Kinetic and Microcalorimetric Analysis of Substrate and Cofactor Interactions in Epoxyalkane:CoM Transferase, a Zinc-Dependent Epoxidase†

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ABSTRACT: Epoxyalkane:CoM transferase (EaCoMT) is a key enzyme of bacterial propylene metabolism, catalyzing the nucleophilic attack of coenzyme M (CoM, 2-mercaptoethanesulfonic acid) on epoxypropane to form the thioether conjugate 2-hydroxypropyl-CoM. The biochemical and molecular properties of EaCoMT suggest that the enzyme belongs to the family of alkyltransferase enzymes for which Zn plays a key role in activating an organic thiol substrate for nucleophilic attack on an alkyl-donating substrate. In the present work, the role of Zn in the EaCoMT-catalyzed reactions is established by removing Zn from EaCoMT, resulting in loss of catalytic activity that was restored upon addition of Zn back to the enzyme, and by expressing an inactive and Zn-deficient form of the enzyme that was activated by addition of $ZnCl_2$ or $CoCl_2$. Site-directed mutagenesis of one of the predicted Zn ligands (C220A) resulted in the formation of a largely catalytically inactive protein (0.06% of wild-type activity) that, when purified, contained a substoichiometric complement of Zn. EaCoMT was kinetically characterized and found to follow a random sequential mechanism with kinetic parameters $K_{m,epoxypropane} = 1.8\ \mu M$, $K_{m,CoM} = 34\ \mu M$, and $k_{cat} = 6.5\ s^{-1}$. The CoM analogues 2-mercaptopropionate, 2-mercaptoethanol, and cysteine substituted poorly for CoM as the thiol substrate, with specific rates of epoxyalkane conjugation that were at best 0.6% of the CoM-dependent rate, while ethanethiol, propanethiol, glutathione, homocysteine, and lipoic acid provided no activity. 2-Mercaptoethanol was a weak competitive inhibitor vs CoM with a K_I of 192 mM. Isothermal titration calorimetry was used to investigate the thermodynamic binding determinants for the interaction of CoM and analogues with holo, Zn-deficient, and C220A EaCoMT variants. The stoichiometry of CoM binding correlated directly with the Zn content rather than monomer content of protein samples, reinforcing the importance of Zn in CoM binding. The binding of CoM to EaCoMT occurred with $\Delta G = -7.5$ kcal/mol ($K_d = 3.8\ \mu M$) and was driven by a large release of enthalpy. The thermodynamic contributors (K_a, ΔG, ΔH, ΔS) to the individual binding of CoM, ethanesulfonate, and ethanethiol were determined and used to assess the contributions of the thiol, alkyl, and sulfonate moieties to total binding energy in the E·CoM binary complex.

Organic thiols are important nucleophiles in biological systems, reacting with diverse electrophilic compounds via nucleophilic substitution or addition to produce alkylated thioether products. The simplest thiol cofactor is coenzyme M (CoM,[1] 2-mercaptoethanesulfonic acid), which was first identified as a methyl group carrier in the process of reductive methane formation in the methanogenic archaea (*1*, *2*). Recently, a second function was discovered for CoM, namely, as the nucleophile for aliphatic epoxide ring opening and as the carrier of intermediates that result in net epoxide carboxylation in alkene-utilizing soil bacteria (*3*). For both methanogens and alkene-oxidizing bacteria, CoM transferases catalyzes the nucleophilic attack of CoM on the electrophilic substrate, although in one instance the reaction is a nucleophilic substitution while the other is an addition as shown in eqs 1 and 2. The biochemical and molecular characterization of these CoM transferases has revealed that they are zinc-containing enzymes (*4*−*7*). Multiple sequence alignments reveal that they contain a characteristic zinc-binding motif that was first identified in cobalamin-independent methionine synthase (MetE) (*8*). MetE is one of several

$$\text{HS-CoM} + [\text{CH}_3\text{-Co}^{3+}]\text{corrinoid protein} \xrightarrow{\substack{\text{methylcobamide:CoM} \\ \text{methyltransferase 2 (MT2)}}} \text{CH}_3\text{-S-CoM} + [\text{Co}^{1+}]\text{corrinoid protein} + \text{H}^+ \quad (1)$$

$$\text{HS-CoM} + \text{epoxypropane} \xrightarrow{\substack{\text{epoxyalkane:CoM} \\ \text{transferase (EaCoMT)}}} \text{2-hydroxypropyl-CoM} \quad (2)$$

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[1] Abbreviations: CoM, coenzyme M (2-mercaptoethanesulfonic acid); EaCoMT, epoxyalkane:CoM transferase [EC 4.2.99.19; systematic name, 2-hydroxypropyl-CoM:2-mercaptoethanesulfonate lyase (epoxyalkane-ring-forming)]; EXAFS, extended X-ray absorption fine structure; GST, glutathione *S*-transferase; ICP-MS, inductively coupled plasma atomic emission mass spectrometry; IPTG, isopropyl thiogalactoside; ITC, isothermal titration calorimetry; MetE, cobalamin-independent methionine synthase; MT2-A, methylcobamide:CoM methyltransferase 2-A (MtbA); MT2-M, methylcobamide:CoM methyltransferase 2-M (MtaA); PAR, 4-(2-pyridylazo)resorcinol; PMPS, *p*-(hydroxymercuri)benzenesulfonic acid.

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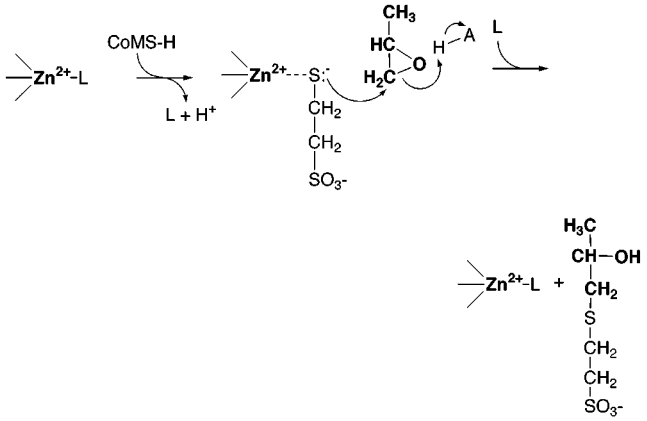
Scheme 1



enzymes that have been shown to activate a thiol for nucleophilic attack by coordination to zinc [other enzymes of this type are cobalamin-dependent methionine synthase (*9*), the ADA protein (*10*, *11*), protein farnesyl transferase (*12*), human betaine−homocysteine methyltransferase (*13*), and *S*-methylmethionine:homocysteine methyltransferase (*14*)]. Mechanistic and spectroscopic studies of these enzymes have allowed the formulation of a general mechanism wherein coordination of the thiol group of the substrate to the active site zinc lowers the pK_a of the thiol, thereby activating it for nucleophilic attack on the electrophilic substrate. Binding of the thiolate involves ligand exchange at the zinc center of MetE, as revealed by a change in zinc coordination from a 2S + 2N/O to a 3S + 1N/O environment, with the dissociable N/O ligand believed to be a water molecule (*15*). Upon thiolate alkylation, the zinc coordination environment in MetE returns to 2S + 2N/O as the alkylated thiol product dissociates from the zinc center and is replaced by the exchangeable ligand (*15*). Recent EXAFS studies of the zinc center of MT2-A have demonstrated an identical ligand rearrangement during reaction of the enzyme with CoM, confirming the role of zinc in activating the thiol group of CoM (*4*).

