ABSTRACT

Title of Dissertation: BENZOYL-COA REDUCTASE:

A BIOLOGICAL BIRCH REDUCTION

Steven Thomas Poole, Doctor of Philosophy, 2003

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Department of Chemistry

Benzoyl-CoA reductase, isolated from the anaerobic bacterium *Thauera aromatica*, catalyzes the ATP-dependent, two-electron reduction of the aromatic ring of benzoyl-CoA. A Birch-like mechanism, which involves two separate one electron and one proton additions to the aromatic ring of benzoyl-CoA, has been previously proposed for benzoyl-CoA reductase. The first electron transfer of this reaction, which produces a radical anion, is thought to be the rate-limiting step. Other mechanisms, such as hydride reduction and catalytic hydrogenation, are possible. In an effort to determine how the enzyme reduces its substrate, several substrate analogues were synthesized and studied using kinetic and/or product analysis. Of the nitrogen-containing, heterocyclic analogues, only picolinoyl-CoA proved to be a substrate for the reductase, having a k_{cat} similar to that of benzoyl-CoA. Nicotinoyl-CoA did not react with the enzyme and isonicotinoyl-CoA was

reduced by the electron donor in the absence of the enzyme. Mass spectrometric analysis of the products formed by the fluorinated analogues, m-fluorobenzoyl-CoA and p-fluorobenzoyl-CoA indicated that both substrates were defluorinated by benzoyl-CoA reductase, supporting a Birch-like mechanism with the first electron being added to the carbonyl functionality of the thioester. Also, benzoyl-CoA reductase only exhibited a small kinetic isotope effect (1.8), arguing against simultaneous hydrogen and electron transfer and hydride transfer. It was also found that under aerobic conditions and without ATP, benzoyl-CoA reductase could carry out the oxidation of its native reduction product reforming the substrate of the reaction, benzoyl-CoA. Since the reduction capability of benzoyl-CoA reductase is quickly and irreversibly inactivated by oxygen, it is thought that the enzyme is degraded under aerobic conditions. However, these finding suggest that benzoyl-CoA reductase may only be partially degraded by oxygen exposure and that some of its subunits may still retain some of its functionality and structure.

BENZOYL-COA REDUCTASE: A BIOLOGICAL BIRCH REDUCTION

by

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DEDICATION

This dissertation is dedicated to my family for their constant support and to my advisor, Dr. David Jollie, for his patience and guidance.

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LIST OF ABBREVIATIONS

A	armstrong
ATP	adenosine triphosphate
CoA	coenzyme A
D_2O	deuterium oxide
DEAE	diethylaminoethyl
ESI	electrospray ionization
FPLC	fast protein liquid chromatography
HPLC	high performance liquid chromatography
kDa	kilodalton
LC	liquid chromatography
LC/MS	liquid chromatography coupled to mass spectrometry
MES	3-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
NaOD	sodium deuteroxide
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
TEA	triethanolamine

UV	ultraviolet
Vis	visible

CHAPTER I. Background and Theoretical Rationale

Background.

Impact of aromatic compounds on the environment. As concerns over the environment grow, interest has been directed toward the fate of chemical toxins released into the environment. One class of environmental toxins are aromatic hydrocarbons, which are commonly found in chemical solvents, pesticides and petroleum-base products. The increased production of these aromatic compounds has led to their widespread dispersion into our environment, which can occur either through their direct use, such as the spraying of crops with pesticide, or by accidental release during the storage, transport, or use of these chemicals. Such occurrences have resulted in the release of more than four hundred organic chemicals into the environment. The presence of these chemicals in the environment can have harmful effects, including increases in cancer risks for humans and a reduction in the population of some species (Young, 1984). However, not all aromatic compounds in the environment are man-made. In fact, aromatic compounds derived from natural sources make up the majority of those found in the environment. These natural sources include the simple aromatics, like those found in amino acids, and large polymeric aromatics, such as lignin. Lignin

makes up approximately thirty percent of the dry weight of vascular plant tissue and is one of the most abundant polymers in the environment (Elder, 1994). The aromatic ring systems present in these compounds provide them with a large resonance energy and a great deal of stability, making them resistant to environmental degradation (Gibson, 1984). In fact, most animal and plants can not metabolize aromatic compounds and for the most part, aromatic compounds are degraded by microorganisms, such as fungi, bacteria, and some algae (Heider, 1997). The actions of these microorganisms prevent the buildup of harmful, potentially carcinogenic, aromatic compounds in the environment and recycle the carbon atoms contained within these compounds back into the carbon cycle so life can continue (Gibson, 1984).

Aerobic metabolism of aromatic compounds. In order to metabolize aromatic hydrocarbons, microorganisms must develop a strategy to overcome the inherent stability of these compounds. One such strategy involves the use of molecular oxygen to hydroxylate and then cleave the aromatic ring (Lipscomb, 1992). These microorganisms can transform a variety of aromatic compounds into a few, central aromatic intermediates (Heider and Fuchs, 1997). These central intermediates have similar characteristics. They all have hydroxyl substituents located on the aromatic ring system and are all capable of undergoing aromatic ring cleavage, chemically and enzymatically. These microorganisms use peripheral pathways, which feed into the metabolic central pathway is to remove any

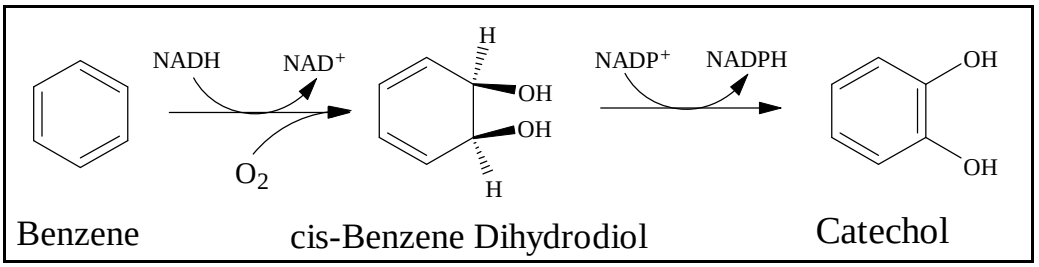


Figure 1. **The initial reactions of the aerobic metabolism of benzene**. Benzene is oxidized to cis-benzene dihydrodiol by benzene dioxygenase. Cis-benzene dihydrodiol dehydrogenase then converts the dihydrodiol to catechol, which can then undergo aromatic ring cleavage. Figure adapted from Gibson, 1984.

substituents from the aromatic ring and replace them with hydroxyl groups (Heider and Fuchs, 1997). The addition of hydroxyl substituents to the aromatic ring is carried out by a variety of oxygenases. As seen in Fig. 1, benzene dioxygenase catalyzes the conversion of benzene to cis-benzene dihydrodiol, which is then oxidized to catechol by a dehydrogenase (Gibson, 1984). These central intermediates then undergo cleavage of the aromatic ring and the resulting product is further broken down to common metabolites, such as acetyl-CoA. The cleavage of the aromatic ring system also requires the use of an oxygenase. Ring cleavage occurs either ortho or meta to an aromatic hydroxyl substituent, as shown in Fig 2 (Heider and Fuchs, 1997). This metabolic pathway employs monooxygenases and dioxygenases to carry out the transformation and cleavage reactions on the aromatic ring. These enzymes use the reactivity of molecular oxygen to overcome the large activation energy needed to cleave the stable aromatic ring structure.

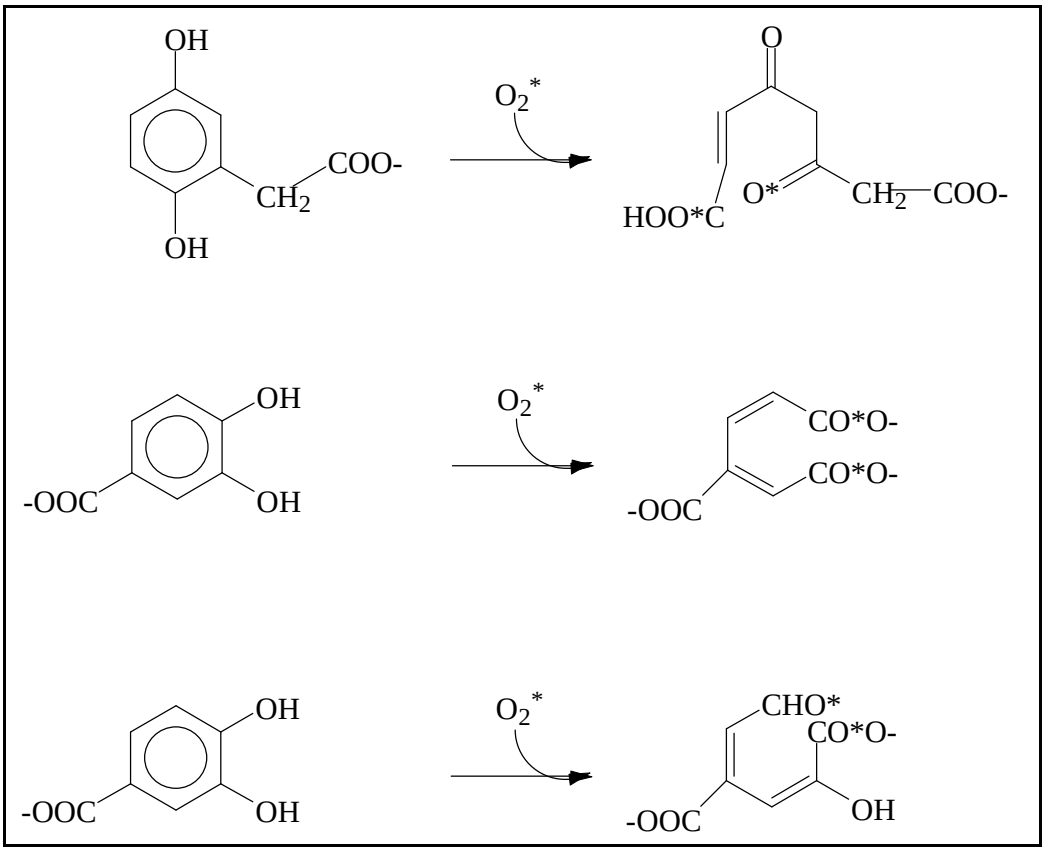


Figure 2. **Dearomatizing reactions in aerobic metabolic pathways**. The reactions shown represent the gentisic acid (top) pathway, the ortho-cleavage pathway (middle), and the meta-cleavage pathway (bottom). Figure adapted from Heider and Fuchs, 1997.

Oxygen is a required cosubstrate of these enzymes and is incorporated into the final product (Lipscomb and Orville, 1992).

Anaerobic metabolism of aromatic compounds. However, in anaerobic environments, where the biological use of oxygen exceeds the rate at which oxygen enters the environment due to a lack of exchange with the environment, or where it is too dark for photosynthesis, this strategy cannot be used to degrade aromatic compounds. Such places occur in the digestive tracts of animals, in waterlogged

and compacted soils, and in sediments found under bodies of water (Young, 1984). In these environments, microorganisms use terminal electron acceptors other than oxygen to carry out respiration, such as nitrate, sulfate, manganese, iron, and carbon dioxide (Bouwer, 1992). Since the previously described pathway requires oxygen, it would be logical to assume that the metabolism of aromatic compounds can only be performed by microorganisms living in oxygen-rich environments. However, it has been shown that simple aromatic compounds do not accumulate in anaerobic environments (Tarvin and Buswell, 1934; Evans, 1977). Therefore, microorganisms living under these conditions must use a different strategy for degrading aromatic compounds that does not require the use of oxygen. Instead of using oxygenases and molecular oxygen to disrupt the stable aromatic character of aromatic compounds, it was proposed that the ring character of aromatic compounds is eliminated by a reduction pathway (Evans, 1977; Evans and Fuchs, 1988).

