at an isocratic flow rate of 1 mL/min. In-line fluorescence was monitored by an Agilent 1100 series detector with excitation at 383 nm and emission at 450 nm.

Nitrate Reductase (NR) Activity. The expression vectors for the MoaD variants were transformed into a *moaD*$^-$ *E. coli* strain [previously termed the *chlM* strain (*20*)] carrying the G80E mutation, which had been lysogenized with the DE3 vector (*12*). The resulting strains were grown for 16 h at 34 °C in 25 mL of LB medium containing 15 mM KNO_3 and 50 μg/mL carbenicillin. Cultures were harvested by centrifugation, resuspended with 5 mL of 50 mM Tris (pH 7.6), and passed through a small French pressure cell. Aliquots of 100 μL of each extract were analyzed for nitrate reductase (NR) activity in a total volume of 2 mL of an assay mixture containing 0.3 mM benzyl viologen, 10 mM KNO_3, and 20 mM Tris (pH 7.6). The reaction was initiated by the injection of sodium dithionite into a sealed cuvette containing the anaerobic assay mixture. The change in absorbance at 600 nm was measured for 1 min, and the units of activity in millimoles of nitrate reduced per minute for each sample were determined using the equation $U = {}^1/_2[\Delta A_{600}/\text{min}][1/\epsilon_{600}(\text{benzyl viologen})][v]$, where the extinction coefficient for benzyl viologen equals 7.4 $\text{mmol}^{-1}\ \text{cm}^{-1}$ and v equals 2 mL. The protein concentration of each extract was determined using the BCA assay (Pierce).

RESULTS

To investigate the role of the C-terminal Gly-Gly motif in MoaD functionality, the following single-amino acid variants in both MoaD and MoaD-SH were generated and purified: G80A, G80S, G80E, G81A, G81Δ, and +82G. All variants exhibited expression and purification characteristics similar to those of the wild-type proteins, indicating that the amino acid changes did not cause significant structural changes that result in protein instability or altered solubility (data not shown).

Analysis of Effects of the MoaD Variants on the Efficiency of Adenylation by MoeB. The effects of the amino acid exchanges at the C-terminus of MoaD on the efficiency of in vitro acyl-adenylate formation were investigated using reaction mixtures containing either [α-^{32}P]ATP or [γ-^{32}P]-ATP with MoeB and one of the MoaD variants. After incubation, the mixtures were passed through a desalting column to remove unbound ATP, AMP, and free inorganic phosphate. As seen in the first column of Table 1, no radioactivity was found in the complexes of reaction mixtures containing [α-^{32}P]ATP and MoaD G81A, G81Δ, or +82G, indicating that no MoaD adenylation occurred in these reactions. On the other hand, substitution of Gly80 with alanine, serine, or glutamate resulted in a 20−50% increase in the level of acyl-adenylate formation compared to that of wild-type MoaD. No radioactivity was detected in any of the protein fractions from reaction mixtures containing [γ-^{32}P]ATP, demonstrating that ATP is only loosely bound to MoeB, even after the formation of the $(\text{MoaD}-\text{MoeB})_2$ complex (data not shown).

Table 2: K_d Values Obtained for Formation of a Complex of MoaD or MoaD-SH with MoaE or MoeB

	K_d (μM)		
	MoaE−MoaD-SH	MoaE−MoaD	MoeB−MoaD
wild type	0.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.4
+82G	0.8 ± 0.1	1.1 ± 0.2	0.3 ± 0.1
G81Δ	3 ± 1	7 ± 2	nd
G81A	1.1 ± 0.1	2.3 ± 0.5	2.1 ± 0.4
G80S	3.5 ± 0.7	6 ± 2	5 ± 2
G80A	2.4 ± 0.4	7 ± 1	3.1 ± 0.3
G80E	nd[a]	nd	13 ± 3

[a] nd − none detected.

