Thermodynamic Analysis of Subunit Interactions in *Escherichia coli* Molybdopterin Synthase†

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ABSTRACT: The molybdopterin (MPT) synthase complex in *Escherichia coli* consists of two MoaE subunits and two MoaD subunits in a heterotetrameric structure with the two MoaE subunits forming a central dimer. Each MoaD subunit binds to a single MoaE molecule to form two identical MoaE/MoaD interfaces. Here we define the thermodynamic properties of the interaction between MoaE and MoaD in MPT synthase using a H/D exchange and matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy based method termed SUPREX (*s*tability of *u*npurified *p*roteins from *r*ates of H/D *ex*change). SUPREX-derived protein folding free energies and *m* values are reported for MoaE in the presence and absence of MoaD and MoaD-SH, the thiocarboxylated form of MoaD that is essential for the catalytic activity of MPT synthase. The protein folding free energy measurements were used to calculate a dissociation constant of 17 ± 7 μM for the binding of MoaD to MoaE in inactive MPT synthase and a dissociation constant of 2.6 ± 0.9 μM for the binding of MoaD-SH to MoaE in active MPT synthase. The increased binding affinity of MoaD-SH for MoaE is consistent with a previously proposed mechanism for the MPT synthase reaction. Using the increased *m* values exhibited by MoaE in the presence of either MoaD subunit, the solvent accessible surface area buried upon formation of the subunit interface in MPT synthase was estimated to be 2378 Å^2 for inactive MPT synthase and 4117 Å^2 for active MPT synthase.

Virtually all organisms have molybdenum- or tungsten-containing enzymes in which the metal is chelated by the dithiolene moiety of a member of the molybdenum cofactor family. Although the structure of these cofactors varies, the basic component in all cases is a phosphorylated, tricyclic pterin molecule termed molybdopterin (MPT).[1] MPT contains the metal-chelating dithiolene group, and during molybdenum cofactor biosynthesis, addition of the two dithiolene sulfur atoms to an MPT precursor termed precursor Z is effected by the MPT synthase protein. Although human genetic defects in molybdenum cofactor biosynthesis are rare, such defects severely impair neurological development and are usually fatal (*1*).

The structure, function, and mechanism of *Escherichia coli* MPT synthase have been the subjects of recent studies, and much of the information gained from these studies is summarized in Figure 1 (*2*−*5*). X-ray crystallographic data on the *E. coli* enzyme revealed that it is a heterotetrameric complex containing a central dimer of two MoaE subunits of 16 845 Da. Each MoaE is bound to a MoaD subunit of 8744 Da to form the elongated MPT synthase complex (*3*, *4*). In active MPT synthase, the carboxy-terminus of each MoaD subunit carries a thiocarboxylate moiety (MoaD-SH) that serves as the source for the sulfur atoms of the dithiolene group of MPT. Following sulfur transfer to precursor Z during MPT formation, the MoaD thiocarboxylate must be regenerated before the subunit can participate in another round of MPT synthesis. While the thiocarboxylate moiety on the MoaD subunit is essential for MPT synthase activity, it is not required for formation of the synthase heterotetramer, since both MoaD and MoaD-SH bind the MoaE homodimer. An interesting feature of the binding of MoaD and MoaD-SH to MoaE is that in both cases, the C-termini of the MoaD subunits are buried in the MoaE dimer to create an active site near each of the two MoaE/MoaD interfaces in the complex (*3*, *4*).

As seen in Figure 1, a similar type of architecture exists in the MoaD/MoeB complex. In *E. coli*, the MoeB protein is necessary for regeneration of the MoaD thiocarboxylate (*6*), and crystallographic studies of this complex also revealed a heterotetrameric architecture with the two MoeB molecules forming a dimer (*7*). Again, the C-termini of the two MoaD molecules are extended into pockets on the MoeB surface to form the active sites. With the exception of this tail, the structures of MoaD complexed with either MoaE or MoeB are quite similar (*3*, *7*). In MPT biosynthesis in *E. coli*, the function of MoeB is to activate MoaD by adenylation of the carboxy terminus. Although the exact mechanism for conversion of this acyl adenylate to a thiocarboxylate is unclear,

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[1] Abbreviations: $C^{1/2}_{SUPREX}$, denaturant concentration at the transition midpoint of a SUPREX curve; SUPREX, stability of unpurified proteins from rate of H/D exchange; GdmCl, guanidinium chloride; MALDI, matrix-assisted laser desorption ionization; MPT, molybdopterin; MoaD-SH, MoaD containing a C-terminal thiocarboxylate moiety; SA, sinapinic acid; ΔASA, change in accessible surface area.