Similar to its aerobic counterpart, the anaerobic scheme of metabolizing aromatic compounds contains several peripheral pathways that funnel into one central metabolic pathway, allowing anoxic organisms to utilize a variety of aromatic substrates, while only having to solve the energy barrier problem for one substrate, benzoyl-coenzyme A (CoA) (Fig. 3). However, while the aerobic peripheral pathways add hydroxyl substituents to the ring system, their anaerobic counterparts remove substituents from the ring and if needed, introduce a single

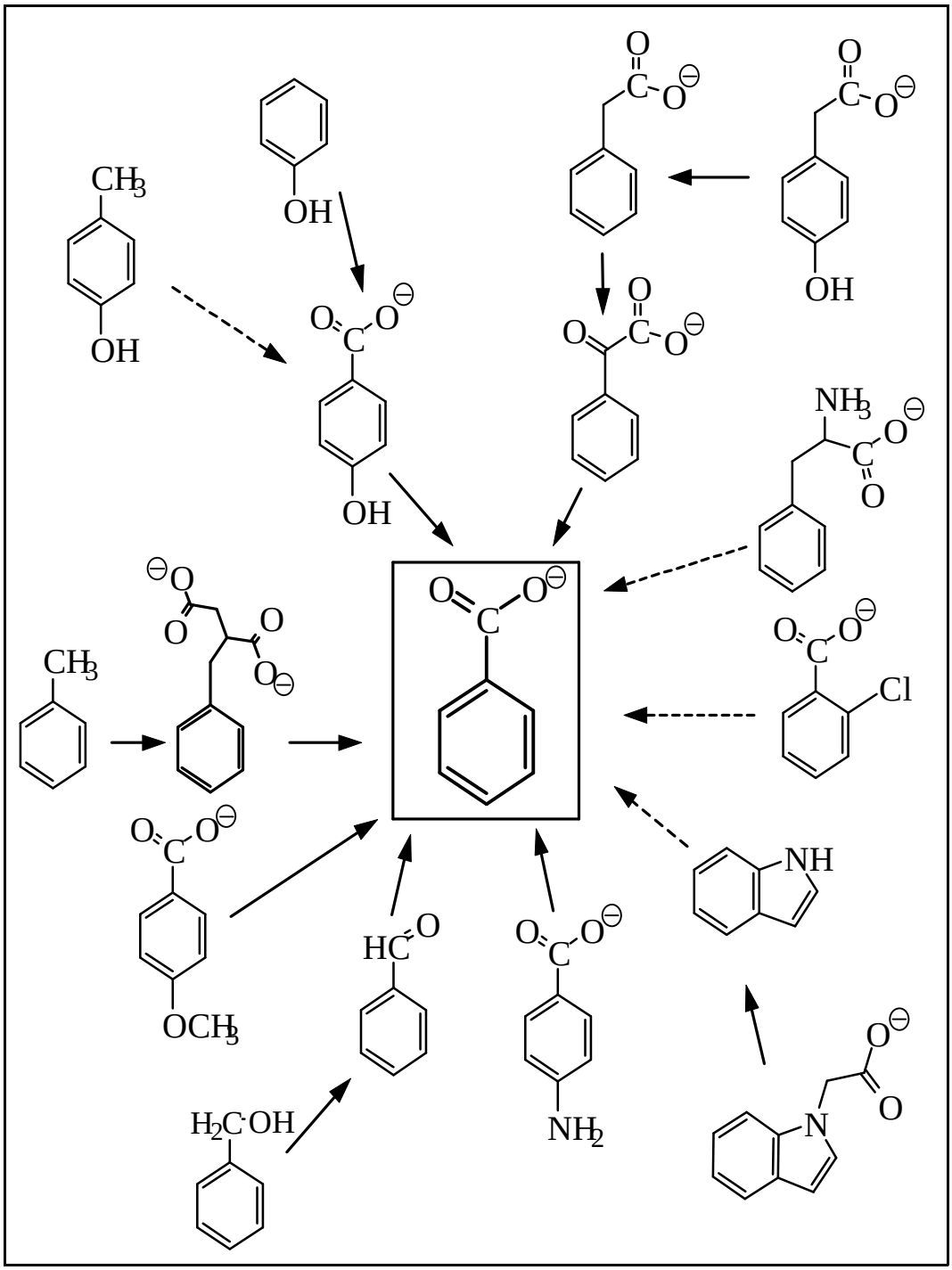


Figure 3. The metabolism of various simple aromatic compounds by anaerobic bacteria converge at a common intermediate, benzoyl-CoA.

carboxylic acid substituent. The carboxylic acid substituent is then reacted with CoA, through the action of a CoA ligase enzyme, to produce a thioester (Heider and Fuchs, 1997). The central intermediate of the pathway, benzoyl-CoA, undergoes an adenosine triphosphate (ATP)-dependent, two-electron reduction of the aromatic ring, producing a cyclohexa-1,5-diene-1-carbonyl-CoA (Heider and Fuchs, 1997). With the aromatic character of the ring gone, the cyclohexadiene is then metabolized further. In the first steps, water is added across each of the two double bonds to form a 2,6-hydroxycyclohexane-1-carbonyl-CoA. This is then followed by the oxidation of one of the hydroxyl groups to yield a keto group, which allows for the subsequent hydrolysis of the bond between carbon-one and carbon-two by a hydrolase. The resulting product, 3-hydroxypimelyl-CoA, is then oxidized to produce three molecules of acetyl-CoA and one molecule of carbon dioxide through a series of reactions similar to those seen in ß-oxidation (Heider and Fuchs, 1997; Evans and Fuchs, 1988). The anaerobic degradation pathway for benzoyl-CoA is shown in Fig. 4.

The enzyme responsible for catalyzing the ATP-dependent, two-electron reduction of the aromatic ring of benzoyl-CoA, is benzoyl-CoA reductase, which was first isolated and purified from the denitrifying bacterium *Thauera aromatica* (formerly known as *Pseudomonas* strain K172) (Boll and Fuchs, 1995). Since the anaerobic metabolic pathways of numerous aromatic compounds feed into the common intermediate, benzoyl-CoA, this enzyme has a central role in the

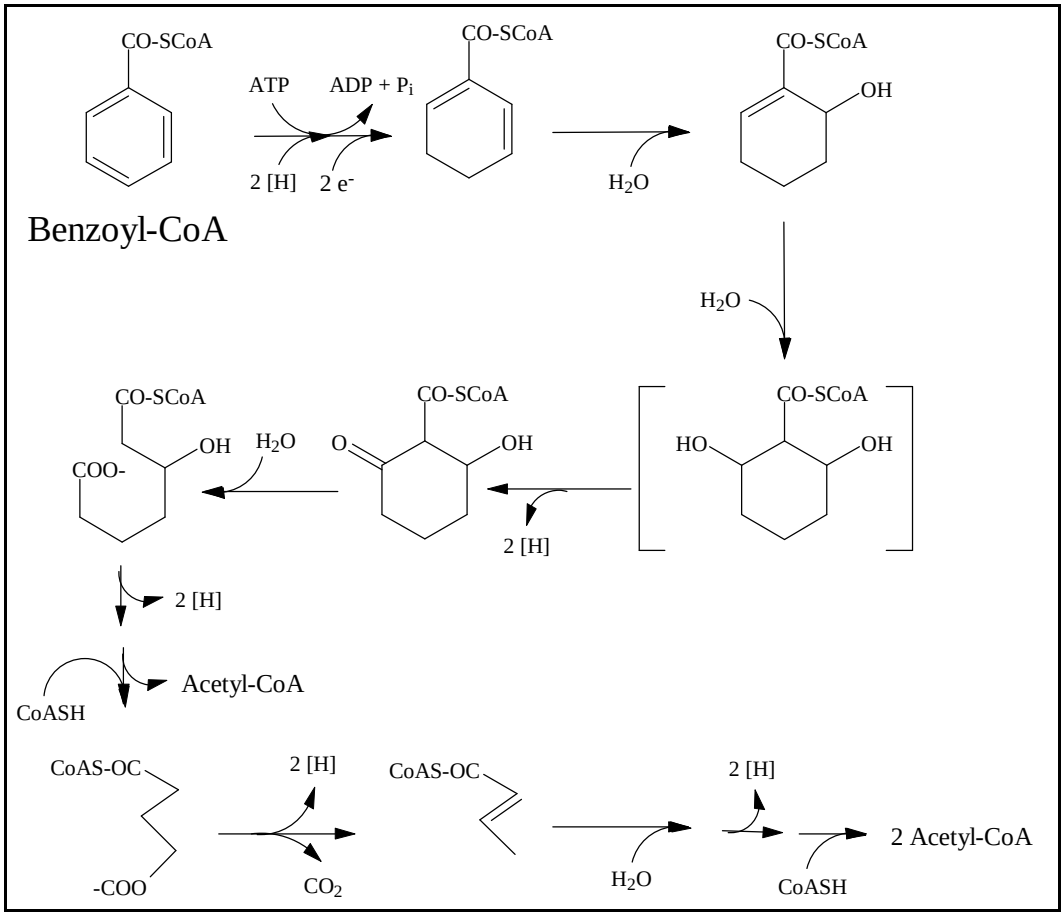


Figure 4. **The anaerobic metabolism of benzoyl-CoA**. Figure adapted from Heider and Fuchs, 1997.

anaerobic metabolic pathway of aromatic compounds (Heider and Fuchs, 1997). The mechanistic details of this enzymatic reduction are still unclear, but what is known about it draws comparisons to the Birch reduction, a synthetic reaction which involves the two-electron reduction of aromatic compounds via a radical mechanism. For this reason, the enzymatic reaction has been affectionately named the "biological Birch reduction" (Heider and Fuchs, 1997).

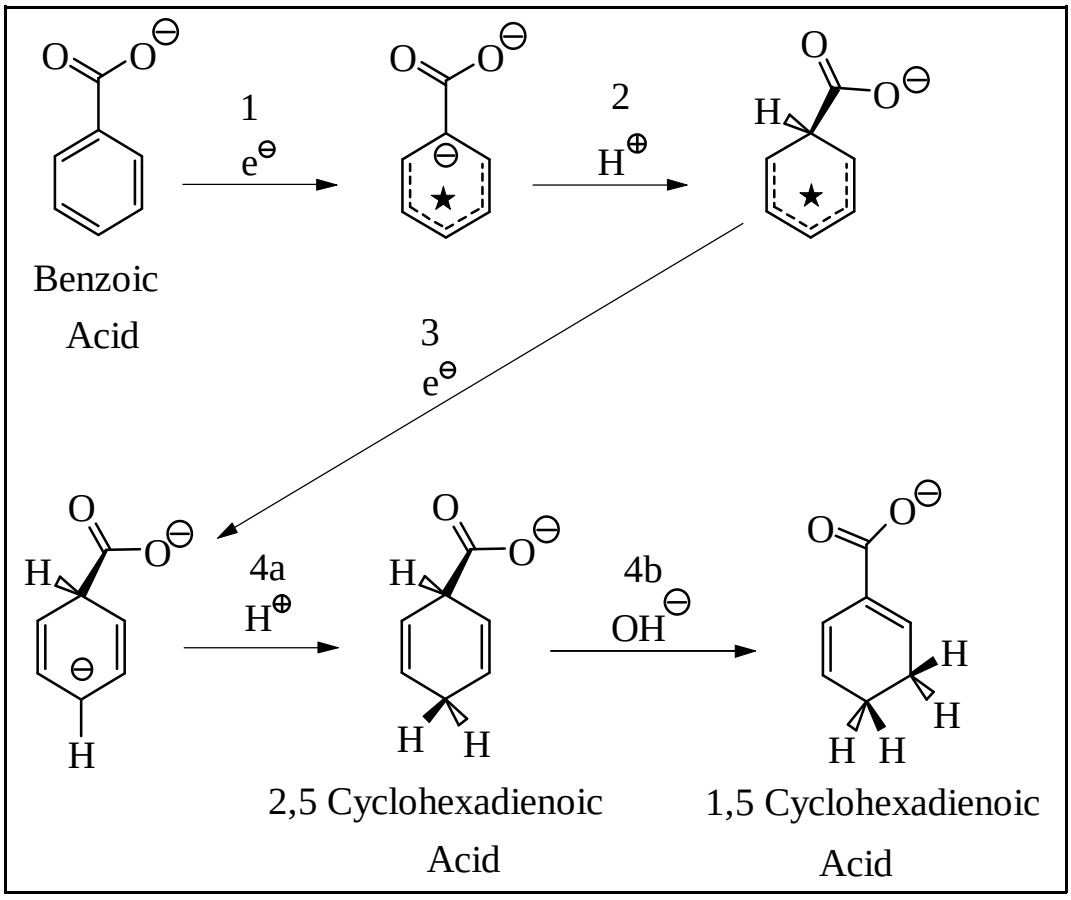


Figure 5. The Birch reduction of benzoic acid.