ITC Studies of the Assembly of MoaD Variants with MoeB or MoaE. To date, the only dissociation constants determined for any of the MoaD−MoaE or MoaD−MoeB complexes were those obtained using the SUPREX method for the binding of MoaE to wild-type MoaD (17 ± 7 μM) and MoaD-SH (2.6 ± 0.9 μM) (*21*). To analyze the effect of altering the C-terminus of MoaD or MoaD-SH on complex formation with both MoeB and MoaE, ITC was employed in this study. In all cases, the binding isotherms showed a complex pattern that was characterized by a short initial increase in the magnitude of the heat released upon injection followed by the expected decrease characteristic of an exothermic binding process, irrespective of whether MoeB or MoaE was used for the assembly with MoaD. Additionally, binding stoichiometries in the range of 0.6−0.8 for MoeB and 0.8−1.4 for MoaE were obtained (data not shown). Table 2 lists the K_d values obtained for the binding of the carboxylated MoaD variants to both MoaE and MoeB in addition to the binding of the MoaD-SH variants to MoaE. Dissociation constants between 0.3 ± 0.1 and 13 ± 3 μM for MoeB and between 0.6 ± 0.1 and 7 ± 2 μM for MoaE with wild-type MoaD and all MoaD variants were obtained (Table 2). With the exception of the +82G variant, the

MoaD−MoaE dissociation constants were higher for all MoaD variants that were tested than for the wild-type protein. In addition, the K_d values for thiocarboxylated MoaD with MoaE were approximately half of those obtained for the same MoaD variant in its carboxylated form, indicating a preference for the binding of MoaE to MoaD-SH (Table 2). While conservative substitutions at either glycine had a minimal effect on the dissociation constants, substitution at the penultimate position with a bulky, charged residue (G80E) completely abolished formation of a complex of both MoaD and MoaD-SH with MoaE.

The dissociation constants for all MoaD variants with MoaE were higher compared to the values obtained with MoeB, showing a preference for the formation of the $(MoeB−MoaD)_2$ complex over the $(MoaE−MoaD)_2$ complex (Table 2). Again, with the exception of the +82G variant, the dissociation constants with MoeB were higher for all the MoaD variants that were tested than for the wild type. MoaD G80E was the only variant for which a significant increase in the K_d value with MoeB was obtained (9-fold). While conservative substitutions at either glycine had some effect on the dissociation constants, deletion of the last glycine (G80Δ) completely abolished formation of a complex with MoeB.

Due to additional isotherms produced by ATP binding and hydrolysis, it was not possible to use ITC to analyze the effect of addition of ATP on the binding of MoaD to MoeB. Therefore, formation of a qualitative complex between MoaD and MoeB in the presence and absence of ATP/Mg^{2+} was analyzed by size exclusion chromatography (Figure 1). With the exception of MoaD G80E and G81Δ, the MoaD variants associated with MoeB regardless of whether ATP was included in the incubations. However, formation of the complex was tighter in the presence of ATP, indicating stronger binding of MoaD and MoeB after acyl-adenylate formation. While substitution of the penultimate MoaD glycine with either alanine or serine did not alter the protein's ability to interact with MoeB, MoaD containing glutamate at this position formed only a stable complex in the presence of ATP (Figure 1C). Consistent with the ITC results obtained in the absence of ATP, the MoaD G81Δ variant also did not form a complex with MoeB in the presence of ATP (Figure 1B).

Analysis of MPT Formation. To determine whether the alterations at the C-terminus of MoaD affected MPT synthase activity, a defined in vitro system consisting of MoaE, purified precursor Z, and one of the MoaD-SH variants was used to measure the rate of conversion of precursor Z to MPT (*4*, *5*, *7*). At varying time points, the MPT produced was converted to its stable oxidation product, form A. The reaction mixtures were subjected to HPLC, and dephospho-form A was quantified by its fluorescence (Figure 2 and Table 1, column 4). Similar reaction mixtures containing wild-type MoaD-SH have been previously reported to reach completion in 2−3 min (*5*), and comparable results were observed in these studies. As seen in Figure 2, replacement of Gly80 with serine or alanine resulted in a moderately slower rate of MPT formation, since a reaction time of approximately 10 min was required for mixtures containing these MoaD-SH variants to produce amounts of MPT comparable to that produced in the wild-type reaction. MoaD-SH G81A and +82G exhibited slower rates of MPT formation, and even after incubation for 40 min, wild-type MPT levels were not obtained (Figure 2). As might be expected from its inability to form a MPT synthase complex with MoaE (Table 2), no MPT production by MoaD-SH G80E was observed (Figure 2). Interestingly, deletion of Gly81 also completely abolished MPT synthase activity, although formation of a MPT synthase complex with this variant was unaffected (Table 2 and Figure 2).