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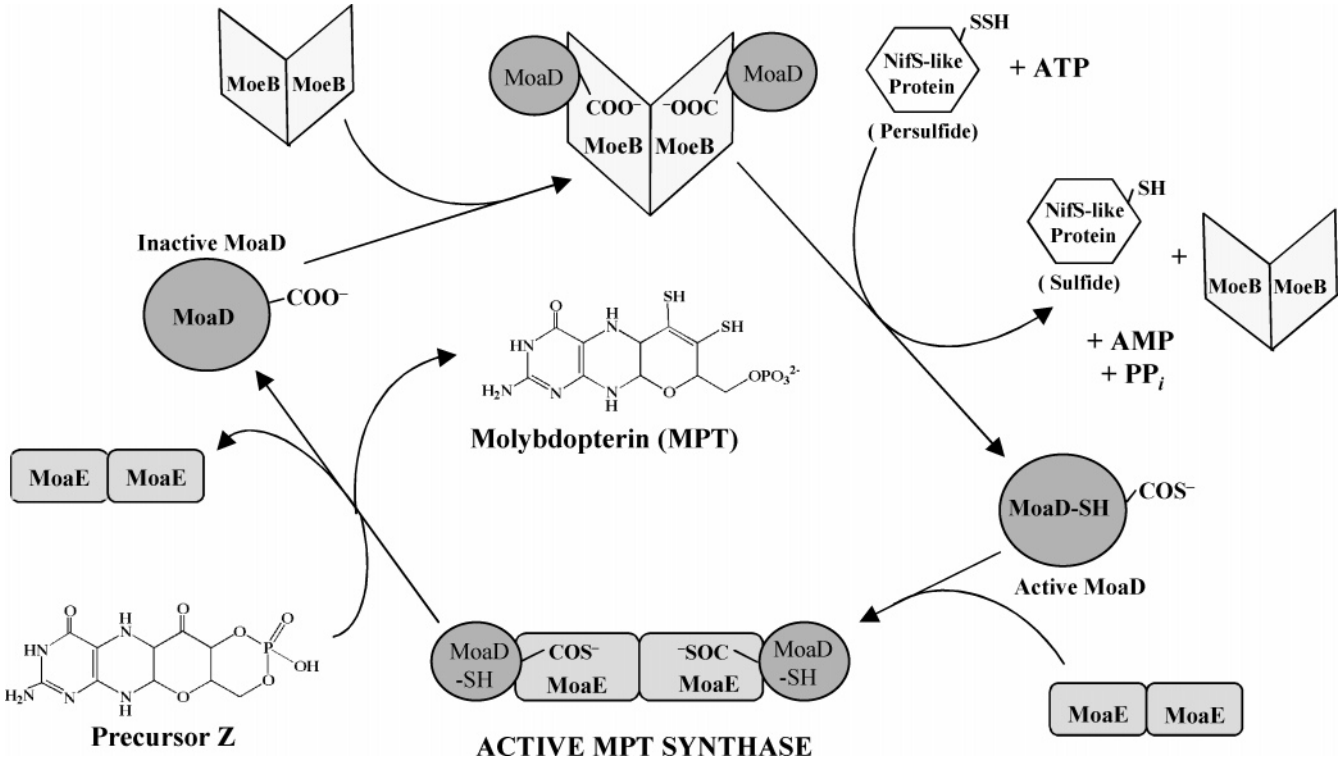

FIGURE 1: Proposed reaction mechanism of *E. coli* MPT synthase.

it is known that L-cysteine is the ultimate sulfur source, that any of several NifS-like sulfur transferases can catalyze this conversion, and that the sulfur is transferred in the form of a persulfide (*6*, *8*).

At present, very little is known about the thermodynamic properties of MPT synthase. In particular, the relative binding affinities of the different subunits in the MPT synthase heterotetramer have not been defined, and it is not clear how the thiocarboxylate moiety on MoaD-SH impacts the association/dissociation properties of the MPT synthase complex. Here we report data for selected thermodynamic properties of the different subunit interactions in MPT synthase obtained using the SUPREX technique. SUPREX (*s*tability of *u*npurified *p*roteins from *r*ate of H/D *ex*change) is a relatively new H/D exchange and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry based method for obtaining thermodynamic measurements of a protein's conformational stability. We and others have successfully utilized SUPREX for the quantitative evaluation of protein folding free energies (ΔG_f values), protein folding *m* values, and the binding affinities of a variety of different protein−ligand complexes (*9*−*18*). As opposed to the traditional spectroscopic methods often used for such analyses, the SUPREX technique does not require specific spectroscopic labels. Additionally, unlike calorimetric methods, only small quantities (picomoles) of protein are required for SUPREX analysis.