By analogy to the documented chemistry of MetE and MT2-A, the zinc center of EaCoMT has been proposed to activate CoM in a similar fashion, in this case for attack at the electrophilic C1 of epoxypropane as shown in Scheme 1 (*7*). Cleavage of the C1−O oxirane bond of the epoxide and protonation of the resultant alkoxide result in addition of the entire substrate molecule to CoM, forming the 2-hydroxypropyl-CoM adduct, a reaction analogous to that of the epoxidase activity of glutathione *S*-transferases (GSTs). Notably, GSTs are not zinc-containing enzymes, and they lower the thiol group pK_a solely through hydrogen-bonding interactions with amino acid residue(s) at the active site (*16*). Thus, two strategies appear to have developed in nature for thiol group activation: one involving zinc coordination and the other involving hydrogen-bonding interactions.

While circumstantial evidence suggests a key role for zinc in thiol group activation by EaCoMT, the role of zinc in CoM binding and activation has not been experimentally verified by spectroscopic and biochemical studies. Furthermore, the elegant simplicity of CoM, as opposed to structurally more complex thiol cofactors such as glutathione, homocysteine, and lipoamide, makes it an ideal candidate for studying the kinetics and thermodynamics of thiol binding to the enzyme. Accordingly, in the present study, we have used a combination of kinetic, biochemical, and calorimetric techniques to elucidate details of CoM binding, activation, and turnover by EaCoMT from the propylene-oxidizing bacterium *Xanthobacter autotrophicus* strain Py2. These studies establish the role for zinc in thiol binding, define the kinetic parameters of the two-substrate system, define the pK_a's of the zinc-bound thiolate and a probable general acid, and define the thermodynamic parameters (K_a, $\Delta G°$, $\Delta H°$, $\Delta S°$) of CoM binding. Furthermore, calorimetric studies of the interaction of CoM and CoM analogues with apo, holo, and mutant CoM transferases have allowed the elucidation of the thermodynamic contributions of zinc and the functional moieties of CoM to ligand binding.

EXPERIMENTAL PROCEDURES

Materials. (*R*)-(+)-1,2-Epoxypropane, racemic propylene oxide (>99%), 2-mercaptoethanesulfonic acid (coenzyme M, CoM), *p*-(hydroxymercuri)benzenesulfonic acid (PMPS), and 4-(2-pyridylazo)resorcinol (PAR) were purchased from Sigma-Aldrich Chemical Co. All other chemicals were of analytical grade.

Site-Directed Mutagenesis of EaCoMT. All oligonucleotides were purchased from Operon. Site-directed mutagenesis of pJK1, the plasmid containing the gene encoding EaCoMT (*17*), was carried out utilizing the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocols. The desired mutation was C220A for which the primers 5′-GCCAAGATCATCGCCCATGTGGCGTGGGG-CAACTGG-3′ and 5′-CCAGAACGGGGACGCCACAT-GGGCGATTGATCTTGGC-3′ were designed. The mutation was confirmed by Big-Dye sequencing at the Utah State University Biotechnology Center.

Expression of Enzymes. Bacteria were grown at 37 °C on standard LB media unless otherwise stated. *Escherichia coli* BL21 (DE3) cells were transformed with pJK1, the expression vector for EaCoMT from *X. autotrophicus* strain Py2, or the corresponding mutant plasmid, as described previously (*17*). For expression of EaCoMT, freshly transformed cells were grown in a 15 L capacity Microferm fermentor (New Brunswick Scientific) containing 12 L of LB-rich media (per liter: 20 g of tryptone, 15 g of yeast extract, 2 g of K_2HPO_4, 1 g of KH_2PO_4, 8 g of NaCl, and 10 g of glucose) and antifoam 204 (0.005% v/v). Cells were grown at 37 °C with stirring at 400 rpm and with forced aeration to an A_{600} between 0.6 and 1.0. At this point the temperature was lowered to 30 °C, and IPTG was added to a final concentration of 1.0 mM. For expression of zinc-containing enzymes, 1 mM $ZnSO_4$ was added with the IPTG. For expression of a zinc-free form of the enzyme, 1 mM EDTA was added in place of $ZnSO_4$. After a 5 h induction period cells were concentrated using a tangential flow filtration system (Millipore Corp.) and pelleted by centrifugation. The cell paste was washed once with 50 mM potassium phosphate buffer, pH 7.2, and then drop frozen in liquid nitrogen and stored at −80 °C.

Purification of Enzymes. Recombinant wild-type EaCoMT was purified using the procedure described previously (*17*). For purification of the zinc-depleted enzyme, all buffers were passed over a column of Chelex-100 (Bio-Rad) to remove trace divalent metal ions, followed by the addition of EDTA (1 mM) to the buffer. Recombinant EaCoMT containing the

mutation C220A was expressed and purified using the same protocol as for the wild type, except that the heat treatment step was eliminated. When necessary, EDTA was removed from protein samples (1 mL) by passage through a column of Sephadex G-25 (1.5 × 10 cm) that had been equilibrated in chelexed buffer [50 mM Tris·HCl (pH 8.0)].

Gas Chromatographic Assay of EaCoMT Activity. Assays (1 mL total volume reagents) were performed at 30 °C in sealed serum vials (9 mL) that contained 2.5 mM epoxypropane, 2 mM CoM, and 50 mM Tris·HCl (pH 8.0). The source of EaCoMT was either clarified cell extract (0.17 mg of protein) or purified protein (0.02−0.42 mg of protein). For assays with cell extracts, the assays also contained 1 mM EDTA and, where indicated, 1.5 mM $CoCl_2$ or $ZnCl_2$ which was preequilibrated with the assay mixture for 15 min. Assays were initiated by the addition of epoxypropane. At the desired time points, samples were removed from the headspace of the assay vials, and the amount of epoxypropane remaining was determined by gas chromatography as described previously (*3*).

Coupled Spectrophotometric Assay of EaCoMT Activity. A real-time spectrophotometric assay was developed for EaCoMT that quantifies 2-(*R*)-hydroxypropyl-CoM by measuring the reduction of NAD^+ to NADH by the coupling enzyme 2-(*R*)-hydroxypropyl-CoM dehydrogenase (*3*, *18*) according to eqs 3 and 4.

$$(R)\text{-epoxypropane} + \text{CoM} \rightarrow 2\text{-}(R)\text{-hydroxypropyl-CoM} \quad (3)$$

$$2\text{-}(R)\text{-hydroxypropyl-CoM} + \text{NAD}^+ \rightarrow 2\text{-ketopropyl-CoM} + \text{NADH} + \text{H}^+ \quad (4)$$

Quartz assay cuvettes (2 mL total volume; 1 cm path length) contained 40 μg of EaCoMT, 250 μg of (*R*)-hydroxypropyl CoM dehydrogenase (*18*, *19*), 3.0 mM NAD^+, 0−0.25 mM CoM, and buffer [50 mM Tris·HCl (pH 8.0) except for pH studies] in a final volume of 1 mL and at 30 °C. The amount of (*R*)-hydroxypropyl-CoM dehydrogenase added to the assay was sufficient to quantitatively convert 2-(*R*)-hydroxypropyl-CoM to 2-ketopropyl-CoM in a non-rate-limiting fashion. Assays were initiated by the addition of (*R*)-epoxypropane (0−2 mM final concentration) from stock solutions freshly prepared in water. NADH formation (ϵ_{340} = 6.22 mM^{-1} cm^{-1}) was monitored on a Shimadzu UV-2401 spectrophotometer interfaced to a computer containing UVPC Kinetics Software. Rates were determined from the linear portions of progress curves and were taken within the first 10 s of the reaction. Kinetic constants (K_m and V_{max}) were calculated by iteratively fitting initial rate data to the form of the Michaelis−Menten equation describing a sequential two-substrate reaction (eq 5) as described by Cleland (*20*) and using the software SigmaPlot.