Functional Complementation of an E. coli moaD⁻ Strain. For in vivo characterization of overall Moco biosynthesis, the MoaD variants were expressed in an *E. coli* strain containing a mutation in the *moaD* gene leading to the G80E substitution. The resulting strains were tested for functional complementation of the Moco-containing protein nitrate reductase (NR). Column 5 of Table 1 lists the NR activity observed in crude cell extracts of the complemented strains. While exchanging the penultimate glycine with either alanine or serine (G80A or G80S, respectively) resulted in only a minor decrease in NR activity, substitution at the terminal glycine (G81A) resulted in a drastic decrease in NR activity and varying the length of the Gly-Gly tail (G81Δ and +82G) abolished activity completely. As expected, complementation with a plasmid carrying the same mutation as that present in the genome (G80E) also did not result in any complementation of NR activity (*20*). The NR activity results for the G80A, G81A, G81Δ, and +82G variants are comparable to those reported by Hänzelmann et al. (*22*) using different growth and assay conditions.

DISCUSSION

In the course of MPT synthesis, MoaD cycles between two heterotetrameric protein complexes. The $(MoaD-SH−MoaE)_2$ MPT synthase complex transfers sulfur from the MoaD C-terminal thiocarboxylate to precursor Z. In the $(MoaD−MoeB)_2$ complex, the C-terminus of MoaD is acyl-adenylated, an essential prerequisite for subsequent regeneration of its thiocarboxylate. The X-ray structures of both *E. coli* MoaD complexes revealed a similar architecture, namely, a central MoaE or MoeB dimer with a MoaD subunit at each end of the dimer. Additionally, in both complexes, the C-terminus of MoaD (residues 76−81) is extended into a pocket on the MoeB or MoaE surface (*3*, *13*, *15*). The interface between MoaD and either MoaE or MoeB in the complexes consists of similar hydrophobic interactions involving four aromatic MoaD amino acids in addition to extensive contacts between the last six C-terminal amino acids of MoaD and residues lining the pocket of the larger proteins (*3*, *15*).

It had been previously shown that wild-type MoaD forms a complex with MoeB both in the presence and in the absence of ATP (*13*). For the studies reported here, ITC analysis and gel filtration were employed to examine formation of a complex between MoeB and the MoaD variants and to obtain K_d values for the various complexes. As seen in Figure 1 and Table 2, with two exceptions, all of the MoaD variants readily formed stable complexes with MoeB regardless of whether ATP was present. These results indicate that neither conservative substitution at either glycine (G80A, G80S, or G81A) nor lengthening the motif by one residue (+82G) adversely affected the formation of the $(MoaD−MoeB)_2$ complex. However, with the exception of

