Structural and Functional Studies Suggest a Catalytic Mechanism for the Phosphotransacetylase from *Methanosarcina thermophila*

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Phosphotransacetylase (EC 2.3.1.8) catalyzes reversible transfer of the acetyl group from acetyl phosphate to coenzyme A (CoA), forming acetyl-CoA and inorganic phosphate. Two crystal structures of phosphotransacetylase from the methanogenic archaeon *Methanosarcina thermophila* in complex with the substrate CoA revealed one CoA (CoA₁) bound in the proposed active site cleft and an additional CoA (CoA₂) bound at the periphery of the cleft. The results of isothermal titration calorimetry experiments are described, and they support the hypothesis that there are distinct high-affinity (equilibrium dissociation constant \( K_D = 20 \mu M \)) and low-affinity (\( K_D = 2 \mM \)) CoA binding sites. The crystal structures indicated that binding of CoA₁ is mediated by a series of hydrogen bonds and extensive van der Waals interactions with the enzyme and that there are fewer of these interactions between CoA₂ and the enzyme. Different conformations of the protein observed in the crystal structures suggest that domain movements which alter the geometry of the active site cleft may contribute to catalysis. Kinetic and calorimetric analyses of site-specific replacement variants indicated that there are catalytic roles for Ser³⁰⁹ and Arg³¹⁰, which are proximal to the reactive sulfhydryl of CoA. The reaction is hypothesized to proceed through base-catalyzed abstraction of the thiol proton of CoA by the adjacent and invariant residue Asp³¹⁶, followed by nucleophilic attack of the thiolate anion of CoA on the carbonyl carbon of acetyl phosphate. We propose that Arg³¹⁰ binds acetyl phosphate and orients it for optimal nucleophilic attack. The hypothesized mechanism proceeds through a negatively charged transition state stabilized by hydrogen bond donation from Ser³⁰⁹.

Acetate is an end product of the energy-yielding metabolism of nearly all fermentative microbes in the domain *Bacteria* in which acetyl coenzyme A (acetyl-CoA) is converted to acetate by phosphotransacetylase (Pta) (equation 1) and acetate kinase (equation 2) coupled to the synthesis of ATP. Acetate also is the growth substrate for methane-producing archaea. Thus, acetate is a major intermediate in the global carbon cycle, and acetate conversion to methane is responsible for the majority of biological methane production (7). In *Methanosarcina* species, Pta and acetate kinase function in the opposite direction to catalyze the ATP-dependent activation of acetate to acetyl-CoA for cleavage of the C—C bond of acetate by the carbon monoxide dehydrogenase/acetyl-CoA synthase, which releases the methyl group for eventual reduction to methane.

\[
\begin{align*}
\text{CH}_3\text{COS-CoA} + P_i & \rightarrow \text{CH}_3\text{COP}_2\text{O}^{2-} + \text{CoA} \quad (1) \\
\text{CH}_3\text{COP}_2\text{O}^{2-} + \text{ADP} & \rightarrow \text{CH}_3\text{COO}^- + \text{ATP} \quad (2)
\end{align*}
\]

The kinetic and catalytic mechanisms of acetate kinase are well characterized; however, Pta has been studied in considerably less detail, although the enzyme from the fermentative anaerobe *Clostridium kluyveri* was purified in the early 1950s (35). Kinetic analyses of Ptas from *C. kluyveri* and *Veillonella alcalescens* have suggested that rather than a ping-pong mechanism, the mechanism likely proceeds through formation of a ternary complex (20, 28). In a reexamination of the *C. kluyveri* Pta workers attempted to detect an acetyl-enzyme intermediate; however, no isotope exchange from labeled acetyl phosphate into either acetyl-CoA or inorganic phosphate was observed in the absence of free CoA, and attempts to isolate an acetyl-Pta intermediate were unsuccessful, which is consistent with a ternary complex mechanism (9). Although numerous genetic and physiological studies have continued to demonstrate the universal function of Pta in acetate metabolism in diverse microbes (1, 4, 6, 8, 15, 17, 24, 25, 27, 30, 32, 38), mechanistic analyses of the enzyme were abandoned until there was an investigation of Pta from the archaeon *Methanosarcina thermophila*, which obtains energy for growth by converting acetate to methane (22).

Cloning of the gene and heterologous expression of Pta from *M. thermophila* allowed the large-scale production of protein required for structural studies, biochemical analyses, and the use of site-specific replacement to analyze the function of specific residues (22). Cys³¹² was predicted to be present in the active site, although it is not essential for catalysis (31). Arg⁸⁷ and Arg³³³ were proposed to interact with the 3' and 5' phosphate groups of CoA, respectively (12), while Arg³¹⁰ was found to be essential for catalysis, although its role was not defined further (31). In spite of the insight gained from the site-specific replacement studies, key questions remained. The architecture of the active site was unknown, and, other than the residues

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listed above, our understanding of interactions between the enzyme and the substrates was incomplete.

The crystal structure of the *M. thermophila* enzyme was the first structure to be solved for a Pta from any organism and remains one of only two examples published to date (13, 42); however, neither of the structures that have been determined contains bound substrates. In the current study we closed this gap by incorporating information deduced from two crystal structures in complex with CoA together with kinetic analyses of site-specific replacement variants in order to propose a catalytic mechanism.

### MATERIALS AND METHODS

**Materials.** CoA-Li salt (yeast), acetyl-CoA-Li salt (enzymatically prepared), and acetyl phosphate-Li salt were purchased from Sigma-Aldrich (St. Louis, MO). Diithiothreitol, NH₄Cl, (NH₄)₂SO₄, HEPES, KCl, KHPO₄, Tris base, and isopropyl-β-D-thiogalactopyranosidase (IPTG) were purchased from Fisher Scientific Company (Newark, DE) and were the highest grade available. *Escherichia coli* (BL21(DE3)) cells were purchased from Novagen Inc. (Madison, WI). All chromatographic resins and supports were purchased from Amersham Biosciences Corporation (Piscataway, NJ).