$$v = VAB(K_{ia}K_b + K_aB + K_bA + AB)^{-1} \quad (5)$$

Kinetic Analysis of 2-Mercaptoethanol Inhibition of EaCoMT. Inhibition studies were performed using the coupled spectrophotometric assay described above. Epoxypropane was present at a fixed saturating level (2.5 mM) while the concentrations of CoM and β-mercaptoethanol were varied. 2-Mercaptoethanol was both a substrate and an inhibitor under these conditions; however, 2-hydroxypropylmercaptoethanol, the product of 2-mercaptoethanol conjugation to epoxypropane, was not a substrate for, nor an inhibitor of, the coupling enzyme (*R*)-hydroxypropyl-CoM dehydrogenase. Thus, EaCoMT activity for these assays reflects only conjugation of CoM and epoxypropane. All assays were performed in duplicate, and the inhibition was evaluated by nonlinear regression computer analysis using the forms of the Michaelis−Menten equation that incorporate the appropriate inhibition terms as described by Cleland (*20*).

pH Dependence of Kinetic Parameters. Kinetic parameters were determined in a buffer mixture consisting of 30 mM each Tris, glycine, phosphate, and succinate that was adjusted to a range of pH values between 6.0 and 10.0 by the addition of HCl or NaOH. In preparing the buffer mixture Goode buffers were purposefully excluded due to possible inhibitory effects arising from the presence of their sulfonate groups. Specific activities determined by the coupled assay at pH 8.0 agreed within 5% for assays conducted in 50 mM Tris vs the buffer mixture. For pH studies, CoM was the variable substrate (0−0.25 mM), and (*R*)-epoxypropane was held at a fixed saturating concentration of 2.5 mM. Kinetic data were iteratively fit in SigmaPlot to eq 6, which is the form of the pH profile where the kinetic constants drop at both high and low pH (*20*):

$$\log y = \log(c(1 + [H^+]/K_a + K_b/[H^+])^{-1}) \quad (6)$$

In this equation, y is the catalytic efficiency (V_{max}/K_m), c is the pH independent value of the parameter y, and K_a and K_b are the dissociation constants of the catalytically important ionizing residues.

Removal of Zinc from Epoxyalkane:CoM Transferase. All glassware used for these procedures was washed with 1 M HNO_3 and 1 M HCl for 16 h followed by rinsing with deionized distilled water and chelexed buffer. Zinc was removed from CoM transferase samples by complexation with PAR in the presence of PMPS using an adaption of the procedure described by Matthews and co-workers (*8*). One milliliter samples of enzyme (400 μM) that had been passed through a column of Chelex-100 were incubated with 1.6 mM PAR and 3.2 mM PMPS at room temperature for 10 min. The Zn−PAR complex, free PAR, and free PMPS were then separated from the enzyme on a 1.5 × 10 cm column of Sephadex G-25 that was equilibrated in 50 mM Tris·HCl (pH 8.0) containing 0.05 mM EDTA. For quantification of the Zn−PAR complex, the published extinction coefficient of 66000 M^{-1} cm^{-1} at 500 nm was used (*21*). PAR- and PMPS-treated samples were assayed for CoM transferase activity by gas chromatography as described above.

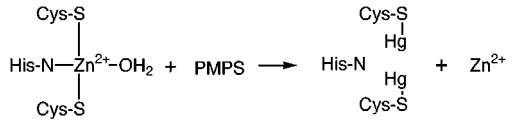
Isothermal Titration Calorimetry. Isothermal titration calorimetry was performed using a MicroCal titration microcalorimeter (Northampton, MA) (*22*, *23*). Solutions were prepared in 50 mM Tris·HCl (pH 8.0) and degassed under vacuum prior to use. The reference offset was 30%, and the reaction temperature was 30 °C with a stirring speed of 100 rpm. The reaction and reference cells had a volume of 1.374 mL. Protein concentration, ligand concentration, and volume of injections are as noted in Table 1. In background data analysis the ligand was injected with only the buffer in the cell. Background reaction heats were subtracted from data before the thermograms were evaluated. Thermogram data

Table 1: Parameters for ITC Experiments

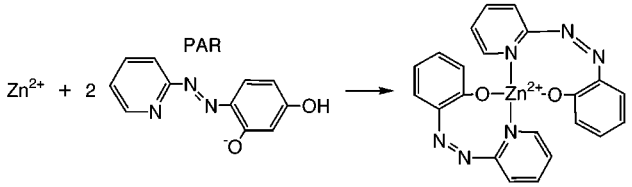
EaCoMT (μM)	Zn content[a]	ligand (mM)	injection volume (μL)
wild type (50)	1.0	CoM (1.0)	8.0
wild type (50)	1.0	ethanesulfonate (75)	8.0
wild type (50)	1.0	ethanethiol (10)	8.0
wild type (50)	1.0	propanethiol (10)	4.0
wild type (50)	1.0	2-mercaptoethanol (1−50)	4.0
wild type (50)	0.2	CoM (1.0)	4.0
C220A (40)	0.6	CoM (1.0)	8.0

[a] Zn contents are reported as mole of Zn per mole of EaCoMT monomer for the indicated protein samples.

Scheme 2



Scheme 3



were integrated using the ORIGIN software supplied by MicroCal Inc. The equilibrium binding constant, K_a, and the enthalpy change, ΔH, were used to calculate ΔG and ΔS using the Gibbs free energy relationship shown in the equation:

$$\Delta G^\circ = -RT \ln [K_a] = \Delta H^\circ - T\Delta S^\circ \qquad (7)$$

where $R = 1.9872$ cal mol^{-1} K^{-1}. Thermodynamic parameters are presented in accordance with the recent recommendations on biochemical thermodynamic data (*24*).

Analytical Procedures. Protein concentrations were determined by means of a modified biuret assay (*25*). Metal analyses were performed by ICP-MS at the Utah State University Veterinary Diagnostics Laboratory.

RESULTS

Zinc Is Required for Epoxyalkane:CoM Transferase Activity. The requirement of zinc in the EaCoMT-catalyzed reaction was explored in two ways: by attempting to remove zinc from the purified holoenzyme by complexation with a zinc-selective chelator and by expressing EaCoMT in the presence of EDTA in hopes of making a stable apo form of the enzyme. Similar procedures have been used to establish the role of zinc in homocysteine activation by MetE and in CoM activation by the methanogenic methyltransferases MT2-A and MT2-M (*4*, *5*, *8*). In their studies, Matthews and co-workers reported that the addition of the zinc chelator PAR alone was not sufficient to remove zinc from MetE (*8*). Instead, it was necessary to first displace zinc from the enzyme by addition of PMPS to compete for the zinc thiol ligands, followed by complexation of the released zinc with PAR as shown in Schemes 2 and 3 (*8*). In our studies of EaCoMT we observed a similar phenomenon, i.e., no formation of a Zn−PAR complex in the absence of PMPS.