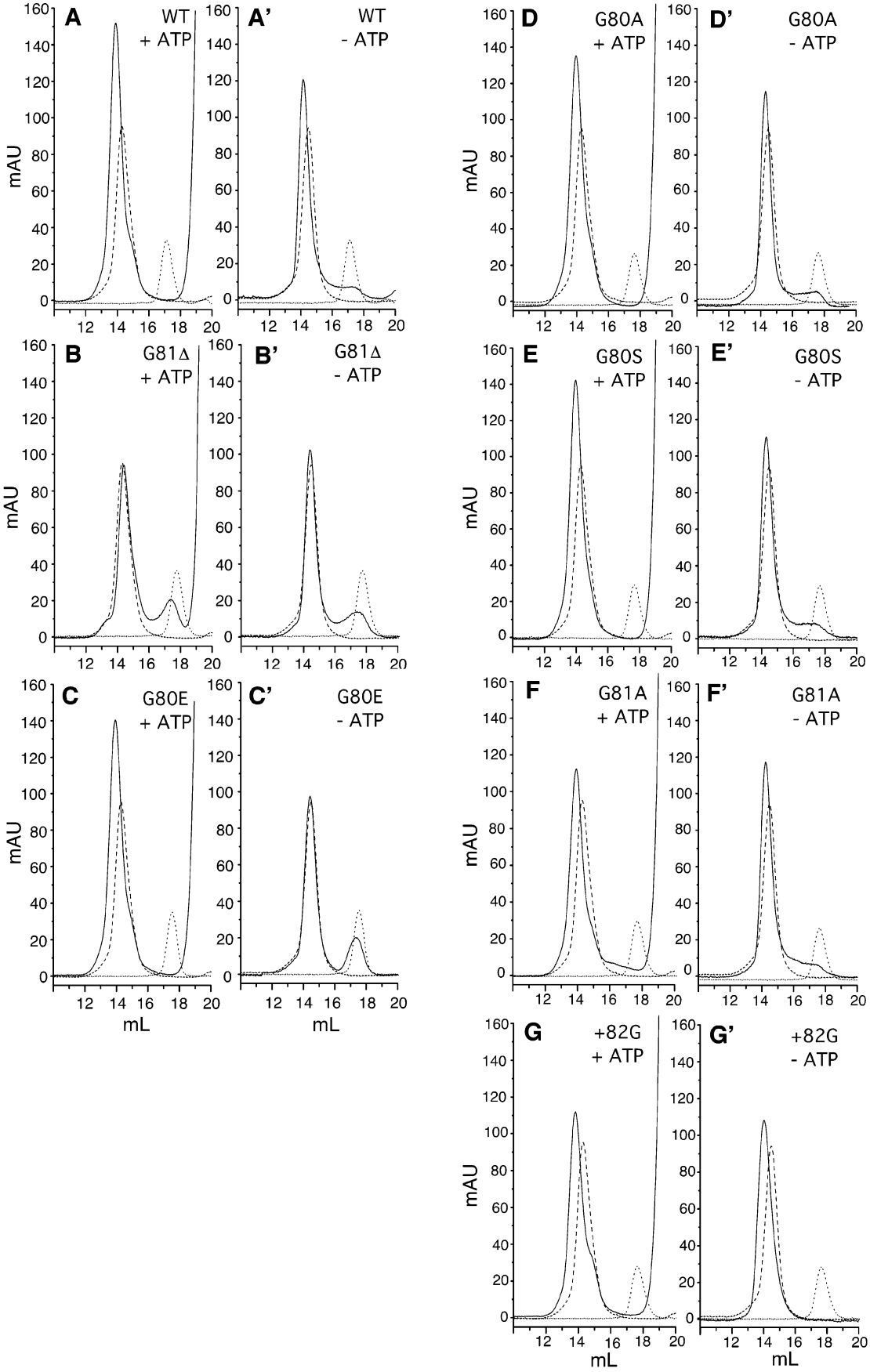

FIGURE 1: Analysis of the in vitro assembly of the *E. coli* (MoaD−MoeB)$_2$ heterotetramer by size exclusion chromatography. Reaction mixtures containing 13−14 μM MoaD and 30−33 μM MoeB were incubated in the presence or absence of 10 μM ATP/$MgCl_2$ and 5 units of pyrophosphatase. Each mixture was then applied to a size exclusion chromatography column. In each panel, the solid line is that of the mixture, the dotted line represents the elution profile for the MoaD variant alone, and the dashed line indicates the elution position of MoeB alone. The large peak eluting after 18 min in the first panel of each set is due to excess ATP.

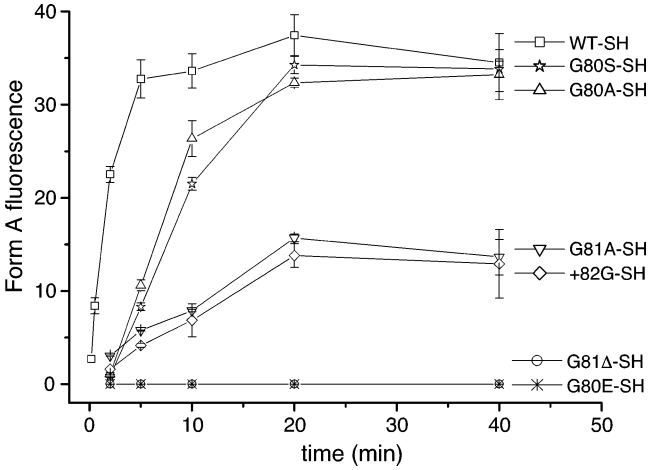

FIGURE 2: HPLC analysis of time-dependent MPT production using in vitro-assembled MPT synthase. For each MoaD variant, multiple reaction mixtures containing 2.5 μM MoaD-SH, 2.5 μM MoaE, and 1.4 μM precursor Z were started simultaneously. After incubation times of 0.17, 0.5, 2, 5, 10, 20, and 40 min, two reactions for each variant were terminated and MPT was converted to form A dephospho which was quantified by fluorescence detection on reverse phase HPLC.