Previous SUPREX studies have largely served to validate the accuracy and precision of the technique through the analysis of a variety of well-characterized model protein and protein−ligand systems (*9*−*16*). The work described here represents one of the first applications of SUPREX in which the technique is used to define the thermodynamic properties of a system largely uncharacterized by conventional methods. The MPT synthase system also represents one of the first protein complexes studied by SUPREX in which there is a relatively large (on the order of 3000 Å^2) interface involved in the binding interactions being examined. In this work, SUPREX-derived binding affinities (K_d values) are reported for the interactions of MoaE with MoaD and MoaD-SH in the MPT synthase complex. The technique was also used to quantitate the surface area that is buried in the binding interface between MoaE and MoaD or MoaD-SH upon formation of the inactive and active forms of MPT synthase.

MATERIALS AND METHODS

Materials. Protonated guanidinium chloride (GdmCl) was purchased from EM Science. Deuterated GdmCl was obtained by repeated (three times) dissolution and lyophilization of the protonated form in D_2O. Trifluoroacetic acid was obtained from Halocarbon, and acetonitrile was from Fisher. D_2O and sinapinic acid (SA) were purchased from Aldrich. Insulin and ubiquitin were obtained from Sigma.

Instrumentation. MALDI mass spectra were acquired on a Voyager DE Biospectrometry Workstation from Perseptive Biosystems. Positive ion mass spectra were collected in the linear mode using a nitrogen laser (337 nm, 3 Hz) with SA as the matrix, and each spectrum represented the sum of the data from 32 laser shots. Spectra were internally calibrated using two proteins of known mass, insulin (5734.6 Da) and cytochrome *c* (12 361.1 Da). GdmCl concentrations were determined with a Bausch & Lomb refractometer as previously described (*19*). Measurements of pH were made using a Jenco 6072 pH meter equipped with a Futura calomel pH electrode from Beckman Instruments. To correct for isotope effects, the pH of each D_2O solution was converted to a pD by adding 0.4 to the measured value (*20*).

Preparation of Protein Samples. MoaE, MoaD, and MoaD-SH were prepared and purified according to previ-

ously described protocols (*5*) that typically yielded 0.7−3.0 mM solutions of each purified protein in buffer containing 50 mM Tris, 50 mM NaCl, pH 8.0. The exact protein concentrations in the stock solutions were determined according to their calculated extinction coefficients at 280 nm using 39 042 M^{-1} cm^{-1} for MoaE and 6782 M^{-1} cm^{-1} for MoaD and MoaD-SH (*5*).

Stock solutions of the MPT synthase complex were prepared by mixing appropriate amounts of the above stock solutions and equilibrating the sample for at least 20 min at room temperature prior to SUPREX analysis. The MoaE concentration in the synthase complex stock solutions was approximately 0.1 mM and the MoaD or MoaD-SH concentrations were approximately 1.0 mM. The large molar excess of MoaD or MoaD-SH was a necessary part of the SUPREX strategy to evaluate the binding affinities of these proteins to MoaE (see below).

SUPREX Data Acquisition and Analysis. The SUPREX protocol and data analysis procedures employed in this work were very similar to those previously described in earlier SUPREX studies (*11*, *14*, *15*). Briefly, aliquots from either the MoaE or MPT synthase stock solutions described above were diluted 10-fold into a series of D_2O-containing H/D exchange buffers comprised of 20 mM sodium acetate, pD 5.8, 20 mM KCl, and increasing amounts of GdmCl. The H/D exchange reactions (in which labile protons in each protein are exchanged for solvent deuterons in each D_2O- and GdmCl-containing buffer) were allowed to proceed for a predetermined amount of time. While this time varied from 5 to 60 min depending on the experiment, the same H/D exchange time was used to collect all the data points in a given SUPREX curve. The H/D exchange reaction in each D_2O- and GdmCl-containing buffer was quenched by 10-fold dilution into a MALDI matrix solution containing saturated SA in water/acetonitrile (50/50 v/v) and 0.1% (v/v) trifluoroacetic acid. In the case of the SUPREX analysis of the 1 μM MoaE solution, each H/D exchange reaction was submitted to a concentration and desalting step (as previously described in ref *15*) using C4 ZipTips (Millipore) prior to MALDI mass spectral analysis.