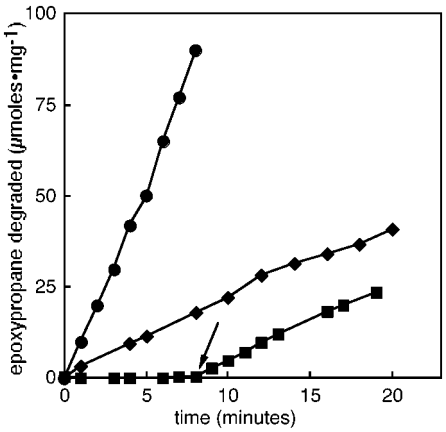


FIGURE 1: PMPS inactivation of EaCoMT activity. Data points represent the average of duplicate measurements. Symbols: (●) untreated EaCoMT, (◆) PMPS-treated EaCoMT containing 20 μM $ZnSO_4$ from the onset of the assay, and (■) PMPS-treated EaCoMT to which 20 μM $ZnSO_4$ was added at the time indicated by the arrow.

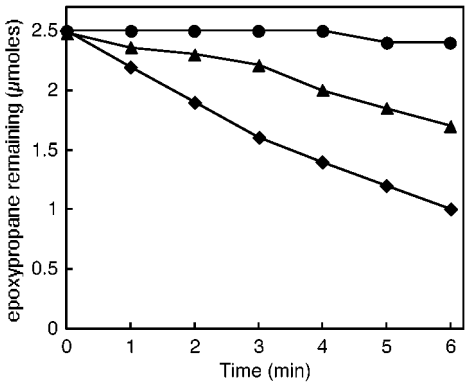


FIGURE 2: Divalent metal ion requirement of recombinant EaCoMT expressed under zinc-free conditions. Assays contained clarified cell extract (170 μg of protein) prepared from cells induced for EaCoMT activity in the absence of zinc as described under Experimental Procedures. Symbols: (●) no metal ion added; (▲) 1.5 mM $CoCl_2$ added; (◆) 1.5 mM $ZnCl_2$ added.

For these experiments, a sample of recombinant EaCoMT was used that had a metal complement of 0.68 Zn/monomer as determined by ICP-MS. This metal complement is slightly lower than that typically associated with recombinant EaCoMT preparations, which range in metal content from 0.8 to 1.2 Zn (data not shown). The sample used in this experiment was passed over a column of Chelex-100 prior to analysis in order to eliminate any interference from adventitious zinc. This treatment lowered the zinc content of the sample by approximately 10% over the nonchelexed protein, suggesting that some adventitious zinc was removed by the treatment or possibly that a small portion of active site zinc was labile to the Chelex treatment. The remaining zinc remained tightly bound when the protein was incubated with PAR or EDTA alone. However, the simultaneous addition of PAR and PMPS resulted in stoichiometric removal of zinc. The absorption of the zinc−PAR complex yielded a zinc stoichiometry of 0.68 Zn released/monomer, the same value as determined by ICP-MS for the untreated protein. In addition, ICP-MS analysis of the treated enzyme showed that it was completely devoid of zinc, confirming the stoichiometric removal by the PAR/PMPS treatment.

As shown in Figure 1, the PAR/PMPS-treated enzyme was completely incapable of catalyzing the addition of CoM to

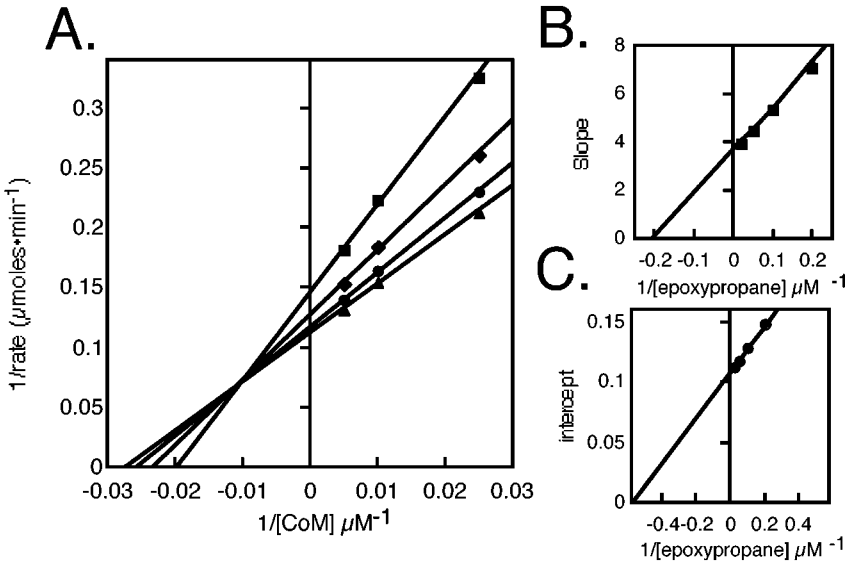


FIGURE 3: Determination of kinetic parameters for EaCoMT. (A) Double reciprocal plot. The lines were drawn from the nonlinear regression fits of each experimental data set to the Michaelis−Menten equation. (B and C) Slope and intercept replots of the data in (A).

epoxypropane. The addition of $ZnSO_4$ resulted in the restoration of some EaCoMT activity (Figure 1). Under optimal conditions, a maximum of 30% of EaCoMT activity could be restored. Presumably, the harsh conditions associated with the PMPS treatment rendered 70% of the enzymes irreversibly inactivated.

To further explore the requirement of zinc in EaCoMT, an apo form of the enzyme was prepared by expressing and purifying the enzyme in the presence of EDTA. Similar strategies have been used to prepare apo forms of the CoM transferases MT2-A and MT2-M (*4*, *5*). As shown in Figure 2, no detectable EaCoMT activity was present in clarified cell extracts prepared from cells induced for EaCoMT in the presence of 1 mM EDTA. However, the addition of either $ZnCl_2$ or $CoCl_2$ to cell extracts resulted in significant stimulation of activity. Under these conditions, the activity observed with $ZnCl_2$ was approximately 2-fold higher than that with $CoCl_2$. While the results indicate that a zinc-free form of EaCoMT is present in cell extracts, the further purification of the enzyme resulted in increase in specific activity and concomitant incorporation of zinc into the protein, despite our attempts to minimize exposure to zinc by chelexing and adding EDTA to the buffers and acid-washing glassware. The purified EaCoMT was found to exist as a hexamer, contained 0.2 mol of zinc/mol of monomer, and had a specific activity of 3.0 units/mg. By comparison, the preparation of holo-EaCoMT used for all subsequent experiments in this paper contained 1.0 mol of zinc/mol of monomer and had a specific activity of 11.5 units/mg. These numbers reveal that the activity of EaCoMT samples is directly proportional to the zinc content of the samples, suggesting that, for the zinc-depleted enzyme, a population of zinc-occupied active sites (20% of total) are catalytically active while the remaining sites (80% of total) are unpopulated and inactive.

