Reinvestigation of the Catalytic Mechanism of Formyl-CoA Transferase, a Class III CoA-transferase*

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Formyl-coenzyme A transferase from Oxalobacter formigenes belongs to the Class III coenzyme A transferase family and catalyzes the reversible transfer of a CoA carrier between formyl-CoA and oxalate, forming oxalyl-CoA and formate. Formyl-CoA transferase has a unique three-dimensional fold composed of two interlaced subunits locked together like rings of a chain. We here present an intermediate in the reaction, formyl-CoA transferase containing the covalent β-aspartyl-CoA thioester, adopting different conformations in the two active sites of the dimer, which was identified through crystallographic freeze-trapping experiments with formyl-CoA and oxalyl-CoA in the absence of acceptor carboxylic acids. The formation of the enzyme-CoA thioester was also confirmed by mass spectrometric data. Further structural data include a trapped aspartyl-formyl anhydride protected by a glycine loop closing down over the active site. In a crystal structure of the dimer, oxalyl-CoA was found bound to the open conformation of the glycine loop. Together with hydroxylamine trapping experiments and kinetic data as well as mutagenesis data, the structures of these formyl-CoA transferase complexes provide new information on the Class III CoA-transferase family and prompt redefinition of the catalytic steps of the reaction mechanism of formyl-CoA transferase proposed here.

CoA-transferases catalyze reversible transfer reactions of coenzyme A carriers from CoA-thioesters to free acids. Most members of the enzyme class are grouped into the well characterized Class I and II CoA-transferases, but recently a third class of enzymes was identified, differing in sequence and three-dimensional structure from the other CoA-transferases (1, 2). Members of this third class are mostly from bacteria, but putative genes have been identified in Archaea and Eukarya as well. Class III enzymes are known to be involved in the metabolism of oxalate, carnitine, tolune, and bile acid and also Stickland fermentation. The first Class III CoA-transferase identified was formyl-CoA transferase from Oxalobacter formigenes (1).

Formyl-Coenzyme A transferase is the first of two enzymes involved in oxalate degradation in the gut-dwelling bacterium O. formigenes (3). Formyl-CoA transferase catalyzes the transfer of a CoA moiety between formyl-CoA and oxalate and thereby activates oxalate in the form of oxalyl-CoA (4, 5). Oxalyl-CoA is then decarboxylated by the second enzyme of the pathway, oxalyl-CoA decarboxylase, which regenerates formyl-CoA (6, 7). Oxalate catabolism has a central role in O. formigenes, where oxalate serves as vital source of energy as well as carbon (3, 8).

The crystal structure of formyl-CoA transferase revealed an interesting new fold composed of two subunits linked together in an interlocked dimer like two rings of a chain (2) (Fig. 1). Later, this fold proved to be characteristic for the Class III family as the crystal structures of the formyl-CoA transferase ortholog in Escherichia coli coded by the yfdW gene (9) and the close homolog γ-butyrobetaine-CoA:carnitine CoA transferase (10, 11) were determined.

The Class I CoA-transferases, including mostly enzymes involved in fatty acid metabolism, have a well established mechanism described in Fig. 2 (top). The formation of covalent intermediates involving a glutamate residue of the enzyme results in a classical ping-pong mechanism with exchanging substrate/product glutamyl-acyl anhydrides and γ-glutamyl-CoA thioesters (12, 13). The γ-glutamyl-CoA thioester was first identified in a Class I transferase in 1968 by electrophoresis and chromatography studies with labeled borohydrate (12) and was recently trapped in a crystallographic study, giving the first structural proof of its existence (14).

The smaller group of Class II CoA-transferases catalyzes a partial reaction in the citrate and citramalate lyase complexes. These reactions do not include covalent enzyme intermediates, and the transfer of a dephospho-CoA, which is covalently bound to an acyl carrier protein in the enzyme complex, is carried out through a ternary complex where a mixed anhydride is formed between the two acids during the transition state (Fig. 2, bottom) (15, 16).