the +82G variant, the dissociation constants with MoeB were higher for all MoaD variants that were tested than for the wild-type protein, indicating weakened binding. The results also verify that, unlike the Ub system, binding of ATP to MoeB is not required for the formation of a stable complex with MoaD (*23*), although the presence of ATP did appear to increase the stability of the complexes (Figure 1). No MoeB complex formation was observed with MoaD G81Δ, indicating that although a specific residue at the Gly81 position is not essential, the presence of some residue at this position is essential for the formation and maintenance of a stable heterotetrameric (MoaD−MoeB)$_2$ complex. MoaD G80E was the only variant for which the ability to form a complex with MoeB was strongly affected by ATP addition since for this variant, complex formation was only observed in the presence of ATP/Mg^{2+} (Figure 1). Comparison of the crystal structure of the MoaD−MoeB apo complex with that of the ATP-bound complex elucidated relatively large shifts in the location of a number of MoeB residues surrounding the MoaD Gly-Gly motif (*12*). Therefore, the most likely interpretation of the differential G80E complex formation is that binding of ATP to MoeB causes a structural change or "relaxation" in the protein that allows passage of the large, charged glutamate side chain through an area of the MoeB fold that would otherwise be inaccessible.

ITC determination of the dissociation constants between the MoaD or MoaD-SH variants and MoaE yielded results similar to those observed for formation of a complex with MoeB. Again, conservative substitutions at either glycine had no effect, while substitution of a glutamate at the penultimate position completely abolished complex formation (Table 2). However, in contrast to the results with MoeB, deletion of the terminal MoaD glycine (G81Δ) had no effect on the protein's ability to form the MPT synthase complex (Table 2), indicating that the interactions between the C-terminus of MoaD and MoaE are substantially stronger than in the MoaD−MoeB complex. Interestingly, the active site pockets in both complexes must be somewhat flexible, since the presence of an additional glycine on the MoaD C-terminus (+82G) strengthens formation of a complex with either protein.

As shown in Table 2, comparison of the ITC data of the various carboxylated and thiocarboxylated MPT synthase complexes reveals that in all cases, binding was tighter between MoaE and the thiocarboxylated variants, indicating preferred formation of the activated form of the MPT synthase complex (MoaD-SH−MoaE)$_2$. When the dissociation constants of the MoaD variants with MoaE are compared to those for binding to MoeB in Table 2, a preference for the formation of the (MoaD−MoeB)$_2$ complex over the (MoaD−MoaE)$_2$ complex was observed. This trend in binding strength within the complexes [(MoaD-SH−MoaE)$_2$ > (MoaD−MoeB)$_2$ > (MoaD−MoaE)$_2$] is mechanistically logical given that during the course of MPT biosynthesis, MoaD-SH first binds to MoaE to form the active MPT synthase complex where transfer of the MoaD-SH thiocarboxylate to precursor Z occurs, yielding MPT and inactive MPT synthase. MoaD must then dissociate from this inactive complex to form a new complex with MoeB, a prerequisite for the regeneration of MoaD-SH. In addition, the (MoaD−MoeB)$_2$ complex is stabilized by ATP addition and the subsequent formation of the acyl-adenylate on MoaD.

