THE MECHANISM OF FORMYL-COENZYME A TRANSFERASE, A FAMILY III COA TRANSFERASE, FROM *Oxalobacter formigenes*

By

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This dissertation is dedicated to my family.
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THE MECHANISM OF FORMYL-COENZYME A TRANSFERASE, A FAMILY III COA TRANSFERASE, FROM Oxalobacter formigenes

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Chair: Nigel G. J. Richards
Major Department: Chemistry

Formyl-Coenzyme A transferase (FRC) is a part of an oxalate-degrading catalytic cycle in Oxalobacter formigenes, a bacterium which colonizes the gastrointestinal tract of many mammals, including humans, symbiotically degrading toxic oxalate ingested with food and produced as a byproduct of normal cellular metabolism. FRC is a member of a recently recognized Family III CoA transferases, which apparently use a novel mechanism of CoA transfer as indicated by the limited kinetic studies that have been published so far.

FRC from O. formigenes was overexpressed in Escherichia coli and purified by anionic exchange and affinity chromatography. The selenomethionine derivative of FRC was also expressed and purified, allowing determination of the X-ray crystal structure of the enzyme. Mutations of the putative main catalytic residue, identified by analysis of the crystal structure of FRC with bound CoA, caused very diminished or complete loss of transferase activity. Steady-state initial rate kinetic studies on the wild-type enzyme
indicate a ternary complex (sequential) mechanism rather than Ping-Pong kinetics, which are observed for the well known Family I CoA transferases, and product inhibition studies strongly support an ordered Bi Bi mechanism. A catalytic mechanism is proposed, based on the crystal structure and kinetic data, where the main catalytic residue forms mixed anhydrides with formate and oxalate during catalytic turnover. One of these proposed intermediates, an aspartyl-oxalyl anhydride, was observed in a crystal structure obtained from crystals of the wild-type FRC grown in the presence of oxalyl-CoA, lending further evidence to the proposed mechanism.
Oxalic Acid Breakdown

Oxalic acid is a byproduct of normal cellular metabolism and is toxic to almost all organisms. Several oxalate-degrading processes have therefore naturally evolved (Figure 1-1):

(A) Oxalate oxidase

\[ \text{Oxalate oxidase} \quad \text{O}_2, 2\text{H}^+ \rightarrow 2\text{CO}_2 + \text{H}_2\text{O}_2 \]

(B) Oxalate decarboxylase

\[ \text{Oxalate decarboxylase} \quad \text{cat. O}_2, \text{H}^+ \rightarrow \text{CO}_2 + \text{H}_2\text{O}_2 \]

(C) Oxalate peroxidase

\[ \text{2 HO}_2\text{C}_2\text{O}_4} \rightarrow 2[(\text{HO}_2\text{C}_2\text{O}_4)\text{Mn}^{3+}]^2 \rightarrow 4\text{CO}_2 \]

(D) Oxalyl-CoA reductase (Glyoxylate dehydrogenase)

\[ \text{Oxalyl-CoA reductase} \quad \text{NAD}(P)^+ \rightarrow \text{Oxalyl-CoA} + \text{CoA}^- \]

(E) Oxalyl-CoA decarboxylase

\[ \text{Oxalyl-CoA decarboxylase} \quad \text{CO}_2 \]

Figure 1-1. Currently recognized classes of enzymes that catalyze the direct or indirect degradation of oxalate in (A) plants and fungi, (B) fungi and bacteria, (C) fungi only, and (D, E) bacteria only.
• (A) Oxalate oxidase, found mainly in plants\(^2\) and more recently in fungi\(^3\), catalyzes the oxidation of oxalate to carbon dioxide with concomitant reduction of dioxygen to hydrogen peroxide.

• (B) Oxalate decarboxylase, found mainly in fungi\(^4\) and more recently in bacteria\(^5\), decarboxylates oxalate in the presence of catalytic amounts of dioxygen.

• (C) Manganese peroxidase, secreted by some fungi, catalyzes the formation of Mn\(^{+3}\) from Mn\(^{+2}\) and hydrogen peroxide. Oxalate-Mn\(^{+3}\) complexes then spontaneously form and break down to Mn\(^{+2}\), carbon dioxide and hydrogen peroxide in the presence of dioxygen.\(^6-8\)

• (D) Oxalyl-CoA reductase, also known as glyoxylate dehydrogenase, found in bacteria able to use oxalate as a source of carbon (oxalotrophic bacteria), catalyzes the NAD(P)H (nicotinamide adenine dinucleotide (phosphate)) dependent reduction of oxalyl-CoA to glyoxylate and free CoA.\(^9,10\)

• (E) Formyl-CoA transferase (FRC)\(^11\) and oxalyl-CoA decarboxylase (OXC),\(^12,13\) also found in oxalotrophic bacteria, form a catalytic cycle that breaks down oxalate to carbon dioxide and formate via formyl- and oxalyl-CoA ester intermediates.

Bacterial enzymes are the least studied of the oxalate-degrading enzymes. Of particular interest are FRC and OXC, which have been purified and characterized from \textit{Oxalobacter formigenes}, an anaerobic Gram-negative bacterium, which colonizes the gastrointestinal tract of many warm-blooded animals, including humans.\(^11,13,14\) \textit{O. formigenes} is unique since oxalate is the only compound that supports its growth, although small amounts of acetate are also required.\(^14\) Approximately 99% of the oxalate consumed by the bacterium is decarboxylated to CO\(_2\) and formate by OXC and FRC (Figure 1-1E) and the rest is used for cell biosynthesis, presumably through the action of oxalyl-CoA reductase (Figure 1-1D).\(^14-17\) OXC and FRC activities are therefore crucial for the bacterium’s survival and play a central role in its oxalate metabolism. Absence of this bacterium from human intestinal flora has been strongly linked to pathological conditions that can arise if oxalate accumulates in the human body, including hyperoxaluria (increased levels of oxalate in urine), the formation of kidney stones (urolithiasis), renal failure,
cardiomyopathy, and cardiac conductance disorders. OXC is a TPP-dependent (thiamine pyrophosphate-dependent) decarboxylase. The mechanisms of TPP-dependent enzymes are generally well known and will not be discussed here. The amino acid sequence of FRC, however, bears no similarity to the well known Family I CoA transferases or Family II CoA transferases. Instead, FRC, by sequence similarity, belongs to a newly recognized Family III CoA transferases (Pfam accession number PF02515), for which only limited kinetic studies and no mechanistic studies have been reported until now.

Coenzyme A Transferases

Overview

The reversible transfer of the CoA moiety (Figure 1-2) from CoA thioesters to free acids is catalyzed by CoA transferases (Figure 1-1E) which were, until recently, grouped into two enzyme families. The well known Family I contains CoA transferases for 3-oxoacids, short-chain fatty acids, and glutaconate. The transfer reaction proceeds via a Ping-Pong mechanism, where a glutamate residue of the enzyme forms covalently bound anhydrides and CoA thioesters during catalysis. These enzymes incorporate $^{18}$O when $^{18}$O-containing CoA acceptor is used in the reaction since one oxygen atom is transferred from the incoming free acid to the main catalytic residue during one catalytic cycle (Figure 1-3A). Family I CoA transferases are inactivated by incubation with hydroxylamine or sodium borohydride (NaBH$_4$) in the presence of CoA thioesters.

Family II includes only the homodimeric $\alpha$-subunits of citrate lyase and citramalate lyase, which catalyze the transfer of an acyl carrier protein (ACP) containing a covalently bound CoA derivative. In a ternary complex mechanism a direct attack of the incoming citrate or citramalate on the acetyl thioester of the acetylated CoA derivative results in the
formation of a mixed anhydride of acetate and citrate or citramalate during catalytic
turnover (Figure 1-3B). No covalent enzyme-substrate intermediates are formed in the
mechanism.37-39

![Figure 1-2. Structure of Coenzyme A.](image)

![Figure 1-3. Previously known enzyme-catalyzed mechanisms of CoA transfer. (A) Ping-
Pong mechanism of Family I CoA transferases. Oxygen atoms from the
incoming free acid are shown in bold font. (B) Ternary complex mechanism of Family II CoA transferases.](image)

The new Family III of CoA transferases currently includes only three characterized
enzymes other than formyl-CoA transferase from *O. formigenes* (FRC). These are BbsF
(succinyl-CoA: (R)-benzylsuccinate CoA transferase from \textit{Thauera aromatica}),\textsuperscript{26,27} FldA ((E)-cinnamoyl-CoA: (R)-phenyllactate CoA transferase from Stickland-fermenting \textit{Clostridia}),\textsuperscript{28,29} and CaiB (butyrobetainyl-CoA: (R)-carnitine CoA transferase from \textit{E. coli} and \textit{Proteus} sp.).\textsuperscript{30,31} The subunits of these enzymes have similar masses, 42–47 kDa, while their quaternary structures include homodimers (FRC and CaiB), $\alpha_2\beta_2$ aggregates (BbsF), and a subunit in a heterotrimeric enzyme complex (FldA).

Additionally, there are many putative proteins that have been identified as probable Family III CoA transferases based on DNA and/or translated amino acid sequence similarities. A ternary mechanism with no covalent enzyme-substrate intermediates, similar to the mechanism of Family II CoA transferases, has been proposed for Family III enzymes based on the limited kinetic data reported that indicate a sequential mechanism rather than Ping-Pong.\textsuperscript{25}

**Formyl-CoA Transferase**

Unmodified native wild-type FRC has 428 amino acids. The monomeric mass is 47.3 kDa and its pI is 5.2 calculated from the amino acid sequence.\textsuperscript{40} FRC catalyzes transfer of the CoA moiety from formyl-CoA to oxalate producing oxalyl-CoA and formate (Figure 1-1E). Previous literature on this enzyme is limited to earlier studies on the oxalate-degrading \textit{Pseudomonas oxalaticus},\textsuperscript{9,12} which were the first indication of the existence of such an enzyme, and more recently the purification and limited characterization of FRC isolated from \textit{Oxalobacter formigenes},\textsuperscript{11} the cloning of the FRC gene from \textit{O. formigenes} and subsequent overexpression of the recombinant FRC in \textit{Escherichia coli}.\textsuperscript{40} In addition, a gene (\textit{yfdW}) from \textit{E. coli} sharing 61\% sequence identity with FRC has been cloned and expressed as a His-tagged gene product.\textsuperscript{41} It is not yet known whether the \textit{yfdW} gene product is an active transferase. There are no reports of
oxalate degradation by *E. coli*, and it is not known if the *yfdW* gene is expressed under standard growth conditions or what factors could induce its expression.