**Site-directed mutagenesis.** Mutagenesis was performed by the oligonucleotide-directed in vitro mutagenesis method (18), using a QuikChange mutagenesis kit (Stratagene) according to the manufacturer's instructions. Plasmid pML702 (22), a derivative of the expression vector pT7-7 (36) containing the *M. ther- mopila* *pta* gene, was the target for mutagenesis using the primers listed in Table 1. Mutations were verified by dye termination cycle sequencing using an ABI PRISM 377 DNA sequencer (Applied Biosystems) at the Nucleic Acid Facility at Pennsylvania State University.

**Heterologous expression and purification of Pta.** *E. coli* BL21(DE3) cells transformed with wild-type or mutated plasmid pML702 were grown to an *A₅₆₂₅* of 0.6 at 37°C with shaking in LB medium containing 100 μg/ml ampicillin. The temperature was reduced to 15°C, and IPTG was added to a final concentration of 1 mM, after which incubation was continued for 12 to 16 h. Cells were harvested by centrifugation, resuspended in 25 mM Tris-HCl (pH 7.2) containing 180 mM KCl at a ratio of 1 g cells/ml, and disrupted by two passages through a French pressure cell (4°C, 20,000 lb/in²). The lysate was clarified by centrifugation (75,000 × g, 2 h), brought to 45% (NH₄)₂SO₄ saturation by slow addition of (NH₄)₂SO₄, and stirred overnight at 4°C. The solution was centrifuged (75,000 × g, 2 h), and the supernatant was applied to a butyl Sepharose column equilibrated with 50 mM HEPES buffer (pH 7.5). The crystals were rapidly transferred into mother liquor containing 5% and 10% glycerol and then cryocooled in liquid N₂. These crystals were soaked with 5 mM CoA overnight, and the resulting complex crystals were transferred into formbin oil prior to cryocooling in liquid nitrogen. Several crystals were tested for diffraction, and all of them belonged to the I4₁4₁4₁ space group. The structure was solved by molecular replacement with the program MOLREP (37) by using apo-PTAs (CD dimer of PDB entry 1QZ7) as the search model. Refinement, including TLS refinement, was carried out with REFMAC (26) and was completed by manual addition of three CoA molecules and two sulfate molecules and automated addition of solvent molecules with ARP (29). Subse- quently, Pta at a concentration of 5 mg/ml was cocrystallized in the presence of 5 mM CoA against a reservoir solution containing 1.1 M sodium citrate and 0.1 M HEPES buffer (pH 7.5). The crystals were rapidly transferred into mother liquor containing 5% and 10% glycerol and then cryocooled in liquid N₂. These crystals belonged to the space group with closely related unit cell dimensions. A data set with 2.15-A˚ resolution was collected from one crystal at beam line X26C at the National Synchrotron Light Source (NSLS) at a wavelength of 1.1 Å with a Quantum IV ADSC charge-coupled device detector. The structure was solved by molecular replacement with the program MOLREP (37) by using apo-PTA (CD dimer of PDB entry 1QZ7) as the search model. Refinement, including TLS refinement, was carried out with REFMAC (26) and was completed by manual addition of three CoA molecules and two sulfate molecules and automated addition of solvent molecules with ARP (29).

**Analytical ultracentrifugation.** Sedimentation equilibrium measurements were obtained using a Beckman Optima XL-A analytical ultracentrifuge equipped with a four-sector rotor with standard six-channel cells. Experiments were performed at 20°C at a speed of 13,500 rpm, and the absorbance-versus-radius distributions were recorded at 280 nm. Samples were considered to have reached equilibrium when scans taken 4 h apart were identical. The partial specific volume of Pta calculated with the Nonlin analysis program (15) using a nonlinear least-squares curve-fitting regression.

**Data collection and structure determination.** Pta was crystallized initially as previously described using the hanging drop vapor diffusion method (14). Pta crystals were soaked with 5 mM CoA overnight, and the resulting complex crystals were transferred into formbin oil prior to cryocooling in liquid nitrogen. Several crystals were tested for diffraction, and all of them belonged to the I4₁ space group. The structure was solved by molecular replacement with the program MOLREP (37) by using apo-PTA (CD dimer of PDB entry 1QZ7) as the search model. Refinement, including TLS refinement, was carried out with REFMAC (26) and was completed by manual addition of three CoA molecules and two sulfate molecules and automated addition of solvent molecules with ARP (29).

**Pta activity assay.** The rate of the reaction was determined at 23°C by monitoring the change in absorbance at 233 nm concomitant with formation of the thioester bond of acetyl-CoA (ε = 4,360 M⁻¹ cm⁻¹) using a 0.1-cm-path-length quartz cuvette in a Hewlett-Packard 8455A diode array spectrophotometer. The stan- dard reaction mixture (200 μl) contained 50 mM Tris-HCl (pH 7.2), 20 mM NH₄Cl, 20 mM KCl, 2 mM diithiothreitol, 0.05 μg/ml Pta, and the appropriate substrate for the experiment. Reactions were initiated by addition of the second substrate.