The Birch reduction. The Birch reduction is a useful and intriguing reaction in organic chemistry. The Birch reduction of benzene is a four-step process in which two electrons and two protons are added to an aromatic ring to produce a cyclohexa-1,4-diene, or in the case of an aromatic acid, a cyclohexa-2,5-diene (Fig. 5). The first, and usually rate-limiting, step of the reaction involves the donation of one electron from a metal with a low reduction potential (such as sodium or lithium) to the aromatic ring forming a radical anion (Zimmerman and Wang, 1993). The transfer of this electron is mediated by liquid ammonia, as it is

a good solvating agent for electrons. Since this step generates a carbanion, an electron withdrawing substituent, such as a thioester, would help stabilize the formation of this species and in turn favor reduction. Thus, in the case of an aromatic acid, such as benzoic acid, carbanion formation at the one position would be favored due to the stabilizing interactions with the electron withdrawing carboxylic acid substituent (Birch and Slobbe, 1976). The second step of the reaction is the protonation of the radical anion by either an added alcohol, or the solvent, forming a radical intermediate. This step can be rate-limiting as the radical anion has a low basicity, requiring the presence of a more acidic proton source than the ammonia solvent (Birch and Slobbe, 1976). This step is quickly followed by the addition of a second electron to the ring and a subsequent protonation of the resulting carbanion to form the unconjugated 2,5-cyclohexadienoic acid (Zimmerman and Wang, 1993). This unconjugated cyclohexadiene is the kinetic product of the reaction, forming first and being higher in energy than the more thermodynamically stable conjugated cyclohexadiene. This kinetic product normally cannot rearrange into the conjugated cyclohexadiene (Fig 5-4a) (Birch and Slobbe, 1976). However, if reversible protonation is allowed to occur, such as through the addition of a strong base, then the kinetic product can isomerize, forming the conjugated thermodynamic product, cyclohexa-1,5-diene-1-carboxylic acid (Fig 5-4b) (Birch

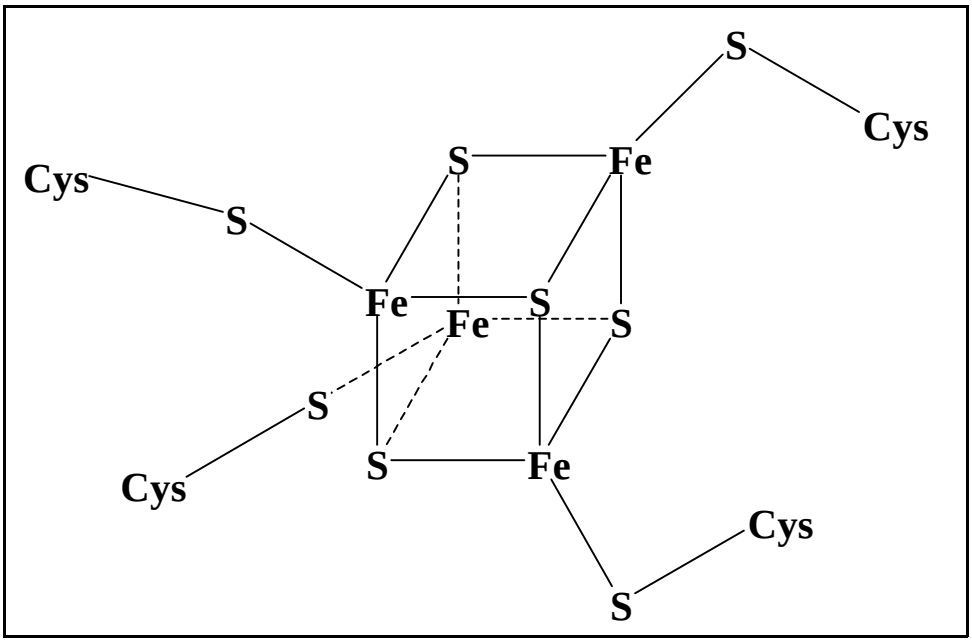


Figure 6. **A cysteine-ligated [4Fe-4S] cluster.**

and Slobbe, 1976; Kuehne and Lambert, 1958). The conjugated cyclohexadiene, unlike the unconjugated product, may undergo further reduction to cyclohexene in the presence of excess metal (Birch and Slobbe, 1976).

The components of benzoyl-CoA reductase. The oxygen-sensitive benzoyl-CoA reductase, the enzyme responsible for catalyzing the "biological Birch reduction", is a 170 kDa iron-sulfur (Fe-S) protein made up of four subunits: 48, 45, 38, and 32 kDa. The subunits have an [abcd] configuration. The enzyme also contains a substoichiometric amount of a flavin. The role of this flavin, if any, or whether it is an artifact of purification, has yet to be determined (Boll and Fuchs, 1995). Electron paramagnetic resonance (EPR) investigations of the protein found that the enzyme contains three cysteine-ligated [4Fe-4S] clusters, two of the

clusters interacting (Boll, Fuchs et. al., 2000). An example of a cysteine-ligated [4Fe-4S] cluster is shown in Fig. 6. Benzoyl-CoA reductase is proposed to have two functionally distinct modules. The first module, consisting of the a and d subunits, contains two binding sites for ATP and a symmetrically coordinated [4Fe-4S] cluster. This module is where the ATP-dependent electron activation is thought to take place. The second module is proposed to be the site of substrate reduction and is made up of the b and c subunits, which contain the two interacting [4Fe-4S] clusters (Unciuleac and Boll, 2001).

Characteristics of Fe-S cluster-containing proteins. Fe-S cluster-containing proteins, such as benzoyl-CoA reductase, can perform a variety of roles in biological systems. Simple Fe-S proteins contain standard Fe-S clusters of the form, $FeCys_4$, Fe_2S_2, Fe_3S_4, or Fe_4S_4. Simple Fe-S proteins primarily carry out electron transfer processes and non-redox catalysis. Complex Fe-S proteins can contain several Fe-S clusters, some of which are of the standard variety and some that are unusual, and additional prosthetic groups, such as flavins. These unusual Fe-S cluster-containing proteins can carry out electron transfer, catalysis, or both electron transfer and catalysis (Holm et. al., 1996). The enzyme nitrogenase, which catalyzes the reduction of dinitrogen to ammonia, is a complex Fe-S protein. Nitrogenase is similar to benzoyl-CoA reductase as they both catalyze the ATP-dependent reduction of a stable substrate (Howard and Rees, 1996). Nitrogenase has two unusual metalloclusters, the P-cluster, consisting of two Fe_4S_4 clusters

joined by two cysteinate bridges, and a cofactor, FeMo-co, consisting of a complex Fe-S cluster containing either a molybedenum, or a vanadium, atom (Long and Holm, 1995). An unusual Fe-S cluster, known as the H-cluster, can also be found in iron-only hydrogenases, which generate molecular hydrogen from solvated protons and an electron donor (Leger et. al., 2002). In both the hydrogenase and nitrogenase systems, unusual Fe-S clusters are required to carry out difficult reactions. EPR studies on benzoyl-CoA reductase have not revealed the presence of any unusual Fe-S clusters (Boll, Fuchs et. al., 2000). The 2-hydroxyglutaryl-CoA dehydratase system of *Acidaminococcus fermentans*, which carries out the ATP-dependent hydration of hydroxyglutaryl-CoA (Fig. 7), shares sequence homology (Unciuleac and Boll, 2001). The proposed catalytic mechanism of 2-hydroxyglutaryl-CoA dehydratase is similar to that of benzoyl-CoA reductase (Unciuleac and Boll, 2001). However, unlike benzoyl-CoA reductase, 2-hydroxyglutaryl-CoA dehydratase carries out a non-redox catalytic function and is thus considered to be a simple iron-sulfur protein. The 2-hydroxyglutaryl-CoA dehydratase system contains similar Fe-S clusters to that of benzoyl-CoA reductase and like benzoyl-CoA reductase, contains a flavin.

Mechanism of the activase/2-hydroxyglutaryl-CoA dehydratase system of *Acidaminococcus fermentans*. As stated previously, it has been found that benzoyl-CoA reductase has an amino acid sequence similar to that of the

activase/2-hydroxyglutaryl-CoA dehydratase system of *Acidaminococcus fermentans*.

Sequence homology studies have shown that the two enzyme systems share 38-52% similarity (Unciuleac and Boll, 2001). 2-hydroxyglutaryl-CoA dehydratase carries out a reaction that has a proposed mechanism similar to that of benzoyl-CoA reductase, shown in Fig. 7 (Heider and Fuchs, 1997). The dehydratase does not actually carry out a redox reaction. Instead, it removes water from a 2-hydroxycarboxylic acid. This is a difficult reaction because the ß-proton is not acidic enough to assist in water elimination. This reaction is much easier for a 3-hydroxy carboxylic acid because the a-proton is more acidic and is a good leaving group (Heider and Fuchs, 1997). Instead of using proton removal to facilitate this reaction, the enzyme transfers an electron to the substrate. This step requires that the substrate be in the CoA-thioester form instead of the free acid and the dehydratase needs to be actively reduced by an activating enzyme. The activating enzyme contains an ATP binding site and a [4Fe-4S] cluster (Locher et. al., 2001). The transfer of the electron from the activating enzyme to the dehydratase requires the hydrolysis of one molecule of ATP, which binds to the activating enzyme (Locher et. al., 2001). The dehydratase contains a [4Fe-4S] cluster at the catalytic site, which mediates electron transfer between the activating enzyme and the substrate. This cluster also accepts an electron from the radical ion formed by the substrate during catalysis (Locher et. al., 2001). After the transfer of the electron to

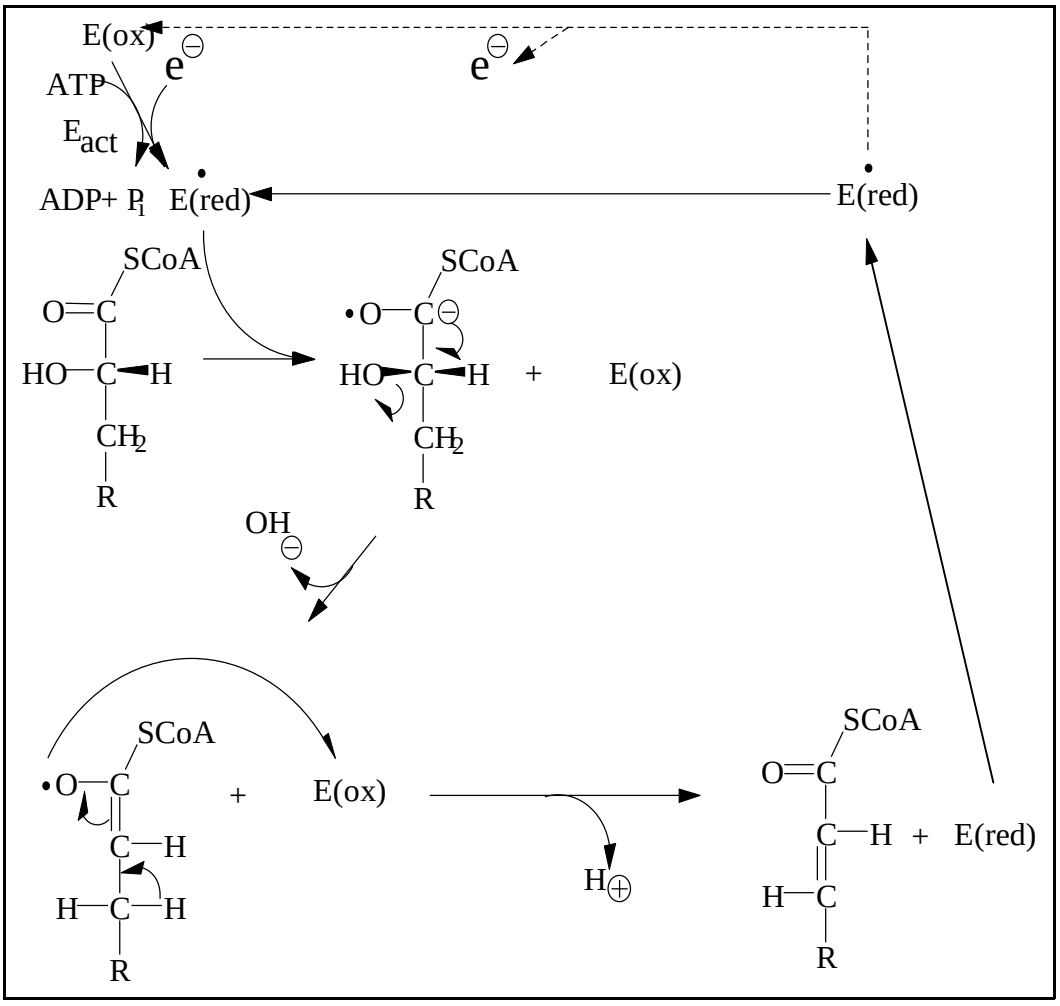


Figure 7. **Proposed reaction mechanism for 2-hydroxyglutaryl-CoA dehydratase from *Acidaminococcus fermentans***. E(ox) and E(red) represent the oxidized and reduced forms of the dehydratase respectively. E_{act} respresents the activating enzyme. After a catalytic cycle, the reduced dehydratase may enter another round of catalysis, or it may lose an electron and go to its oxidized form, requiring reactivation by the activating enzyme in order to resume catalysis. Figure adapted from Heider and Fuchs, 1997.