It is important to note that for all SUPREX analyses of either MoaE alone or one of the MPT synthase complexes, only the MoaE subunit mass was monitored to determine the number of deuterons that had exchanged into the protein. This number of deuterons was defined as the ΔMass value, and it was determined by subtracting the molecular mass of fully protonated MoaE (16 845 Da) from the molecular mass of the protein ascertained in each MALDI analysis. Five replicate MALDI mass spectra were acquired from each MALDI sample, and the average of the five MoaE ΔMass values for each GdmCl-containing H/D exchange buffer was determined. These averages were used to generate MoaE SUPREX curves (plots of ΔMass versus [GdmCl]) at each predetermined H/D exchange time. Unless noted, exchange reactions were performed in triplicate, yielding three MoaE SUPREX curves for each exchange time.

The data points in each MoaE SUPREX curve were fit to a sigmoidal curve using SigmaPlot (SYSTAT Software, Inc.) in order to extract a $C^{1/2}_{SUPREX}$ value (the denaturant concentration at the transition midpoint). The three $C^{1/2}_{SUPREX}$ values determined at each exchange time were averaged, and this average was plotted as a function of the H/D exchange time according to eq 1 as described by Powell and Fitzgerald (*14*).

$$-\Delta G_f^{\,0} = mC^{1/2}_{SUPREX} + RT \ln \frac{\left(\frac{\langle k_{int}\rangle t}{0.693} - 1\right)}{\left(\frac{n^n}{2^{n-1}}[P]^{n-1}\right)} \quad (1)$$

In this equation, R is the gas constant, T is the temperature in Kelvin, $\langle k_{int}\rangle$ is the estimated average intrinsic exchange rate of an unprotected amide proton, t is the H/D exchange time used in SUPREX, n is the multimeric state of the protein, [P] is the concentration of protein in n-mer equivalents, m is defined as $\delta\Delta G/\delta$[denaturant], and ΔG_f^0 is the standard-state folding free energy of the protein in the absence of denaturant. A linear least-squares analysis was used to determine the slope and y-intercept of the plots of $RT \ln((((\langle k_{int}\rangle t/0.693) - 1)/n^n[P]^{n-1}/2^{n-1})$ vs $C^{1/2}_{SUPREX}$ for each protein system. These values correspond to the protein folding m value and ΔG_f^0 value, respectively. Since all experiments were performed at room temperature, T was set at 293 K for all calculations involving eq 1. The $\langle k_{int}\rangle$ value used in our calculations was 0.175 s^{-1}. This value was determined using the SPHERE program (*21*, *22*) and the entire primary amino acid sequence of MoaE.

K_d Value Determinations. Dissociation constants (K_d values) for MoaD and MoaD-SH binding to MoaE were determined from SUPREX-derived binding free energy ($\Delta G_{Binding}$) values using eq 2.

$$\Delta G_{Binding} = -NRT \ln[1 + L/K_d] \quad (2)$$

In this equation, N is the number of binding sites, L is the concentration of free ligand, $\Delta G_{Binding}$ is defined as the difference in MoaE folding free energies measured in the absence and presence of ligand (MoaD or MoaD-SH), and R and T are as defined above. It has been previously established that the MoaE homodimer contains two equivalent and independent MoaD/MoaD-SH binding sites (*3*, *5*). Thus, in our K_d calculations using eq 2, N was set at 2. Since the concentration of ligand (MoaD or MoaD-SH) used in our studies was always 10-fold greater than the concentration of MoaE, the total concentration of ligand in each sample was used for L.

ΔASA Calculations. The SUPREX-derived changes in accessible surface area (ΔASA) for the binding of MoaD or MoaD-SH to MoaE were calculated using the following relationship between protein folding m value and ΔASA that was empirically derived by Myers et al. (*23*).

$$m = 953 + 0.23\Delta ASA \quad (3)$$

In eq 3, the m value unit is cal mol^{-1} M^{-1} and the ΔASA unit is $Å^2$. The m values used in calculations of ΔASA corresponded to the increased m value that was measured upon ligand binding. These are reported as Δm values in Table 2.

RESULTS AND DISCUSSION

SUPREX Analysis of MoaE Alone. Figure 2 shows typical SUPREX curves for the self-association of MoaE using an H/D exchange time of 20 min and protein concentrations of

Table 1: Summary of Transition Midpoints ($C^{1/2}_{SUPREX}$ Values) Obtained by SUPREX

protein system	H/D exchange time, *t* (min)	$C^{1/2}_{SUPREX}$ (M)[a]
MoaE (1 μM)	10	0.39[b]
MoaE (10 μM)	5	0.98[b]
MoaE (37 μM)	5	1.26 ± 0.01
	10	1.08 ± 0.07
	20	0.94 ± 0.13
	30	0.86 ± 0.03
MoaE (213 μM)	20	1.33[b]
MoaE (15 μM) + MoaD (152 μM)	5	1.33 ± 0.01
	10	1.21 ± 0.06
	20	1.12 ± 0.08
	30	1.08 ± 0.03
MoaE (10 μM) + MoaD-SH (95 μM)	5	1.52 ± 0.02
	10	1.39 ± 0.02
	20	1.31 ± 0.01
	60	1.20 ± 0.05

[a] The average value and standard deviation obtained from three replicate SUPREX curves are reported, except where noted. [b] The value obtained from a single SUPREX curve is reported.