Determination of Kinetic Constants for EaCoMT. Previous assays of EaCoMT activity have in most instances used racemic epoxypropane, which is a mixture of the *R*- and *S*-enantiomers, as the substrate, and used gas chromatographic measurements of epoxypropane consumption to measure specific rates (*3*, *17*). For simplification of the kinetic analysis, we chose to focus on the *R*-enantiomer, reported to be a better substrate for EaCoMT (*3*), and developed a coupled spectrophotometric assay that allows EaCoMT activity to be measured in real time and by product formation rather than substrate depletion. To accomplish this, NAD^+ and 2-(*R*)-hydroxypropyl-CoM dehydrogenase (*18*, *19*) were included in assays, such that 2-(*R*)-hydroxypropyl-CoM is immediately oxidized to 2-ketopropyl-CoM with concomitant NADH formation, as shown in eqs 1 and 2. The amounts of 2-(*R*)-hydroxypropyl-CoM dehydrogenase and NAD^+ added to the assays were sufficient to quantitatively and rapidly convert 2-(*R*)-hydroxypropyl-CoM to 2-ketopropyl-CoM over the range of substrate concentrations and pH values used in the kinetic analyses.

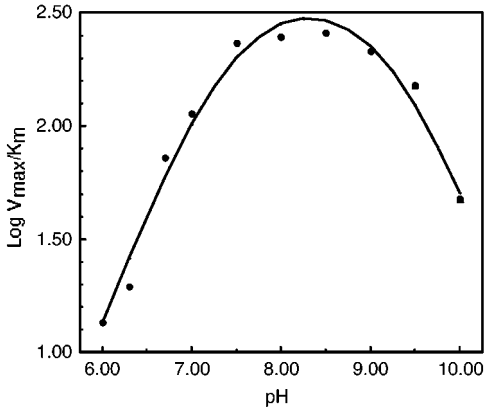


FIGURE 4: pH dependence of epoxyalkane:CoM transferase activity. V_{max} is reported as units per milligram, and K_m is reported as millimolar concentration of CoM. The line was drawn from the fit of the experimental data to eq 6.

As shown in Figure 3, the two-substrate kinetic analysis of EaCoMT conforms graphically to the Michaelis−Menten model for a two-substrate sequential mechanism. By fitting the data to eq 5, the following kinetic parameters were obtained: $K_{m,epoxypropane} = 1.8 \pm 0.17$ μM, $K_{m,CoM} = 33.6 \pm 1.6$ μM, and $k_{cat} = 6.5$ s^{-1} (on the basis of active sites).

pH Dependence of Kinetic Parameters. As shown in Scheme 1, a key feature of the proposed mechanism of EaCoMT involves the deprotonation of the thiol of CoM,

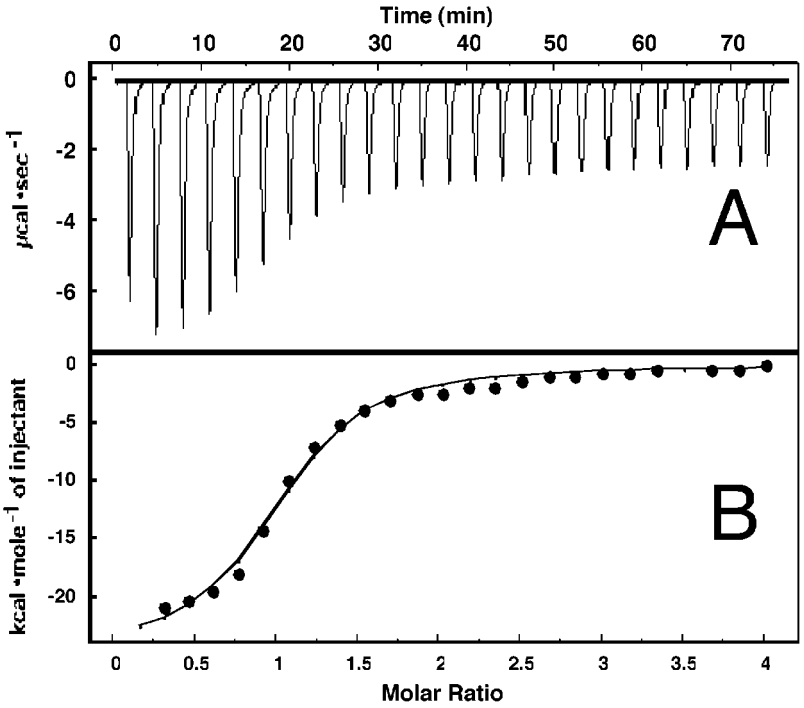


FIGURE 5: ITC titration data for CoM binding to holo-EaCoMT. Run parameters were as described in Table 1. Panel A shows the differential power signal recorded in the course of the experiment. Panel B shows the integrated heats plotted against the molar ratio of CoM to EaCoMT. The solid line represents the nonlinear least-squares fit of the data to a ligand binding model with a single interacting site.

Table 2: Thermodynamic Binding Parameters for CoM Binding to EaCoMT Isoforms

protein	n_P[a]	n_{Zn}[a]	K_a (M^{-1})	ΔH (kcal·mol^{-1})	$T\Delta S$ (kcal·mol^{-1})	ΔG (kcal·mol^{-1})
wild type	1.00 ± 0.02	1.00 ± 0.02	$(2.65 \pm 0.37) \times 10^5$	-24.6 ± 0.8	-17.1	-7.5
zinc depleted	0.21 ± 0.05	0.97 ± 0.06	$(1.14 \pm 0.26) \times 10^5$	-13.3 ± 3.7	-6.2	-7.1
C220A	0.66 ± 0.09	1.09 ± 0.15	$(3.64 \pm 0.44) \times 10^4$	-29.1 ± 0.5	-22.8	-6.3

[a] *n* value in relation to the concentration of protein, n_P, or zinc, n_{Zn}. Final parameters (K_a, ΔH, $T\Delta S$, and ΔG) were evaluated with n_{Zn}. Parameters for the ITC run were as shown in Table 1. Errors for individual titration runs were obtained from the direct nonlinear regression fit of the data to the binding isotherms using the software ORIGIN.

thereby activating CoM for nucleophilic attack on epoxypropane. Additionally, the C−O oxirane bond cleavage that occurs in concert with thiolate attack must necessarily form an alkoxide in the transition state, an intermediate that most likely requires either charge stabilization or facilitated protonation (Scheme 1). With these considerations in mind, the pH dependence of the reaction was investigated in order to gain insights into the nature of the requisite ionizable group(s).

As shown in Figure 4, the plot of log V_{max}/K_m was bell shaped with a pH optimum centered between pH 8 and pH 8.5. A fit of the experimental data to eq 6 provided pK values of 7.4 ± 0.06 (pK_a) and 9.2 ± 0.07 (pK_b). The acidic slope of the pH profile was determined to have a value of 0.99 by linear regression of the data points from pH 6.0 to pH 7.0, suggesting that a single ionizable residue is involved. This ionization most likely represents that of CoM, which must be in the thiolate form for catalysis to occur. The unperturbed pK_a of CoM has been reported to be 9.1 (*26*); thus, binding of CoM to EaCoMT results in a lowering of the pK_a by 1.7 units.