the substrate, the resulting radical ion then rearranges, eliminating the hydroxyl group and generating the neutral 1-enoxy radical (Heider and Fuchs, 1997). The formation of this 1-enoxy radical increases the acidity of the ß-protons and makes it possible for one of the protons to leave, creating a second radical anion. This new radical anion then transfers an electron back to the enzyme, which can then carry out another round of catalysis (Heider and Fuchs, 1997). Other similarities between 2-hydroxyglutaryl-CoA dehydratase and benzoyl-CoA reductase include that they are both deactivated by oxygen exposure and require a CoA-thioesterified substrate (Heider and Fuchs, 1997).

Proposed mechanism for benzoyl-CoA reductase. Although the details on how benzoyl-CoA reductase carries out its reaction are unclear, some clues about its mechanism have been found through EPR analysis. EPR analysis of the dithionite-reduced enzyme in the presence of benzoyl-CoA and ATP found that the iron-sulfur clusters, which act to transfer an electron from the electron donor to the substrate, were mostly in their oxidized state, suggesting either ATP hydrolysis or the reduction of the enzyme as the rate limiting step (Boll et al., 1997). EPR has also shown the presence of an organic radical during the steady-state enzymatic reduction of benzoyl-CoA, supporting the idea that the reduction occurs via a radical mechanism, similar to that of the Birch reduction. (Boll et. al., 1997). However, EPR studies have not shown that a phenyl radical forms during the reduction of benzoyl-CoA by the enzyme (Boll et al., 1997). This observation

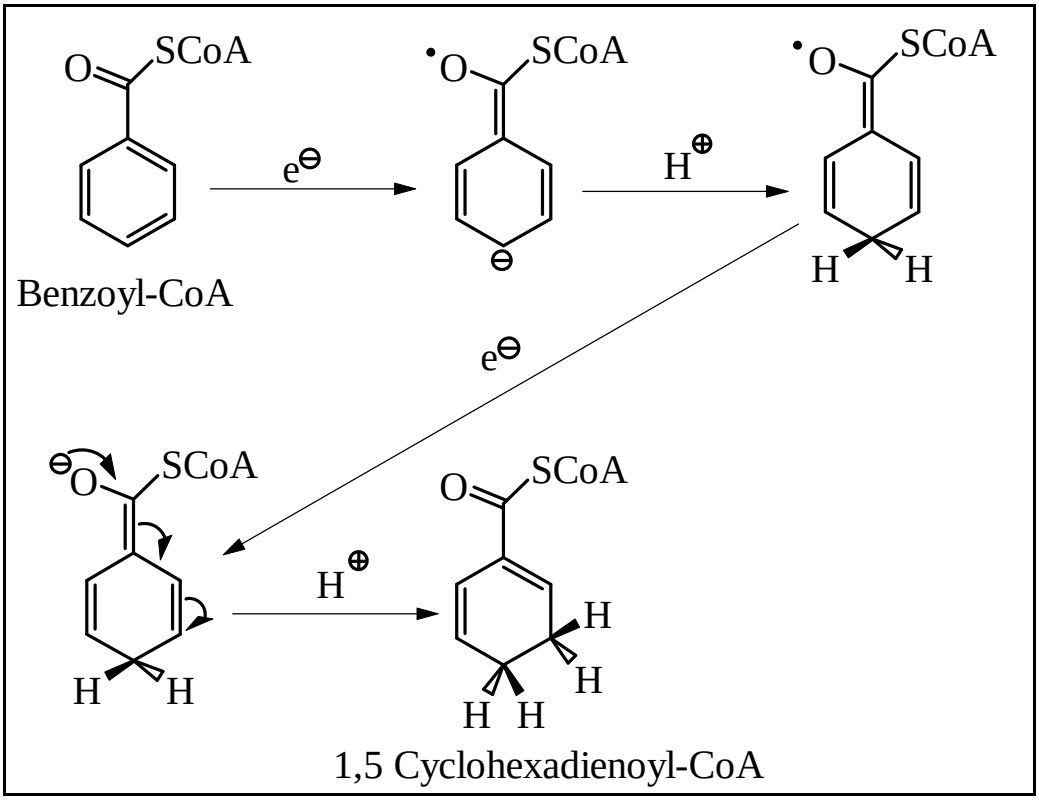


Figure 8. **The current proposed mechanism for benzoyl-CoA reductase**. The stereochemistry of the protonation steps is not known.

refuted the presence of a radical on the aromatic ring, lending support to a previously proposed mechanism where the radical is a ketyl radical located on the carbonyl group of the thioester (Buckel and Keese, 1995). This alternate mechanism, shown in Fig. 8, yields the conjugated cyclohexa-1,5-diene-1-carbonyl-CoA without the need for an additional isomerization step and is similar to the mechanism of 2-hydroxyglutaryl-CoA dehydratase shown in Fig 7. (Heider and Fuchs, 1997). However, studies on the metabolism of benzoyl-CoA by the phototropic bacterium *Rhodopseudomonas palustris* under anaerobic conditions

found the presence of two cyclic diene intermediates, a cyclohexa-1,4-diene-1-carboxylate and a cyclohexa-2,5-diene-1-carboxylate, which suggest that isomerization may occur. Furthermore, it was also found that the organism could use either of these compounds anaerobically as a carbon source (Gibson and Gibson, 1992).

Energetics of the "biological Birch reduction." Unlike the Birch reduction, the "biological Birch reduction" occurs under physiological conditions and it requires the involvement of CoA and ATP. Benzoyl-CoA reductase will not reduce benzoate and requires the formation of the benzoyl-CoA thioester (Boll and Fuchs, 1995). The formation of the benzoyl-CoA thioester significantly reduces the midpoint potential for the addition of the first electron, from -3.15 V for benzene, to -1.9 V. The formation of these CoA thioesters are catalyzed by CoA ligases and several such CoA ligases have been isolated and purified (Heider and Fuchs, 1997). The "biological Birch reduction" also requires ATP, suggesting that the energy produced by ATP hydrolysis is used to overcome the high activation energy barrier for reducing the chemically stable bonds of an aromatic ring system (Boll and Fuchs, 1995). The hydrolysis of one molecule of ATP can theoretically lower the redox potential of an electron by about 500 mV under physiological conditions (Boll and Fuchs, 1998). However, the actual amount ATP can lower the redox potential of an Fe-S cluster is probably much smaller. In the case of the Fe protein component of nitrogenase, the binding of ATP to the protein only lowers the redox

potential of the Fe-S cluster approximately 100 mV (Howard and Rees, 1996). Benzoyl-CoA reductase requires two molecules of ATP to reduce one molecule of benzoyl-CoA. The electrons transferred to the benzoyl-CoA by its natural electron donor, ferredoxin, have a midpoint potential of -0.45V. With the midpoint potential for the addition of the first electron to benzoyl-CoA being -1.9 V and the theoretical limit that ATP can lower the potential of an Fe-S cluster being -0.5 mV, there is still about -0.4 V difference that needs to be overcome in order to make the reduction of the aromatic ring favorable (Boll and Fuchs, 1998). The details on how ATP hydrolysis is used to assist in the reduction of benzoyl-CoA in benzoyl-CoA reductase are still unclear, but ATP hydrolysis could be causing conformation changes in the protein. Fe-S clusters can fluctuate between redox potentials depending on conditions in the surrounding environment (Stephens et al., 1996). Thus, the ATP-hydrolysis-mediated alteration of the interactions between the protein and a Fe-S cluster, or solvent and the cluster, could affect the redox potential of the cluster. In cases where electron and proton transfer are coupled, the redox potential of the cluster may be pH dependent (Holm et. al., 1996). For the enzyme nitrogenase, which is analogous to benzoyl-CoA reductase as both enzymes catalyze the ATP-dependent reduction of a stable substrate, it is thought that conformational changes in the enzyme, induced by the binding and hydrolysis of ATP, are essential for allowing electron transfer from the Fe-S clusters of the protein to the substrate (Howard and Rees, 1996). In fact, it has been observed that

the redox potential of the Fe-S cluster in the Fe protein component of nitrogenase drops by about 200 mV upon the formation of the Fe protein-MoFe protein complex (Lanzilotta and Seefeldt, 1997). Although, nitrogenase and benzoyl-CoA do not share any sequence homology, this does not imply that the two enzymes can't use the same strategy when directing electron transport.(Heider and Fuchs, 1997). It is thought that benzoyl-CoA reductase facilitates the reduction of benzoyl-CoA by rapidly protonating the hypothesized radical anion intermediate that forms after the first electron addition (Boll and Fuchs, 1998). This differs from the Birch reduction, for which the reaction conditions can make the protonation of the radical anion intermediate slow and rate-limiting (Zimmerman and Wang, 1993).

Theoretical rationale.