### TABLE 1. Mutagenic oligonucleotide primers

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primer sequences*</th>
</tr>
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<tbody>
<tr>
<td>Ser³⁰⁹Ala</td>
<td>5’ CCA ATT AAC GAT CGT GCC AGA GGC TGC AGC GAC 3’</td>
</tr>
<tr>
<td>Ser³⁰⁹Cys</td>
<td>5’ CCA ATT AAC GAT CGT TGC AGA GGC TGC AGC GAC 3’</td>
</tr>
<tr>
<td>Ser³⁰⁹Thr</td>
<td>5’ CCA ATT AAC GAT CGT ACC AGA GGC TGC AGC GAC 3’</td>
</tr>
<tr>
<td>Arg³¹⁰Ala</td>
<td>5’ CCA ATT AAC GAT CGT TCC GCC GGC TGC AGC GAC 3’</td>
</tr>
<tr>
<td>Arg³¹⁰Gln</td>
<td>5’ CCA ATT AAC GAT CGT TCC GAG GGC TGC AGC GAC 3’</td>
</tr>
<tr>
<td>Arg³¹⁰Lys</td>
<td>5’ CCA ATT AAC GAT CGT TCC AAA GGC TGC AGC GAC 3’</td>
</tr>
<tr>
<td>Asp³¹⁶Glu</td>
<td>5’ GCC TGC AGC GAC GAA GAA ATT GTC GGT GCC GTT 3’</td>
</tr>
<tr>
<td></td>
<td>5’ AAC GGC ACC GAC AAT TTC TTC GTC GCT GCC GAC 3’</td>
</tr>
</tbody>
</table>

* The mutated codon in each primer is indicated by boldface type.

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Isothermal titration calorimetry. Prior to each experiment a 2-ml aliquot of Pta was dialyzed against 4 liters of filtered buffer containing 50 mM Tris-Cl (pH 7.2), 20 mM KCl, and 20 mM NH₄Cl at 4°C overnight. The dialysis buffer was subsequently used as the diluent to prepare the ligand solution for titration. The cell volume was 1.42 ml, and the syringe volume was 300 μl. To analyze CoA binding to wild-type Pta, CoA was titrated in 59 5-μl injections into Pta concentrations ranging from 30 to 100 μM to obtain final molar ratios of CoA to Pta ranging from 2:1 to 4:1 at the end of the experiment. Three experiments were performed with Pta and CoA at 30°C. Blank injections of CoA into buffer were performed to estimate the heat of injection, mixing, and dilution, and the results revealed that the heat effects were less than 0.5% of the Pta-CoA binding heat.

To analyze acetyl phosphate binding to wild-type and variant Ptas, protein and ligand samples were prepared as described above, and each titration was performed in duplicate at 25°C. The ligand was titrated into the samples as follows: 630 μM acetyl phosphate in 59 5-μl injections into 90 μM wild-type Pta, 5 mM acetyl phosphate in 30 × 10-μl injections into Arr310Gln, 650 μM acetyl phosphate in 59 5-μl injections into Arr310Gln, and 1 mM acetyl phosphate in 59 5-μl injections into 70 μM Arr310Lys. Blank injections of acetyl phosphate into buffer were performed to estimate the heat of injection, mixing, and dilution, and the results revealed that the heat effects were approximately 5% of the Pta-acetyl phosphate binding heat, which were subtracted from the baseline for each titration curve. For every injection the binding enthalpy (kcal/mol of injectant) was calculated by integration of the peak area using the ORIGIN software provided by Microcal. The binding enthalpy was plotted as a function of the molar ratio and was fitted to equations describing a single binding site for acetyl phosphate or two binding sites. The binding enthalpy was fitted to equations describing a single binding site for acetyl phosphate or two binding sites.

Table 2. Data collection statistics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Soaked structure</th>
<th>Co-crystallized structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
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<td>1.1</td>
</tr>
<tr>
<td>Resolution limits (Å)</td>
<td>50–2.7</td>
<td>50–2.15</td>
</tr>
<tr>
<td>Unit cell dimensions (Å)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>114.5</td>
<td>116.5</td>
</tr>
<tr>
<td>c</td>
<td>127.8</td>
<td>127.5</td>
</tr>
<tr>
<td>Completeness</td>
<td>0.950 (0.967)</td>
<td>0.984 (0.991)</td>
</tr>
<tr>
<td>Mean redundancy</td>
<td>4.48</td>
<td>5.81</td>
</tr>
<tr>
<td>R_mer</td>
<td>0.052 (0.379)</td>
<td>0.065 (0.444)</td>
</tr>
<tr>
<td>(I/σ(I))</td>
<td>24.2 (3.1)</td>
<td>28.7 (2.6)</td>
</tr>
</tbody>
</table>

* R_mer = Σ_hkl |Fo| - |Fc| |Σ_hkl|Fo|, where I is the ith measurement and J is the weighted mean of all measurements of J. (I/σ(I)) indicates the average intensity divided by the standard deviation. The numbers in parentheses are the highest resolution data shell in each dataset.

**RESULTS AND DISCUSSION**

Gross architecture of Pta-CoA complexes. Crystals of Pta with bound CoA were obtained by soaking apo-Pta crystals with CoA and also by co-crystallization with CoA (Tables 2 and 3). The structure of the Pta-CoA complex obtained by soaking (PDB identifier 2AF3) was refined at a resolution of 2.7 Å to an R-factor of 0.215 (R_free, 0.290). The crystals belonged to space group I4₁, and contained one dimer per unit cell (with subunits referred to as monomers A and B). The crystals of the previously described M. thermophila apo-Pta structure (13) belong to space group P4₁, and contain two dimers per unit cell (with subunits referred to as monomers A-B and C-D). The Cα trace of each monomer of the soaked structure revealed two α/β/α domains; residues 1 to 144 and 301 to 333 form domain I, while domain II is composed of residues 145 to 300. This structure is identical to the previously described apo-Pta structure (Fig. 1A). The two domains are arranged side by side and form an almost continuous β-sheet. This arrangement creates a prominent cleft between the two domains, which, based on sequence conservation and kinetic analysis of site-directed variants, was previously proposed to contain the active site (13). Domain II is responsible for dimerization, and relative to this core, the orientations of domain I in the A-B dimer of the apo-Pta structure have been reported to be in open (monomer A) and closed (monomer B) conformations that differ by a 20° rotation of domain I relative to domain II. Both monomers of the C-D dimer of the apo-Pta structure are in an intermediate conformation (not shown). Superimposition of the monomers in the soaked structure reported here revealed that they differ by an 8° rotation of domain I, resulting in a root mean square (RMS) deviation of 1.05 Å (not shown). This shift in domain I leads to differences in the geometry of the proposed active site cleft between the two domains, which result in an open conformation for monomer A and a partially closed conformation for monomer B (Fig. 1B). Monomer B of the soaked structure exhibited significantly higher B-factors (114.1 Å² for monomer B, compared with 63.6 Å² for monomer A), and long stretches in domain I of monomer B appear to be highly flexible based on the poor quality of the electron density maps.