During initial studies of the Class III CoA-transferases, steady state kinetics showed that the reaction is not consistent with a ping-pong mechanism as in the Class I CoA-transferases. The mechanism was instead interpreted to proceed through a ternary complex, where both formyl-CoA and oxalate need to be bound to the enzyme before catalysis (17–19). The crystal structure of an aspartyl-oxalyl mixed anhydride led to the sug-
gestion that the reaction was initiated in the ternary complex with both substrates by the formation of an aspartyl-formyl anhydride and CoA-S-\(^{11002}\). The CoA-S-\(^{11002}\) was then kept bound in the active site as a spectator while oxalate replaced formate, before attacking the aspartyl-oxalyl anhydride yielding oxalyl-CoA (18).

We show here from a freeze-trapped crystal structure that the enzyme-\(^{9252}\)-aspartyl-CoA thioester intermediate is also formed during catalysis by formyl-CoA transferase, a finding leading to reassessment of the catalytic mechanism of Class III CoA-transferases. We also complement the mechanistic investigation with the crystal structure of a trapped aspartyl-formyl anhydride similar to the previously characterized aspartyl-oxalyl anhydride complex (18) and two mutant protein structures, where one contains the complex with \(\beta\)-aspartyl-CoA and oxalate. Central to the catalyzed reaction is a glycine-rich loop that adopts two different conformations controlling the accessibility of the active site. Mutations in the loop seriously affect the activity, proving its importance during catalysis. A modified mechanism in concordance with all information obtained is proposed, where catalysis includes formation of both the aspartyl-formyl and -oxalyl anhydrides and the \(\beta\)-aspartyl-CoA thioester and where the carboxylate product remains bound to the enzyme until release of the acceptor thioester.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis and Protein Production**—The Q17A, G259A, and G260A mutant variants were prepared by QuikChange site-directed mutagenesis (Stratagene) with the formyl-CoA transferase gene (20) in the pET-9a vector (Novagen) with the following primers: 5'-Q17A, 5'-GCT TGA CTT TAC CCA CAG GGC GGC CAG TCC TGC CTG TAC ACA GAT CAT GGG; 3'-Q17A, 3'-CCC ATC ATC TGT GTA CAG GCA GGA CCT GCC GCC ACG TGG GTA AAG TCA AGC; 5'-G259A, 5'-GCT CAG GGC GGC CAG CCA GCC TGG; 3'-G259A, 3'-GCC CGC ACC TGC GTT ACC ACC ACG TGG; 5'-G260A, 5'-GCC GGC GGC CAG CCA GCC TGG ATG CTG; 3'-G260A, 3'-GCC CGC GCC ACC TGC GTT ACC ACC ACG TGG. PCR primers were obtained from Integrated DNA Technologies, Inc. (Corvalle, IA). DNA sequencing was performed by the DNA
Sequencing Core of the Interdisciplinary Center for Biotechnology Research at the University of Florida.

Recombinant formyl-CoA transferase and mutant proteins were produced and purified following the procedure previously described (18). In short, E. coli strain BL21(DE3) (Novagen) was transformed with the plasmids, and the genes were expressed. Purification was then carried out sequentially by four steps of chromatography: DEAE anion exchange, Blue-Sepharose fast flow affinity, Sephadex G-250 size exclusion, and QHP anion chromatography: DEAE anion exchange, Blue-Sepharose fast flow affinity, Sephadex G-250 size exclusion, and QHP anion chromatography. The final purified enzymes were stored at −80 °C in 500 mM HCl. The reaction mixture was allowed to incubate for 30 s at 303 K with varied concentrations of formyl-CoA (0.14–188 μM). As before, the reaction mixture was treated only with NH₂OH or with oxalate and then NH₂OH incubated for 30 s at 303 K with varied concentrations of oxalate (0.001–10 mM).

Enzyme Kinetic and Inhibition Studies—Formyl-CoA transferase activity was assayed by monitoring the formation of oxalyl-CoA by an HPLC² point assay developed by Jonnson (18). Formyl-CoA and oxalyl-CoA were prepared by previously described methods (18). Reaction mixtures containing 60 mM potassium phosphate, pH 6.7, ~80 ng of enzyme, and appropriate amounts of formyl-CoA and oxalate were prepared in a total volume of 200 μl. Reactions were started by the addition of formyl-CoA and quenched by the addition of 30% acetic acid. The formation of oxalyl-CoA was measured by separating the CoA-derivatives by reverse-phase chromatography, monitoring the absorbance at 260 nm, and integrating the area under the oxalyl-CoA peak. The effects of contaminating CoA were controlled by first determining the inhibitory effect of CoA against varied concentrations of formyl-CoA. Kinetic constants $V_{max}$, $K_{f(\text{CoA})}$, and $K_m(\text{formyl-CoA})$ were then used to fit initial velocity plots of varied oxalate concentration at constant formyl-CoA concentrations to determine the apparent $K_m(\text{oxalate})$ and the $K_I(\text{app})$ for formyl-CoA assuming a sequential kinetic mechanism.