Possible mechanisms for aromatic ring reduction. There are least three possible reduction mechanisms that could be employed by benzoyl-CoA reductase to reduce the stable aromatic ring of benzoyl-CoA. The first, is a mechanism similar to the Birch reduction, consisting of two alternating one-electron transfer and protonation steps. This mechanism has been discussed earlier in this work and is the most feasible based on chemical precedence and what is known about the enzyme. Two other possible mechanisms: hydride reduction and "catalytic" hydrogenation, shown in Fig. 9, would be unusual for carbon-based aromatic rings, but cannot be dismissed. In the case of hydride reduction, two electrons are added

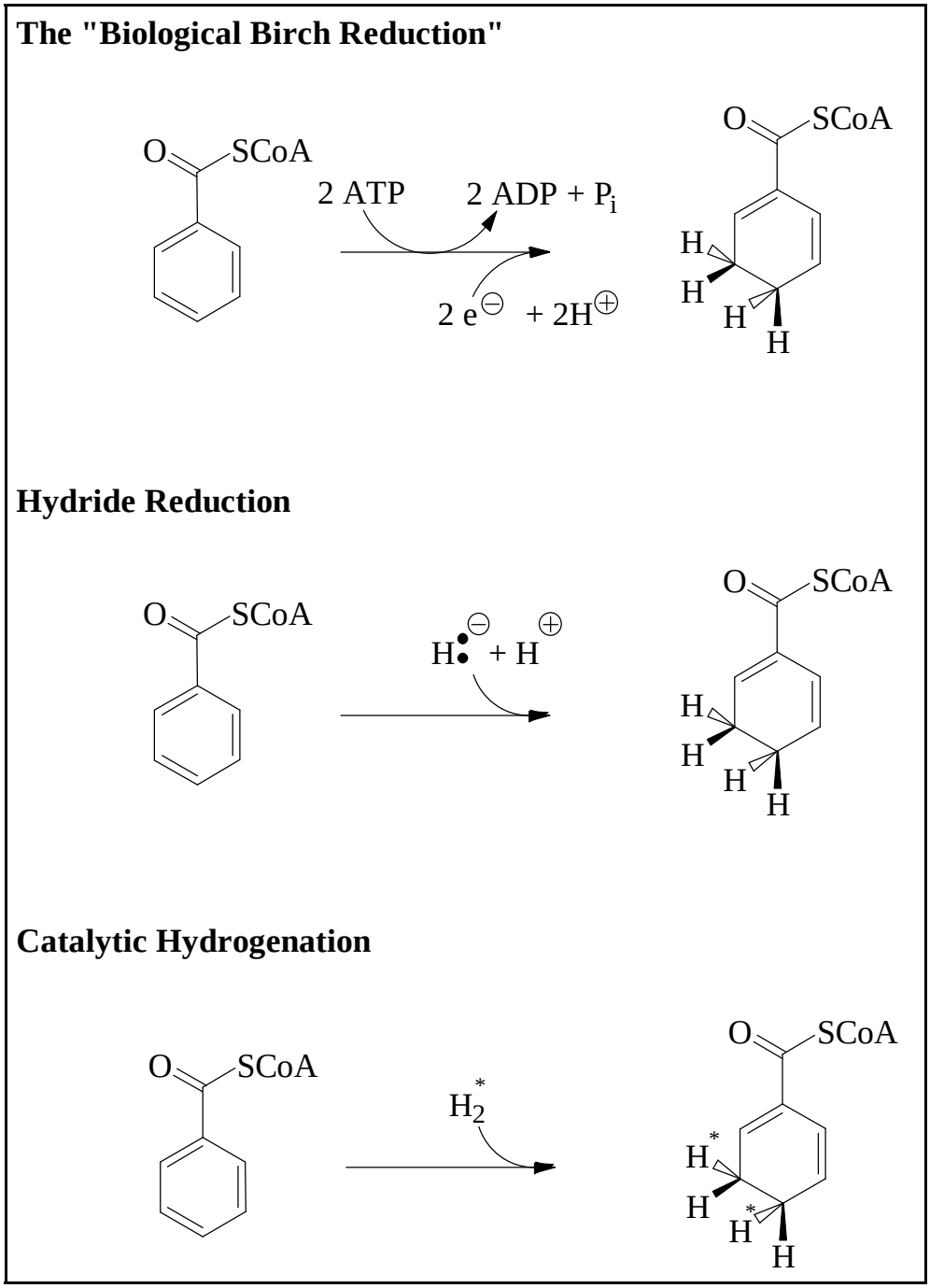


Figure 9. **Three possible mechanisms for aromatic ring reduction**. In the case of catalytic hydrogenation, the hydrogen atoms are usually added to the same face of the double bond. The stereochemistry for protonation in the other two mechanisms may be syn, or anti.

to the aromatic ring simultaneously in the form of a hydride ion. This step is then followed by protonation of the ring, to give the reduced aromatic ring. Hydride reductions usually require a cofactor, such as a nicotinamide (NAD(P)H), or a flavin (FAD, FMN). An enzymatic hydride reduction where the hydride transfer between the cofactor and the substrate is the rate limiting step will exhibit a significant deuterium isotope effect when the cofactor is transferring a deuteride instead of a hydride (Bridge et al., 1995). In cases where the hydride transfer is not the rate-limiting step and some other step during catalysis, such as cofactor/substrate binding and release, is deuterium isotope effect when the cofactor is transferring a deuteride instead of a hydride (Bridge et. al., 1995). The deuterium isotope effects for NAD(P)+ dependent alcohol dehydrogenases, where hydride transfer is the rate limiting step, have been observed to be around 3 to 5, when dividing the catalytic rate observed for hydride transfer (k_h) by the catalytic rate observed for deuteride transfer(k_d). In cases where the hydride transfer is not the rate-limiting step and some other step during catalysis, such as cofactor/substrate binding and release, is influencing the enzymatic reaction rate, there is little or no deuterium isotope effect ($k_h/k_d = 1$). For catalytic hydrogenation, two hydrogen atoms, in the form of a hydrogen molecule (H_2), are added in a concerted fashion to the same face of a double bond. This mechanism does not allow for isomerization and it requires the presence of dissolved hydrogen gas molecules. This mechanism seems unlikely, as dissolved hydrogen is not an

apparent requirement for the enzymatic reduction of benzoyl-CoA. However, some enzymes, like hydrogenase, are capable of generating molecular hydrogen from solvated protons and an electron donor (Leger et. al., 2002). It might then be possible that benzoyl-CoA reductase could generate molecular hydrogen, which could then be used to reduce the aromatic ring.

Use of substrate analogues to study the mechanism of benzoyl-CoA reductase. Benzoyl-CoA reductase has previously been shown to have activity on benzoyl-CoA analogues with small substituents on the aromatic ring, such as m-fluorobenzoyl-CoA, o-fluorobenzoyl-CoA, and p-fluorobenzoyl-CoA (Boll and Fuchs, 1995). The identification of the products of the reactions with benzoyl-CoA reductase and benzoyl-CoA analogues may provide an insightful look into which mechanism the enzyme uses to carry out the reduction of its native substrate, benzoyl-CoA, and is the focus of this investigation. With the fluorinated anologues, the enzyme activity is highest with the fluorine in the ortho position, about 78 percent as active as with the native substrate, and lowest with the fluorine in the para position, about ten percent as active as with the native substrate (Boll and Fuchs, 1995). Birch reductions carried out on fluorobenzoic acids can result in the elimination of the fluorine atom from the aromatic ring as a fluoride ion, especially if the fluorine substituent is either ortho, or para to the carboxylic acid (Rabideau, 1992). The removal of a fluoride yields benzoic acid, which can undergo further reduction to the 2,5-cyclohexadienoic acid. However, Birch

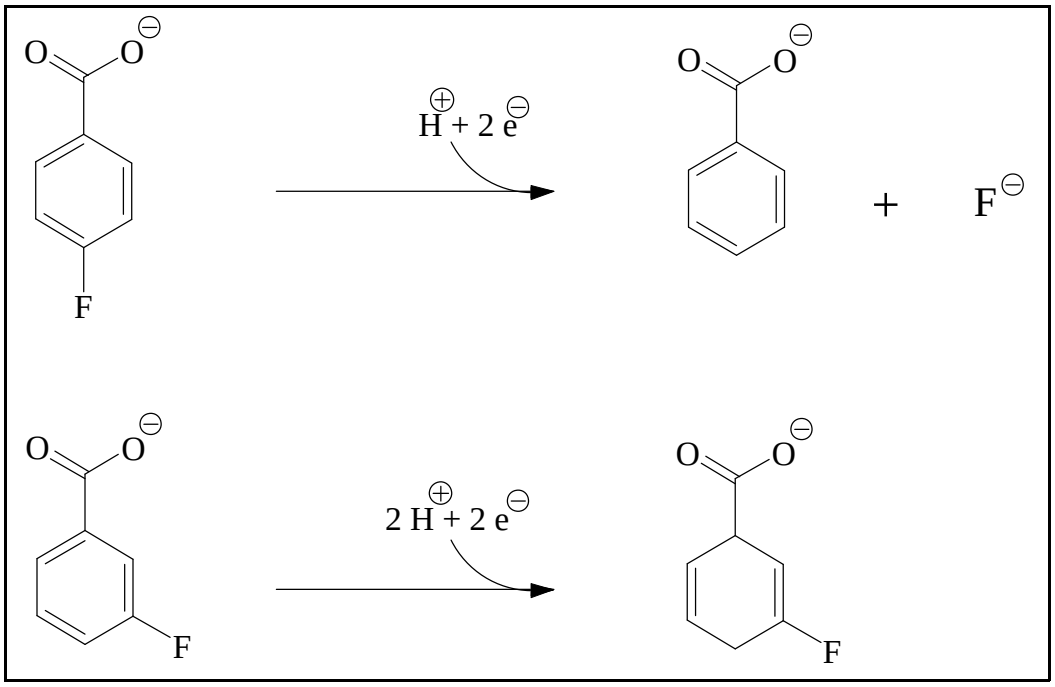


Figure 10. **The Birch reductions of p-fluorobenzoic acid (top) and m-fluorobenzoic acid (bottom).**

reductions on m-fluorobenzoic acids usually do not result in the loss of fluorine and the aromatic ring is reduced directly to the 2,5-cyclohexadiene (Fig. 10) (Rabideau, 1992). So, if benzoyl-CoA reductase follows the "biological Birch reduction", then its action on a p-fluorobenzoyl CoA substrate would result in the elimination of fluorine from the aromatic ring and the production of benzoyl-CoA. By the same logic, the enzymatic reduction of the m-fluorobenzoyl CoA substrate, would yield 4-fluorocyclohexa-1,5-diene-1-carbonyl-CoA. Nitrogen-containing, aromatic heterocycles, such as nicotinic acid, isonicotinic acid, and picolinic acid (Fig. 11) have not yet been used as substrates for benzoyl-CoA reductase, but if the CoA-thioester derivatives of these compounds are found to be substrates for the

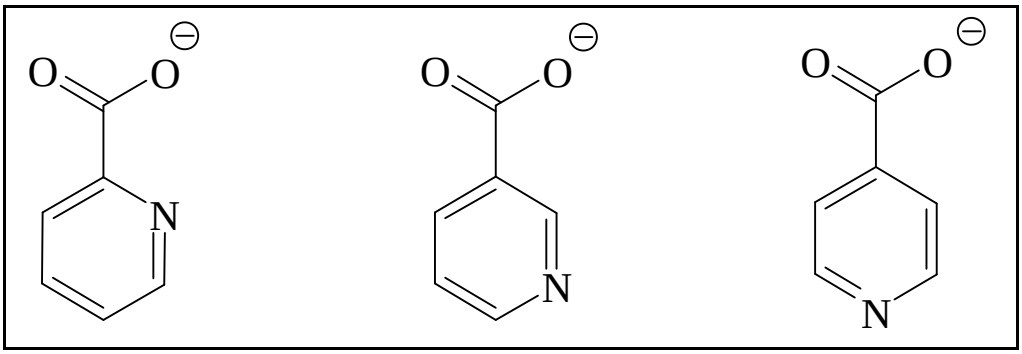


Figure 11. **Nitrogen-containing aromatic heterocycles: picolinic acid(left), nicotinic acid (middle), isonicotinic acid (right).**

enzyme, they may also provide some clues about the enzyme's mechanism. Carbon-nitrogen double bonds have a higher affinity for electrons than carbon-carbon double bonds. Nitrogen has a higher electron-affinity than carbon, allowing it to support a negative charge easier and making the first reduction step of the Birch reduction more favorable (Birch and Slobbe, 1976). If the presence of nitrogen in the aromatic ring facilitates a Birch reduction of the aromatic ring and benzoyl-CoA reductase follows a similar mechanism as the Birch reduction, then the enzyme might catalyze the reduction of the CoA thioester derivatives of these compounds at a faster rate than that of its native substrate, benzoyl-CoA.

Reversibility of electron transfer by benzoyl-CoA reductase. As seen earlier with 2-Hydroxyglutaryl-CoA dehydratase, the enzyme adds an electron to its substrate, but at end of the catalytic cycle, that electron is transferred back to the enzyme (Locher et. al., 2001). Since benzoyl-CoA reductase and 2-hydroxyglutaryl-CoA dehydratase share sequence homology, it could then be

possible that the electrons are transferred back to benzoyl-CoA reductase from its reduction product. This possibility will be examined by isolating the cyclohexa-1,5-diene-1-carbonyl-CoA product from benzoyl-CoA reductase enzymatic reaction on benzoyl-CoA and reintroducing the product to the enzyme under oxidizing conditions. If benzoyl-CoA reductase is able to take the product and form benzoyl-CoA under these conditions, then it suggests that the enzyme can oxidize its reduction product, the cyclohexadienoyl-CoA, back to form benzoyl-CoA, the substrate for the "reductase" activity. This would mean that benzoyl-CoA reductase can carry out this process even though its reduction capability has been irreversibly inactivated by oxygen.