Crystals of Pta with CoA bound were also obtained by co-crystallization (PDB identifier 2AF4), and these crystals belong to the same I4₁ space group as the soaked structure, with only slightly modified unit cell dimensions (Table 2). The co-crystallized structure was refined at a resolution of 2.15 Å to an R-factor of 0.203 (R_free, 0.272). Compared to the soaked structure, the two monomers are more similar to each other in this structure, as shown by an overall RMS deviation of 0.8 Å for the Cα atoms of monomers A and B and RMS deviations for the Cα atoms of residues in domains I (residues 3 to 140) and
II (residues 151 to 300) of 0.64 Å and 0.32 Å, respectively. As a consequence, the cleft between domains I and II has similar dimensions in the two monomers, and both monomers are in the open conformation, similar to monomer A of the apo structure (not shown). As in the soaked structure, domain I of monomer B of the cocrystallized structure exhibits considerable flexibility, as shown by high B-factors (110.4 Å² for domain I and 91.4 Å² for the entire monomer B, compared to 75.8 Å² for domain II of monomer B and 65.0 Å² for the entire monomer A) and correspondingly poorer electron density for monomer B (especially domain I) compared to monomer A, despite the higher resolution.

In the report of the initial purification and characterization of *M. thermophila* Pta, the authors concluded that the enzyme existed in solution as a monomer (24); however, Pta was found to be a dimer in the previously reported apo-Pta crystal structures (13, 42) and both structures described here. Therefore, the oligomeric state of the enzyme in solution was reexamined. The calculated molecular mass of the Pta monomer is 35,198 Da, and the enzyme migrated in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel as a single band with an apparent mass of 36 kDa (not shown). Wild-type Pta and the variants described in this paper consistently eluted from a size exclusion column in a single symmetrical peak corresponding to a molecular mass of 70.7 kDa, approximately twice the calculated molecular mass of the Pta monomer. The apparent dimerization of wild-type Pta was investigated further using sedimentation equilibrium analytical ultracentrifugation. The sedimentation data fit a single-species model with no evidence of sample aggregation (not shown). Analysis yielded an apparent molecular mass of 69.5 kDa, which corresponds well to the molecular mass revealed by size exclusion chromatography (70.7 kDa). Thus, we concluded that *M. thermophila* Pta is a dimer in solution, which is consistent with the dimeric states reported for the Ptas from *V. alcalescens*, *E. coli*, and *Streptococcus pyogenes* (28, 33, 42).

Active site analysis of the Pta-CoA complexes. The cocrystallized structure revealed one CoA molecule bound per monomer located in the interdomain cleft that was previously predicted (13) to contain the active site (Fig. 2A). The CoA molecule is contacted by several residues that were determined to be conserved or type conserved (Table 4) by alignment of 32 Pta sequences (13), and the CoA in this site is referred to as CoA¹. Figure 2B shows CoA¹ bound to monomer A of the cocrystallized structure, and the CoA¹ bound to monomer B was positioned similarly (not shown). When we considered only distances of ≤3.5 Å, we found that 13 direct hydrogen bonds are formed between the protein and CoA¹ in monomers A and B (Table 4 and Fig. 2B). Of these 13 hydrogen bonds, 11 are present in both monomers (Table 4). In addition, there is a slightly longer polar interaction between the sulfur of Met¹⁷⁴ and N-6 of the adenine ring. The electron density appears to be weak in the vicinity of the amide group proximal to the sulfur of CoA, presumably due to a lack of interactions between the enzyme and this region of the cofactor (Fig. 2C). In addition, the sulfur atom of CoA¹ is located 2 Å from the side chain of Cys³¹², and it is covalently linked to this side chain via a disulfide bridge (Fig. 2B). The existence of this linkage is considered a crystallization artifact resulting from cocrystallization under nonreducing conditions. Finally, two water molecules mediate interactions between CoA¹ and the enzyme (Fig. 2B). One of the water molecules forms hydrogen bonds to N-1 of the adenine ring and also to Asn²⁰⁶ and Asp³⁰⁷. The other water molecule forms hydrogen bonds to the second amide oxygen of the pantetheine moiety and also to Lys²⁸³, Gln²⁸⁶, and Arg²⁸⁷. Three cis peptides involving residue pairs Ala¹⁷⁰-Asp¹⁷¹, Gln²⁴¹-Glu²⁴², and Gly²⁹⁵-Pro²⁹⁶ could be identified in this structure, and the cis peptide involving Gly²⁹⁵-Pro²⁹⁶ was already known from the apo structure (13). The cocrystallized Pta-CoA complex revealed that the Gly²⁹⁵-Pro²⁹⁶ pair forms part of the CoA¹ binding pocket and that a trans peptide in this position would lead to steric interference with the pantetheine of the bound CoA¹ (not shown). In addition, this cis peptide appears to be important for CoA¹ binding.
as both the backbone O and N of Gly295 form hydrogen bonds to the pantetheine of CoA1 (Table 4). In contrast, the other cis peptides are distant (11 and 27 Å, respectively) from the active site.