Table 1-1. Previously reported properties of native wild-type formyl-CoA transferase from *Oxalobacter formigenes*. One unit (U) equals one micromole of CoA transferred per minute.11,40

<table>
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<th>Properties</th>
<th>Values / Observations</th>
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<tr>
<td>$K_m$ (formyl-CoA) vs. excess succinate</td>
<td>3.0 ± 0.5 mM</td>
</tr>
<tr>
<td>$V_{max}$ (formyl-CoA) vs. excess succinate</td>
<td>30 U/mg</td>
</tr>
<tr>
<td>$K_m$ (oxalate) vs. formyl-CoA</td>
<td>5.1 ± 0.5 mM</td>
</tr>
<tr>
<td>$V_{max}$ (oxalate) vs. formyl-CoA</td>
<td>6.4 U/mg</td>
</tr>
<tr>
<td>$K_m$ (succinate) vs. formyl-CoA</td>
<td>2.3 ± 0.6 mM</td>
</tr>
<tr>
<td>$V_{max}$ (succinate) vs. formyl-CoA</td>
<td>19 U/mg</td>
</tr>
<tr>
<td>Maximum specific activity for transfer of CoA from formyl-CoA to oxalate</td>
<td>2.2 U/mg</td>
</tr>
<tr>
<td>pH optimum</td>
<td>6.5 – 7.5</td>
</tr>
<tr>
<td>Isoelectric point (calculated)</td>
<td>5.2</td>
</tr>
<tr>
<td>Isoelectric point (experimental)</td>
<td>4.7</td>
</tr>
<tr>
<td>Molecular weight (calculated)</td>
<td>47.3 kDa</td>
</tr>
<tr>
<td>Molecular weight (experimental)</td>
<td>44.7 kDa (active as monomer)</td>
</tr>
<tr>
<td>Acceptable CoA acceptors</td>
<td>Oxalate, succinate</td>
</tr>
<tr>
<td>Not acceptable CoA acceptors</td>
<td>Acetate, malonate</td>
</tr>
<tr>
<td>Acceptable CoA donors</td>
<td>Formyl-CoA, succinyl-CoA</td>
</tr>
<tr>
<td>Not acceptable CoA donors</td>
<td>Acetyl-CoA</td>
</tr>
<tr>
<td>Inhibition</td>
<td>20% by 1.0 mM <em>N</em>-ethylmaleimide</td>
</tr>
<tr>
<td></td>
<td>91% by 1.0 mM <em>p</em>-chloromercuribenzoate</td>
</tr>
<tr>
<td>No inhibition</td>
<td>10 mM EDTA, 10 mM Ca$^{2+}$, 1.0 mM TPP</td>
</tr>
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In the previous characterization the enzyme was assayed with a continuous, coupled enzyme assay in which formate dehydrogenase (FDH) was used to detect the rate of formate production by monitoring the formation of NADH (reduced form of nicotinamide adenine dinucleotide) spectrophotometrically. Findings of the previous characterization of the native wild-type FRC are summarized in Table 1-1.11 Although formyl-CoA and succinyl-CoA can both act as CoA donors, there is no report of oxayl-
CoA acting as one, so there is so far no evidence of FRC being able to catalyze the reverse reaction (transfer of CoA from oxalyl-CoA to formate).

Figure 1-4. Crystal structure of the interlocked FRC dimer with bound CoA. For clarity, the protein monomers are colored red and green and represented by molecular ribbons. The bound CoA molecules are shown as space-filling models, which are colored using the following scheme: H - white; C - grey; N - blue; O - red; S - yellow; P - purple.

The structure of recombinant FRC from *O. formigenes* has recently been published and represents the first crystal structure of a member of the new Family III CoA transferases. The structure is a novel fold where two circular shaped monomers are interlocked like two links in a chain creating a tightly packed homodimer. Each dimer has two active sites located on opposite sides of the structure in a cleft between the monomers (Figure 1-4). The structure, with bound coenzyme A molecules, allowed D169 to be identified as the most likely main catalytic residue (see Chapter 3). The *yfdW* gene from *E. coli* yields a protein with the same structure.
Kinetics of Bisubstrate Enzymes

Overview

The majority of enzymes catalyze reactions between two or more substrates yielding two or more products. Enzymes that use two substrates and yield two products, such as FRC, employ one of several possible Bi Bi mechanisms (using Cleland’s nomenclature where Bi Bi refers to two substrates and two products). The three main forms of Bi Bi mechanisms are (A) sequential random Bi Bi, where substrates bind in random order and products are released in random order, (B) sequential ordered Bi Bi, where substrates bind in an ordered manner and products are released in a specific order, and (C) Ping-Pong Bi Bi, where the first product is released before binding of the second substrate (Figure 1-5). The sequential mechanisms are also known as ternary complex mechanisms. Some variations of these general scenarios exist, but are not relevant to this discussion.

Furthermore, the above systems can be under either rapid equilibrium conditions or steady-state conditions. Rapid equilibrium conditions refer to when all binding and dissociation steps are very rapid compared to the catalytic step, and the rate-limiting step is the breakdown of EAB to E + P + Q. This describes some random binding systems well, but the steady-state approach is preferred for sequential ordered and Ping-Pong mechanisms. The steady-state approach describes systems where the isomerization of the central complex (EAB) and product release are so rapid that E, EA, and EAB never attain equilibrium, but are kept at near-constant, or steady-state levels (Figure 1-5). This is generally the case when the substrate concentrations and the values of $K_m$ (Michaelis constant) for the substrates greatly exceed the enzyme concentration.
Initial Velocity Studies

The first step in determining the kinetic mechanism of an enzyme is usually initial velocity studies. Initial velocity refers to the rate of the catalyzed reaction in the absence of products. Generally, the reaction is said to be at initial velocity when less than 10% of the substrates have been used and product formation is still linear. Exceptions from this rule include cases where the equilibrium constant for the catalyzed reaction is very small or one of the products is removed from the assay mixture, for example if the product is CO₂. The velocity data are usually plotted in double reciprocal plots (Lineweaver-Burk plots) as 1/v vs. 1/[A] or 1/[B] with the other substrate at a fixed concentration. Velocity equations for multisubstrate enzymes have been derived and the microscopic rate constants (k₁, k⁻₁, etc.) grouped into kinetic constants (Vₘₐₓ, Kₘ, etc.) that can be determined by experiments. The ordered Bi Bi mechanism, for example, has ten individual rate constants that can be combined in such a way that the initial forward
velocity in the absence of products can be described by an equation containing only four kinetic constants (Figure 1-6).

\[
E + A \xrightleftharpoons[k_{-1}]{k_1} \text{EA} + B \quad \text{EA} + \text{P} \xrightleftharpoons[k_{-3}]{k_3} \text{EQ} + \text{P} \xrightleftharpoons[k_{-2}]{k_2} \text{EPQ} + \text{P} \xrightleftharpoons[k_{-4}]{k_4} \text{E} + \text{Q}
\]

\[
V_{\text{max}} = \left[\frac{[E]_i}{k_1} \left(\frac{k_2 k_3 k_4 k_p}{k_1 k_4 + k_2 k_p + k_3 k_p + k_4 k_p}\right)\right] ; \quad K_{ii} = \frac{k_{-1}}{k_1} ;
\]

\[
K_{mA} = \frac{k_3 k_4 k_p}{k_1 (k_3 k_4 + k_3 k_p + k_4 k_p + k_4 k_p)} ; \quad K_{mA} = \frac{k_4 (k_2 k_3 + k_2 k_p + k_3 k_p)}{k_2 (k_3 k_4 + k_3 k_p + k_4 k_p + k_4 k_p)}
\]

\[
\frac{v}{V_{\text{max}}} = \frac{[A][B]}{K_{ii} K_{mB} + K_{mB} [A] + K_{mA} [B] + [A][B]}
\]

Figure 1-6. Ordered Bi Bi mechanism showing the individual rate constants (top), and the equation for the initial forward velocity in the absence of products (bottom).

The double reciprocal forms of the velocity equation from Figure 1-6 are

\[
\frac{1}{v} = \frac{K_{mA}}{V_{\text{max}}} \left(1 + \frac{K_{ii} K_{mB}}{K_{mA} [B]} \right) \frac{1}{[A]} + \frac{1}{V_{\text{max}}} \left(1 + \frac{K_{mB}}{[B]} \right) \quad \text{when [A] is varied and [B] is constant,}
\]

and

\[
\frac{1}{v} = \frac{K_{mB}}{V_{\text{max}}} \left(1 + \frac{K_{ii}}{[A]} \right) \frac{1}{[B]} + \frac{1}{V_{\text{max}}} \left(1 + \frac{K_{mA}}{[A]} \right) \quad \text{when [B] is varied and [A] is constant.}
\]

Therefore, except in the rare case when \(K_{ii}\) is very small compared to \(K_{mA}\), the data should give intersecting lines, since both the slope and intercepts of the 1/v vs. 1/[substrate] plots are affected by the value of the constant substrate concentration. The
lines will intersect above, on, or below the x-axis, depending on the ratio of $K_{mA}/K_{ia}$ (Figure 1-7A).

The random Bi Bi mechanism gives more complex equations describing nonlinear double reciprocal plots unless one substrate is saturating, and equations for the Ping-Pong mechanism describe parallel lines in the double reciprocal plots (Figure 1-7B).

![Figure 1-7. Lineweaver-Burk plots of initial velocity data for a bisubstrate enzyme. (A) Intersecting lines indicating a random or ordered sequential (ternary complex) mechanism. (B) Parallel lines suggesting Ping-Pong kinetics. The same patterns appear when the other substrate is varied.](image)

**Product Inhibition Studies**

Taking the reverse of the catalyzed reaction into consideration, complete velocity equations have been derived and written for A or B as the varied substrate in the presence of P or Q. The effects of P and Q on the slopes and intercepts of the lines in double reciprocal plots can be read from the equations and the type of inhibition predicted. Three main types of inhibitors are recognized in enzyme kinetics: (A) Competitive inhibitors only affect the slope, but not the $1/v$-axis intercept, (B) uncompetitive inhibitors only affect the intercept, but have no effect on the slope, and (C) mixed-type inhibitors (also known as noncompetitive inhibitors) affect both the slope and intercept (Figure 1-8).

[^46]: Reference text
[^43]: Reference text
[^48]: Reference text
Figure 1-8. Lineweaver-Burk plots showing inhibition patterns in enzyme kinetics. (A) Competitive inhibition, (B) uncompetitive inhibition, and (C) mixed-type (noncompetitive) inhibition. For product inhibitions I is either P or Q and [A] is varied at constant [B] or vice versa.

The complete velocity equation for the ordered Bi Bi mechanism, rearranged to show inhibition by P vs. A, at constant [B] is

\[
\frac{v}{V_{\text{max}}} = \frac{[A]}{K_{m_A} \left[ 1 + \frac{K_m}{[B]} \left( 1 + \frac{K_{m_Q}}{K_{i_q}^\text{K}_m^\text{P}} \right) \right] + \left[ B \left( 1 + \frac{K_m^P}{K_{i_q}^\text{K}_m^P} \right) + \frac{P}{K_{i_p}} \right]}
\]

where the indicated slope and intercept factors will be factors in the slope and intercept of the reciprocal equation. Therefore, at low (unsaturating) [B], the inhibition by P is a mixed-type inhibition since both the slope and intercept factors are a function of [P]. At very high (saturating) [B], the slope factor approaches unity, but the intercept factor is still dependent on [P], so the inhibition is uncompetitive.46-48

Rearranged to show inhibition by P vs. B, at constant [A], the equation becomes

\[
\frac{v}{V_{\text{max}}} = \frac{[B]}{K_{m_B} \left[ 1 + \frac{K_m}{[A]} \left( 1 + \frac{K_{m_Q}}{K_{i_q}^\text{K}_m^P} \right) \right] + \left[ B \left( 1 + \frac{K_m^P}{K_{i_q}^\text{K}_m^P} \right) + \frac{P}{K_{i_p}} \right]}
\]

showing that P is a mixed-type inhibitor at all concentrations of A, since very high (saturating) [A] leaves [P] terms in both slope and intercept factors.46-48
Inhibition by Q vs. A, at constant [B] is described by

$$\frac{v}{V_{\text{max}}} = \frac{[A]}{K_{m_A} \left( 1 + \frac{[Q]}{K_{iq}} \right) \left( 1 + \frac{K_{iA} K_{mB}}{K_{m_A} [B]} \right) + [A] \left( 1 + \frac{K_{mB}}{[B]} \right)}$$

which shows that Q is a competitive inhibitor at all concentrations of B, since [Q] has an effect on the slope factor at any [B], but has no effect on the intercept factor.\(^{46-48}\)

Finally, the inhibition by Q vs. B, at constant [A] is described by

$$\frac{v}{V_{\text{max}}} = \frac{[B]}{K_{m_B} \left( 1 + \frac{K_{iA}}{[A]} \left( 1 + \frac{[Q]}{K_{iq}} \right) \right) + [B] \left( 1 + \frac{K_{m_A}}{[A]} \left( 1 + \frac{[Q]}{K_{iq}} \right) \right)}$$

showing that Q is a mixed-type inhibitor at relatively low (unsaturating) concentrations of A with both the slope and intercept factors being functions of [Q]. At very high [A] (saturating) there is no inhibition since the [Q] terms are eliminated from both factors.\(^{46-48}\)

Velocity equations for other possible Bi Bi mechanisms show different product inhibition patterns, making it possible to distinguish between mechanisms solely by product inhibition studies.\(^{46-48}\) Some examples are shown in Chapter 2 (Table 2-5).