CHAPTER II. Materials and Methods

Materials and bacterial strain. Chemicals were obtained from Sigma, Acros Organics, Bio-Rad, Sigma, Fisher, and Aldrich. All liquid chromatography and fast protein liquid chromatography (FPLC) materials and equipment were from Pharmacia or BioRad. High performance liquid chromatography (HPLC) materials and equipment were from Shimadzu. *Thauera aromatica* strain K172 (DSM 6984) was obtained from Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). All ultraviolet (UV) and visible (Vis) spectroscopy was performed using a HP 8453 UV-visible photo-diode array spectrophotometer unless otherwise noted.

Growth of bacterial cells. The minimal salts medium contained 11 mM Na_2HPO_4, 9 mM NaH_2PO_4, 1 ml/l of selenite tungstate solution (Tschech and Pfennig, 1984), and 1ml/l of trace element solution SL7 (Widdel and Pfennig, 1981). The medium was autoclaved and cooled under an atmosphere of nitrogen. $MgCl_2 \cdot 6\ H_2O$, $CaCl_2 \cdot 2\ H_2O$, and $NaHCO_3$ were then added to the medium as described (Tschech and Fuchs, 1987). The pH of the medium was adjusted to 7.2 by the addition of 1M HCl or NaOH. *T. aromatica* was grown in this medium at 28 degrees Celsius. 5 mM benzoic acid served as the carbon source and 20 mM

potassium nitrate served as the terminal electron acceptor. These two chemicals were added to the bacterial cell cultures periodically from sterile, oxygen-free stock solutions to maintain these concentrations. Growth of the bacterial cell cultures was monitored by measuring the optical density in 1 cm cuvettes at 650 nm. Consumption of benzoic acid, nitrate, and nitrite were measured by taking the UV absorption spectra of the aqueous layer of a chloroform-extracted, centrifuged sample of the culture liquid. The UV absorption of this solution was measured at 272 nm, 302 nm, and 360 nm in order to determine the concentration of benzoate, nitrate, and nitrite respectively. These values were compared to a standard curve constructed from solutions containing known concentrations of benzoate, nitrate, and nitrite.

Harvesting of bacterial cells. Bacterial cells were harvested under nitrogen using either centrifugation, or flow-through centrifugation at 6000 rpm. Bacterial cells were then stored on liquid nitrogen.

Preparation of cell extracts. Cell-free extracts were prepared anaerobically at 4 degrees Celsius by re-suspending bacterial cells in a buffer (2ml/g cells) containing 20 mM triethanolamine (TEA), 0.25 mM dithionite, and 1mM dithioerythritol. DNAse (0.2mg/g cells), RNAse (0.2 mg/g cells), lysozyme (0.5 mg/g cells), and 1%(volume/volume) Trition X-100 were then added to the buffer and mixed for one hour. This preparation was then centrifuged at 12000 rpm for one hour. 2 mM

$MgCl_2$, 10% glycerol, and 0.2 mM Fe(II) were then added to the cell extract to stabilize the Fe-S cluster containing proteins.

Purification of benzoyl-CoA reductase. Purification was performed at 4 degrees Celsius under a nitrogen atmosphere in a glove box. All buffers contained 0.25 mM dithionite and 1 mM dithioerythritol as reducing agents. The purification started with the cell extract from 80 grams (wet cell mass) of *T. aromatica*. Benzoyl-CoA reductase activity was determined using the spectrophotometric assay described below.

Diethylaminoethyl (DEAE)-Sepharose chromatography. 150 ml of the cell-free extract (12000 rpm supernatant) was applied to a FPLC column (diameter 2.6 cm., volume 120 ml.) of DEAE CL-6B resin (fast-flow, Pharmacia). The column was equilibrated and run, using a flow rate of 1 ml min^{-1}, as described (Boll and Fuchs, 1995). A greenish-brown protein band eluted from the column with 115 mM KCl in a volume of 135 ml.

Hydroxyapatite chromatography. The combined fractions from the DEAE-Sepharose column containing benzoyl-CoA reductase activity were applied to a FPLC column (diameter 1.6 cm, volume 30 ml.) of Macro-Prep (ceramic hydroxyapatite type I with a diameter of 40 µm, Biorad). The column was equilibrated and run, using a flow rate of 1 ml min^{-1}, as described (Boll and Fuchs, 1995). Activity eluted from the column in a volume of 75 ml. The pooled fractions containing benzoyl-CoA reductase activity were concentrated

anaerobically with an ultra-filtration stirred cell apparatus (Amicon) to a volume less than 5 ml.

Gel filtration. The concentrated fractions from the hydroxyapatite column were applied to a FPLC Sephacryl 300 column (Pharmacia; diameter 2.6 cm, volume, 130 ml.). The column was equilibrated and run, using a flow rate of 0.6 ml min^{-1}, as described (Boll and Fuchs, 1995). Activity was eluted from the column in a volume of 70 ml. The fractions containing benzoyl-CoA reductase were pooled and concentrated to a volume less than 5 ml as described previously.

DEAE-Sepharose chromatography. The concentrated fractions from the gel filtration column were applied to a FPLC column (diameter 1.6 cm., volume 5 ml.) of DEAE CL-6B resin (fast-flow, Pharmacia). The column was equilibrated at a flow rate of 1 ml min^{-1} using a buffer containing 20 mM TEA at pH 7.8, 4 mM $MgCl_2$, and 10% glycerol (referred to as buffer 1). The column was then washed with four bed volumes of buffer 1. Benzoyl-CoA reductase activity was eluted with a linear 0-300 mM KCl gradient (10 bed volumes) formed from buffer 1 and 300 mM KCl in buffer 1. Benzoyl-CoA reductase activity eluted from the column at about 110-120 mM KCl in a volume of 20 ml. The resulting enzyme solution was concentrated to a volume of 2 ml and stored under liquid nitrogen until use.

Sodium dodecyl sulfate (SDS)/polyacrylamide gel electrophoresis (PAGE) and Bradford assay. SDS/PAGE (10% polyacrylamide) was performed as described

(Neville, 1971). Protein concentration was determined by the Bradford method using a bovine serum albumin standard (Bradford, 1976).

Assay of benzoyl-CoA reductase activity. Two separate assays were used at 25 degrees Celsius under strictly anaerobic conditions. The spectrophotometric assay was primarily used to assay for enzymatic activity, while the HPLC assay was mainly used to observe the products formed by the enzymatic reaction. The spectrophotometric assay was carried out in a sealed quartz cuvette and the HPLC assay was carried out in 5 ml sealed glass vials. All containers were evacuated and refilled with nitrogen gas at least four times to remove any dissolved oxygen.

*Spectrophotometric assay***.** The ATP and substrate-dependent oxidation of either reduced methyl viologen, or titanium (Ti) (III) citrate by benzoyl-CoA reductase was monitored continuously by a spectrophotometer as described (Boll and Fuchs, 1995). The 1 ml standard assay mixture contained 150 mM 3-(N-morpholino)propanesulfonic acid (MOPS)/KOH pH 7.3, 10 mM $MgCl_2$, 5 mM ATP, 1 mM methyl viologen or 0.8 mM Ti (III) citrate, 0.2 mM benzoyl-CoA, and 5-100 µl of enzyme solution (Boll and Fuchs, 1995). For methyl viologen, the assay was observed at 730 nm (e_{730} = 2.4 mM^{-1} cm^{-1} (Fraisse and Simon, 1988)). For the assays using titanium citrate, a 80 mM Ti (III) citrate stock solution was prepared and the reactions were monitored at 340 nm (e_{340} = 0.73 mM^{-1} cm^{-1} (Seefeldt and Ensign, 1994)). 0.8 mM Ti(III) citrate was used in the assay in place of methyl viologen. The enzymatic assays were started by adding either the benzoyl-CoA,

ATP, or the enzyme solution. Due to the high background rate of redox dye oxidation by the cell extract in the absence of substrate, it was difficult to measure the activity of the enzyme during purification until the cell extract had been passed over both the DEAE-Sepharose and the hydroxyapatite column. The data from the spectrophotometric assays were collected and analyzed using the Advanced UV-Visible Software G1116AA Rev. A.02.05 by Hewlett Packard.

HPLC assay. A 0.5 ml assay mixture was prepared as described previously for the Ti(III) citrate spectrophotometric assay. 100 µl samples were taken at several time increments during the assay and 10 µl of 1 M H_2SO_4 was added. The samples were then centrifuged to removed the denatured protein. 20 µl of the supernatant was passed over a C-18 reverse-phase HPLC column (All-tech Platinum; diameter, 4.6 mm; length 150 mm; 100 A pore size; 5 µM particle size) which was equilibrated with 97% 50 mM ammonium acetate at pH 5.5 and 3% acetonitrile at a flow rate of 1 ml min^{-1}. The components of the assay were then separated by applying a linear gradient of 3-40% acetonitrile over twenty minutes at a flow rate of 1 ml min^{-1}. CoA thioesters eluting from the column were observed using a dual-channel UV-Vis diode array detector (Shimadzu) set to monitor both 260 nm and 232 nm in order to detect any species containing an adenosine group, such as a CoA thioester. This procedure was also used to monitor product formation during the spectrophotometric assay. This was done by removing 100 µl samples of the assay mixture at time points during the spectrophotometric assay.

The samples were added to 10 µl of 1M H_2SO_4, centrifuged, and applied to the HPLC column as described previously. The Class VP chromatography data system version 4.2, supplied by Shimadzu, was used to collect and process the HPLC assay data.

Synthesis and purification of coenzyme A thioesters. Benzoyl-CoA was synthesized from CoA and benzoic acid anhydride (Schachter and Taggart, 1976). All other thioesters were synthesized by a method based on that of Gross and Zenk. (Gross and Zenk, 1966). The thioesters were by reacting the acid chloride, prepared from the respective carboxylic acid, with free CoA . This was done by first dissolving 220 µmol of the carboxylic acid (isonicotinic, nicotinic, picolinic, m-fluorobenzoic, or p-fluorobenzoic acid) in 10 ml of tetrahydrofuran containing 10 mg of Na_2SO_4 as a drying agent. This solution was passed through a short column of Na_2SO_4 and into a dry, sealable container. The container was then sealed to prevent water vapor from entering the container and to prevent the organic reagents from evaporating. After sealing the container, 22 µl of ethyl chloroformate and 30 µl of triethylamine was added. After reacting for one hour, the solution was passed through a glass wool filter and into 10 ml of a degassed solution at pH 7.5 containing 50 mM potassium phosphate and 32 mg of coenzyme A (CoA). After one hour, the pH was adjusted to between 3 and 4 using HCl. The resulting solution is passed through a glass wool filter and extracted with $CHCl_3$ to remove any contaminant. For further purification, the reaction mixture was

applied to a Sephadex G-10 gel filtration column (Pharmacia, 1.6 cm diameter, 140ml), equilibrated with 50 mM 3-(N-morpholino)ethanesulfonic acid (MES) at pH 5.6. Fractions containing the CoA-thioester were identified by spectrophotometric detection at 260 nm. These fractions were combined and freeze-dried. The synthesized thioester, which was produced with a 70% yield, was then stored in 50 mM MES buffer at pH 5.6 at a concentration of 20 mM. The fractions were analyzed for purity by HPLC using the column and gradient as the HPLC assay described above and the identity of the thioester was confirmed using electrospray mass spectrometry on a Thermo-Finnigan LCQ with a quadrupole ion trap. The atomic masses of the ions that formed were observed using either the negative or positive ion channel. The synthesis of the thioester was further confirmed by proton nuclear magnetic resonance spectroscopy (NMR) using a Bruker 400 MHz NMR spectrometer. The proton NMR spectrum for CoA and benzoyl-CoA is shown in Fig. 12B. An observed downfield shift in the -CH_2-S signal was indicative of a substitution on the sulfur atom and the formation of a thioester (Mieyal et al., 1974). Observed signals in the region between 6 ppm and 8 ppm were indicative of phenyl protons belonging to the respective aromatic substituent (Fig. 12). The NMR spectra of picolinoyl-CoA, nicotinoyl-CoA, m-fluorobenzoyl-CoA, and p-fluorobenzoyl-CoA are located in the appendix. The amount of thioester present was determined by measuring the amount of UV absorption by the adenosine, contained in the CoA functionality, at 260 nm. The