The downward slope at higher pH values represents ionizable group(s) which must be protonated for catalysis; likely candidates would be amino acid residue(s) that serve as the proton donor(s) to the alkoxide intermediate (Scheme 1). The slope between the two highest data points on the basic side was −1.0, suggesting that a single ionizable residue is responsible for this effect as well. Equations 8 and 9 summarize the ionizable groups identified in this analysis and their respective pK_a values.

$$\text{CoM-SH} \rightleftharpoons \text{COM-S}^- + \text{H}^+ \qquad \text{p}K_a = 7.4 \qquad (8)$$

$$\text{A-H} \rightleftharpoons :\text{A}^- + \text{H}^+ \qquad \text{p}K_a = 9.2 \qquad (9)$$

Thermodynamics of CoM−EaCoMT Interactions. The simplicity of CoM makes it an ideal molecule for studying the thermodynamic contributors to enzyme−thiol cofactor binding. Isothermal titration calorimetry is currently the method of choice for studying the thermodynamics of protein−ligand interactions (*22*, *23*, *27*). Accordingly, ITC was employed to gain insights into the features of CoM−EaCoMT binding. Figure 5 shows a representative thermogram and integrated data from the titration of CoM into a solution of holo-EaCoMT (parameters described in Table 1). The analysis of the experimental data (*22*; Table 2) provided an "*n*" value of 1.0 for the ratio of CoM/EaCoMT monomer, demonstrating a single CoM binding site per monomeric unit. No cooperativity for ligand binding was observed, demonstrating that each CoM binding site in the α_6 hexamer binds

Table 3: Alternative Thiols Investigated as Substrates for EaCoMT[a]

substrate tested	formula	specific activity (milliunits/mg)	% activity
CoM	$HSCH_2CH_2SO_3^-$	11800 ± 900	100
3-mercaptopropionate	$HSCH_2CH_2COO^-$	70.0 ± 1.6	0.59
2-mercaptoethanol	$HSCH_2CH_2OH$	60.4 ± 0.5	0.51
cysteine	$HSCH_2CH(NH_3^+)COO^-$	13.4 ± 0.5	0.11
ethanethiol	$HSCH_2CH_3$	BDL[b]	<0.008
2-mercaptoethylamine	$HSCH_2CH_2NH_3^+$	ND[c]	0
propanethiol	$HSCH_2CH_2CH_3$	ND	0
homocysteine	$HSCH_2CH_2CH(NH_3^+)COO^-$	ND	0
thiocyanate	HSCN	ND	0
glutathione	γ-Glu-Cys-Gly	ND	0
lipoic acid	$HSCH_2CH_2CH(HS)R$	ND	0

[a] Gas chromatographic assay of epoxypropane consumption was performed as described under Experimental Procedures. The assays contained epoxypropane (2.5 mM), EaCoMT (0.02−1 mg of protein), and the indicated organic thiol (10 mM). One unit of activity is defined as 1 μmol of epoxypropane degraded per minute at 30 °C. [b] BDL, below detectable limits; confident detectable limits were set at >1.0 milliunits/mg. [c] ND, no detectable activity with 1.0 mg of CoM transferase and 10 mM thiol compound.

CoM independent of the other sites. Further analysis of the data (Table 2) provided a thermodynamic association constant (K_a) for CoM binding of 2.65×10^5 M^{-1}, which corresponds to a K_d of 3.8 μM. By comparison, the true K_m for CoM, determined by the two-substrate kinetic analysis (Figure 3), was 33.6 μM. As shown in Table 2, CoM binding to holo-EaCoMT was driven by a large increase in enthalpy while the binding was entropically disfavored.

Zinc Requirement for CoM Binding. Zn-depleted EaCoMT was analyzed by ITC in order to define the importance of zinc in CoM binding. As shown in Table 2, when the binding of CoM was analyzed in terms of protein monomers, a stoichiometry of 0.22 CoM/protein monomer was obtained. When the data were reanalyzed on the basis of Zn, a stoichiometry of 0.97 CoM/Zn was obtained, and thermodynamic parameters in the range of those determined for the holoenzyme were obtained (Table 2).

Interaction of CoM with a Site-Directed Mutant of EaCoMT. EaCoMT contains the proposed "Zn-binding motif" first identified by Matthews and co-workers in their studies of MetE and also present in the CoM transferases (e.g., MT2-A) of the methanogenic archaea (*8*). This motif consists of a Cys residue and His residues separated by a single hydrophobic residue, followed by an additional Cys residue approximately 175 residues away (*8*). Biochemical and spectroscopic studies of MetE and MT2-A support the assignment of these amino acid residues as permanent zinc ligands, with the fourth coordination site being occupied by the exchangeable ligand (water or CoM) (*4*, *15*). For EaCoMT, the proposed permanent ligands in the primary sequence are His218, Cys220, and Cys341. Thus, as an initial step to defining the ligand environment of EaCoMT, we mutagenized Cys220 to Ala and purified and partially characterized the mutant enzyme.

The C220A mutant had a specific activity (0.007 unit/mg) that was only 0.06% of the wild-type enzyme, consistent with an important function for this residue. Metal analysis of the enzyme revealed the presence of 0.6 Zn/monomer. When CoM binding to the C220A mutant was analyzed by ITC, the mutant enzyme was found to bind 0.66 CoM/monomer, which translates to 1.09 CoM/Zn in the mutant enzyme (Table 2). The K_a value for CoM binding (Table 2) was an order of magnitude lower than for the wild-type enzyme, showing that the perturbed Zn environment in the mutant leads to decreased binding affinity for CoM.

Table 4: Binding and Thermodynamic Parameters for CoM and CoM Analogues[a]

ligand	K_a (M^{-1})	ΔH (kcal·mol^{-1})	$T\Delta S$ (kcal·mol^{-1})	ΔG (kcal·mol^{-1})
ethanethiol	$(3.05 \pm 0.52) \times 10^3$	−0.6 ± 0.2	4.2	−4.8
ethanesulfonate	$(2.50 \pm 0.10) \times 10^2$	−3.6 ± 0.2	−0.3	−3.3
propanethiol	$(1.18 \pm 0.18) \times 10^4$	−0.20 ± 0.01	5.4	−5.6
2-mercapto-propionate	BDL[b]	BDL	BDL	BDL
2-mercapto-ethanol	BDL	BDL	BDL	BDL
cysteine	BDL	BDL	BDL	BDL

[a] Thermodynamic parameters were determined by setting $n = 1$ as described in the text. Values represent the average of duplicate measurements. [b] BDL, below detectable limits; confident detectable limits for the ITC instrumentation required a K of $>10^2$.

Interaction of CoM Analogues with EaCoMT. Our success in using ITC to establish the importance of Zn in CoM binding by EaCoMT led us to investigate the structural determinants of CoM important to ligand binding and catalysis. For this analysis, a number of organic thiols with varying degrees of structural similarity to CoM were first investigated as possible substrates for EaCoMT. As shown in Table 3, EaCoMT was able to catalyze the conjugation of 3-mercaptopropionate, 2-mercaptoethanol, and cysteine to epoxypropane, although the specific rates of the transformations were at best 0.6% of the rate with CoM. Other organic thiols, including the important biomolecules glutathione, homocysteine, and lipoic acid, and the simple thiols, ethanethiol and thiocyanate, were not substrates for the enzyme.