Surprisingly, the soaked structure revealed that three CoA molecules are bound per Pta dimer. Two CoA molecules are bound to monomer A (Fig. 1A and 3A), and one is bound to monomer B (not shown); one binding site is common to both monomers. The common binding site is different from the CoA1 site in the cocrystallized structure and is referred to as the CoA2 site below. The CoA2 site is located near the entrance to the interdomain cleft (Fig. 3A) in a region where significant positive surface potential is accumulated. In monomer A of the soaked structure, the position of the other CoA is similar to the position of CoA1 in the cocrystallized structure; however, in monomer B of the soaked structure, there is insufficient space for CoA binding to the CoA1 site (Fig. 1B). This is due to movement of domain I, particularly the side chain of Ser307, which prevents CoA1 binding by sterically interfering with the N-3, C-4, C-5, C-6, and N-6 atoms of CoA1.

The interactions between CoA1 and Pta are closely related in the two structures (Table 4), but they are somewhat more extensive in the soaked structure. Applying a 3.5-Å cutoff resulted in 16 direct hydrogen bonds between CoA1 and the protein. In addition, the slightly longer polar interaction involving the sulfur of Met174 and N-6 of the adenine ring observed in the cocrystallized structure is also present. One key difference is that the disulfide linkage between CoA1 and Cys312 in monomer A of the cocrystallized structure is replaced by a hydrogen bond between the pantetheine sulfur and the backbone N of Ser309 in the soaked structure (not shown).

In the soaked structure, CoA1 has an average B-factor of 82.8 Å², while CoA2 exhibits considerably higher conformational flexibility (average B factor, 100.8 Å²). As judged by the number of contacts with the enzyme, the CoA2 molecule does not interact with Pta as tightly as CoA1, although there are significant interactions between Pta and CoA2 (Fig. 3B). The 3′/H11032 phosphate group of CoA2 has ionic and hydrogen-bonded interactions with Arg87, while the α-phosphate of the pyrophosphate linkage is stabilized by electrostatic interactions with Arg133, but at a distance that is too great for the formation of hydrogen bonds (Fig. 3B). The interactions observed for Arg87 and Arg133 are in excellent agreement with kinetic analyses of site-specific variants, which predicted that Arg87 interacts with the 3′ phosphate and Arg133 interacts with one of the 5′ phosphates (12). Atoms N-1 and N-6 of the adenine base in CoA2 were observed to hydrogen bond to Ala148 in both monomers, and these interactions were the only hydrogen bond interactions observed between CoA2 and monomer B. N-6 is also close (~4.2 and 4.8 Å in monomers A and B, respectively) to the S atom of Met174, the same residue which also interacts with N-6 of CoA1. In monomer A, O-7 and O-8 of the 3′ phosphate hydrogen bond to Arg87, and the pantetheine sulfur hydrogen bonds with the backbone N and Oe1 of Glu176. The

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**FIG. 2.** Structural features of Pta cocrystallized with CoA. (A) Structure of the Pta dimer. Monomer A is shown in a ribbon representation; α-helices are red, β-strands are yellow, and loops are gray. Monomer B is shown in a surface representation; domain I is violet, and domain II is slate blue. The CoA molecules are shown in a van der Waals representation in monomer A and in a stick representation in monomer B and are indicated by arrows. The view is approximately along the twofold axis of the dimer. (B) Stereo diagram of the CoA1 binding site of monomer A. Monomers A and B (green and yellow, respectively) are shown in a loop representation, and residues involved in hydrogen-bonded interactions with the CoA are labeled. In addition, the proposed catalytic residues Arg310 and Asp316, as well as Cys312, which forms the disulfide linkage with CoA, are labeled. Residues Phe4, Leu5, Tyr294, Ile297, and Ile323 (proposed to interact with the methyl group of acetyl phosphate) are near the top and do not have labels. Hydrogen bonds are indicated by dashed lines. Two water molecules, which mediate interactions between the protein and the cofactor, are indicated by red spheres. (C) Omit electron density map of bound CoA1. The difference omit map is red at a contour level of three times the RMS deviation. Weak density is apparent in the vicinity of the amide group proximal to the sulfur of CoA, presumably due to

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Waals interactions between CoA₂ and nearby residues, which mobility of this moiety. However, there are multiple van der either Arg87 or Arg133 led to a considerable increase in the relevance for the enzyme since site-specific replacement of is that these two residues preorient the CoA molecule before for CoA (12). One possible explanation for this observation of accessible surface area is buried in the complex. In the case that the high B-factors for CoA₂ are largely attributable to the lack of hydrogen bonding to the pantetheine moiety suggests contribution to binding, which is reflected by the fact that 780 Å² that CoA₁ is bound more tightly to the enzyme than CoA₂. Despite by negligible entropic contributions. Based on interactions ob-

The observation of two CoA binding sites in the soaked structure was unexpected; thus, the binding of CoA to Pta in solution was examined by isothermal titration calorimetry (Fig. 4, top panel). The experimental data could not be modeled with a single binding site (Fig. 4, middle panel); however, a significant improvement in the data fit was obtained by assuming that there are two sequential and independent binding sites (Fig. 4, bottom panel). One binding site was characterized by a dissociation constant of ~20 μM and an associated enthalpy of about 10 kcal/mol. The other site displayed a dissociation constant of approximately 1 mM, but the enthalpy could not be accurately determined. Both binding events were characterized by negligible entropic contributions. Based on interactions observed in the cocrystallized and soaked structures, it can be assumed that the higher-affinity binding site corresponds to CoA₁ and the lower-affinity site corresponds to CoA₂. Despite its lower binding affinity, the CoA₂ site must have functional relevance for the enzyme since site-specific replacement of either Arg⁸⁷ or Arg¹³³ led to a considerable increase in the \( K_a \) for CoA (12). One possible explanation for this observation is that these two residues preorient the CoA molecule before it is transferred into the high-affinity binding site, where catalysis occurs. Analysis of the CoA₁ and CoA₂ sites suggests that CoA₁ binds at the site of catalysis; thus, for further mechanistic interpretations we focused on the CoA₁ position.