A report describing a C-terminal G76A variant of Ub indicated that this protein can be acyl-adenylated and quantitatively converted to a thioester (*24*). However, it will not sustain a second round of adenylate synthesis such as that which occurs with wild-type Ub (*24*). In contrast, our results showed that MoaD G81A was completely impaired in adenylate formation (Table 1). Since the same was also true for both MoaD G81Δ and +82G, it appears that both the specificity of the terminal residue and the length of the extended C-terminus are crucial for adenylation during MPT biosynthesis. In contrast, all three MoaD variants with substitutions at Gly80 actually exhibited higher levels of production of the MoaD−AMP species than the wild-type protein (Table 1). Since the penultimate glycine is conserved in all Ubl proteins, it is possible that this particular residue may be more important for the subsequent reaction of thioester or acyl-disulfide formation with an E1 protein. Unfortunately, this hypothesis is difficult to validate since analysis of variants at the penultimate glycine of Ub or other Ubl proteins has not been reported to date. Additionally, while ThiF has been directly implicated in the creation of the ThiS C-terminal thiocarboxylate (*16*, *25*), there is no evidence of a similar involvement of MoeB beyond MoaD acyl-adenylation. The adenylation reaction has been extensively studied in the Ub−E1 system, where it has been determined that the reaction proceeds via an absolutely ordered mechanism with ATP binding to E1 preceding Ub binding, followed by Ub-adenylate formation (*9*, *26*−*28*). Ub residue Arg72 and the terminal glycine (Gly76) are involved in interactions between Ub-AMP and its E1, and Arg72 plays a particularly critical role in determining Ub specificity with respect to its E1 (*24*, *29*, *30*). In contrast, the formation of the (MoaD−MoeB)$_2$ heterotetramer clearly occurs in the absence of ATP. The fact that MoaD G81A and +82G form stable MoeB complexes that do not support adenylation and the observation that none of the proteins show evidence of γ-labeled ATP binding suggest that ATP is only loosely bound to the complex even in the presence of MoaD, but in the presence of competent MoaD variants,

the stably bound acyl-adenylate is formed. The differential binding of MoaD G80E to MoeB based on the presence or absence of ATP can also be attributed to the much stronger association of the acyl-adenylate with the MoeB dimer. The crystal structure of the ternary complex of MoeB, MoaD, and ATP has been reported (*13*). The complex was generated by soaking the MoeB−MoaD crystal with ATP in the absence of Mg^{2+}, demonstrating that MoaD can bind to MoeB in the absence of ATP and that ATP can subsequently bind to the complex. Further, soaking the MoeB−MoaD crystal with ATP and Mg^{2+} resulted in the formation of the acyl-adenylate, indicating that the MoeB−MoaD complex is in fact catalytically competent.

When the MoaD-SH variants were analyzed for their ability to support transfer of sulfur to precursor Z in the MPT synthase reaction, the MoaD G81A and +82G variants exhibited greatly reduced activity and the MoaD G81Δ variant was completely inactive (Figure 2). These results indicate that while the specificity of the terminal residue is important, the presence of a terminal residue at this position is essential for MPT synthase activity. This is somewhat in a contrast to the results obtained for adenylation where all changes at the terminal glycine abolished activity (Table 1). Conservative substitution at the penultimate MoaD-SH position (G80S or G80A) had a moderate effect on the MPT synthase activity. As seen in Figure 2, although the rate of MPT production with these variants was slower, the final quantity of MPT produced was comparable to the amount of wild type. The total lack of MPT formation by MoaD-SH G80E can be attributed to its inability to form the MPT synthase complex with MoaE (Table 2). These results clearly show that the terminal glycine is more important for MoaD acyl-adenylate formation in complex with MoeB than for the subsequent transfer of sulfur to precursor Z in the MPT synthase complex.

There are a number of bacterial NR proteins, all of which require Moco for activity. To ascertain the net effect of the designed changes in the Gly-Gly motif on overall in vivo Moco biosynthesis, plasmids expressing the MoaD variants were transformed into an *E. coli* moaD$^-$ strain. The resulting strains were then tested for functional complementation of NR activity. This assay tracks the cumulative effects of the amino acid exchanges on both the MoaD adenylation−thiocarboxylation step and the sulfur transfer step in MPT biosynthesis. As seen in the last column of Table 1, with regard to the MoaD G80E variant, the inability to form the MPT synthase complex leads to a complete lack of MPT synthase activity that translates into a loss of NR activity despite a higher-than-normal adenylation value. Similarly, the higher adenylation rates for the MoaD G80A variant are compensated by its slower rate of transfer of sulfur to precursor Z such that the in vivo result is slightly reduced NR activity. As shown by the G80S variant (the residue present in *A. nidulans* MoaD), it appears that the identity of the penultimate residue of MoaD does not play an important role in overall MoaD function as long as the residue does not inhibit formation of either complex. MoaD G81A and +82G exhibit similar results in all the in vitro assays, and due to their inactivity in the adenylation assay, it would be expected that neither would exhibit NR activity. While this was true for the +82G variant, complementation with MoaD G81A resulted in a measurable amount of NR activity, implying that in vivo, MoaD adenylation of this variant by MoeB must have occurred to at least some extent. Removal of Gly81 from MoaD abolished all the MoaD functions with the exception of complex formation with MoaE; thus, it was not surprising that this variant exhibited no NR activity.