The inhibition of formyl-CoA transferase by chloride ions was determined at different oxalate concentrations at saturating concentration of formyl-CoA (2.4 μM) and 10 μM CoA and KCl concentrations of 5, 15, and 30 mM.

Hydroxylamine and Sodium Borohydride Trapping Experiments—The experiments were carried out in a reaction volume of 500 μl, containing 6.2 μg of recombinant wild-type formyl-CoA transferase in 60 mM potassium phosphate buffer, pH 6.7, and 77 mM oxalate. The reaction was started by the addition of 173 μM formyl-CoA and was allowed to run for 10 s before treatment with 15 mM hydroxylamine at pH 7 for 30 s at 293 K. Small molecules were immediately removed from the reaction solution by size exclusion chromatography (5 ml of G-25), after which the residual specific activity of formyl-CoA transferase was assayed using the normal HPLC point assay. The trapping experiment was repeated in the absence of oxalate with protein incubated for 30 s at 303 K with varied concentrations of formyl-CoA (0.14–188 μM). As before, the reaction mixture was separated by gel filtration chromatography 30 s after the addition of hydroxylamine. Residual activities of the protein treated only with NH₂OH or with oxalate and then NH₂OH were also assayed.

Borohydride trapping experiments were carried out as above with the exception that the reaction was trapped with the addition of NaBH₄ (1 mM NaBH₄ in 1 mM NaOH) to a final concentration of 33 mM, immediately followed by the addition of an equal volume of 1 M HCl. The reaction mixture was allowed to incubate at room temperature for 30 min prior to gel filtration.

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² The abbreviations used are: HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; r.m.s., root mean square.
Crystallization and Freeze Trapping—Formyl-CoA transferase was crystallized by the hanging drop vapor diffusion method using conditions previously optimized for the wild type enzyme (2). 2 μl of the protein solution containing 7.5 mg/ml formyl-CoA transferase in 25 mM MES buffer, pH 6.2, and 10% glycerol was mixed with 2 μl of precipitant solution and set up to equilibrate against 1 ml of well solution at 293 K. A precipitant solution of 21–25% polyethylene glycol 4000, 0.1 M HEPES buffer, pH 7.2–7.5, and 0.5 M MgCl₂ resulted in 0.1 × 0.1 × 0.2-mm single crystals that grow to full size within 48 h.

The freeze-trapping experiments were performed by transferring the crystals to a drop containing a modified well solution (30% polyethylene glycol 4000, 0.5 M MgCl₂, 0.1 M HEPES buffer, pH 7.2) mixed in a 1:1 ratio with 20 mM formyl-CoA or oxalyl-CoA in 50 mM sodium acetate buffer, pH 5.0. The crystals were flash frozen in liquid nitrogen after the desired reaction times. The crystals, diffracting to 2.0 Å resolution, belong to space group I4 with an asymmetric unit containing two 47-kDa formyl-CoA transferase monomers, comprising the biological dimer.

Crystals where the aspartyl-formyl anhydride complex was trapped were obtained by a new crystallization condition devoid of chloride ions. An optimized well solution of 1.35 M sodium citrate and 0.1 M HEPES buffer, pH 7.2–7.5, was used when setting up the crystallization experiments using the same protein solution and mixing conditions as above. The crystals belong to the same space group and were isomorphous with the previous ones. In order to form the anhydride complex, 2–3 μl of a formyl-CoA solution was slowly added to the crystals in the drop, and crystals were then transferred to an ethylene glycol cryosolution (1M sodium citrate, 75 mM HEPES buffer, pH 7.2, and 25% ethylene glycol) after ~10 min. The formyl-CoA solution was prepared by mixing equal volumes of 20 mM formyl-CoA in 50 mM sodium acetate buffer, pH 5.0, and well solution.