When analyzed by ITC, only two of the organic thiols that were tested as alternate substrates (i.e., ethanethiol and propanethiol) were found to bind to EaCoMT with affinities above the confident detection limits of the instrumentation ($K_a > 10^2$; Table 4). Interestingly, neither of these compounds was found to be an alternate substrate for EaCoMT, while those that were (3-mercaptopropionate, 2-mercaptoethanol, and cysteine) must have sufficiently low association constants to make calorimetric analysis of their binding to EaCoMT impossible. To verify this, we evaluated the binding of 2-mercaptoethanol to EaCoMT by traditional means, i.e., by kinetically characterizing 2-mercaptoethanol as an inhibitor of EaCoMT. As shown in Figure 6, 2-mercaptoethanol was

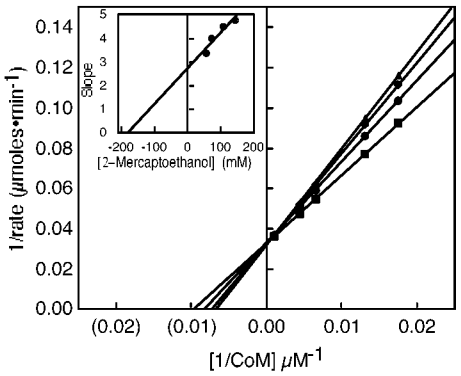


FIGURE 6: 2-Mercaptoethanol inhibition of epoxyalkane:CoM transferase activity. Double reciprocal plots of v vs [CoM] are presented for the following fixed concentrations of 2-mercaptoethanol: (■) 57.2 mM, (●) 71.5 mM, (◆) 107.3 mM, and (▲) 143 mM. The inset shows the slope replot of the data.

a very weak competitive inhibitor of the conjugation of CoM to epoxypropane, with a K_I value of 192 mM. Thus, while 2-mercaptoethanol is capable of substituting for CoM in the conjugation assay, its affinity is 5700 times lower than for CoM and too low to allow ITC quantification.

The striking differences in the affinities of CoM and 2-mercaptoethanol for EaCoMT suggest that the sulfonate moiety of CoM is an important determinant in organic thiol binding and catalysis. However, as shown in Table 2, the presence of zinc in the enzyme is the primary determinant in substrate binding, and on the basis of the model of thiol coordination to zinc (Scheme 1), the thiol should contribute most of the binding energy to the enzyme−CoM complex. To investigate this further, the binding of ethanesulfonate (CoM lacking the thiol group) was evaluated by ITC. Titration of ethanesulfonate into EaCoMT yielded thermograms that were above the confident detection limits of the instrumentation. To facilitate the evaluation of the experimental data, we evaluated the binding of both ethanethiol and ethanesulfonate by assuming that $n = 1$ for each compound, an assumption validated by the experimentally derived n value for CoM binding (Table 2).[2] When the data were thus analyzed, the thermodynamic parameters and associated standard errors reported in Table 4 were obtained.

The K_a for ethanethiol binding was about 100-fold lower than that for CoM and about 10-fold higher than for ethanesulfonate (Table 4). The binding of either compound occurred with much less enthalpy release than for CoM, and the binding of ethanethiol was actually entropically driven. Analysis of propanethiol binding to EaCoMT yielded a similar trend; i.e., the binding was entropically rather than enthalpically driven (Table 4).

Analysis of CoM Binding Determinants. An examination of the structure of CoM shows three potential groups that could contribute to binding: the thiol, sulfonate, and alkyl moieties (Figure 7). With thermodynamic data in hand for ethanesulfonate and ethanethiol binding to EaCoMT, we examined the respective contributions of the three groups to binding EaCoMT. For this analysis, the experimentally

[2] As described by Wiseman et al. (*22*), for calorimetric data near the edge of the K window, the deconvolution of isotherms is greatly simplified by constraining n to the expected value, in this case 1, and floating only K and ΔH. We have made this assumption for analyzing the isotherms of ethanesulfonate, ethanethiol, and propanethiol.

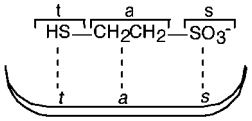


FIGURE 7: Model for CoM binding to EaCoMT. The binding of CoM is represented by association of the thiol (t), alkyl (a), and sulfonate (s) groups with corresponding binding sites (t', a', s') on the enzyme surface.

Table 5: CoM Thermodynamic Binding Determinants[a]

binding determinant	K_a (M^{-1})	ΔH (kcal·mol^{-1})	$T\Delta S$ (kcal·mol^{-1})	ΔG (kcal·mol^{-1})
HS (thiol)	1070	−21.0	−16.8	−4.2
SO_3^- (sulfonate)	86	−24.0	−21.3	−2.7
CH_2CH_2 (alkyl)	2.9	20.4	21.0	−0.6
total binding energy	2.65×10^5	−24.6	−17.1	−7.5

[a] Thermodynamic parameters were derived from the experimental data presented in Table 4 by the addition/subtraction of eqs 10−12 as described in the text. The values for total binding energy are the product (K_a) or sum (ΔH, $T\Delta S$, ΔG) of the individual determinants; these values are identical to those measured and reported in Table 2.

determined free energies of ligand binding were dissected into the potential contributions from the alkyl (a), thiol (t), and sulfonate (s) groups to the enzyme (E) according to the model of Figure 7 and as shown in eqs 10−12. Subtraction

model for CoM binding

$$E_{t'} + E_{a'} + E_{s'} + t + a + s \rightleftharpoons E_{t'}\cdot t + E_{a'}\cdot a + E_{s'}\cdot s \qquad \Delta G = -7.5 \text{ kcal/mol} \quad (10)$$

model for ethanesulfonate binding

$$E_{a'} + E_{s'} + a + s \rightleftharpoons E_{a'}\cdot a + E_{s'}\cdot s \qquad \Delta G = -3.3 \text{ kcal/mol} \quad (11)$$

model for ethanethiol binding

$$E_{t'} + E_{a'} + t + a \rightleftharpoons E_{t'}\cdot t + E_{a'}\cdot a \qquad \Delta G = -4.8 \text{ kcal/mol} \quad (12)$$

of eq 11 from eq 10 provides the theoretical free energy contribution of the thiol moiety to CoM binding, while subtraction of eq 12 from eq 10 provides the free energy contribution of the sulfonate:

$$E_{t'} + t \rightleftharpoons E_{t'}\cdot t \qquad \Delta G = -4.2 \text{ kcal/mol} \quad (13)$$

$$E_{s'} + s \rightleftharpoons E_{s'}\cdot s \qquad \Delta G = -2.7 \text{ kcal/mol} \quad (14)$$

The contribution of the alkyl moiety to binding can be calculated either from the subtraction of eq 13 from eq 12 or from subtraction of eq 14 from eq 11. As shown in eq 15, both analyses provide the same value of ΔG (−0.6 kcal/

$$E_{a'} + a \rightleftharpoons E_{a'}\cdot a \qquad \Delta G = -0.6 \text{ kcal/mol} \quad (15)$$

mol) for alkyl group binding, a result that enhances our confidence that the thermodynamic contributors to CoM binding can be accurately deduced from the calorimetric data obtained for CoM, ethanesulfonate, and ethanethiol. The complete set of derived thermodynamic constants for binding of CoM to EaCoMT is summarized in Table 5.

DISCUSSION

CoM is striking in its structural simplicity and narrow scope of reactions. To date, the only defined roles for this cofactor are as the methyl group carrier in archaeal methanogenesis (*1*) and as the nucleophile for epoxide ring opening and carrier of intermediates in bacterial alkene metabolism (*7*). While these functions are quite different, a conserved strategy is used to initiate CoM reaction, namely, its activation as a nucleophile for attack on an electrophilic substrate (eqs 1 and 2). The available biochemical, genetic, and spectroscopic data suggest that for both classes of CoM transferases Zn is the essential cofactor in activating CoM (*4*, *5*). Thus, CoM shares the common feature of Zn-mediated activation with other organic thiols that participate in nucleophilic displacement reactions (*28*).