**Research Objective**

The main objective of this project was to obtain a thorough understanding of the mechanism of CoA transfer by formyl-CoA transferase, a key enzyme in oxalate breakdown by *Oxalobacter formigenes*. A mechanism is proposed based on kinetic and crystallographic data (Figure 3-8). This is the first detailed mechanistic study of a Family III CoA transferase, and suggests a novel mechanism of CoA transfer likely employed by all members of this class of enzymes.
CHAPTER 2
EXPRESSION, PURIFICATION, AND KINETIC STUDIES OF FORMYL-COA TRANSFERASE

The results of kinetic studies and all requirements for the enzyme kinetics are covered in this chapter, including protein expression, protein purification, synthesis of thioester substrates, and enzyme assay development.

Expression and Purification of Recombinant Formyl-CoA Transferase

FRC was produced by IPTG (isopropyl-β-D-thiogalactopyranoside) induction of *Escherichia coli*, carrying the FRC gene in a pET-9a vector. This bacterial strain, commonly used for protein expression, has the advantage of being deficient in proteases that might degrade the desired protein, and it carries a gene for T7 RNA polymerase, whose transcription is controlled by the IPTG inducible lacUV5 promoter.\(^{49,50}\) Upon induction by IPTG the bacterium starts producing T7 RNA polymerase which recognizes a T7 promoter located shortly upstream of the FRC gene in the pET-9a plasmid. Thus the bacterium starts producing FRC after addition of IPTG.

The purification of FRC was based on a procedure developed by Kjell Eriksson and Billi Herzer (Fast Trak process development, Amersham Pharmacia Biotech, Inc., currently known as Amersham Biosciences) for Ixion Biotechnology, Inc (personal communication). The enzyme was purified from crude lysate of harvested cells by two steps of anion exchange, one step of affinity chromatography, and a buffer exchange step. Various adjustments were made to the original procedure to optimize the yield of active enzyme. The enzyme proved stable enough to do all the FPLC (fast performance liquid
chromatography) work at ambient temperature if it was kept on ice between column runs, and the first three purification steps were performed on the same day. The yield of purified FRC was typically 10-15 mg of highly pure protein from each liter of culture (Table 2-1 and Figure 2-1). See Appendix A for examples of FPLC chromatograms of the FRC purification.

Table 2-1. Purification table of FRC showing typical yield and purification level. Some activity measurements were not reliable due to precipitation of protein in the samples prior to assaying, and are omitted from the table.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Total activity (U)</th>
<th>Yield (%U)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>1150</td>
<td>0.51</td>
<td>587</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>DEAE anion exchange</td>
<td>333</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BlueFF affinity chromatography and buffer exchange</td>
<td>67</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Q Sepharose High Performance (QHP) anion exchange</td>
<td>63</td>
<td>6.11</td>
<td>385</td>
<td>66</td>
<td>12</td>
</tr>
</tbody>
</table>

Figure 2-1. SDS-PAGE gels showing the expression level and purification of FRC. (A) 1. Cell pellet at time of induction; 2. Cell pellet 150 minutes after induction; 3. Molecular weight markers (kDa). (B) 4. Pooled FRC fractions from DEAE column; 5. Empty lane; 6. BlueFF flowthrough fractions; 7. BlueFF low salt wash; 8. MW markers (same as lane 3A); 9. Pooled FRC fractions after buffer exchange; 10. BlueFF NaOH wash; 11. QHP low salt wash; 12. Fully purified FRC from QHP. Both gels were stained with Coomassie Blue.

The selenomethionine derivative of FRC, which was needed to solve the protein crystal structure, was prepared by inhibiting the natural biosynthesis of methionine by the
expression strain grown in minimal medium, and adding selenomethionine before IPTG induction.\textsuperscript{51} The selenomethionine derivative was purified as described above for the recombinant wild-type FRC. Mass spectrometry showed full incorporation of selenomethionine.

No formyl-CoA transferase activity was detected in a cell lysate of expression strain cells, containing a pET-9a plasmid without the FRC gene, grown under the same conditions as when FRC was expressed. FRC was therefore assumed to be responsible for all the CoA transferase activity isolated from the expression strain.

**Synthesis of CoA Esters**

The CoA ester substrate and product were synthesized using a thioester exchange reaction between CoA and an aromatic thiol ester of the appropriate acid (Figure 2-2).

\[ \text{SH} \quad \text{HO} \quad \text{OO} \quad \text{S} \quad \text{O} \quad \text{H} \quad \text{CoASH} \quad \text{CoASH} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{S} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{CoASH} \quad \text{O} \quad \text{H} \]

\[ \begin{align*}
\text{(A)} & \quad \text{HO} + \text{COO} \\
& \quad \text{45°C, 2.5 hrs} \\
\text{SH} & \quad \text{HOO} \quad \text{O} \quad \text{S} \quad \text{O} \quad \text{H} \quad \text{CoASH} \\
& \quad \text{cat. pyridine} \\
\text{SH} & \quad \text{HOO} \quad \text{O} \quad \text{S} \quad \text{O} \quad \text{H} \quad \text{CoASH} \\
& \quad \text{pH 8} \\
\text{(B)} & \quad \text{SH} \quad \text{HOO} \quad \text{O} \quad \text{S} \quad \text{O} \quad \text{H} \quad \text{CoASH} \\
& \quad \text{pH 8} \\
\end{align*} \]

Figure 2-2. Synthetic schemes for formyl-CoA and oxalyl-CoA. A) Synthesis of formyl-CoA from CoA and formylated thiophenol. B) Synthesis of oxalyl-CoA from thiocresol oxalate and CoA.

**Formyl-CoA**

Formyl-CoA was produced by allowing CoA to react with an excess of formylthiophenyl ester as described by Sly and Stadtman.\textsuperscript{52} The formylthiophenyl ester was made by formylating thiophenol\textsuperscript{53} using a formylating reagent made from acetic
anhydride and formic acid as described by Stevens and van Es\textsuperscript{54} (Figure 2-2A). The formylation of CoA was verified by mass spectrometry, and the expected change in retention time on reverse phase C-18 column by HPLC was observed (peaks 3 and 4 in Figure 2-6).

**Oxalyl-CoA**

Oxalyl-CoA was synthesized by a similar method, except the precursor was thiocresol oxalate monoester made previously by Dr. Jianqiang Wang by the method of Stolle\textsuperscript{55} (Figure 2-2B). The synthesis was verified by mass spectrometry and by HPLC (Figure 2-3).

![Figure 2-3](image)

Figure 2-3. Three HPLC chromatograms showing the hydrolysis of oxalyl-CoA by KOH. (A) Crude oxalyl-CoA reaction mixture. (B) Same as (A), but spiked with CoA. (C) Same as (A), but after hydrolysis with KOH. Peak assignments: 1 - Oxalyl-CoA; 2 - CoA; 3 - Thiocresol oxalate monoester; 4 - Thiocresol. The chromatograms show absorbance at 260 nm vs. time and all have same or similar scale. Peaks were assigned by coinjecting standards. The slight discrepancy in retention times of peak 3 in (A) and (B) is due to instrumentation problems.

**Purification**

The excess aromatic thiol esters were removed from the crude reaction mixtures by multiple ether extractions, adjusting the pH of the aqueous phase to 3.0 prior to each
extraction (Figure 2-4). Both CoA esters were further purified by preparative reverse phase HPLC followed by freeze-drying to minimize the amount of CoA in their stock solutions.

![Figure 2-4](image)

**Figure 2-4.** Three HPLC chromatograms showing extraction of thiocresol oxalate monoester from oxalyl-CoA reaction mixture. (A) Crude reaction mixture. (B) After four extractions with ether. (C) After eight extractions with ether. Peak assignments: 1 - Oxalyl-CoA; 2 - Thiocresol oxalate monoester. The chromatograms show absorbance at 260 nm vs. time and all have same or similar scale. Peaks were assigned by coinjecting standards. The slight discrepancy in retention times of peak 2 in (A) and (B) is due to instrumentation problems.

**Stability**

Stabilities of the CoA esters under the assay conditions were determined by measuring the pseudo first-order rate for the uncatalyzed hydrolysis of these compounds at pH 6.7 and 30°C. The concentrations of the CoA esters were measured by HPLC as described below. The half-lives were calculated from the pseudo first-order rate constants taken as the slope of the best-fit line of the plots in Figure 2-5, as described by the first-order rate equation: \( \ln[A] = \ln[A_0] - kt \). This gave an estimate of 150 min for the half-life of formyl-CoA, which is in reasonable agreement with a literature value of 300 min in aqueous solution at room temperature and neutral pH.\(^{52}\) The measured half life for oxalyl-CoA was about 10 days, which is also consistent with previous reports that
solutions of oxalyl-CoA at pH 6.5 are stable for weeks when stored at –15°C. The large difference in the rate of uncatalyzed hydrolysis of the two thioesters can likely be attributed to the presence of the negatively charged carboxylate group in oxalyl-CoA, which will destabilize the tetrahedral adduct formed by nucleophilic attack of water on the thioester carbonyl.

![Graphs for determining the rate of hydrolysis of the CoA esters. (A) Formyl-CoA and (B) oxalyl-CoA.](image)

**Assay Development**

The only other previous study on FRC used a coupled enzyme assay to determine the rate of formate production by monitoring the formation of NADH from NAD$^+$ by formate dehydrogenase (FDH). A more direct approach of determining the rate of oxalyl-CoA formation by HPLC was developed for the studies described here. The FDH coupled assay consistently produced lower rate values than the HPLC-based assay. Since NAD$^+$ was suspected to inhibit FRC due to its structural similarity to the CoA esters, the effect of 0.5 and 1.0 mM NAD$^+$ in the HPLC assay was studied. Rapid hydrolysis of formyl-CoA was observed and very little production of oxalyl-CoA was detected. The hydrolysis of formyl-CoA in the presence of NAD$^+$ may explain the high value of $K_m$ for formyl-CoA (3.0 mM) published in the original study of FRC. This value is closer to
the $K_m$ value of formate for the FDH used (13 mM),\textsuperscript{56} than the published $K_m$ values for CoA esters in two other Family III CoA transferases (3 and 40 µM).\textsuperscript{27,29}

![HPLC chromatograms of assay mixture aliquots quenched after 5 min (A), 15 min (B), and 55 min (C). Peak assignments: 1 - Oxalate; 2 - Oxalyl-CoA; 3 - CoA; 4 - Formyl-CoA. The chromatograms show absorbance at 260 nm vs. time and all have same or similar scale. Peaks were assigned by injecting standards.](image)

Figure 2-6. HPLC chromatograms of assay mixture aliquots quenched after 5 min (A), 15 min (B), and 55 min (C). Peak assignments: 1 - Oxalate; 2 - Oxalyl-CoA; 3 - CoA; 4 - Formyl-CoA. The chromatograms show absorbance at 260 nm vs. time and all have same or similar scale. Peaks were assigned by injecting standards.