The identification of CoA₁ as the catalytically relevant site is based on several convergent lines of reasoning. The CoA₁ site is occupied in at least one monomer of both structures described here, and CoA₁ interacts more extensively with Pta than CoA₂ interacts with Pta. Furthermore, the CoA₁ site is located in the most highly conserved region of the proposed active site cleft (13). Although CoA₂ interacts with Arg⁸⁷ and Arg¹³³, residues previously shown to be important for binding CoA (12), the reactive sulfhydryl of CoA₂ is pointed out to-ward the solvent (Fig. 3B). The reactive sulfhydryl group of CoA₁, however, is located in the active site cleft proximal to residues either implicated in catalysis or predicted to be present in the active site. The guanidino group of Arg³¹⁰ is located 5 Å and 8 Å from the 3’ phosphate of CoA₁ in the cocrystallized and soaked structures (Fig. 5). In the soaked structure, Arg³¹⁰ was found to coordinate a sulfate ion originating from the mother liquor. The interaction with the sulfate ion in the soaked structure orients Arg³¹⁰ differently relative to its position in the cocrystallized structure, demonstrating the mobility of this residue. In a previous study, replacement of Arg³¹⁰ with a glutamine was found to decrease the kₗₐₜ₅ₐ₆-, showing that Arg₃¹⁰ plays a crucial role in catalysis (31). Cys³¹², which is located 2 Å and 3.5 Å from the —SH of CoA₁ in the cocrystallized and soaked structures, was predicted to be present in the active site based on modification of this residue and inhibition of activity by N-ethylmaleimide, which could be alleviated by preincubation with substrates (31). This result is also consistent with previous reports that the activity of other Ptas can be affected by thiol-

### Table 4. Hydrogen bonds between CoA₁ and residues in the Pta-CoA complexes obtained by cocrystallization and soaking

<table>
<thead>
<tr>
<th>CoA moiety</th>
<th>CoA atom</th>
<th>Residue</th>
<th>Residue atom</th>
<th>Monomer A</th>
<th>Monomer A</th>
<th>Monomer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine ring</td>
<td>N-1</td>
<td>Thr⁴⁰⁸</td>
<td>Oy1</td>
<td>3.4</td>
<td>3.6</td>
<td>3.6</td>
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<tr>
<td></td>
<td>N-3</td>
<td>Asp⁴⁰⁷</td>
<td>O8</td>
<td>3.2</td>
<td>3.3</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>N-6</td>
<td>Thr⁴⁰⁸</td>
<td>Oy1</td>
<td>2.8</td>
<td>2.6</td>
<td>3.0</td>
</tr>
<tr>
<td>α-Phosphate</td>
<td>O-1</td>
<td>Asn⁴⁰⁷</td>
<td>N82</td>
<td>3.4</td>
<td>2.6</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>O-1</td>
<td>Gln⁴⁰⁴b,d</td>
<td>Ne2</td>
<td>3.4</td>
<td>3.2</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>O-2</td>
<td>Tyr⁴⁰⁶b</td>
<td>OH</td>
<td>2.6</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>β-Phosphate</td>
<td>O-4</td>
<td>Lys⁴⁰⁸c</td>
<td>Nε</td>
<td>3.0</td>
<td>2.7</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>O-5</td>
<td>Lys⁴⁰⁸c</td>
<td>Nε</td>
<td>2.6</td>
<td>2.7</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>O-5</td>
<td>Lys⁴⁰⁸c,d</td>
<td>Nε</td>
<td>3.0</td>
<td>2.7</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>O-5</td>
<td>Gln⁴⁰⁴b,d</td>
<td>Ne2</td>
<td>4.0</td>
<td>3.5</td>
<td>3.3</td>
</tr>
<tr>
<td>3’ Phosphate</td>
<td>O-8</td>
<td>Ser⁴³⁰b</td>
<td>Oy</td>
<td>2.9</td>
<td>4.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Ribose</td>
<td>O-2’</td>
<td>Ser⁴³⁰b</td>
<td>Oy</td>
<td>2.9</td>
<td>3.6</td>
<td>2.3</td>
</tr>
<tr>
<td>Second amide nitrogen of pantetheine</td>
<td>N-8</td>
<td>Gly⁴⁰⁵</td>
<td>O</td>
<td>5.4</td>
<td>3.1</td>
<td>2.9</td>
</tr>
<tr>
<td>First amide oxygen of pantetheine</td>
<td>O-5</td>
<td>Gly⁴⁰⁵</td>
<td>N</td>
<td>3.1</td>
<td>3.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Second amide oxygen of pantetheine</td>
<td>O-9</td>
<td>Gln⁴⁰⁴b</td>
<td>Ne2</td>
<td>3.1</td>
<td>3.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Hydroyl of pantetheine</td>
<td>O-10</td>
<td>Tyr⁴⁰⁶b</td>
<td>OH</td>
<td>2.9</td>
<td>3.7</td>
<td>3.8</td>
</tr>
<tr>
<td>Sulfur of pantetheine</td>
<td>S-1</td>
<td>Ser⁴³⁰a</td>
<td>N</td>
<td>3.0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>S-1</td>
<td>Cys³¹²</td>
<td>S</td>
<td>NA</td>
<td>2.0f</td>
<td>2.0f</td>
</tr>
</tbody>
</table>

- Distance that are ≤3.5 Å are considered hydrogen bonds.
- Type-conserved residue.
- Strictly conserved residue.
- Residue originating from adjacent monomer.
- NA, not applicable.
- The sulfur of pantetheine is covalently linked via a disulfide bridge to Cys³¹², an artifact resulting from nonreducing cocrystallization conditions.
modifying reagents (9, 34). These results indicate that CoA binds at the probable catalytic site, and based on a closer inspection of the enzyme architecture surrounding CoA we identified Ser309 and Asp316 in addition to Arg310 as potential catalytic residues to target for functional analyses (Fig. 5).