Crystals of the G260A and Q17A mutant variants of formyl-CoA transferase were obtained using the same conditions as for the aspartyl-formyl anhydride complex. Crystals of the G260A mutant protein were directly frozen in liquid nitrogen after transfer through silicon oil, whereas crystals of Q17A were used for complex formation. For the oxalate complex, the drops containing the Q17A mutant protein crystals were supplemented with formyl-CoA as for the wild type aspartyl-formyl anhydride complex, followed by the addition of 1 μl of 40 mM potassium oxalate mixed into the well solution. For this complex, the ethylene glycol cryosolution was supplemented with 40 mM potassium oxalate. Crystals of the Q17A mutant protein belong to the space group I4 with unit cell dimensions \(a = b = 153.6\) Å and \(c = 98.1\) Å, whereas the G260A mutant protein crystallized in space group P4\(_3\)2\(_1\)2 with cell dimensions of \(a = b = 97.3\) Å and \(c = 193.4\) Å.

Data Collection, Structure Determination, and Refinement—Data were collected at beamlines ID14 eh1 and ID23 eh2 at the European Synchrotron Research Facility (Grenoble, France) and at beamline 1911-2 at MAX-lab (Lund, Swe-
Data collection and refinement statistics are summarized in Table 1. All images were integrated with MOSFLM (22) and further processed using SCALA (23). Phases from the originally determined apoenzyme (Protein Data Bank accession code 1p5h) (2) were used to solve the structures by molecular replacement using MOLREP (24). Refinement by the maximum likelihood method was carried out in REFMAC5 (25) interspersed with manual model building in WinCoot (26), where water molecules were assigned and checked. The quality of the final structures was validated using PROCHECK (27) and WinCoot (26), and annealed omit maps calculated in CNS (28) were used to confirm the conformations in the active sites. All images of protein molecules were generated using PYMOL (29).

Mass Spectrometric Analysis—A sample of formyl-CoA transferase incubated with formyl-CoA in the absence of oxalate was prepared according to an experiment by Lloyd and Shoolingin-Jordan (30). A 125-μl reaction mixture containing 0.153 mM formyl-CoA transferase in 25 mM MES buffer, pH 6.2, with 10% glycerol and 0.596 mM formyl-CoA was incubated for 1 min at room temperature. The reaction mixture was then immediately desalted at 277 K into 1 mM HCl using a prepacked NAP-5 column (Amersham Biosciences). The protein elution of 1 ml was mixed with an equal volume of 98% acetonitrile and 2% formic acid. Data were immediately acquired in positive mode on a QTOF ULTIMA API instrument (Waters Corp., Milford, MA) equipped with the standard Z-spray source with a capillary voltage of 1.5 kV. The instrument was calibrated between 300 and 1400 m/z with myoglobin prior to the run. The sample was introduced with a metal-coated borosilicate glass capillary needle (Proxeon Biosystems A/S, Odense, Denmark). Data were collected over a mass range between 300 and 2500 m/z and with a scan time of 1 s for about 5 min. The spectra were combined and deconvoluted to zero charged ions with MaxEnt 1 in the Masslynx software (Waters Corp., Milford, MA).

| TABLE 3 |
|------------------|------------------|------------------|
| Formyl-CoA dependence of inactivation of formyl-CoA transferase (0.52 μM) by hydroxylamine trapping under turnover conditions with saturating oxalate (77 mM) and varied concentration of formyl-CoA | | |
| [Formyl-CoA] | Residual activity | Residual activity |
| μM | % | units/mg |
| 0.0 | 100 ± 7 | 7.4 ± 0.5 |
| 5.5 | 81 ± 2 | 6.0 ± 0.1 |
| 10.0 | 75 ± 6 | 5.6 ± 0.3 |
| 38.0 | 33 ± 3 | 2.4 ± 0.1 |
| 77.0 | 12 ± 18 | 0.9 ± 0.2 |
RESULTS

Wild-type Formyl-CoA Transferase Activity—By first determining the inhibitory effects of free CoA (Table 2), a ubiquitous contaminant resulting from the hydrolysis of formyl-CoA, we were able to improve the kinetic parameters for formyl-CoA transferase and mutant variants. The values obtained for formyl-CoA transferase by this method were similar to previously reported values (18) and reflect the relatively high $K_i^{(CoA)}$ of 16.7 ± 0.7 mM.