The facile separation of CoA and its esters by reverse phase HPLC\textsuperscript{57} is the basis for the enzymatic assay used in the work described here. The rate of oxalyl-CoA production by the enzyme can be determined from the amount of oxalyl-CoA present in assay mixtures quenched at different timepoints as demonstrated in Figure 2-6. Similar conditions were used as in the previous study, except no other enzyme reaction was coupled to the one being studied. The enzyme, substrates, and inhibitors when appropriate, were added to phosphate buffer at pH 6.7, adding formyl-CoA last to initiate the reaction. Aliquots of the assay mixture were quenched with acetic acid at two
different timepoints, typically after 1.0 and 1.5 minutes, stored on ice, and analyzed by HPLC within 60 minutes.

The efficacy of the quench was verified by performing the assay at pH 3-4 instead of pH 6.7. No oxalyl-CoA had formed after 28 min, nor after overnight incubation at ambient temperature. Furthermore, the formyl-CoA peak area did not decrease appreciably over the first 28 min, but had dropped somewhat overnight due to hydrolysis as evidenced by an equal increase in the CoA peak area. After quenching, the pH is between 3 and 4, which is favorable for the stability of CoA esters, thus there was no detectable decrease in oxalyl-CoA or formyl-CoA content in quenched samples stored on ice for up to 60 minutes.

Free CoA and CoA esters were quantitated by HPLC using a standard solution of CoA. The assumption was made that the extinction coefficients of the CoA esters at 260 nm, the detection wavelength, were the same as for CoA. This assumption was successfully validated for formyl-CoA by measuring its concentration in solution using the hydroxylamine method, and for oxalyl-CoA by measuring the concentration of oxalate in an oxalyl-CoA solution before and after hydrolysis by base using an oxalate oxidase-based detection kit (Sigma-Aldrich Corp., St. Louis, MO).

The rate of the catalyzed reaction increased linearly with increasing enzyme concentration (Figure 2-7). Diluting the enzyme stock solution excessively before assaying, however, resulted in lowered specific activity (Table 2-2). This most likely resulted from dissociation of the homodimeric enzyme into its subunits upon dilution, a proposal supported by size exclusion chromatography studies discussed in Chapter 3. A 20-fold dilution of a 0.90 mg/mL stock enzyme solution retained its specific activity well
over a few days when stored at 4°C, while 40-fold and 80-fold diluted solutions lost their specific activity rapidly. Typically the 20-fold diluted stock solution lost only about 15 % of its transferase activity when stored at 4°C for 3 weeks.

![Graph showing linear increase of initial rate with increasing enzyme concentration.](image)

**Figure 2-7.** Linear increase of initial rate with increasing enzyme concentration.

**Table 2-2.** Effect of excessive dilution of the enzyme stock solution on specific activity. Protein concentration of the original stock was 0.90 mg/mL. The same amount of enzyme was used in all assays.

<table>
<thead>
<tr>
<th>Dilution (fold)</th>
<th>Storage time at 0-4°C (hours) before assaying</th>
<th>Relative specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>20X</td>
<td>24</td>
<td>1.0</td>
</tr>
<tr>
<td>40X (made from 20X)</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>40X (made from 1X stock)</td>
<td>24</td>
<td>0.6</td>
</tr>
<tr>
<td>80X (made from 40X)</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>80X (made from 20X)</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td>80X (made from 20X)</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>80X (made from 20X)</td>
<td>3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

No transferase activity was detected when one of the substrates or the enzyme was not present in the assay mixture, and no activity was detected when enzyme denatured by incubation in boiling water for 5 minutes was used.

The detection limit of the HPLC assay depends on the amount of enzyme in the assay mixture, and the length of incubation. Long incubation times are not desirable for initial rate measurements due to the lability of formyl-CoA, but can be used if the goal is
merely to see if there is some measurable activity or not, such as when screening for alternative substrates or checking mutant enzymes for transferase activity.

**Equilibrium Constant**

The equilibrium constant for the FRC-catalyzed CoA transfer was estimated by measuring the equilibrium concentrations of formyl-CoA and oxalyl-CoA incubated with FRC and known initial amounts of formate and oxalate. The value of $K_{eq}$ was determined to be $32 \pm 3$, favoring oxalyl-CoA and formate to formyl-CoA and oxalate. This value is similar to the equilibrium constant determined for the reaction catalyzed by succinyl-CoA:acetoacetate transferase.\textsuperscript{61}

**Alternative Substrates**

Results of the former study identifying succinate and succinyl-CoA as alternative substrates, but not acetyl-CoA,\textsuperscript{11} were confirmed and a variety of other potential substrates were screened. Somewhat surprisingly, the natural substrates, formyl-CoA and oxalate, did not yield the highest rate of CoA transfer. The highest specific activities were observed for CoA transfer from succinyl-CoA to formate and from formyl-CoA to succinate. CoA transfer from formyl-CoA to glutarate was also faster than to oxalate, while CoA transfer to maleate was slower. Malonyl-CoA, methylmalonyl-CoA, acetyl-CoA, and propionyl-CoA were not used as CoA donors by FRC with oxalate or formate as acceptors. Propionate, oxamate, and pyruvate were not accepted as substrates (Figure 2-8 and Table 2-3).

Acetate did not inhibit the enzyme at concentrations of up to 60 mM, while acetyl-CoA inhibited the rate of CoA transfer by 25% at 100 µM and 45% at 200 µM concentration in the presence of 35 µM formyl-CoA and 100 mM oxalate. There was no indication of an irreversible inhibition by acetyl-CoA, since the enzyme was preincubated
with the inhibitor before adding formyl-CoA. Acetyl-CoA therefore appears to bind to FRC but the thioester is not lysed by the enzyme. This is not surprising since the homologous protein from *E. coli* has been crystallized with bound acetyl-CoA. The remaining alternative substrates were not assayed for inhibition, but it seems likely that any CoA ester will inhibit the enzyme by competing for the CoA binding site with the natural substrate even if the thioester moiety cannot be used as substrate.

Table 2-3. Summary of alternative substrates screening. CoA ester concentrations were 80-200 µM and free acid concentrations were 62.5-125 mM.

<table>
<thead>
<tr>
<th>CoA Donor</th>
<th>Acceptor (free acid)</th>
<th>Approx. specific activity* (µmol/min•mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formyl-CoA</td>
<td>Oxalate</td>
<td>5.5 (forward reaction)</td>
</tr>
<tr>
<td>Oxalyl-CoA</td>
<td>Formate</td>
<td>0.7 (reverse reaction)</td>
</tr>
<tr>
<td>Succinyl-CoA</td>
<td>Oxalate</td>
<td>4.5</td>
</tr>
<tr>
<td>Succinyl-CoA</td>
<td>Formate</td>
<td>40</td>
</tr>
<tr>
<td>Formyl-CoA</td>
<td>Succinate</td>
<td>50</td>
</tr>
<tr>
<td>Formyl-CoA</td>
<td>Glutarate</td>
<td>15</td>
</tr>
<tr>
<td>Formyl-CoA</td>
<td>Maleate</td>
<td>2</td>
</tr>
<tr>
<td>Formyl-CoA</td>
<td>Oxamate</td>
<td>0</td>
</tr>
<tr>
<td>Formyl-CoA</td>
<td>Pyruvate</td>
<td>0</td>
</tr>
<tr>
<td>Formyl-CoA</td>
<td>Acetate</td>
<td>0</td>
</tr>
<tr>
<td>Oxalyl-CoA</td>
<td>Acetate</td>
<td>0</td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>Formate</td>
<td>0</td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>Oxalate</td>
<td>0</td>
</tr>
<tr>
<td>Methylmalonyl-CoA</td>
<td>Formate</td>
<td>0</td>
</tr>
<tr>
<td>Methylmalonyl-CoA</td>
<td>Oxalate</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>Formate</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>Oxalate</td>
<td>0</td>
</tr>
<tr>
<td>Propionyl-CoA</td>
<td>Formate</td>
<td>0</td>
</tr>
<tr>
<td>Propionyl-CoA</td>
<td>Oxalate</td>
<td>0</td>
</tr>
</tbody>
</table>

*The limit of detection was approximately 0.001 µmol/min•mg

The overall selectivity rule for CoA transfer by FRC based on the observations above appears to be a requirement for a negative charge on the thioester end of the incoming CoA ester substrate, and the free acid substrate needs to be a dianion. The only exceptions to this rule are formate/formyl-CoA, which are accepted, and (methyl)malonyl-CoA, which are not accepted as substrates. The exceptions can be explained by the small size of formate, which allows it to position itself correctly in the
active site, and by the lack of flexibility of (methyl)malonyl-CoA, making it unable to attain a favorable conformation in the active site. In this regard the large difference in the transfer rates of CoA to succinate and maleate also indicates a requirement of flexibility in the CoA acceptor molecule allowing it to make favorable interactions in the active site. The lack of CoA transfer from propionyl-CoA and acetyl-CoA are consistent with these substrates not being able to attain a favorable conformation in the active site, presumably due to their hydrophobic methyl and ethyl groups. Finally, the absence of CoA transfer to acetate, oxamate, and pyruvate are all indications that without a second negative charge these molecules are not able to make favorable interactions in the active site.

![Chemical structures](image)

**Figure 2-8.** Structures of free acids and ester parts of CoA esters used for alternative substrate screening (see Table 2-3). (A) Accepted by FRC as substrates. (B) Not accepted by FRC as substrates.

**Kinetics**

As discussed in Chapter 1, kinetic studies are a powerful tool to explore possible mechanisms of enzyme catalysis. In the case of bisubstrate enzymes yielding two products, such as FRC, the pattern of lines in a Lineweaver-Burk plot ($1/v$ vs. $1/[S]$) of initial velocity data are a good indication of what type of mechanism the enzyme uses. Intersecting lines are indicative of a sequential (ternary complex) mechanism, while
parallel lines suggest Ping-Pong kinetics. In the case of ternary complex mechanisms, random and ordered ones can be distinguished by product inhibition patterns, and the order of substrate binding and product release can be deduced in the case of ordered.

**Initial Rates**

Initial rate measurements in the absence of products were designed so the same assays would provide data that could be plotted as $1/v$ versus $1/[\text{formyl-CoA}]$ and $1/v$ versus $1/[\text{oxalate}]$ (Figures 2-9 and 2-10). The results were clearly indicative of a sequential (ternary complex) mechanism and ruled out Ping-Pong kinetics. The kinetic constants $k_{\text{cat}}$, $V_{\text{max}}$, $K_m(\text{formyl-CoA})$, $K_m(\text{oxalate})$, and $K_{\text{ia}}$ (dissociation constant for the EA complex) were calculated from the slope and intercept replots (Figures 2-9 and 2-10 inserts), and are summarized in Table 2-4. The $K_m$ of formyl-CoA in this study is about 400-fold lower than that reported for the native FRC, while the $K_m$ of oxalate is comparable (Table 1-1). Descriptions of how to calculate the kinetic constants from best-fit lines of double reciprocal plots and their replots are accessible in Segel’s book on enzyme kinetics. Error values were calculated by standard error propagation rules from the original standard errors in slopes and intercepts of the best-fit straight lines fitted by using KaleidaGraph (v. 3.5, Synergy Software).

Table 2-4. Kinetic constants of FRC calculated from initial rates data (Figures 2-9 and 2-10). One unit (U) is defined as one micromole of CoA transferred per minute.

<table>
<thead>
<tr>
<th>Constant</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{\text{cat}}$</td>
<td>$4.3 \pm 0.1$ s$^{-1}$</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>$5.5 \pm 0.2$ U/mg</td>
</tr>
<tr>
<td>$K_m(\text{formyl-CoA})$</td>
<td>$8.0 \pm 0.3$ µM</td>
</tr>
<tr>
<td>$K_m(\text{oxalate})$</td>
<td>$3.9 \pm 0.3$ mM</td>
</tr>
<tr>
<td>$K_{\text{ia}}$</td>
<td>$16 \pm 2$ µM</td>
</tr>
</tbody>
</table>
Figure 2-9. Lineweaver-Burk plot of initial rates at three fixed formyl-CoA concentrations (■ 7 µM; ○ 14 µM; ● 65 µM), each at four oxalate concentrations (5 – 75 mM). Slope and intercept replots are shown as inserts. Each data point represents an average of two rate measurements.