37 or 213 μM. A number of additional SUPREX curves were acquired for MoaE using different H/D exchange times and protein concentrations, and the $C^{1/2}_{SUPREX}$ values extracted from these curves are listed in Table 1. The SUPREX curves in Figure 2 and the data in Table 1 reveal that the $C^{1/2}_{SUPREX}$ values for MoaE are dependent on both the H/D-exchange time and the MoaE concentration. Both of these dependencies can be explained by eq 1, which predicts that a plot of $RT\ \ln((((\langle k_{int}\rangle t/0.693) - 1)/n^n[P]^{n-1}/2^{n-1})$ vs $C^{1/2}_{SUPREX}$ will be linear and that the *y*-intercept and slope of the resulting linear plot will correspond to the ΔG_f^0 and *m* value, respectively, of the protein's folding reaction (*13*, *14*, *16*). A linear least-squares analysis of the plot of $RT\ \ln((((\langle k_{int}\rangle t/0.693) - 1)/n^n[P]^{n-1}/2^{n-1})$ vs $C^{1/2}_{SUPREX}$ for MoaE using *n* values of 1, 2, 3, and 4 was performed, and as shown in Figure 3, the best fit of the data was obtained when *n* was set at 2. Moreover, the relatively high correlation coefficient obtained in this analysis when $n = 2$ (0.9987) indicates that the data are well-described by eq 1. The *y*-intercept and slope values generated by this analysis of MoaE self-association were -12.1 ± 0.1 kcal mol^{-1} and 2.8 ± 0.1 kcal mol^{-1} M^{-1}, respectively.

The accurate evaluation of a protein's ΔG_f^0 and *m* value by SUPREX using eq 1 requires that the denaturant-induced equilibrium unfolding properties of the protein under study be reversible and well-modeled by a two-state (folded and unfolded) process. It is also necessary that the protein under study exhibit so-called EX2 exchange behavior (i.e., the folding rate is much faster than the intrinsic exchange rate of an unprotected amide proton, k_{int}) (*22*−*24*). The H/D exchanged MoaE molecules in these experiments exhibited one ion signal in the MALDI mass spectral analyses. (Data not shown.) This is consistent with the protein being under EX2 rather than EX1 exchange conditions, as EX2 conditions are expected to generate H/D exchanged molecules of only one distinct mass over the time course of an exchange reaction. This is in contrast to EX1 conditions, which generate two distinct populations of H/D exchanged molecules (*22*−*24*).

The denaturant-induced equilibrium unfolding properties of MoaE have not been previously studied. Therefore, it is not known if MoaE is a two-state folder. The good fit of our SUPREX data to eq 1 when using an *n* value of 2 provides some evidence that the GdmCl-induced equilibrium unfolding/refolding of MoaE may be well-modeled by a two-state process involving a folded homodimer and 2 unfolded monomers. This apparent homodimeric structure of MoaE's folded state is consistent with X-ray crystallographic data on the protein (*3*). However, the *m* value determined by SUPREX analysis for MoaE of 2.8 ± 0.1 kcal mol^{-1} M^{-1} is smaller than that predicted by Myers and co-workers for the cooperative, two-state folding reaction of a 300 amino acid protein complex like the MoaE homodimer (*23*). By their analysis, the *m* value for a complex of this size should be on the order of 7.8 kcal mol^{-1} M^{-1}. The relatively low *m* value determined here for MoaE may indicate the presence of partially folded intermediate states in the unfolding reaction for MoaE, suggesting the possibility of non-two-state folding. While further biophysical studies are needed to more definitively define the number of molecular states that are significantly populated in MoaE's equilibrium folding/unfolding reaction, such studies are not required for the thermodynamic analyses described here. This is because the absolute accuracy of the SUPREX-derived ΔG_f^0 or *m* values is not expected to impact the analysis of the relative thermodynamic properties of MoaE in the presence and absence of MoaD and MoaD-SH.