Enzyme-$\beta$-aspartyl-CoA Thioester Complexes—Crystallographic freeze-trapping experiments were performed in order to gain more information about the catalytic steps and the intermediates of CoA transfer in formyl-CoA transferase. Crystals, produced with a precipitant mixture of polyethylene glycol and magnesium chloride, were soaked with formyl-CoA for 1, 5, and 10 min, respectively, and with oxalyl-CoA for 2, 4, and 10 min, respectively. Inspection of the crystal structures from different soaking times revealed that all formyl-CoA-soaked crystals contained the same intermediate, and all oxalyl-CoA-soaked crystals contained the same intermediate, with no difference over time. The best data set of each, formyl-CoA-soaked for 2 min and oxalyl-CoA-soaked for 5 min, were used for further analysis.

Close inspection of the freeze-trapped formyl-CoA and oxalyl-CoA intermediates shows that the formyl as well as the oxalyl moieties are cleaved off by the enzyme, and a covalent bond is formed between the carboxyl group of Asp-169 and the thiol group of the CoA carrier. The resulting intermediates from the formyl-CoA and oxalyl-CoA soaks are thus highly similar, and the dimeric structures superimpose with an r.m.s. deviation of 0.28 Å over 851 C atoms.

An intriguing feature is that in both complexes, the two subunits of the dimer adopt different active site conformations with the pantetheine arm of the CoA molecule bound in different orientations (Fig. 3). Several residues show different conformations in the two subunits of the dimer. Tyr-139 is centrally positioned in the active site and moves with the side chain hydroxyl group shifted −3 Å, in order to give space for the two different orientations of the pantetheine moiety. Lys-137 is positioned on the same side of the CoA molecule and shows a shift of 4.5 Å at the side chain amino group.

Residues Arg-38 and His-15 also adopt different side chain conformations in order to adapt to the two CoA conformations. Finally, Gln-17 takes on two different rotamer conformations, with a position behind Asp-169 in subunit A and above the thioester bond in subunit B. As was observed already for the apoenzyme (2), the side chain conformation of Trp-48 is flipped 90° between the two monomers, and the glycine loop

### Table 4

| [Formyl-CoA] | Hydroxylamine residual activity | Sodium borohydride residual activity |
|-------------|---------------------------------|-------------------------------------|
|             | %                               | %                                   |
| 0.0         | 100 ± 3                          | 100                                 |
| 0.1         | 71 ± 7                           | 93                                  |
| 0.2         | 32 ± 2                           |                                     |
| 0.3         | 19 ± 1                           |                                     |
| 1.1         | 12 ± 0.2                         |                                     |
| 5.0         | 13 ± 1.4                         |                                     |
| 24.0        | 14.8 ± 0.3                       |                                     |
| 188.0       | 15 ± 7                           |                                     |
| 262.0       | 1                                |                                     |

FIGURE 5. A, overlay of the aspartyl-formyl (green) and aspartyl-oxalyl (light blue) anhydride active sites. The glycine loop is in the closed conformation in both structures. The Ca trace of the enzyme is displayed. $F_o - F_c$ electron density map contoured at 5σ, calculated with the aspartyl-formyl anhydride and CoA molecule omitted from the structure. B, overlay of the aspartyl-formyl anhydride active site (green) and the Q17A formyl-CoA transferase mutant enzyme active site with the aspartyl-CoA thioester and oxalate bound to the open glycine loop (pink).
The $B$-factors show a clear difference in the region of the small domain comprising the two loops 230–247 and 282–347 between the two subunits (Fig. 1). In subunit B, this region is much more flexible, and in the $\beta$-aspartyl-CoA thioester complex obtained from formyl-CoA, residues 286–316 have no interpretable electron density and are modeled with zero occupancy. Inspection of the crystal packing reveals that the corresponding region of subunit A forms crystal contacts with the adjacent molecule, whereas this region in subunit B is freely exposed to solvent.

**Inhibition of Formyl-CoA Transferase by Chloride Ions**—The identification of chloride ions bound in the active sites was followed up by kinetic measurements showing that chloride has an inhibitory effect on the transferase activity in formyl-CoA transferase. Chloride is a weak competitive inhibitor to oxalate with $K_{i(\text{cat})}$ of 3 ± 2 mM (Fig. 4A).