Figure 2-10. Lineweaver-Burk plot of initial rates at four fixed oxalate concentrations (□ 5 mM; ■ 10 mM; ○ 25 mM; ● 75 mM), each at three different oxalate concentrations (7 – 65 µM). Slope and intercept replots are shown as inserts. Each data point represents an average of two rate measurements.
Product Inhibition

The appropriate product inhibitions by formate and oxalyl-CoA that would support or rule out the proposed mechanism (Figure 3-8) were studied. The results are summarized in Tables 2-5 and 2-6 and discussed below.

Table 2-5. Product inhibition patterns observed for various mechanisms of bisubstrate enzymes. The inhibitions observed in this study are shown in bold italic letters. Substrates and products are \( A = \text{formyl-CoA}, \ B = \text{oxalate}, \ P = \text{formate}, \) and \( Q = \text{oxalyl-CoA}. \) The types of inhibitions are \( C = \text{competitive}, \ MT = \text{mixed-type (noncompetitive)}, \ UC = \text{uncompetitive}, \) and \(- = \text{no inhibition}. \) Table reproduced from Segel’s book on enzyme kinetics.46

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>Inhibitor</th>
<th>Unsaturated A with B</th>
<th>Saturated A with B</th>
<th>Unsaturated B with A</th>
<th>Saturated B with A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steady-state Ordered</td>
<td>P</td>
<td>( MT )</td>
<td>( UC )</td>
<td>( MT )</td>
<td>MT</td>
</tr>
<tr>
<td>Bi Bi</td>
<td>Q</td>
<td>C</td>
<td>C</td>
<td>MT</td>
<td>-</td>
</tr>
<tr>
<td>Steady-state Random</td>
<td>P</td>
<td>MT</td>
<td>MT</td>
<td>MT</td>
<td>MT</td>
</tr>
<tr>
<td>Bi Bi</td>
<td>Q</td>
<td>MT</td>
<td>MT</td>
<td>MT</td>
<td>MT</td>
</tr>
<tr>
<td>Steady-state Ping Pong</td>
<td>P</td>
<td>MT</td>
<td>-</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>Q</td>
<td>C</td>
<td>C</td>
<td>MT</td>
<td>-</td>
</tr>
<tr>
<td>Rapid equilibrium</td>
<td>P</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td>Random Bi Bi</td>
<td>Q</td>
<td>C</td>
<td>-</td>
<td>C</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2-6. Inhibition constants for formate and oxalyl-CoA.*

<table>
<thead>
<tr>
<th>Inhibition constant</th>
<th>Value</th>
<th>Slope (( K_{ii} )) and intercept (( K_{ia} )) composition of ( K_{i} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_i(\text{formate vs. formyl-CoA}) )</td>
<td>130 ± 20 mM</td>
<td>N/A (pure uncompetitive)</td>
</tr>
<tr>
<td>( K_i(\text{formate vs. oxalate}) )</td>
<td>350 ± 30 mM</td>
<td>( K_{ii} = 17 ± 1 \text{ mM} ) ( K_{ia} = 380 ± 40 \text{ mM} )</td>
</tr>
<tr>
<td>( K_i(\text{oxalyl-CoA vs. oxalate}) )</td>
<td>21 ± 7 \text{ mM}</td>
<td>( K_{ii} = 150 ± 50 \text{ µM} ) ( K_{ia} = 280 ± 90 \text{ µM} )</td>
</tr>
</tbody>
</table>

*Inhibition constants were calculated as described by Segel and error values were calculated as described above.

Product inhibition by formate was measured under three different conditions (Figures 2-11, 2-12, 2-13). Only one set of conditions was practical for measuring product inhibition by oxalyl-CoA (Figure 2-14), that is varying oxalate concentration at fixed, unsaturating formyl-CoA concentration. Making a stock solution with high
enough concentration of formyl-CoA to achieve saturation in the assay mixture is difficult, and the experiment would waste large amounts of the relatively expensive substrate without giving much information about the mechanism (Table 2-5). Oxalyl-CoA could not be studied as a product inhibitor when varying formyl-CoA concentrations since the initial rate of formation of oxalyl-CoA is being measured, only a relatively large increase in its concentration can be detected when the assay mixture already contains it. This is not possible at low formyl-CoA concentrations because it would require using 20-100% of the substrate, and the reaction would therefore not be at initial velocity.

**Inhibition by formate**

Formate caused mixed-type inhibition when the concentration of formyl-CoA was varied at a fixed unsaturating oxalate concentration of 350 mM (Figure 2-11), but the inhibition became uncompetitive with oxalate saturating (1.25 M) (Figure 2-12).

![Lineweaver-Burk plot showing product inhibition by formate vs. formyl-CoA at fixed unsaturating oxalate concentration. Formate concentrations were ● 0 mM, ○ 75 mM, and ■ 150 mM. Formyl-CoA concentrations were 5.5 – 110 μM. The initial concentration of oxalate was 350 mM in all assays. Each data point represents an average of two rate measurements.](image-url)
Figure 2-12. Lineweaver-Burk plot showing product inhibition by formate vs. formyl-CoA at fixed saturating oxalate concentration. Formate concentrations were ● 0 mM, ○ 20 mM, and ■ 40 mM. Formyl-CoA concentrations were 5.5 – 110 µM. The initial concentration of oxalate was 1.25 M in all assays. Intercept replot is shown as insert. Each data point represents an average of two rate measurements.

Figure 2-13. Lineweaver-Burk plot showing product inhibition by formate vs. oxalate at fixed unsaturating formyl-CoA concentration. Formate concentrations were ● 0 mM, ○ 50 mM, and ■ 100 mM. Oxalate concentrations were 2.5 – 230 mM. The initial concentration of formyl-CoA was 105 µM in all assays. Slope and intercept replots are shown as inserts. Each data point represents an average of two rate measurements.
Formate also caused mixed-type inhibition when the concentration of oxalate was varied in the presence of fixed unsaturating formyl-CoA concentration (Figure 2-13). The inhibition was close to being pure competitive, since the slope effect was much greater than the intercept effect (Table 2-6).

**Inhibition by oxalyl-CoA**

Oxalyl-CoA functioned as a mixed-type inhibitor when the concentration of oxalate was varied in the presence of fixed unsaturating formyl-CoA concentration (Figure 2-14). Relatively high concentrations of oxalyl-CoA were needed to see any inhibition, which indicates the inhibition will disappear at saturating formyl-CoA concentration, although this is hard to confirm since very high formyl-CoA concentration is likely needed.

![Figure 2-14](image.png)

*Figure 2-14. Lineweaver-Burk plot showing product inhibition by oxalyl-CoA vs. oxalate at fixed unsaturating formyl-CoA concentration. Oxalyl-CoA concentrations were ● 0 µM, ○ 90 µM, and ■ 180 µM. Oxalate concentrations were 2.5 – 75 mM. The initial concentration of formyl-CoA was 100 µM in all assays. Intercept replot is shown as insert. Each data point represents an average of two rate measurements.*

The kinetic measurements of FRC detailed above strongly support an ordered ternary complex mechanism where formyl-CoA binds first, followed by oxalate, with
formate then being released before oxalyl-CoA. Other possible kinetic mechanisms are effectively ruled out by the results above (Table 2-5). The mechanism is discussed in more detail in Chapter 3.

Inhibition by Coenzyme A

CoA was of interest as a potential inhibitor, since inhibition by CoA would indicate binding of it to the active site, and therefore a possibility of seeing it bound in the crystal structure of FRC incubated with CoA. This would be an example of dead-end inhibition assuming CoA binds to the free enzyme in the same way formyl-CoA does, but yielding an enzyme-inhibitor complex that is unable to do catalysis. A mixed-type inhibition pattern was observed when CoA was used as an inhibitor against oxalate at fixed unsaturating formyl-CoA concentration is shown in Figure 2-15. This is the expected pattern when the inhibitor binds only to the free enzyme, and further supports an ordered Bi Bi mechanism for FRC.\textsuperscript{62}

Figure 2-15. Lineweaver-Burk plot showing inhibition by CoA vs. oxalate at fixed unsaturating formyl-CoA concentration. CoA concentrations were ● 35 µM, ○ 75 µM, and ■ 115 µM. Oxalate concentrations were 2.5 – 50 mM. The initial concentration of formyl-CoA was 75 µM in all assays. Each data point represents an average of two rate measurements.
CHAPTER 3
STRUCTURE AND MECHANISM OF FORMYL-COA TRANSFERASE

At the start of this research project, Family III CoA transferases had not been recognized as a new class of enzymes and protein structures were not available. Crystal structures of FRC as an apoenzyme and in complex with CoASH became available as a result of collaboration with a research group at the Karolinska Institute in Stockholm, Sweden. Further crystallization experiments yielded a crystal structure of an acylenzyme intermediate formed when FRC was incubated with oxalyl-CoA. These structures and those of three mutant enzymes* are discussed below and how they, combined with kinetic data from Chapter 2, lend strong support for a novel mechanism of CoA transfer.

Structure

The crystal structure of FRC reveals a homodimer with a unique assembly of the subunits. Each monomer consists of a large and a small domain where residues from both the N- and C-termini of the subunit are part of the large domain. The linkers between the domains give the subunit an oval shape with a 13 Å by 22 Å hole in the middle. In the homodimer the second monomer is threaded through the hole in the first one and vice versa like two links in a chain (Figure 3-1).

* Structure factors and coordinates have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/) under accession codes 1P5H (FRC), 1P5R (FRC/CoA complex), 1VGQ (D169A mutant), 1VGR (D169E mutant), 1T3Z (D169S mutant) and 1T4C (FRC/oxalyl-CoA complex).
The interlocked dimer that is observed in the crystal structure of FRC is inconsistent with previous claims that this enzyme exists as a monomer in solution.\textsuperscript{11} The oligomeric state of recombinant wild-type FRC was therefore examined using size exclusion chromatography.

Figure 3-1. Monomer and homodimer structures of recombinant formyl-CoA transferase. (A) One monomer of FRC revealing a large hole in the oval structure. The protein is shown as molecular ribbons with the N-terminus in blue and gradual color change through cyan, green, and yellow to the C-terminus shown in red. (B) The structure of the interlocked FRC homodimer with bound CoA. For clarity, the protein monomers are colored red and green and represented by molecular ribbons. The bound CoA molecules are shown as space-filling models, which are colored using the following scheme: H - white; C - grey; N - blue; O - red; S - yellow; P - purple.

When 7 µg of FRC were injected on the size exclusion column a broad peak with a retention time corresponding to a molecular weight of 53.8 kDa resulted. Larger amounts (30 µg) of FRC, however, yielded a sharp peak matching a mass of 81.0 kDa (Figure 3-3B). The smaller mass (53.8 kDa) is reasonably consistent with the mass of a monomer (47.2 kDa) with the deviation between measured and theoretical mass arising from the open structure of the monomer, and the broadness of the peak probably a result of various
states of unfolding (Figure 3-1A). The larger mass, similarly, is consistent with the enzyme being a homodimer with the deviation between measured (81.0 kDa) and theoretical mass (94.4 kDa) arising from the tightly interlocked dimer structure (Figure 3-1B). This is consistent with the observation that excessive dilution of FRC causes decreased specific activity of the enzyme (see Chapter 2), and most likely means the homodimer is disassociating on the size exclusion column when small amounts of sample are injected.