In summary, of the two glycine residues in the highly conserved MoaD C-terminal Gly-Gly motif, only the terminal glycine is essential for proper function of the *E. coli* MoaD protein. It was shown that this glycine is essential for MoaD-AMP formation by the E1-like protein MoeB, while for the subsequent transfer of sulfur to precursor Z, other residues can at least partially substitute for this glycine. It remains to be seen if this is true for all other members of the Ubl family of proteins.

ACKNOWLEDGMENT

We thank Susan Stager and Ashley Carpenter (Duke University, Durham, NC) for their assistance in protein purification. We thank Prof. Seckler (University of Potsdam) for help with the ITC experiments.

REFERENCES

1. Pitterle, D. M., and Rajagopalan, K. V. (1989) Two proteins encoded at the chlA locus constitute the converting factor of *Escherichia coli chlA1*, *J. Bacteriol. 171*, 3373−3378.
2. Pitterle, D. M., and Rajagopalan, K. V. (1993) The biosynthesis of molybdopterin in *Escherichia coli*. Purification and characterization of the converting factor, *J. Biol. Chem. 268*, 13499−13505.
3. Rudolph, M. J., Wuebbens, M. M., Rajagopalan, K. V., and Schindelin, H. (2001) Crystal structure of molybdopterin synthase and its evolutionary relationship to ubiquitin activation, *Nat. Struct. Biol. 8*, 42−46.
4. Gutzke, G., Fischer, B., Mendel, R. R., and Schwarz, G. (2001) Thiocarboxylation of molybdopterin synthase provides evidence for the mechanism of dithiolene formation in metal-binding pterins, *J. Biol. Chem. 276*, 36268−36274.
5. Wuebbens, M. M., and Rajagopalan, K. V. (2003) Mechanistic and mutational studies of *Escherichia coli* molybdopterin synthase clarify the final step of molybdopterin biosynthesis, *J. Biol. Chem. 278*, 14523−14532.
6. Unkles, S. E., Heck, I. S., Appleyard, V. C. L., and Kinghorn, J. R. (1999) Eukaryotic molybdopterin synthase. Biochemical and molecular studies of *Aspergillus nidulans cnxG* and *cnxH* mutants, *J. Biol. Chem. 274*, 19286−19293.
7. Pitterle, D. M., Johnson, J. L., and Rajagopalan, K. V. (1993) *In vitro* synthesis of molybdopterin from precursor Z using purified converting factor. Role of protein-bound sulfur in formation of the dithiolene, *J. Biol. Chem. 268*, 13506−13509.
8. Schwartz, D. C., and Hochstrasser, M. (2003) A superfamily of protein tags: Ubiquitin, SUMO and related modifiers, *Trends Biochem. Sci. 28*, 321−328.
9. Haas, A. L., and Rose, I. A. (1982) The mechanism of ubiquitin activating enzyme. A kinetic and equilibrium analysis, *J. Biol. Chem. 257*, 2543−2548.
10. Hochstrasser, M. (2000) Evolution and function of ubiquitin-like protein-conjugation systems, *Nat. Cell Biol. 2*, E153−E157.
11. Pickart, C. M. (2001) Mechanisms underlying ubiquitination, *Annu. Rev. Biochem. 70*, 503−533.
12. Leimkühler, S., Wuebbens, M. M., and Rajagopalan, K. V. (2001) Characterization of *Escherichia coli* MoeB and its involvement in the activation of MPT synthase for the biosynthesis of the molybdenum cofactor, *J. Biol. Chem. 276*, 34695−34701.
13. Lake, M. W., Wuebbens, M. M., Rajagopalan, K. V., and Schindelin, H. (2001) Mechanism of ubiquitin activation revealed by the structure of a bacterial MoeB-MoaD complex, *Nature 414*, 325−329.
14. Leimkühler, S., and Rajagopalan, K. V. (2001) An *Escherichia coli* NifS-like sulfurtransferase is required for the transfer of cysteine sulfur in the *in vitro* synthesis of molybdopterin from precursor Z, *J. Biol. Chem. 276*, 22024−22031.
15. Rudolph, M. J., Wuebbens, M. M., Turque, O., Rajagopalan, K. V., and Schindelin, H. (2003) Structural studies of molybdopterin