**Hydroxylamine and Sodium Borohydride Trapping**—Class I CoA-transferases are inactivated by hydroxylamine and sodium borohydride in the presence of donor CoA-thioesters (12, 13). In the Class I enzymes, treatment with hydroxylamine gives formation of a hydroxamate at the glutamate bound in the $\gamma$-glutamyl-CoA thioester, whereas sodium borohydride reduces glutamyl-CoA to the corresponding alcohol. Previous experiments on Class III transferases have yielded ambiguous results. The $(E)$-cinnamyl-CoA:(R)-phenyllactate CoA transferase from Clostridium sporogenes was not inactivated by hydroxylamine and retained 50% activity when treated with NaBH$_4$ (17). Activity of succinyl-CoA:(R)-benzylsuccinate CoA transferase from Thauera aromatica was also unaffected by hydroxylamine but could be reduced to 3.5% in the presence of benzylsuccinyl-CoA and 10 mM NaBH$_4$ (19). We tested the effect of both of these inhibitors on formyl-CoA transferase preincubated with formyl-CoA at different concentrations.

Formyl-CoA transferase incubated with oxalate and formyl-CoA was subsequently quenched with hydroxylamine. Since any activated acyl groups are expected to be trapped as hydroxylamine adducts, oxalyl- and formyl-acyl as well as acyl-thioester intermediates in the transferase reaction should be trapped. The enzyme showed a reduced activity after removing (G$^{258}$GG$^{259}$G$^{260}$GGGQ$^{261}$) then assumes the open and closed conformations in subunits A and B, respectively (Fig. 3A).

Neither formate nor oxalate was detected in the active site. However, strong remaining spherical electron densities were interpreted as bound chloride ions due to the high concentration of chloride present in the crystallization. Refinement of these ions resulted in $B$-factors that were similar to the surrounding region. Interestingly, subunit A contains one chloride ion, which is bound behind the active site residue Asp-169, at a position occupied by residue Gln-17 in the other subunit, and subunit B has two chloride ions bound, one on each side of the pantetheine arm of CoA, where one chloride ion is interacting with the closed glycine loop and the other with the main chain amides of Gln-17 and Ala-18 (Fig. 3). In both complexes, Val-16 is positioned in the disallowed or generously allowed part of the Ramachandran plot in both monomers, which was also observed in the complex of formyl-CoA transferase with bound CoA reported earlier (2). Inspection of the structures reveals that Val-16 adopts a strained conformation in order to fit the CoA moiety. In the $\beta$-aspartyl-CoA thioester complex, Glu-140 is in the disfavored part of the Ramachandran plot in subunit B, which can be explained by the structure adopted by the adjacent residue Tyr-139, enforced by the different conformation of the CoA moiety in that subunit.

**TABLE 5**

| Variant  | $k_{\text{cat}}$ | $k_{\text{cat}}/(F-CoA)$ | $K_{m}(F-CoA)$ | $k_{\text{cat}}/(K_{m}(F-CoA))$ |
|----------|-----------------|--------------------------|---------------|-------------------------------|
| Wild type| 5.3 ± 0.1       | 2.0 ± 0.3                | 2.7 ± 0.4 × 10$^6$ | 3.9 ± 0.3                     |
| G259A    | 1.9 ± 0.1       | 4.7 ± 0.8                | 4.1 ± 0.6 × 10$^6$ | 12.1 ± 0.5                    |
| G260A    | 0.23 ± 0.02     | 18 ± 3                  | 1.3 ± 0.2 × 10$^4$ | 18.0 ± 1.6                    |
| Q17A     | 0.12 ± 0.1      | 3.3 ± 0.5                | 3.6 ± 0.6 × 10$^4$ | 13.2 ± 0.6                    |
|          | $K_{m}(\text{oxalate})$ | 1 M | $K_{m}(\text{oxalate})$ | 1 M |
| Wild type| 0.8 ± 0.02      | 10$^6$                   | 3.3 ± 0.6 × 10$^4$ | 10$^4$ |
| G259A    | 0.6 ± 0.02      | 10$^5$                   | 1.3 ± 0.2 × 10$^4$ | 10$^3$ |
| G260A    | 0.5 ± 0.02      | 10$^4$                   | 3.6 ± 0.6 × 10$^4$ | 10$^2$ |
| Q17A     | 0.2 ± 0.02      | 10$^3$                   | 13.2 ± 0.6
|          | $K_{i(\text{cat})}$ | 1 M | $K_{i(\text{cat})}$ | 1 M |
| Wild type| 160 ± 7         | 10$^7$                   | 160 ± 7         | 10$^7$ |
| G259A    | 12 ± 1          | 10$^5$                   | 12 ± 1          | 10$^5$ |
| G260A    | 10 ± 1          | 10$^4$                   | 10 ± 1          | 10$^4$ |
| Q17A     | 8.7 ± 0.9       | 10$^3$                   | 8.7 ± 0.9       | 10$^3$ |