![Graph showing size exclusion chromatography data](image)

**Figure 3-2.** Size exclusion chromatography data used to calculate the molecular mass of FRC. Retention coefficients (K_D) of molecular weight standards are shown by filled circles. Dotted lines show the observed K_D for samples of FRC and the value of log10(MW) calculated from the equation of the best-fit line through the filled circles. Open circles indicate the placement of FRC samples on the calibration line. K_D is calculated as (elution volume – void volume)/(column volume – void volume).

![HPLC chromatograms](image)

**Figure 3-3.** Size exclusion HPLC chromatograms of FRC. (A) Broad peak from smaller amounts of FRC corresponding to 53.8 kDa. (B) Sharp peak from larger amounts of FRC corresponding to 81.0 kDa.
Active Site

There are two equivalent CoA binding sites in the FRC homodimer, located at the interface between the large domain of one subunit and the small domain of the other subunit (Figure 3-1B). The adenine part of CoA is wedged into a thin cleft and buried from solvent while the ribose, ribose phosphate, and pyrophosphate are solvent-exposed. The pantetheine chain of CoA is buried in a cleft formed mainly by the large domain. The small domain participates with a loop composed of residues 258-261 closing off the cleft where the sulfhydryl group on the pantetheine arm of CoA is bound. In the apoenzyme structure, this loop adopts an open conformation in one subunit, but has a closed conformation in the other. In the FRC-CoA complex, this loop is in the closed conformation in both monomers, leaving insufficient space for an oxalate molecule to bind in the active site. There is however a suitable binding site for oxalate in the vicinity of the sulfhydryl group when the loop is in the open conformation (Figure 3-7).

Figure 3-4. Stereo picture of Coenzyme A in its binding site. Amino acids involved in hydrogen bonds with CoA are colored according to atom type (C atoms cyan for FRC and silver for CoA).
Figure 3-5. CLUSTAL W (1.82) multiple sequence alignment of Family III CoA transferases. FRC (formyl-CoA transferase from *O. formigenes*), YfdW (the FRC homolog from *E. coli*), BbsF (succinyl-CoA: (*R*)-benzylsuccinate CoA transferase from *Thauera aromatica*), FldA (*E*-cinnamoyl-CoA: (*R*)-phenyllactate CoA transferase from *Clostridium sporogenes*), CaiB (butyrobetainyl-CoA: (*R*)-carnitine CoA transferase from *E. coli*).
The residues involved in binding of CoA are identified in Figure 3-4. Asp169 and Gln17 are closest to the sulphydryl end of CoA and Asp169 is in a position where it could attack the carbonyl group of a bound CoA ester. Multiple sequence alignment of Family III CoA transferases is shown in Figure 3-5. YfdW from *E. coli* is likely a formyl-CoA transferase since it shares 60% sequence identity with FRC. FRC shared considerably lower sequence identity with the other transferases, or 26%, 23%, and 20% with BbsF, FldA, and CaiB respectively (see abbreviations in Figure 3-5 legend). Asp169 was identified as a residue potentially playing a critical role in catalysis by FRC due to its position in the active site and because it is conserved in Family III CoA transferases. The only other residue close to the sulphydryl group of bound CoA that is fully conserved is Pro20, which is likely needed for structural purposes creating a critical loop in the structure. Tyr59 and Tyr139, which are also close to the active site, are conserved in four of five sequences shown in Figure 3-5. Tyr59 has been suggested to participate in stabilizing the oxyanion tetrahedral intermediates that may be formed in the transfer reaction. Tyr139 makes hydrophobic contacts with the dimethyl group of the pantetheine chain of bound CoA. Asn96, which contacts CoA, is also conserved in four of the five sequences.

The relatively low conservation of residues in the active site of Family III CoA transferases reflects the broad range of substrates that these enzymes use. The location of the active site, in a cleft between a large domain and a small domain with the sulphydryl end of CoA pointing towards the small domain (Figure 3-1B), suggests that the difference in substrate selectivity between the members of Family III CoA transferases may be linked to structural differences in the small subunits. This would allow the same overall
protein fold for all members, while creating flexibility in active site structure. As described above, the small subunit of FRC is part of the active site structure via a flexible tetruglycine loop consisting of residues 258-261. This moiety is also seen in YfdW, but not in the other Family III CoA transferases in Figure 3-5, where there is a gap in the sequence alignment with FRC. This suggests a large difference in the active site structures, which would explain the difference in natural substrates between these enzymes.

**Point Mutation Studies**

Point mutations of FRC were done where Asp169 was replaced with alanine, glutamate, and serine to give D169A, D169E, and D169S mutants, respectively. The mutations were achieved by using mutagenic DNA primers in a polymerase chain reaction (PCR) with the wild-type FRC in a pET-9a plasmid isolated from the bacterial expression strain as template. The mutant proteins exhibited the same chromatographic properties as the wild-type FRC during their purifications. The mutations were confirmed by DNA sequencing of the mutant plasmids and, later, by X-ray crystallography (Figure 3-6).

The catalytic activity of the mutated proteins was then assayed using the HPLC-based assay described in Chapter 2. The sensitivity of the assay allowed up to a 30,000-fold reduction of CoA transfer rate, relative to wild-type FRC, to be detected. As expected, the mutations dramatically decreased the transferase activity of the protein. Surprisingly though, while both the D169E and D169S FRC mutants exhibited no activity above the detection limit, the specific activity of the D169A mutant was decreased only 1,300-fold compared to the wild-type FRC. No increase in the rate of formyl-CoA hydrolysis was detected in these assays, indicating the enzyme had not been
changed to a CoA ester hydrolase by the mutations. Finally, to ensure the loss of CoA-transferase activity was not due to incorrect folding or quaternary structure of the FRC mutants, X-ray crystal structures of all three mutants with bound CoA were obtained. These studies showed that the mutants were correctly folded and formed interlocked dimers like wild-type FRC. In addition, none of the complexes showed any significant difference in structure from that observed for the wild-type FRC/CoA structure (Figure 3-6).

![Figure 3-6. Stereo images of active site structures of FRC mutants complexed with CoA. Superposition of the active sites of the CoA complexes of wild-type FRC and (A) D169A, (B) D169S, (C) D169E. The carbon atoms in the wild-type FRC/CoA complex are drawn in cyan, and the mutants in purple.](image)

In the complexes involving the D169A and D169S FRC mutants (Figure 3-6A/B), the sulfhydryl group of CoA appeared to be oxidized, resulting in hydrogen-bonding with Glu140 rather than Gln17 and Ala18 as observed in the wild-type FRC/CoA complex.
The reason for this observation only in these two complexes is probably because removal of the Asp169 carboxyl group leaves the thiol group exposed to oxidation. The significant loss of transferase activity in these two mutants most likely arises from their inability to form the key anhydride intermediates (Figure 3-8). The most surprising observation was the detection of any transferase activity by D169A, which lacks the active site carboxylate that is apparently critical for normal activity. Since control experiments ruled out contamination by other CoA transferases, the simplest explanation for this observation is that the transfer reaction proceeds by a different mechanism, similar to the one observed for Family II CoA transferases (Figure 1-3B). Thus, oxalate directly attacks formyl-CoA in a ternary complex to give an oxalyl-formyl anhydride, which then reacts with bound CoA to yield oxalyl-CoA and formate. No rate increase of formyl-CoA hydrolysis was observed when this substrate was incubated with oxalate and D169A, which is consistent with the absence of water molecules in the active site of D169A observed in its crystal structure. Moreover, without wild-type FRC or the mutant, no oxalyl-CoA formation was detected when formyl-CoA and oxalate were incubated under the standard assay conditions. This finding is consistent with previous model studies on the rate of reaction of carboxylic acids with thioesters.65

The conformation of CoA in the active site of the D169E mutant was the most similar to that observed for the wild-type FRC/CoA complex. However, since the glutamate side chain is bulkier than that of aspartate the pantotheine group was displaced slightly (Figure 3-6C), and caused some uncertainty in positioning of the thiol. The lack of activity in the D169E mutant is probably associated with problems in positioning the
formyl-CoA correctly to permit anhydride formation by reaction of the thioester with the carboxylate moiety (Figure 3-8).

**Mechanism**

The mechanism for Family III CoA transferases proposed by Heider was based on the mechanism of Family II enzymes (Figure 1-3B). The enzyme was proposed to catalyze direct nucleophilic attack of oxalate on formyl-CoA to yield a mixed oxalyl-formyl anhydride intermediate and free CoA. Addition of CoA at the other end of the mixed anhydride would then complete the acyl transfer. Notably, no covalent intermediates would be formed by reaction of the substrates with active site residues in such a mechanism, the function of the catalyst being primarily to bring the reactants together in the correct orientation. The crystal structure of wild-type FRC is, however, not supportive of this mechanism since the position of the side chain of Asp169 appears to prevent a direct attack of oxalate on formyl-CoA (Figure 3-7).  

![Figure 3-7. Stereo picture of the end of the pantetheine chain of CoA and amino acids in the surrounding active site. Superimposed to loop 258-261 in the closed conformation is the same loop in the open conformation as seen in the apoenzyme structure (grey). The two conformations correspond to different rotamer conformations of Trp48. The cavity formed when the loop is in the open conformation is shown as a light green cloud. A model of bound oxalate, in magenta, is included but its orientation is unknown.](image-url)
A new hypothesis for the catalytic mechanism of FRC, which includes the direct involvement of Asp169, has consequently been proposed (Figure 3-8).\textsuperscript{42,64} Formyl-CoA binds to the active site in the initial step and the substrate thioester reacts with the Asp169 side chain to form a covalent formyl-Asp169 anhydride intermediate and CoA. Oxalate, entering through the cavity formed when the loop of residues 258-261 is in the open conformation (Figure 3-7), reacts to generate a new enzyme anhydride intermediate (oxalyl-Asp169 anhydride) and formate. Subsequent attack of bound CoA on the mixed anhydride then yields oxalyl-CoA and regenerates the carboxylate moiety of Asp169. Since free formate is not produced until after oxalate is bound, kinetic plots cannot assume the form observed for classical Ping-Pong mechanisms like in the case of Family I CoA transferases.
The proposed mechanism of Family III enzymes predicts incorporation of $^{18}$O into the main catalytic residue (Figure 3-8) as for Family I enzymes (Figure 1-3A) if an $^{18}$O-labeled CoA acceptor would be used. This has not yet been verified for any Family III CoA transferase. An important difference between the proposed mechanism of Family III CoA transferases and the Family I CoA transferase mechanism is the absence of covalent enzyme-CoA intermediates in the former. Family I CoA transferases are completely inactivated when incubated with hydroxylamine in the presence of one of their CoA ester substrates. The inactivation is a result of the reaction of hydroxamic acids, formed from the hydroxylamine and the thioester substrate, with the CoA ester of the main catalytic residue (a glutamic acid) (Figure 1-3A). Studies on two Family III enzymes support the absence of covalent enzyme-CoA intermediates in the mechanism of Family III CoA transferases. Neither BbsF (succinyl-CoA: (R)-benzylsuccinate CoA transferase) nor FldA ((E)-cinnamoyl-CoA: (R)-phenyllactate CoA transferase) are inactivated by hydroxylamine in tests with and without the CoA ester substrate.27,29

NaBH$_4$ has also been shown to inactivate Family I enzymes by reacting with the covalent enzyme-CoA intermediate reducing the glutamic acid residue to an alcohol.33,35 Family III CoA transferases are at least partially inactivated by NaBH$_4$, although inactivation requires higher concentration of NaBH$_4$ than for Family I enzymes. The activity of BbsF decreased by 75% after incubation with 0.25 mM benzylsuccinyl-CoA and 1 mM NaBH$_4$ at pH 7.5 for 10 minutes and the activity of FldA decreased by 50% after incubation with 49 µM cinnamoyl-CoA and 10 mM NaBH$_4$ at pH 7 for 15 minutes.27,29
The simplest interpretation of these observations is that there is no covalent enzyme-CoA intermediate present during catalytic turnover of Family III CoA transferase enzymes, which would explain them not being inactivated by hydroxylamine. Furthermore, the relatively slow inactivation by NaBH₄ is then due to the reduction of a covalent enzyme-substrate anhydride intermediate that is less reactive and/or not as accessible as the covalent enzyme-CoA thioester intermediates in the Family I mechanism. This may be possible to confirm using tritiated sodium borohydride as has been done for Family I enzymes. NaBH₄ has been used to reduce mixed anhydrides of carboxylic acids and carbonic acids, and cyclic carboxylic acid anhydrides.