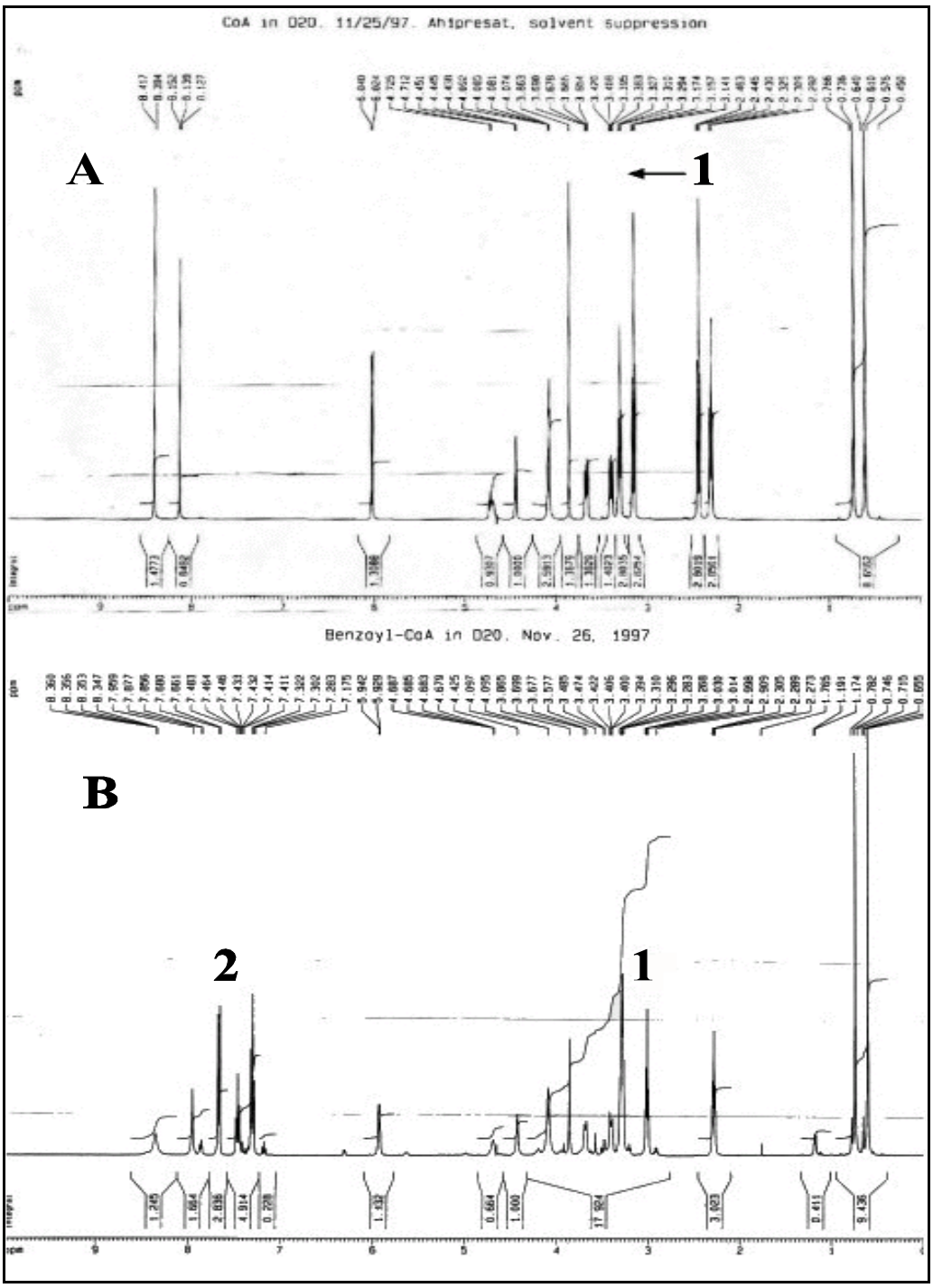


Figure 12. Proton nuclear magnetic resonance spectroscopy of CoA (A) and benzoyl-CoA (B). The formation of the thioester was indicative of a shift in the -CH2S signal of CoA (1). The appearance of phenyl protons from benzoate was also observed (2).

extinction coefficient for the adenosine group on CoA at 260 nm is 15.4 mM^{-1} cm^{-1}. For benzoyl-CoA, the extinction coefficient 21.1 mM^{-1} cm^{-1} was used (Webster et al., 1973). The UV absorption of the synthesized thioester was also taken at 232 as CoA and thioester have different ratios of 232 nm/260 nm absorptions (Webster et al., 1973).

Enzymatic studies on benzoyl-CoA reductase using thioester analogues. As with the enzyme's normal substrate, benzoyl-CoA, each of the substrate analogues, m-fluoro, p-fluoro, nicotinoyl, picolinoyl, and isonicotinoyl-CoA, were used as possible substrates for the spectrophotometric assay and the HPLC assay.

Spectrophotometric assay. The continuous spectrophotometric assay, with either Ti(III) citrate or dithionite-reduced methyl viologen as the reducing agent, was performed as described previously, using one of the thioester analogues in place of benzoyl-CoA at concentrations from 0.2 mM to 2 mM.

HPLC assay. The HPLC assay was performed as described previously, using one of the thioester analogues in place of benzoyl-CoA at concentration from 0.4 mM to 2 mM.

Determination of the catalytic properties of benzoyl-CoA reductase.

Determination of K_m and relative V_{max} values for benzoyl-CoA in H_2O and D_2O. The initial rates of the reductase reaction were measured using the spectrophotometric assay described previously using methyl viologen as the reducing agent. The substrate for these reactions was benzoyl-CoA in

concentrations ranging from 0-150 µM. 10 µl of the enzyme solution was used in each reaction. Two separate studies were carried out. The first study was carried out using a 150 mM MOPS/KOH buffer solution at pH 7.3 made in deionized water. The second study used a 150 mM MOPS/KOD buffer solution at pH 7.3 made in D_2O. In each case, the enzyme solution was the same in order to allow for comparisons of the kinetic data. The reaction rates of these assays were plotted versus the concentrations of the substrate used. The K_m and V_{max} values were then determined from a hyperbolic fit of the resulting plot.

Determination of K_m and relative V_{max} values for picolinoyl-CoA. The initial rates of the benzoyl-CoA reductase reaction were measured with the spectrophotometric assay described previously using methyl viologen as the reducing agent. The substrate for these reactions was picolinoyl-CoA in concentrations ranging from 60 µM to 2 mM. 10 µl of the enzyme solution was used in each reaction. The enzyme used for these reactions was from the same enzyme solution used to determine the catalytic properties for benzoyl-CoA reductase using benzoyl-CoA as the substrate. This was done to prevent impurities in the enzyme solution, such as oxygen-inactivated benzoyl-CoA reductase, from interfering with the comparison between the catalytic properties of the reductase for benzoyl-CoA and those for picolinoyl-CoA. The reaction rates of these assays were plotted versus the concentrations of the substrate used. The K_m and V_{max} values were then determined from a hyperbolic fit of the resulting plot.

Separation of the products of the benzoyl-CoA reductase catalyzed reduction of on benzoyl-CoA and studies on the effect of oxygen on benzoyl-CoA reductase. The products of the benzoyl-CoA reductase reaction on benzoyl-CoA were isolated using the HPLC assay describe previously. Using the chromatogram, the fractions containing the products were collected separately and freeze-dried to remove the liquid. Each product was then redissolved in 100 µl of 150 mM MOPS/KOH at pH 7.3. Each 100 µl solution was divided into two equal solutions. The first solution served as a time 0 for the reaction and 2 µl of enzyme was added to the second solution and incubated for 15 minutes. 5 µl of 1M H_2SO_4 was added to stop the reaction and the solution was centrifuged to remove the precipitated protein. The assays did not contain any reducing agents and contained dissolved oxygen. The assays were then applied to the HPLC using the same procedure described previously. The effect of oxygen exposure on the benzoyl-CoA reductase reaction was also studied by running a HPLC assay using benzoyl-CoA for the substrate for 15 minutes. After 15 minutes, the HPLC assay was uncapped and exposed to oxygen. The assay contents were mixed gently every five minutes for a total of 40 minutes. 100 µl samples were taken from the assay at the start of the assay, after 15 minutes (before the oxygen exposure), and then after the 40 minutes of oxygen exposure. 10 µl of 1M H_2SO_4 was added to each sample and the samples were centrifuged to remove the precipitated protein. The samples were then applied to the HPLC using the same procedure described for the HPLC assay.

Identification of enzymatic products by mass spectrometry. The enzymatic products formed by benzoyl-CoA reductase acting on its substrate and synthesized analogues were identified using either electrospray ionization (ESI) or liquid chromatography/mass spectrometery (LC/MS), where a Waters 2690 HPLC was coupled to the ESI source.

Electrospray (ESI). Electrospray on substances previously separated by HPLC was carried out using a Thermo-Finnigan LCQ with a quadrupole ion trap. The atomic masses of the ions that formed were detected either by monitoring the positive or negative ion channel.

Liquid chromatography/mass spectrometry (LC/MS). A 20 µl sample of the enzymatic assay of interest was applied to a reverse-phase HPLC column before carrying out electrospray using the Thermo-Finnigan LCQ. The atomic masses of the ions that formed were detected by monitoring either the positive or negative ion channel. The reverse-phase column used was either a Zorbax C-18SB reverse-phase HPLC column (Micro-Tech Scientific; diameter, 1.0 mm; length 150 mm; 100 A pore size; 5 µM particle size), or a Platinum C-18 reverse-phase HPLC column (All-tech; diameter, 4.6 mm; length 150 mm; 100 A pore size; 5 µM particle size). CoA thioesters eluting off the HPLC column were detected by monitoring 260 nm with a Waters 996 Photodiode Array Detector.

The Zorbax C-18SB column was equilibrated with 95% 50 mM ammonium acetate at pH 5.5 and 5% acetonitrile at a flow rate of 50 µl min^{-1}. The

components of the assay were then separated by applying a linear gradient of 5-80% acetonitrile over twenty five minutes at a flow rate of 50 µl min^{-1}.

The Platinum C-18 column was equilibrated with 97% 50 mM ammonium acetate at pH 5.5 and 3% acetonitrile at a flow rate of 1 ml min^{-1}. The components of the assay were then separated by applying a linear gradient of 3-40% acetonitrile over twenty minutes at a flow rate of 1 ml min^{-1}.

CHAPTER III. Results

Enzymatic assays. Two enzymatic assays were developed in order to monitor the enzymatic activity of benzoyl-CoA reductase, a spectrophotometric assay and a HPLC assay.

The spectrophotometric assay involved measuring the ATP and substrate-dependent oxidation of either reduced methyl viologen, or titanium (III) citrate by benzoyl-CoA reductase. This assay allowed for the measurement of the rate of electron usage by benzoyl-CoA reductase as it acted enzymatically on its substrate. This assay, however, did not allow for the visualization of any enzymatic products.

The HPLC assay contained the same components as the spectrophotometric assay, except that samples were taken at several time increments during the assay. These samples were then passed over a reverse-phase HPLC column. Any CoA thioesters eluting from the column were observed using a dual-channel UV-Vis diode array detector set to monitor 260 nm. This assay allowed for the visualization of the consumption of substrate and the production of enzymatic products at time points during the enzymatic reaction. This assay yielded no kinetic information about the enzymatic reaction and could only be used to visualize the presence of substrate and any enzymatic products.