The studies presented in this paper establish for the first time the requirement of Zn in activating CoM as a nucleophile for epoxide attack in this most recently defined example of an enzyme exhibiting epoxidase activity. The other class of epoxidase enzymes is the family of glutathione *S*-transferases, which exhibit a high degree of selectivity for glutathione as the nucleophile for epoxide attack (*16*). Glutathone *S*-transferases activate glutathione as a nucleophile via hydrogen-bonding interactions that lower the pK_a of glutathione (unperturbed pK_a = 9.0) (*16*). In contrast, the Zn cofactor of EaCoMT is apparently crucial to lowering the pK_a of the thiol of CoM: as determined in this work, the pK_a of the thiol of CoM is lowered from a value of 9.1 to 7.4 upon enzyme binding. A second ionizable group (pK_a = 9.2), believed to be the proton donor to the alkoxide in the transition state, is also essential to EaCoMT activity (Figure 4 and eqs 8 and 9).

While the turnover number of EaCoMT is modest (k_{cat} = 6.5/s^{-1}), the affinity of the enzyme for its substrates is quite impressive (K_m values of 1.8 and 34 μM, repectively). When k_{cat} is related to the epoxide substrate, a respectable catalytic efficiency is apparent (3.6×10^6 M^{-1} s^{-1}). To our knowledge, no GSTs exhibit such a high affinity for an epoxide substrate. This is not surprising, since GSTs are typically broad-substrate specificity detoxification enzymes that conjugate glutathione to a wide range of biomolecules and hence have low built-in affinity for a specific electrophile (*16*). The low K_m for epoxypropane exhibited by EaCoMT is probably of physiological significance, given that aliphatic epoxides are highly toxic and mutagenic alkylating agents that react abiotically with proteins and DNA to form covalent adducts (*29*). The low K_m for epoxypropane and high level of expression of EaCoMT in propylene-grown cell cultures [~4% of soluble cell protein (*6*)] correlate with our observation that epoxypropane never accumulates to detectable levels in cultures of *X. autotrophicus* growing with propylene as the carbon source. With regard to CoM, the intracellular concentration of this metabolite is not known, but concentrations as high as 5 μM have been shown to accumulate in the extracellular media of propylene-grown cultures (*17*). Thus, it is expected that the intracellular concentration is somewhat higher and in line with the K_m determined in these studies.

The calorimetric studies presented in this paper show that Zn is essential for the formation of a high-affinity E·CoM complex. For all three protein variants studied herein (holo, Zn-depleted, and C220A mutant) the stoichiometry of CoM binding correlated exactly with the Zn complement of the protein (Table 2). While the C220A mutant still bound CoM with a reasonably high affinity, the mutant was essentially catalytically inactive (0.06% of wild-type specific activity). These results highlight the importance of the proper Zn ligand environment in both binding of CoM and activation of bound CoM as a thiolate and nucleophile. As noted earlier, the "MetE-like Zn-binding motif" (**Cys**-X-**His**-X$_n$-**Cys**, with liganding amino acid residues in bold) is conseved among MetE homologues, putative MetE enzymes, and the methanogenic CoM transferases (*8*), suggesting that this particular combination of 2S and 1N ligands to the Zn center is crucial in lowering the pK_a of the substrate thiol ligand (homocysteine or CoM).

On the basis of the studies of CoM and analogue binding to wild-type EaCoMT, the coordination of the thiol group of CoM to Zn is predicted to contribute 4.2 kcal/mol of the total 7.5 kcal/mol of binding energy in the E·CoM binary complex. Turning to the other end of the molecule, the sulfonate is predicted to contribute an additional 2.7 kcal/mol of binding energy, while the ethyl linker contributes an additional 0.6 kcal/mol. As highlighted by the respective contributions of the functional groups to the overall K_a for E·CoM complex formation (Table 5), the contribution of the thiol to CoM binding is fully an order of magnitude greater than that of the sulfonate. Both thiol binding and sulfonate binding were entropically disfavored and enthalpically favored, while ethyl group binding was entropically favored (Table 5). The Zn−thiol interaction presumably is the major contributor to enthalpy release for thiol binding, while specific protein−sulfonate interactions, e.g., hydrogen bonding or ionic interactions with polar and/or charged amino acid residue(s), provide the enthalpy release associated with sulfonate binding.

Interestingly, the substitution of the sulfonate of CoM by a carboxylate (2-mercaptopropionate) or hydroxyl (2-mercaptoethanol) group resulted in sufficiently weak binding to EaCoMT that the interactions could not be confidently analyzed by ITC (Table 4). This contrasts the results of substitution of the sulfonate with a hydrogen (ethanethiol, as discussed above) or a methyl group (propanethiol), in which cases reliable binding data were obtained (Table 4). Thus, the carboxylate and hydroxyl groups are poor mimics of the sulfonate and actually interfere with high-affinity binding, a somewhat surprising result. These results are reinforced by our kinetic studies, in which we found that the inhibition constant for 2-mercaptoethanol was fully 5700 times greater than the K_m for CoM (Figure 6). A possible explanation for these observations is that the carboxylate or hydroxyl group competes with the thiol moeity for binding to the Zn center, resulting in some instances in the formation of a fundamentally different type of E−ligand complex. Alternatively, a nonproductive interaction of the hydroxyl or carboxylate with another portion of the enzyme may prevent formation of the proper Zn−thiol interaction.

While 2-mercaptopropionate and 2-mercaptoethanol were not high-affinity ligands for EaCoMT, they were, along with cysteine, poor substrates for the enzyme (Table 3). In contrast, ethanethiol and propanethiol, which formed reasonably high-affinity complexes with EaCoMT, exhibited no activity as substrates. These results suggest that mimics of

the sulfonate of CoM can increase reactivity of the thiol despite exhibiting an inhibitory effect on thiol binding. The sulfonate itself provides a huge acceleration of thiol reactivity over that observed for any of the alternative substrates (Table 3). Presumably, specific protein−sulfonate interactions contribute to increased reactivity by helping to orient CoM properly for nucleophilic attack on the epoxide substrate. Additionally, the electron-withdrawing sulfonate contributes to a lowering of the pK_a of the thiol of CoM relative to that of ethanethiol [unperturbed pK_a values of 9.1 and 10.5, respectively (*26*)], and this effect may contribute to increased reactivity as well. However, the pK_a of 2-mercaptoethanol (9.5) is similar to that of CoM while that of cysteine (8.3) is actually lower, demonstrating that it is not simply the inherent pK_a of the thiol that controls reactivity.

Concluding Remarks. In addition to providing a foundation of new information on the biochemical and kinetic properties and substrate specificity of EaCoMT, this work provides the first ever thermodynamic characterization of the interaction of CoM with an enzyme-bound Zn center. The structural simplicity of CoM, the availability of analogues thereof, and the ability to prepare Zn-depleted and site-directed mutant forms of EaCoMT make this an excellent model system for dissecting the thermodynamic contributors to enzyme−CoM binding. The studies presented here provide a framework for evaluating and interpreting spectroscopic and structural studies of EaCoMT that are currently in progress. We are hopeful that the methodologies and results herein will prove useful in parallel studies of the methanogenic CoM methyltransferases currently being conducted by other researchers (*4*, *5*).

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