![Figure 3-9. Stereo picture showing the interactions of the oxalyl-aspartyl anhydride with residues in the FRC dimer. The letter designation (A or B) in the numbering scheme indicates the FRC monomer in which the residue is located.](image)

Obtaining protein crystals of FRC with bound formyl-CoA or oxalyl-CoA proved elusive due to the lability of the thioesters. Surprisingly, after extensive screening studies, the product of reaction between FRC and oxalyl-CoA, i.e. the putative oxalyl-Asp169 anhydride intermediate (Figures 3-8 and 3-9), was crystallized by Stefano Ricagno, a graduate student in the laboratory of Dr. Ylva Lindqvist, the crystallography collaborator. This structure lends very strong support to the participation of mixed
anhydrides of Asp169 in the catalytic mechanism of FRC, and along with the kinetic data presented in Chapter 2 strongly supports the proposed ordered Bi Bi mechanism shown in Figure 3-8.
All materials were of the highest purity available and, unless stated otherwise, obtained from Fisher Scientific International, Inc. (Hampton, NH) or Sigma-Aldrich Corp. (St. Louis, MO). Protein concentrations were determined by the Lowry method as modified by Hartree\textsuperscript{69} using bovine serum albumin as a standard. DNA sequencing was performed by the DNA Sequencing Core of the Interdisciplinary Center for Biothechnology Research at the University of Florida. The BL21(DE3) *Escherichia coli* expression strain transformed with pET-9a plasmid carrying the gene for wild-type FRC was supplied by Dr. Harmeet Sidhu (Ixion Biotechnology, Inc., Alachua, FL). Mutagenic PCR (polymerase chain reaction) primers were obtained from GenoMechanix LLC (Gainesville, FL) (D169A and D169E) and Integrated DNA Technologies, Inc. (Coralville, IA) (D169S).

**Expression and Purification of FRC**

Wild-type formyl-CoA transferase (FRC) from *Oxalobacter formigenes* was overexpressed in BL21(DE3) *Escherichia coli* and purified by anion exchange and affinity chromatography. The enzyme was expressed by growing an overnight culture (100 mL) in Luria-Bertani broth containing 30 µg/mL kanamycin (LBK) at 37°C and 200-250 rpm, which was then used to inoculate 4-6 L of fresh LBK. FRC expression was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) when the optical density of the culture, grown at 37°C and 200 rpm, reached 0.6-0.8 at 600 nm (4 mL of 0.1 M IPTG added per liter of culture). The cells were harvested 2.5-4.0 hours after induction
by centrifugation at 4°C. The cell pellets were resuspended in 50-150* mL lysis buffer (100 mM potassium phosphate, 1 mM dithiothreitol (DTT), pH 7.2) and lysed by passing two times through a French press or by sonication (ten 10 second pulses with 30 second intervals). After centrifuging, the lysate supernatant was loaded on a 120 mL DEAE Fast Flow anion exchange column equilibrated with buffer A (25 mM sodium phosphate, 1 mM DTT, pH 6.2) and FRC eluted by stepping to 35% buffer B (25 mM sodium phosphate, 1.0 M NaCl, 1 mM DTT, pH 6.2) at 5 mL/min. The FRC-containing fractions were loaded on a 20 mL Blue FF (Blue Sepharose™ 6 Fast Flow) affinity column equilibrated with buffer A. The column was then washed with 50% buffer B and FRC finally eluted with buffer C (25 mM glycine, 1 mM DTT, 20% isopropanol, pH 9.0) at 4 mL/min. The FRC-containing fractions from the affinity column were immediately buffer-exchanged by passing through a 135 mL G-25 desalting column equilibrated with buffer A at 10 mL/min. The protein solution was then injected on a 60 mL Q-Sepharose high performance anion exchange (QHP) column equilibrated with buffer A and the FRC eluted at 5 mL/min by stepping to 20% buffer B for 1-2 column volumes followed by a linear gradient to 35%B over 6-7 column volumes, with FRC eluting close to the center of the linear gradient. Glycerol was added to achieve a concentration of 10% to stabilize the protein since it has a tendency to precipitate. The resulting protein solution was stored at -80°C. All purification steps were run at ambient temperature and the FRC fractions stored on ice between purification steps. The purification was verified by SDS-PAGE with Coomassie Blue staining and activity measurements of the fully purified enzyme. See Appendix A for typical FPLC chromatograms from each purification step.

* Smaller volumes used when lysing with French press. Larger volumes used when lysing by sonication.
The pooled FRC-containing fractions after the first three chromatography steps have a tendency to form a protein precipitate, resulting in loss of CoA transferase activity. This loss is minimized by using 1 mM dithiothreitol (DTT) in all buffers and storing the collected fractions on ice until the next purification step. For best yields, the first three steps should be performed within 12 hours. The fully purified FRC eluted off the QHP column also has a slight tendency to precipitate, but addition of glycerol to a concentration of 10% stabilizes the protein so no precipitate is visible after freezing and thawing. The Blue FF affinity column resin has a lifetime of about one year when storage instructions are followed and using a column that has started to degrade dramatically decreases yields.

**Expression of Selenomethionine Derivative of Wild-Type FRC**

The selenomethionine derivative of wild-type FRC (SeMet FRC) was prepared using literature procedures for expression of SeMet proteins in nonauxotrophic strains of *E. coli*.51 The bacteria were grown in M9 medium (2 mM MgSO4, 0.1 mM CaCl2, 48 mM Na2HPO4, 22 mM KH2PO4, 9 mM NaCl, 19 mM NH4Cl, 4 g/L glucose) at 37°C and 200 rpm until the optical density of the culture at 600 nm reached 0.6-0.8. Methionine biosynthesis by the bacteria was then downregulated by addition of lysine (100 mg/L), threonine (100 mg/L), phenylalanine (100 mg/L), leucine (50 mg/L), isoleucine (50 mg/L), valine (50 mg/L), and proline (50 mg/L). Selenomethionine (50 mg/L) was then added to the culture, which was incubated for 15 minutes before inducing with IPTG as described for wild-type FRC above. The cells were harvested 4-5 hours after induction and the SeMet FRC purified as described for wild-type FRC.
Site-Directed Mutagenesis

Mutagenic primers were designed using Gene Runner v. 3.05 (Hastings Software, Inc.). The pET-9a plasmid with the FRC gene insert was isolated from BL21(DE3) *E. coli* with the Wizard® Plus Minipreps DNA Purification System (Promega, Madison, WI) and used as a template for PCR with the mutagenic primers using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The desired mutations were verified by DNA sequencing of the FRC gene inserts of the mutated pET-9a plasmids isolated from transformed XL-1 or XL-10 Gold supercompetent cells (Stratagene, La Jolla, CA). BL21(DE3) competent cells (Novagen, Madison, WI) were then finally transformed with the mutated plasmids and the mutated FRC proteins expressed and purified as described for wild-type FRC above.

Synthesis of CoA Esters

**Formyl-CoA**

Formylthiophenyl ester was made by formylating thiophenol using a formylating reagent made from acetic anhydride and formic acid. Formic acid (6.9 g; 150 mmols; 5.8 mL) was added dropwise to stirred acetic anhydride (7.7 g; 75 mmols; 7.1 mL) and the resulting mixture stirred at 45 °C for 2.5 hours. Pyridine (59 mg; 0.75 mmols; 61 µL) was then added, immediately followed by thiophenol (5.5 g; 50 mmols; 5.1 mL), and the reaction mixture stirred at room temperature for 24 hours and then stored at 7 °C overnight after purging with dry nitrogen gas. Thin layer chromatography (TLC) (1:1 chloroform:hexanes) showed the product at \( R_f = 0.53 \) and no reactant (\( R_f = 0.72 \)) with a faint spot at \( R_f = 0.0 \). The remaining acids and anhydrides were removed from the reaction mixture by vacuum distillation (40 – 50°C/20 mmHg) and collected in a cold-finger trap immersed in liquid nitrogen. A water aspirator was used, connected via a drying tube to
the reaction flask. When the volume had decreased to 6-8 mL the product was washed with an equal amount of deionized water and then dried over anhydrous magnesium sulfate. TLC, after washing with water, indicated minor decomposition of the product. The product was finally vacuum distilled (115-117 °C/23 mmHg), yielding 3.1 g or 45 % of pure product. The literature values are 101 °C/15 mmHg and 87 % yield. $^1$H-NMR (CDCl$_3$, 300 MHz) $\delta$ 10.22 (s, 1), $\delta$ 7.46 (m, 5). $^{13}$C-NMR (CDCl$_3$, 75.5 MHz) $\delta$ 190.0, 134.1, 129.9, 129.5, 126.0. See NMR spectra in Appendix B.

Formyl-CoA was prepared by a method based on the procedure by Sly and Stadtman.$^{52}$ Sodium salt of CoA (80 mg, 96 µmols CoA) was dissolved in 2-8 mL of ice-cold deionized water and the pH adjusted to 7.0 with 0.1 M KOH or NaOH while cooling on ice. Formylthiophenyl ester (80 mg, 580 µmols) was dissolved in ice-cold dry ether (1-2 mL) and added quickly to the cold CoA solution while stirring, resulting in pH decrease to 6.6. The pH was adjusted to 7.5-8.0 with 1 M KHCO$_3$ or NaHCO$_3$ at pH 8.0 (0.25-1.0 mL) and the reaction mixture stirred on ice for 2-3 minutes. The pH was then carefully adjusted to 3.0 with 0.1 M HCl under rapid stirring, and the solution washed three times with two volumes of ice-cold ether. The pH was finally adjusted to 5.5 with 0.1 M KOH or NaOH and stored at -80°C. Typical yields were about 70-90%.

LC/(+/-)ESI-MS (Flow Injection Analysis) calculated for C$_{22}$H$_{36}$N$_7$O$_{17}$P$_3$S: 795.5; found: 795.1 (+)ESI-MS and 795.6 (-)ESI-MS.

Oxalyl-CoA

Thiocresol oxalate (25 mg, 0.13 mmol) dissolved in ice-cold anhydrous ether (5-10 mL) was added slowly to an ice-cold solution of CoA (sodium salt) (25 mg, 0.031 mmol) in water (10 mL) at pH 7.5 (adjusted with 0.1 M NaHCO$_3$). The reaction mixture was stirred on ice for about 20 min before removing the aqueous layer and acidifying it
carefully to pH 3.5 with 0.1-1.0 M HCl followed by washing with two 12 mL portions of ice-cold ether. When complete removal of thiocresol oxalate was desired the aqueous phase was washed eight times with ether adjusting the pH of the aqueous phase to 3.0 between washes. The resulting solution was stored at -80 °C. Typical yields were 80-100%. C-18 HPLC/UV(260 nm)/(+)ESI-MS calculated for C_{23}H_{36}N_{7}O_{19}P_{3}S: 839.5; found: 839.4.