**Proposed role for Arg310.** Arg310 is strictly conserved in an alignment of 32 Pta sequences (13), and it has been proposed that this residue plays a role in catalysis based on kinetic analyses of an Arg310Gln variant (31). Furthermore, different orientations of the side chain observed in the structures presented here suggest that its conformational flexibility may play a role in positioning one or both substrates. To address the function of Arg310, the kinetic parameters of Arg310Ala, Arg310Gln, and Arg310Lys variants were determined. The variants had \( k_{cat} \) values that were lower than the value for wild-type Pta (Table 5), further supporting the hypothesis that this residue has a catalytic role. A previous mechanistic analysis of the *C. kluyveri* Pta suggested that a residue with a pKa of >9 may polarize the carbonyl group of acetyl phosphate and make it more susceptible to nucleophilic attack (19). This is one potential role for Arg310, and in this case a lysine residue should be able to substitute for this function; however, the Arg310Lys variant had the lowest \( k_{cat} \). Another possible function for Arg310 is to orient one or both substrates for optimal nucleophilic attack via bidentate interactions with the phosphate groups. If Arg310 also has this function, the greater steric bulk of glutamine or lysine compared to alanine may have interfered with the proper positioning and could account for the more profound decreases in \( k_{cat} \) observed for the Arg310Gln and Arg310Lys variants. If the positively charged Arg310 guanidino group binds the phosphate groups of either substrate in a bidentate manner, the
positive charge at position 310 in the Arg\textsuperscript{310}Lys variant may substitute for binding but force the substrate into a catalytically incompetent orientation, explaining why this variant had the lowest $k_{\text{cat}}$.

Although the side chain of Arg\textsuperscript{310} is near the 3′ phosphate of CoA\textsuperscript{1} (Fig. 5), only modest increases in the $K_m$ values for CoA were observed for each of the variants, arguing against a role for Arg\textsuperscript{310} in the interaction with CoA. The $K_m$ values for acetyl phosphate, however, were greater than the value for the wild type (Table 5), which is consistent with a role for Arg\textsuperscript{310} in the interaction with this substrate. Therefore, the binding of acetyl phosphate to wild-type Pta and Arg\textsuperscript{310} variants was examined by isothermal titration calorimetry. The titration curve for each enzyme fit an equation describing a single binding site per monomer (Fig. 6). The corresponding $K_D$ values were very similar to the $K_m$ values and indicated that the binding of acetyl phosphate to Pta is an enthalpically driven process with minimal entropic contributions (Table 6). The binding to wild-type Pta was predictably the most energetically favorable binding ($\Delta G = -5.1$ kcal mol\textsuperscript{-1}), followed by binding to the Arg\textsuperscript{310}Gln variant ($\Delta G = -4.3$ kcal mol\textsuperscript{-1}) and the Arg\textsuperscript{310}Lys variant ($\Delta G = -4.1$ kcal mol\textsuperscript{-1}).

Binding of acetyl phosphate to Arg\textsuperscript{310}Ala could not be detected by isothermal titration calorimetry, consistent with the large $K_m$ value observed for this variant. The profound increase in the $K_m$ for acetyl phosphate and the inability to calorimetrically detect acetyl phosphate binding to the Arg\textsuperscript{310}Ala variant support the hypothesis that Arg\textsuperscript{310} has a role in binding this substrate. The $K_m$ for acetyl phosphate observed for the Arg\textsuperscript{310}Lys variant was 42-fold lower than the $K_m$ for the Arg\textsuperscript{310}Ala variant, implying that a positive charge at this position is important for acetyl phosphate binding. However, the Arg\textsuperscript{310}Lys variant had $K_m$ and $K_D$ values for acetyl phosphate that were threefold and sixfold higher than the values for the wild type, suggesting that the bidentate charge of arginine may be necessary to properly bind the substrate.

Together, the kinetic and calorimetric data support the hypothesis that Arg\textsuperscript{310} has roles in facilitating catalysis and also binding acetyl phosphate. We propose that Arg\textsuperscript{310} binds acetyl phosphate via a bidentate interaction of its positively charged guanidino side chain with the phosphate dianion moiety of acetyl phosphate. In both structures described here, Arg\textsuperscript{310} is oriented with its side chain pointed away from the active site cleft; however, this residue is located on a flexible loop of the protein, and rotation about the backbone would orient the side chain toward the active site cleft close to the reactive sulfhydryl of CoA\textsuperscript{1}. A hydrophobic pocket formed by highly conserved hydrophobic residues (Phe\textsuperscript{4}, Leu\textsuperscript{5}, Phe\textsuperscript{294}, Ile\textsuperscript{297}, and Ile\textsuperscript{323}) previously identified in the apo-Pta structure (13) is located in the vicinity of the reactive sulfhydryl of CoA\textsuperscript{1} (Fig. 5). This pocket is spatially positioned to accept the methyl group of acetyl phosphate and would place the scissile bond of acetyl phosphate adjacent to the sulfhydryl of CoA\textsuperscript{1}. The methyl group would presumably have some mobility within the hydro-
phobic pocket, and Arg310 may facilitate catalysis by optimizing the position of acetyl phosphate and polarizing the carbonyl group for nucleophilic attack by CoA1.