The goal of this work was not to characterize the absolute thermodynamic properties of the MoaE homodimer. Rather, it was to analyze the relative binding of MoaD and MoaD-SH to MoaE. Such an analysis only requires the calculation of $\Delta\Delta G_f^0$ (or $\Delta G_{Binding}$) values and Δm values. It has previously been shown that SUPREX-derived ΔG_f^0 and *m* values obtained on both two-state and non-two-state folding protein systems can lead to the calculation of reasonably precise and accurate $\Delta G_{Binding}$ values (*13*, *14*, *16*−*18*). Two of the most important assumptions in the calculation of such $\Delta G_{Binding}$ values using the SUPREX technique are that the movement of a protein's $C^{1/2}_{SUPREX}$ value with H/D exchange time in the SUPREX experiment be well-described by eq 1 and that the protein and ligand of interest solely interact with each other in their native, folded states (i.e. no binding occurs in the denatured state or in partially folded intermediate states) (*18*). The experimental results in Figure 3 suggest that the first assumption is valid in the case of MoaE. The latter assumption is generally valid for most protein−ligand systems.

SUPREX Analysis of MoaE in MPT Synthase. Figure 4 shows typical SUPREX curves obtained for MoaE in complex with MoaD or MoaD-SH as well as a curve obtained for MoaE alone under nearly identical conditions. It is noteworthy that the pretransition regions of the SUPREX curves obtained in the presence of MoaD and MoaD-SH are approximately 10 Da lower than the pretransition baseline of the MoaE SUPREX curve. This is likely due to the presence of more globally protected MoaE amide protons in the MPT synthase complex than in the MoaE homodimer. It is also noteworthy that in the presence of MoaD and MoaD-SH, the MoaE SUPREX curve transitions are shifted to higher denaturant concentrations (see Figure 4). Such a shift is consistent with an increase in conformational stability of MoaE upon binding of these proteins. Furthermore, the transition of the MoaE SUPREX curve is shifted to a higher

Table 2: Summary of SUPREX-Derived Thermodynamic Parameters

protein system	ΔG_f^0 (kcal mol^{-1})[a]	$\Delta G_{Binding}$ (kcal mol^{-1})[b]	m (kcal mol^{-1} M^{-1})[a]	Δm (kcal mol^{-1} M^{-1})[b]
MoaE	-12.1 ± 0.1	0	2.8 ± 0.1	0
MoaE+MoaD	-14.8 ± 0.4	-2.7 ± 0.4	4.3 ± 0.3	1.5 ± 0.3
MoaE+MoaD-SH	-16.4 ± 0.8	-4.3 ± 0.8	4.7 ± 0.6	1.9 ± 0.6

[a] Values were obtained from the linear least-squares analysis of the data in Figures 3 and 5. The reported values are "apparent" as the two-state folding assumption made for MoaE in these analyses may not be valid (see the text). Errors are the standard errors of fitting generated by SigmaPlot. [b] Values were obtained from the preceding column and are relative to MoaE.

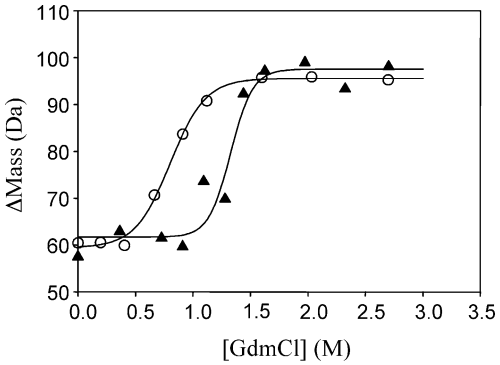

FIGURE 2: Typical SUPREX curves obtained for MoaE self-association. The MoaE protein concentrations used were either 37 μM (○) or 213 μM (▲). The data in both curves were generated using an exchange time of 20 min. The lines represent the best fit of each data set to a four-parameter sigmoidal equation using SigmaPlot.

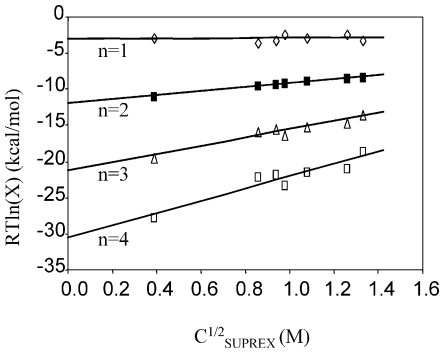

FIGURE 3: Plots of $-RT$ ln(X) vs $C^{1/2}_{SUPREX}$ for MoaE alone. X represents $[((\langle k_{int}\rangle t/0.693) - 1)/(n^n[P]^{n-1}/2^{n-1})]$, and the solid lines are the results of linear least-squares analyses of the data when the n value was defined as 1, 2, 3, or 4. The correlation coefficient obtained for each fit was 0.00854, 0.9987, 0.9653, and 0.9455, respectively.