The proposed reaction mechanism for formyl-CoA transferase.

All complexes observed in crystal structures are boxed.
all small molecules (i.e. excess CoA and hydroxylamine) by gel filtration. Table 3 shows that the addition of 77 \( \mu \text{M} \) formyl-CoA followed by hydroxylamine reduces the activity about 88%. The same experiment performed without the addition of oxalate also displayed a trapping effect by hydroxylamine (Table 4). A clear dependence on formyl-CoA concentration was discovered for the inactivation of formyl-CoA transferase by hydroxylamine.

Verification of the Covalent Enzyme-CoA Thioester Intermediate by Mass Spectrometry—Mass spectrometry was utilized in order to verify the existence of the \( \beta \)-aspartyl-CoA thioester intermediate identified by x-ray crystallography. Interpretation of the data from formyl-CoA transferase incubated with formyl-CoA showed that all of the 47,196-Da formyl-CoA transferase polypeptides had formed the thioester with CoA (748 Da), and a homogenous peak at the expected mass 47,927 ± 1 Da was observed (Fig. 4B). This is the first confirmation of the existence of the \( \beta \)-aspartyl-CoA thioester intermediate in catalysis for a Class III CoA-transferase, but similar mass spectroscopic analyses have been performed for several Class I enzymes (13, 14, 30).

Comparison with Previous Formyl-CoA Transferase Complex Structures—Previously determined structures of wild type formyl-CoA transferase include the apoenzyme structure (Protein Data Bank code 1p5h) (2), an inhibitory complex with CoA bound in the active site (Protein Data Bank code 1p5r) (2), and a structure where co-crystallization with oxalyl-CoA resulted in a crystal structure where CoA is bound in both subunits but where one subunit also contains the aspartyl-oxalyl mixed anhydride (Protein Data Bank code 1t4c) (18). Superimposition of these three structures with the \( \beta \)-aspartyl-CoA thioester intermediate structures results in r.m.s. deviations of 0.4 – 0.5 Å over 854 Ca atoms for the dimer. The differences between the
structures are mainly found in the flexible segments of the small domain in subunit B (residues 230–247 and 282–347) and among the active site residues that shift orientations in the two active sites. The orientation of the CoA moiety observed in subunit B of the β-aspartyl-CoA thioester complex (Fig. 3, A and C) has not been observed before and most probably represents a new state in the catalytic cycle. This conformation will be referred to as the “activated” conformation of CoA, whereas the conformation in subunit A is described as the “resting” one.

Aspartyl-formyl Anhydride Complex—Crystallization conditions devoid of chloride were established due to the finding that chloride inhibits formyl-CoA transferase. The new crystals were used for further freeze-trapping experiments. The structure of formyl-CoA transferase in complex with the aspartyl-formyl anhydride was obtained in the absence of chloride and oxalate ions upon flash-freezing a crystal 10 min after the addition of formyl-CoA. At a resolution of 1.87 Å, subunit A of the dimer was interpreted to contain the covalent β-aspartyl-CoA thioester, and subunit B was interpreted to contain the β-aspartyl-formyl anhydride and free CoA (Fig. 5B). The formyl part of the aspartyl-formyl anhydride was modeled at occupancy 0.6 to best fit the observed electron density. In the subunit containing the trapped mixed anhydride, the active site is nicely shielded by the glycine loop, which adopts the closed conformation. The other subunit has an open glycine loop, and noise in the electron density map indicates flexibility/disorder in the active site, especially in the region of the glycine loop and Tyr-139. The CoA moieties are found in the resting conformation in both subunits. Superposition of the aspartyl-formyl anhydride and aspartyl-oxalyl anhydride (Protein Data Bank code 1t4c) complex structures results in an r.m.s. deviation of 0.44 Å over 427 Cα atoms of the monomer (Fig. 5A). The structures show very small changes in the enzyme core, and the active sites are highly similar, whereas the flexible solvent-exposed areas display some differences.
Catalytic Mechanism of Formyl-CoA Transferase