**Proposed role for Ser309.** Ser309 is also strictly conserved in the alignment of 32 Pta sequences (13) and is located 4 Å from the sulphydryl group of CoA1; thus, Ser309 was targeted for site-specific replacement to determine if this residue participates in catalysis. The variants had kcat values that were greatly decreased relative to the value for the wild type, while the relative differences in the Km values were only minor for both substrates (Table 5). These results indicate that Ser309 is essential for catalysis and does not participate in substrate binding. One possible catalytic role for Ser309 is to act as a nucleophile in a ping-pong mechanism; however, all previously described kinetic analyses of Ptas suggested that the mechanism proceeds through formation of a ternary complex (20, 28), and attempts to isolate an acetyl-Pta intermediate were unsuccessful (9).

Carnitine acetyltransferase and chloramphenicol acetyltransferase catalyze base-facilitated transfers of an acetyl group between CoA and carnitine or chloramphenicol, analogous to the acetyl transfer catalyzed by Pta. For each of these enzymes, a serine has been proposed to function as a hydrogen bond donor to stabilize the negatively charged transition state of the reaction (23, 41). The kinetic data for the Ser309 variants are consistent with a similar role for Ser309 in stabilizing the transition state of the reaction catalyzed by Pta. The inability of threonine or cysteine to substitute for serine in this role was unexpected but not inexplicable. It was expected that the —SH side chain of cysteine could also serve as a hydrogen bond donor; however, if the side chain of the Ser309Cys variant were deprotonated in the enzyme active site, this residue would be unable to function in this role. While the —OH side chain of the Ser309Thr variant would certainly be protonated, steric constraints could prevent threonine from substituting for serine in catalysis.

**Proposed role for Asp316.** The mechanism of Ptas from several species has been proposed to proceed via a concerted attack on the carbonyl carbon of acetyl phosphate by CoA, rather than via a ping-pong mechanism involving an acetyl-enzyme intermediate (9, 10, 19, 20, 28). The results of steady-state kinetic studies of the M. thermophila Pta also support a mechanism proceeds through base-facilitated catalysis, rather than via a ping-pong mechanism involving an acetyl-phosphate group coordinated by the catalytically essential residue Arg310 and its methyl group located in the hydrophobic pocket formed by Phe4, Leu5, Phe20, Ile209, and Ile213. In Fig. 7 CoA is shown in its catalytically relevant orientation (CoA1 site) with the adenine ring located in a pocket formed by Ser129, Ala150, Gly73. Pro306, and Asp307, and hydrogen bonds between CoA1 and the protein are indicated. The proposed mechanism proceeds through base-facilitated catalysis, with Asp316 abstracting the sulphydryl proton from CoA1, enabling the thiolate anion to directly attack the carbonyl carbon of acetyl phosphate (Fig. 7A). This mechanism involves the formation of a negatively charged transition state that could theoretically be stabilized by Ser309 (Fig. 7B). Once acetyl-CoA has been formed (Fig. 7C), the resulting PO3− ion abstracts the proton from Asp316, balancing one of the negative charges of the phosphate to make it a better leaving group and returning Asp316 to a deprotonated state for another round of catalysis. A similar mechanism has been proposed for carnitine and chloramphenicol acetyltransferases, which catalyze analogous reactions (23, 41).

**Conclusions.** In summary, this study was the first investigation of a Pta to incorporate mechanistic analyses with structural information and to propose a model of catalysis consistent with the results of previous kinetic analyses (9, 10, 39). Analysis of the crystal structures of M. thermophila Pta in complex with CoA allowed us to identify the active site of the enzyme, to ascertain which residues are in reasonable proximity to participate in catalysis, and to analyze and propose func-
tions for these residues. Kinetic studies of Pta variants have identified Ser^{309} as a catalytically essential residue in the Pta active site and have clarified the role of Arg^{310} interacting with acetyl phosphate. Furthermore, the structural analysis indicated that Asp^{316} is present in the active site and could participate in catalysis. The different conformations of monomers observed in the Pta structures raise intriguing questions about the impact that domain movements may have on catalysis. The apparent closure of the active site cleft could position the substrates and residues for catalysis or could exclude water from the active site, preventing abortive hydrolysis of acetyl phosphate. The true functional relevance of the CoA^{2} site is

FIG. 6. Isothermal titration calorimetry of acetyl phosphate binding to Pta. (A) Wild type. (B) Arg^{310}Gln. (C) Arg^{310}Lys. In each case the raw data are shown in the top panel, and the data fit to a single-binding-site model is shown in the bottom panel.


TABLE 6. Thermodynamic parameters of acetyl phosphate binding to wild-type and variant phosphotransacetylases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$n^a$</th>
<th>$K_D$ (µM)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$\Delta G$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1</td>
<td>180 ± 10</td>
<td>$-8.1 \pm 0.2$</td>
<td>$-5.1$</td>
</tr>
<tr>
<td>Arg$^{101}$Gln</td>
<td>1</td>
<td>670 ± 20</td>
<td>$-12.0 \pm 3$</td>
<td>$-4.3$</td>
</tr>
<tr>
<td>Arg$^{101}$Lys</td>
<td>1</td>
<td>1,010 ± 20</td>
<td>$-10.0 \pm 0.1$</td>
<td>$-4.1$</td>
</tr>
</tbody>
</table>

$^a$ The values extracted from the data sets for $“n”$ ranged from 0.8 to 1.3 and were fixed at 1 for consistent curve fitting.

unknown. This site may be either a loading site to preorient CoA or a regulatory site to control Pta activity, although the measured $K_D$ for this binding site (1 mM) may be greater than the concentration of CoA available in the cell. While no data are available for a Methanosarcina species, intracellular CoA concentrations ranging from 180 to 860 µM have been reported for other archaea (11). While much information was derived from the results described here, additional structural and kinetic data are required to address these outstanding questions and to confirm the proposed mechanism.

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REFERENCES