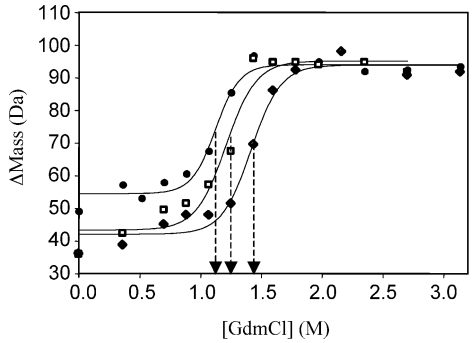

FIGURE 4: Typical SUPREX curves obtained from 37 μM MoaE (●), 15 μM MoaE in the presence of 152 μM MoaD (□), and 10 μM MoaE in the presence of 95 μM MoaD-SH (◆). All data were generated using an H/D exchange time of 10 min, and the lines represent the best fit of each data set to a four-parameter sigmoidal equation using SigmaPlot. Arrows mark the $C^{1/2}_{SUPREX}$ value of each curve. The standard deviations of the ΔMass measurements used to generate each curve were typically between 2 and 4 Da.

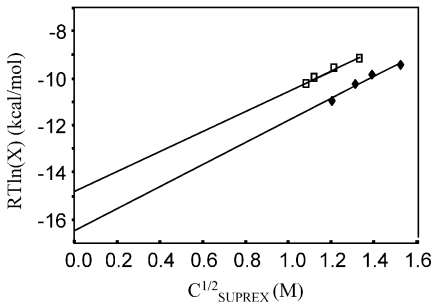

FIGURE 5: Plots of $-RT$ ln(X) vs $C^{1/2}_{SUPREX}$ for MoaE in the presence of MoaD (□) or MoaD-SH (◆). As in Figure 3, X represents $[((\langle k_{int}\rangle t/0.693) - 1)/(n^n[P]^{n-1}/2^{n-1})]$. The solid lines are the result of linear least-squares analysis of the data using an n value of 2. The correlation coefficients obtained for the MoaD and MoaD-SH data sets were 0.9935 and 0.9847, respectively.

denaturant concentration in the presence of MoaD-SH than it is in the presence of MoaD. This is observed despite the slightly lower protein concentrations used to generate the MoaE/MoaD-SH SUPREX curve. This result indicates that the binding affinity of MoaD-SH to MoaE is greater than that of MoaD to MoaE.

To quantify these binding affinities, a series of additional SUPREX curves were obtained for MoaE in the presence of MoaD or MoaD-SH using a range of H/D exchange times. The $C^{1/2}_{SUPREX}$ values extracted from these additional SUPREX curves are summarized in Table 1. In Figure 5 these $C^{1/2}_{SUPREX}$ values are plotted as a function of H/D exchange time according to eq 1. This equation adequately describes the H/D exchange-time dependence of the $C^{1/2}_{SUPREX}$ values obtained for both of the MPT synthase complexes (active and inactive) as evidenced by the high correlation coefficients observed (0.9935 and 0.9847, respectively). In each case it was also possible to extract reasonably precise ΔG_f^0 and m values from these data, as summarized in Table 2. The ΔG_f^0 and m values in Table 2 are listed as "apparent", since the absolute accuracy of the ΔG_f^0 and m values in Table 2 is largely dependent on the validity of our two-state folding assumption for MoaE, and currently there is no biophysical data either supporting or refuting such a two-state folding model for MoaE. A preliminary K_d value of 4.5 μM determined by isothermal titration calorimetry was previously reported for the binding of MoaD to MoaE (*5*). When the $\Delta G_{Binding}$ values in Table 2 were used in eq 2 to calculate a K_d for the binding of MoaD to MoaE, a value of 17 ± 7 μM was obtained. This value is in reasonable agreement with the preliminary K_d value previously reported for this complex (*5*), and it serves to validate the use of the SUPREX technique for determination of dissociation constants in the MPT synthase system.