synthase provide insights into its catalytic mechanism, *J. Biol. Chem. 278*, 14514−14522.
16. Lehmann, C., Begley, T. P., and Ealick, S. E. (2006) Structure of the *Escherichia coli* ThiS-ThiF complex, a key component of the sulfur transfer system in thiamin biosynthesis, *Biochemistry 45*, 11−19.
17. Kinsland, C., Taylor, S. V., Kelleher, N. L., McLafferty, F. W., and Begley, T. P. (1998) Overexpression of recombinant proteins with a C-terminal thiocarboxylate: Implications for protein semisynthesis and thiamin biosynthesis, *Protein Sci. 7*, 1839−1842.
18. Wuebbens, M. M., and Rajagopalan, K. V. (1993) Structural characterization of a molybdopterin precursor, *J. Biol. Chem. 268*, 13493−13498.
19. Johnson, J. L., Hainline, B. E., Rajagopalan, K. V., and Arison, B. H. (1984) The pterin component of the molybdenum cofactor. Structural characterization of two fluorescent derivatives, *J. Biol. Chem. 259*, 5414−5422.
20. Johnson, M. E., and Rajagopalan, K. V. (1987) Involvement of chlA, E, M, and N loci in *Escherichia coli* molybdopterin biosynthesis, *J. Bacteriol. 169*, 117−125.
21. Tong, Y., Wuebbens, M. M., Rajagopalan, K. V., and Fitzgerald, M. C. (2005) Thermodynamic analysis of subunit interactions in *Escherichia coli* molybdopterin synthase, *Biochemistry 44*, 2595−2601.
22. Hänzelmann, P., Schwarz, G., and Mendel, R. R. (2002) Functionality of alternative splice forms of the first enzymes involved in human molybdenum cofactor biosynthesis, *J. Biol. Chem. 277*, 18303−18312.
23. Huang, D. T., Walden, H., Duda, D., and Schulman, B. A. (2004) Ubiquitin-like protein activation, *Oncogene 23*, 1958−1971.
24. Pickart, C. M., Kasperek, E. M., Beal, R., and Kim, A. (1994) Substrate properties of site-specific mutant ubiquitin protein (G76A) reveal unexpected mechanistic features of ubiquitin-activating enzyme (E1), *J. Biol. Chem. 269*, 7115−7123.
25. Xi, J., Ge, Y., Kinsland, C., McLafferty, F., and Begley, T. P. (2001) Biosynthesis of the thiazole moiety of thiamin in *Escherichia coli*: Identification of an acyldisulfide-linked protein-protein conjugate that is functionally analogous to the ubiquitin/E1 complex, *Proc. Natl. Acad. Sci. U.S.A. 98*, 8513−8518.
26. Haas, A. L., Warms, J. V., Hershko, A., and Rose, I. A. (1982) Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation, *J. Biol. Chem. 257*, 2543−2548.
27. Haas, A. L., Warms, J. V., and Rose, I. A. (1983) Ubiquitin adenylate: Structure and role in ubiquitin activation, *Biochemistry 22*, 4388−4394.
28. Hershko, A., Heller, H., Elias, S., and Ciechanover, A. (1983) Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown, *J. Biol. Chem. 258*, 8206−8214.
29. Burch, T. J., and Haas, A. L. (1994) Site-directed mutagenesis of ubiquitin. Differential roles for arginine in the interaction with ubiquitin-activating enzyme, *Biochemistry 33*, 7300−7308.
30. Larsen, C. N., and Wang, H. (2002) The ubiquitin superfamily: Members, features, and phylogenies, *J. Proteome Res. 1*, 411−419.

BI062011W