DISCUSSION

Proposed Catalytic Mechanism—Based on all available data, we now propose the reaction mechanism for formyl-CoA transferase presented in Fig. 6. The reaction catalyzed by both Class I and III of CoA-transferases includes the formation of aspartyl (Class III) or glutamyl (Class I) mixed anhydride intermediates with the oxyacids as well as covalent thioester intermediates to the CoA moiety (Figs. 2 and 6). A distinction between the two families is that the Class I enzymes catalyze a classical ping-pong reaction, whereas the kinetics of Class III enzymes do not agree with this, and release of donor oxyacid is not observed prior to binding of the acceptor oxyacid. This leaves two possibilities, either the requirement of a ternary complex for catalysis or the completion of the reaction before any product can be released.

It can be settled from the kinetic trapping experiments and crystal structures presented above that hydrolysis of both formyl-CoA and oxalyl-CoA as well as formation of the mixed anhydride can be accomplished in formyl-CoA transferase in the absence of acceptor carboxylic acid. The reaction thus does not need the formation of a ternary complex to proceed, and the most probable interpretation of the kinetic data (18) is that the leaving oxyacid remains bound in the enzyme and is released together with the acceptor thioester. Based on the results presented above and the known structural and kinetic data (18), we propose the following catalytic scenario (Fig. 7).

The glycine loop (258GGGGQ261) plays a central role during catalysis in formyl-CoA transferase, and together with Gln-17, it protects the different intermediates from hydrolysis. We propose that upon binding of formyl-CoA, the CoA carrier adopts the resting conformation observed in most structures, including the mixed anhydride complexes. The glycine loop is presumed to close down upon formation of the aspartyl-formyl anhydride complex (1) in Fig. 6. The Gln-17 side chain is positioned behind Asp-169.

During the next catalytic step, CoAS− performs a nucleophilic attack on the mixed anhydride, resulting in the β-aspartyl-CoA thioester (2). Now the glycine loop opens up, and Gln-17 flips its side chain out above the thioester, protecting it from hydrolysis. The released formate molecule binds to the open glycine loop at the site where oxalate was observed in the Q17A mutant protein structure (Fig. 5C).

As the loop closes, formate is pushed down in the active site simultaneously as the CoA moiety reorganizes (3) into the here observed activated conformation and Gln-17 moves back above Asp-169. The thioester is at this stage protected from hydrolysis by the closed glycine loop, and formate is bound in one of the anion sites identified in subunit B of the β-aspartyl-CoA thioester complex (C11B in Fig. 3, A and C). Binding of formate at this site can result in hydrogen bonds to both the pantetheine arm and the main chain amide of Gln-262 (Fig. 8). The activated CoA conformation creates a cavity below the β-aspartyl-CoA thioester with connection to the surface, where oxalate can enter and bind in the second anion site identified in subunit B of the β-aspartyl-CoA thioester complex (C12B in Fig. 3, A and C). Manual modeling of oxalate at this site results in hydrogen bonds to the amides of Gln-17 and Ala-18, and minor shifts would also place His-15 and Asn-96 within hydrogen bonding distances, allowing hydrogen bonds to all four oxygen atoms of oxalate (Fig. 8). With a favorable orientation and a distance of ~3.7 Å to Cγ of Asp-169, oxalate is perfectly aligned for a nucleophilic attack at the β-aspartyl-CoA thioester (4).

The second mixed anhydride, the aspartyl-oxalyl anhydride, results, and CoAS− shifts back to its resting conformation. The final attack by CoAS− at the oxalyl moiety regenerates the aspartate together with oxalyl-CoA (5). Opening of the glycine loop allows the acceptor thioester to leave the active site together with formate.

We find it probable that all Class III CoA-transferases have a mechanism very similar to that described in the legend to Fig. 6. However, other members of the family will have different ways of shielding the intermediates, since the glycine loop is not strictly conserved in the family (10, 11).

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