Using the SUPREX data, a K_d of 2.6 ± 0.9 μM was obtained for the binding of MoaD-SH to MoaE. This value represents the first quantitative measurement of the binding affinity between the subunits in activated MPT synthase. When compared to the SUPREX-derived K_d of 17 ± 7 μM for the MoaD/MoaE pair, the binding affinity in the active MPT synthase complex is measurably stronger than in the inactive complex. This result is consistent with the previously proposed MPT synthase mechanism (*2*, *3*, *5*). Conversion of one molecule of precursor Z to MPT involves the addition of two sulfur atoms to the pterin side chain to form the dithiolene moiety. Since each MoaD-SH carries a single thiocarboxylate, two MoaD-SH molecules are required for each MPT molecule produced. However, each MPT synthase active site can only accommodate a single MoaD-SH molecule. Therefore, after transfer of the thiocarboxylates from the first set of MoaD-SH proteins to the precursor Z molecules, the resulting MoaD at each active site must dissociate from the complex to be replaced by a second molecule of MoaD-SH whose thiocarboxylate will be used to finish the conversion of precursor Z to MPT. Finally, both the newly synthesized MPT and the second set of MoaD molecules must dissociate from the MoaE dimer in order to begin a new round of MPT synthesis. The previous isolation and characterization of a monosulfurated intermediate of the MPT synthase reaction supports this mechanism (*5*), as does the finding here that there is tighter binding in the MoaD-SH/MoaE complex (active MPT synthase) than in the MoaD/MoaE complex (inactive MPT synthase).

The *m* values obtained in our SUPREX analyses of MoaE in the presence of MoaD and MoaD-SH were significantly larger than the *m* value obtained for MoaE alone. It has previously been shown that there is a strong correlation between the magnitude of a protein's *m* value and its ΔASA, the amount of hydrophobic surface area that is buried upon folding (*23*). Using the correlation established by Myers and co-workers, the Δ*m* values of 1.5 and 1.9 kcal mol^{-1} M^{-1} obtained for the MoaD/MoaE and MoaD-SH/MoaE complexes (respectively) can be converted to ΔASA values of 2378 and 4117 Å^2, respectively. That is to say, approximately 2378 Å^2 of solvent accessible surface area is buried in the binding interaction between MoaD and MoaE, while 4117 Å^2 is buried upon binding of MoaD-SH to MoaE. The SUPREX-derived ΔASA value for MoaD binding to MoaE is within 20% of the value of 3000 Å^2 that was calculated directly from the X-ray crystallographic structure of inactive MPT synthase (*3*). The increased ΔASA value obtained for active MPT synthase suggests that there may be a larger and/or more rigid binding interface in this complex as compared to inactive synthase. This result correlates well with the greater affinity for MoaE exhibited by MoaD-SH.

In conclusion, we have analyzed the thermodynamic properties of the MPT synthase complex using the SUPREX technique. This analysis yielded a binding affinity for inactive MPT synthase that was comparable to that previously determined by isothermal titration calorimetry (*5*), and it also facilitated the first determination of the binding affinity between MoaD-SH and MoaE in active MPT synthase. Comparison of the two SUPREX-derived MPT synthase binding constants indicated a tighter binding in the MoaD-SH/MoaE pair, which is consistent with the previously proposed mechanism of action of MPT synthase. The SUPREX technique also proved useful for quantitative measurements of the protein surface area that is buried upon formation of active and inactive MPT synthase.

The MPT synthase system represents one of the first protein−protein systems to be studied by SUPREX. In a protein−protein system the denaturant used in SUPREX can denature both the protein and the protein ligand. The general agreement of our SUPREX-derived K_d value for the MoaE/MoaD complex with a previously reported literature value obtained by isothermal calorimetry (*5*) suggests that appreciable denaturation of the MoaD subunit did not occur during the SUPREX experiments performed here. Therefore, it is likely that the $C^{1/2}_{SUPREX}$ values obtained for the MPT synthase complexes were at low enough denaturant concentrations to not significantly denature the protein ligand.

Now that the MPT synthase system has proven to be amenable to SUPREX analysis, a host of further SUPREX experiments examining other aspects of MPT biosynthesis can be envisioned. Given the role of the MoeB protein in regeneration of the MoaD-SH thiocarboxylate, it would be interesting to use SUPREX to examine the relative binding of MoaD and MoaD-SH to MoeB. In this case, one would predict a reversal of the binding affinities observed with the MoaE protein, since MoaD would be expected to exhibit the higher affinity for MoeB. SUPREX on MoeB alone could also clarify whether the protein exists as a monomer or dimer in solution, since previous attempts to determine the oligomeric structure of MoeB have yielded inconclusive results. Additionally, a number of naturally occurring mutations in the human MPT biosynthesis proteins have been identified in patients suffering from molybdenum cofactor deficiency (*28*). Some of these mutations are located in the coding sequences for the MOCS2A and MOCS2B proteins, the human equivalents of *E. coli* MoaD and MoaE, respectively (*7*, *28*). SUPREX analysis of the human protein variants or the comparable *E. coli* variants could help determine if the in vivo decrease in MPT synthase activity caused by these mutations can be attributed to disruption of MPT synthase complex formation.

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