**Analysis of CoA Esters**

Analysis of the CoA esters was based on literature procedures using C-18 reversed-phase HPLC (Dynamax Microsorb 60-8 C18, 250 x 4.6 mm) with a single-wavelength detector at 260 nm. The column was equilibrated with 86% buffer A (25 mM NaOAc, pH 4.5) and 14% buffer B (20 mM NaOAc, pH 4.5, 20% CH₃CN) running at 1.0 mL/min. Immediately after injection a 12 minute gradient to 34% buffer B was started followed by a step to 100% buffer B for 2 minutes before returning to 14 % buffer B. Oxalyl-CoA eluted at 6.5 minutes, free CoASH at 11.5 minutes, and formyl-CoA at 12.5 minutes. The CoA esters were quantitated by integration of their peaks in the HPLC chromatograms using free CoASH as a quantitative standard. The validity of this method was confirmed for formyl-CoA by independently measuring its concentration in solution using the hydroxylamine method, and for oxalyl-CoA by measuring the concentration of oxalate in an oxalyl-CoA solution before and after complete hydrolysis by base using an oxalate detection kit (Sigma-Aldrich Corp., St. Louis, MO).

**Purification of CoA Esters**

The CoA esters were purified using a preparative C-18 reversed-phase column (Dynamax 60A C18, 250 x 21.4 mm). The column was equilibrated with 88% mobile phase A (10 mM sodium phosphate at pH 5.0) and 12 % mobile phase B (mobile phase A
with 20% acetonitrile) at 10 mL/min. Two minutes after injecting the sample the fraction of mobile phase B was increased linearly to 38% over 17 minutes. The absorbance of the eluent at 260 nm was monitored and fractions collected manually on ice. Oxalyl-CoA eluted at about 5 min, CoA at 12 min, and formyl-CoA at 13 min. The fractions were analyzed as described above and lyophilized in small (1-2 mL) aliquots.

Enzymatic Assay

The recombinant wild-type FRC was assayed by measuring the initial rate of oxalyl-CoA formation by HPLC analysis of quenched aliquots. The assay mixture contained 60 mM potassium phosphate (pH 6.7), FRC (90 ng, 9.5 nM), and variable amounts of substrates and inhibitors (if desired) in a total volume of 200 µL. The reaction was started by addition of formyl-CoA after incubating the other components at 30 °C for about 30 seconds. Aliquots of the reaction mixture (90 µL) were typically taken after 60 s and 90 s and quenched with 10% acetic acid** (10 µL) before quantitating oxalyl-CoA by reversed-phase HPLC using a shorter version of the analytical procedure described above that separates only oxalate and oxalyl-CoA from the rest of the mixture components. No formation of oxalyl-CoA was detected in control experiments when the enzyme or either substrate was omitted, or when FRC denatured by incubation in boiling water was used. The limit of detection of the assay as described is about 0.05 µM of oxalyl-CoA when 75 µL of quenched assay mixture is injected on HPLC column.

The specific activities of the D169A, D169E, and D169S FRC mutants were assayed using an identical procedure except that reaction mixtures were incubated for up

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* Small aliquots minimized hydrolysis of the CoA esters during freeze-drying.

** The pH of the quenched aliquots should be 3-4, which is ideal for CoA ester stability. Oxalyl-CoA concentration in quenched samples remains unchanged for several hours when stored on ice.
to 60 minutes prior to quenching. In addition, because of the much lower activity of the FRC mutants, the amount of enzyme in each assay was increased to 2 µg, and the initial concentrations of oxalate and formyl-CoA were 100 mM and 200 µM, respectively.

Alternative substrates (Table 2-3) were screened using the assay described above, replacing the natural substrates with the substrates being tested. CoA ester concentrations were in the range of 80-200 µM and free acid concentrations were 62.5-125 mM in these experiments.

**Equilibrium Constant Determination**

Wild-type FRC (18 µg) was incubated at 22°C with 73 µM formyl-CoA, 50 µM oxalate, 13 µM formate, and 13 µM free CoA (introduced by formyl-CoA stock solution) in 60 mM potassium phosphate buffer at pH 6.7 (200 µL total volume). Aliquots (45 µL) were withdrawn after 10, 27, and 52 minutes, and quenched with 10% acetic acid (5 µL). The concentrations of oxalyl-CoA, free CoA (and therefore formate), and formyl-CoA in each sample was measured by HPLC as described above. Equilibrium concentrations were reached after 27 minutes, giving $K_{eq} = 32 \pm 3$.

**Size Exclusion Chromatography (SEC)**

A BIOSEP SEC-S2000 column (300 x 7.8 mm with 75 x 7.8 mm guard column) (Phenomenex, Torrance, CA) was equilibrated with 100 mM potassium phosphate at pH 6.6 running at 1.0 mL/min. The column was calibrated using lysozyme (14.4 kDa), carbonic anhydrase (29.0 kDa), peroxidase (44.0 kDa), bovine serum albumin (66.0 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa). The void volume of the column was measured by injecting blue dextran. Samples of FRC (7 µg and 30 µg) were injected on the column and the molecular weights (53.8 kDa and 81.0 kDa) calculated from the retention times.
This research project was originally aimed at determining whether the mechanism of FRC was a Ping-Pong mechanism, used by almost all known CoA transferases known at the time. However, upon discovering that FRC belongs to a new class of CoA transferase enzymes, apparently using a novel mechanism of CoA transfer, the focus of the project turned to deciphering that mechanism. The unique crystal structure of FRC and sequence alignments of Family III CoA transferase enzymes identified a putative main catalytic residue, which was confirmed by its mutation and the resulting loss of activity, thus refuting a mechanism like the one used by Family II CoA transferases as proposed previously. Following synthesis of substrates and development of an enzymatic assay, steady-state kinetic studies and product inhibition patterns led to the proposal of a novel mechanism of CoA transfer, which includes covalent enzyme-substrate anhydride intermediates. One of these putative intermediates was observed by X-ray crystallography of FRC crystals grown in the presence of oxalyl-CoA, providing further evidence for the proposed mechanism. Since the main catalytic residue is conserved in known Family III CoA-transferases, the catalytic mechanism of formyl-CoA transferase is almost certainly employed by all other members of this enzyme family.

Although no direct evidence for the proposed covalent enzyme-substrate anhydride intermediates exist for Family III CoA transferases the crystal structure data presented herein are all supportive of such intermediates. An $^{18}$O-exchange experiment using $^{18}$O-
oxalate with formyl-CoA or \(^{18}\)O-formate with oxalyl-CoA should provide definitive evidence for these intermediates (Figure 3-8) as it has for Family I CoA transferases.\(^{32}\) So far, no convenient proteolytic conditions, which are necessary for such experiments, have been found for FRC.

The possibility of a covalent enzyme-CoA thioester in the mechanism of FRC, as observed for Family I CoA transferases (Figure 1-3A), can be tested by using the methods of Hersh and Jencks.\(^{33}\) After incubation of the enzyme with a CoA ester substrate and subsequent removal of small molecules by size exclusion filtration the presence of free CoA can be assayed after allowing the putative enzyme-CoA intermediate to hydrolyze. Incubation of the enzyme with free CoA serves as a control reaction, since no CoA should be detected in the protein fraction after the size exclusion filtration.

Some other questions remain regarding the proposed mechanism, such as the timing of the mixed anhydride formation, which could take place before or after oxalate binding, although the observation of the aspartyl-oxalyl anhydride intermediate in the crystal structure would suggest this happens in the absence of the free acid substrate, since there was no oxalate or formate present in the solution the crystals were grown in. It is not known whether CoA stays bound after the mixed anhydride formation or if it diffuses into solution. In theory, at least the pantotheine moiety may have to move out of the active site to allow the incoming free acid to attack the mixed anhydride, unless the attack comes from the other side of the anhydride intermediate. One of the biggest questions concerns the substrate selectivity of FRC. The features of the active site responsible for the high selectivity towards formate, oxalate, and succinate, while acetate,
oxamate, and pyruvate are not accepted as substrates, are not yet known. Perhaps computational modeling can provide some answers to these questions, identifying favorable or unfavorable interactions between active site residues and the bound molecules. More site-directed mutagenesis studies aimed at residues in the active site known or suspected of interacting with the bound substrates, such as Gln17, Tyr59, and Tyr139, would also undoubtedly be beneficial to the understanding of the mechanism.

Another issue is the discovery of the yfdW gene in *E. coli* and the expression of its protein product, whose crystal structure is the same as FRC’s. *E. coli* has never been shown to degrade oxalate, although the presence of this gene and the orthologous gene of oxalyl-CoA decarboxylase from *O. formigenes* would indicate that it is capable, given that these proteins have the same function as in *O. formigenes*. An interesting experiment would be to examine whether *E. coli* can be induced to express these genes and become an active oxalate degrader. Furthermore, comparing the activities and substrate specificities of the enzymes from these two sources would be of value.

Given that FRC and oxalyl-CoA decarboxylase (OXC) combined constitute up to 20% of the total protein content of *O. formigenes*, and the important function they serve in the cell, it is not unreasonable to suggest that these enzymes may interact somehow, perhaps creating oxalate-degrading complexes inside the cell. This would be beneficial to the organism, especially since it would minimize spontaneous hydrolysis of the labile formyl-CoA, which would effectively stop the catalytic cycle of oxalate breakdown. The fact that the adenine part of CoA is firmly bound to FRC while the rest of the molecule is solvent accessible could mean that the pantetheine arm of CoA esterified with oxalate can swing out of the active site of FRC directly into the active site
of OXC and return to FRC as a formyl ester after decarboxylation. This hypothesis could be tested by fluorescence labeling and/or immunohistochemistry methods.

Finally, understanding the folding mechanism of the remarkable interlocked dimer of FRC could be a large contribution to the field of protein folding, and collaboration in that regard is currently under way.
APPENDIX A
PROTEIN PURIFICATION CHROMATOGRAMS

The following figures show typical chromatograms of the four purification steps used to purify the wild-type, selenomethionine, and mutated formyl-CoA transferases.

Figure A-1. FPLC chromatogram of the DEAE anion exchange purification step. Blue = Absorbance at 280 nm (0-3000 mAu\*). Red = Conductivity (scale 0-75 mS/cm). Black = Percent concentration of buffer B (scale 0-100%). The arrow points to the FRC containing peak.

\* mAu = milli absorbance unit
Figure A-2. FPLC chromatogram of the BlueFF affinity purification step. Blue = Absorbance at 280 nm (0-1700 mAu). Red = Conductivity (scale 0-45 mS/cm). Black = Percent concentration of buffer B (scale 0-50%). The arrow points to the FRC peak.

Figure A-3. FPLC chromatogram of the buffer exchange purification step. Blue = Absorbance at 280 nm (0-750 mAu). Red = Conductivity (scale 0-22 mS/cm).
Figure A-4. FPLC chromatogram of the QHP anion exchange purification step. Blue = Absorbance at 280 nm (0-120 mAu). Red = Conductivity (scale 0-75 mS/cm). Black = Percent concentration of buffer B (scale 0-100%). The arrow points to the FRC peak.
APPENDIX B
NMR SPECTRA OF FORMYLTHIOPHENYL ESTER

The following figures in show the $^1$H-NMR and $^{13}$C-NMR spectra of formylthiophenyl ester, the formylating reagent used to prepare formyl-CoA. See experimental section (Chapter 4) for chemical shifts.

Figure B-1. $^1$H-NMR spectrum of formylthiophenyl ester.
Figure B-2. $^{13}$C-NMR spectrum of formylthiophenyl ester.

Figure B-3. Close-up on relevant peaks of Figure A-2.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Stefán Jónsson was born in Reykjavík, Iceland, in 1972. He holds a B.Sc. degree in biochemistry from the University of Iceland and an M.Sc. degree in chemistry from the same school. After working one year as a research scientist for deCODE Genetics, Inc in Reykjavik he started graduate studies in the Chemistry Department of the University of Florida in August 1999, where he joined Dr. Nigel G. J. Richards’ research group in May 2000.