Purification of benzoyl-CoA reductase. Benzoyl-CoA reductase was purified from *Thauera aromatica* as described previously (Boll and Fuchs, 1995). The cell-free extract was passed through a DEAE-cellulose anion exchange column and the fractions containing benzoyl-CoA reductase were eluted from the column at 115 mM KCl. After the protein sample passed over the hydroxyapatite column, the substrate dependent oxidation of methyl viologen was detectable in fractions eluting off the column at approximately 15 mM potassium phosphate. These fractions, which had a light brown-green color, were pooled, concentrated and applied to a gel filtration column. After gel filtration, the pooled protein fractions containing benzoyl-CoA reductase activity were applied to a second DEAE-cellulose anion exchange column in order to remove impurities that may have remained after the first DEAE-cellulose anion exchange column. Due to a high background rate of methyl viologen oxidation in the absence of substrate by the protein sample, the spectrophotometric determination of benzoyl-CoA reductase activity could only be used after passing the cell free extract over both a DEAE-cellulose anion exchange column and a hydroxyapatite column. As a result, it was difficult to ascertain the efficiency of the purification until after the protein sample was passed over the hydroxyapatite column. For this reason, benzoyl-CoA reductase was eluted from the first DEAE-anion exchange column based primarily on previously published information regarding the salt concentration at which the

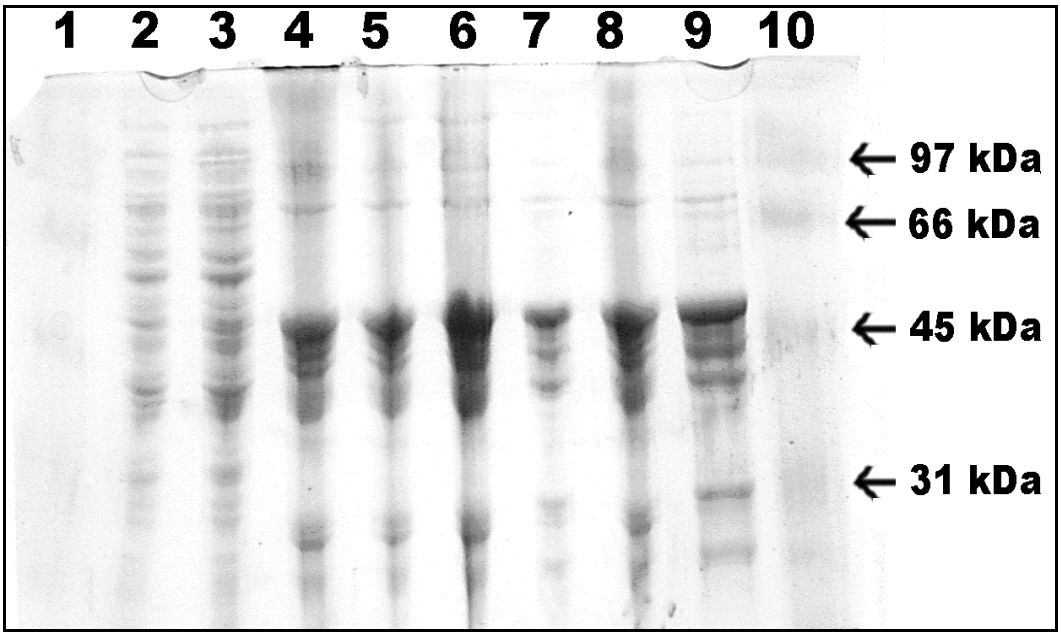


Figure 13. SDS/PAGE overview of a typical benzoyl-CoA reductase purification procedure. Lanes 1 and 10 contain 10 µl and 20 µl of a low range protein standard (Biorad) respectively. Lanes 2 and 3 contain 25 µg and 50 µg of protein from the cell free lysate respectively. Lane 4 contains 25 µg of protein from the active pooled fractions eluted from the gel filtration column. Lanes 5 and 6 contain 25 µg and 50 µg of protein from the active pooled fractions eluted from the hydroxyapatite column respectively. Lane 7 and 8 contain 25 µg and 50 µg of protein eluted from the second DEAE column respectively. Lane 9 contains 50 µg of protein eluted from the second DEAE column which was treated with a small additional amount of SDS in an attempt to flatten out the protein bands.

enzyme elutes off of the column and on the characteristic brownish-green color of the reduced protein (Boll and Fuchs, 1995).

A SDS/PAGE following the purification of the enzyme is shown in Fig. 13. The final preparation coming off of the second DEAE column shows the presence of five major protein bands. The four subunits with the sizes of approximately 48, 45, 38, and 32 kDa correspond to that of benzoyl-CoA reductase can be seen in

lane nine of Fig 13 (Boll and Fuchs, 1995). The additional protein band at around 29 kDa most likely corresponds to the protein cyclohexa-1,5-diene-1-carbonyl-CoA hydratase, which has a mass of approximately 28 kDa and has recently been reported as a co-purifying protein when following this purification protocol (Boll et. al., 2000).

A typical protein purification yielded approximately 35 mg of protein from 70 g of wet cells. Using dithionite-reduced methyl viologen as the electron donor and under saturating concentration of both ATP and benzoyl-CoA, the activity of the final enzyme preparation had a specific activity of 0.024 µmol benzoyl-CoA min^{-1} mg^{-1} at 25 degrees Celsius, with the activity expressed in terms of µmol benzoyl-CoA reduced per minute. This is a factor of 23 smaller than that of 0.55 µmol benzoyl-CoA min^{-1} mg^{-1} previously determined for benzoyl-CoA reductase at 37 degrees Celsius (Boll and Fuchs, 1995). The final protein sample, containing the benzoyl-CoA reductase activity, had a dark brownish-green color, corresponding to the color of reduced benzoyl-CoA reductase (Boll and Fuchs, 1995). The oxidation of methyl viologen by benzoyl-CoA reductase is dependent on the presence of both benzoyl-CoA and ATP, as shown in Fig. 14.

HPLC separation and mass spectrometry analysis of products from the benzoyl-CoA reductase reaction on benzoyl-CoA. The action of benzoyl-CoA reductase on benzoyl-CoA in the presence and absence of the electron donor

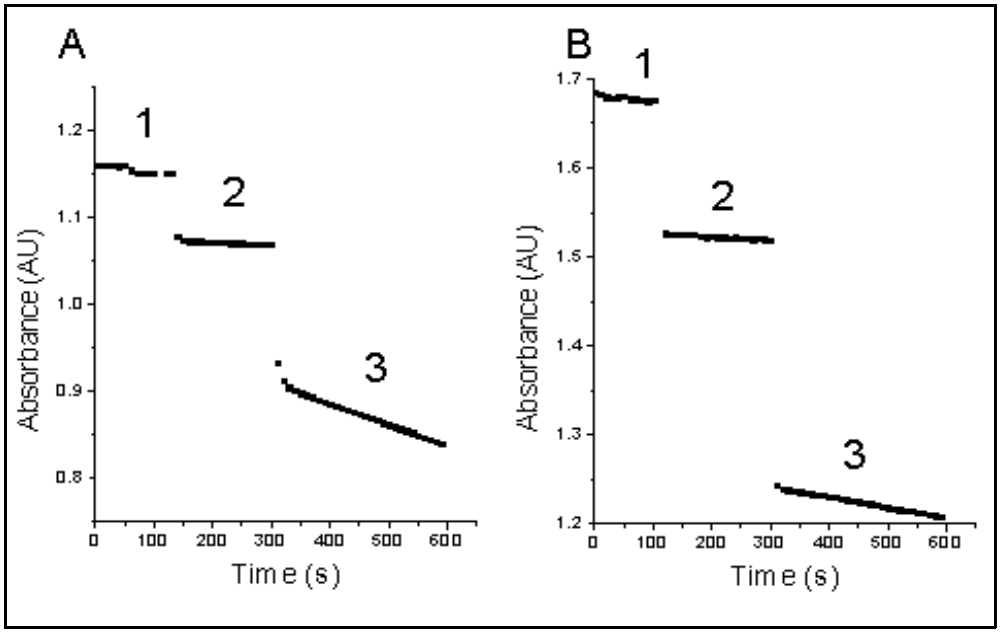


Figure 14. The effect of ATP and benzoyl-CoA on the oxidation of reduced methyl viologen by benzoyl-CoA reductase. The oxidation of methyl viologen was observed at 730 nm. (**A**) ATP dependence of the reaction. After 10 µl of enzyme solution is incubated with 1 mM dithionite-reduced methyl viologen (1), 0.2 mM benzoyl-CoA is added (2), followed by the addition of 5 mM ATP (3). (**B**) Substrate dependence of the reaction. After the enzyme is incubated with 1 mM dithionite-reduced methyl viologen (1), 5 mM ATP is added (2), followed by the addition of 0.2 mM benzoyl-CoA (3). Note: The large observed drop in absorbance at the beginning of each addition is caused by the presence of trace amounts of oxygen in the stock solutions and by dilution.

titanium (III) citrate was studied using a Platinum C-18 reverse-phase HPLC column (Figs. 15 and 16).

In the absence of titanium(III) citrate, there was no evidence of enzymatic activity. The benzoyl-CoA peak, which eluted off from HPLC at 13 minutes, showed very little change in amount after 15 minutes of assay time. Also, the

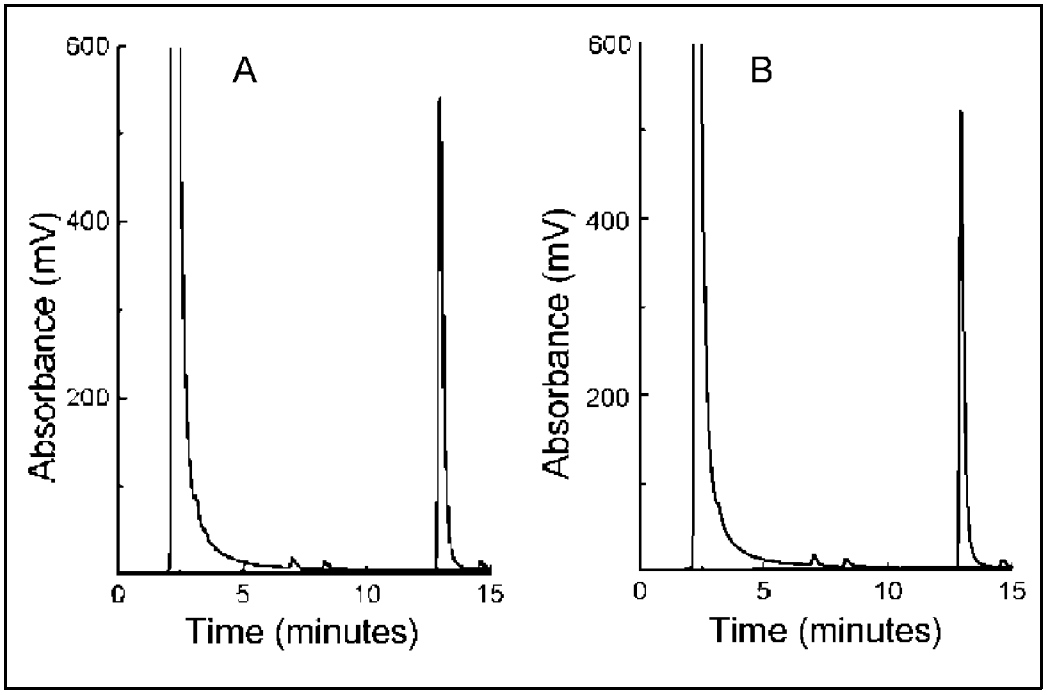


Figure 15. HPLC traces of a benzoyl-CoA reductase assay using benzoyl-CoA as the substrate, without the electron donor titanium(III) citrate. (A) HPLC trace of the assay after one minute of incubation with the enzyme. (B) HPLC trace of the assay after fifteen minutes of incubation with the enzyme. The peak at 13 minutes represents the substrate, benzoyl-CoA. Absorbance was measured at 260 nm, which allowed for the detection of molecules containing adenosine, such as CoA. The anaerobic assay was degassed and started by the addition of the enzyme.

absence of any new peaks on the chromatogram indicated that no new CoA thioesters were produced (Fig. 15).

In the presence of titanium(III) citrate, there was evidence of an enzymatic reaction (Fig. 16). The thirteen minute peak, benzoyl-CoA, is significantly reduced after 15 minutes of incubation with ATP and titanium(III) citrate (Fig. 16: **1**). This decrease coincided with the appearance of two new peaks (Fig. 16: **2,3**). The first peak was more polar than the substrate peak, eluting at around 10.5 minutes, and

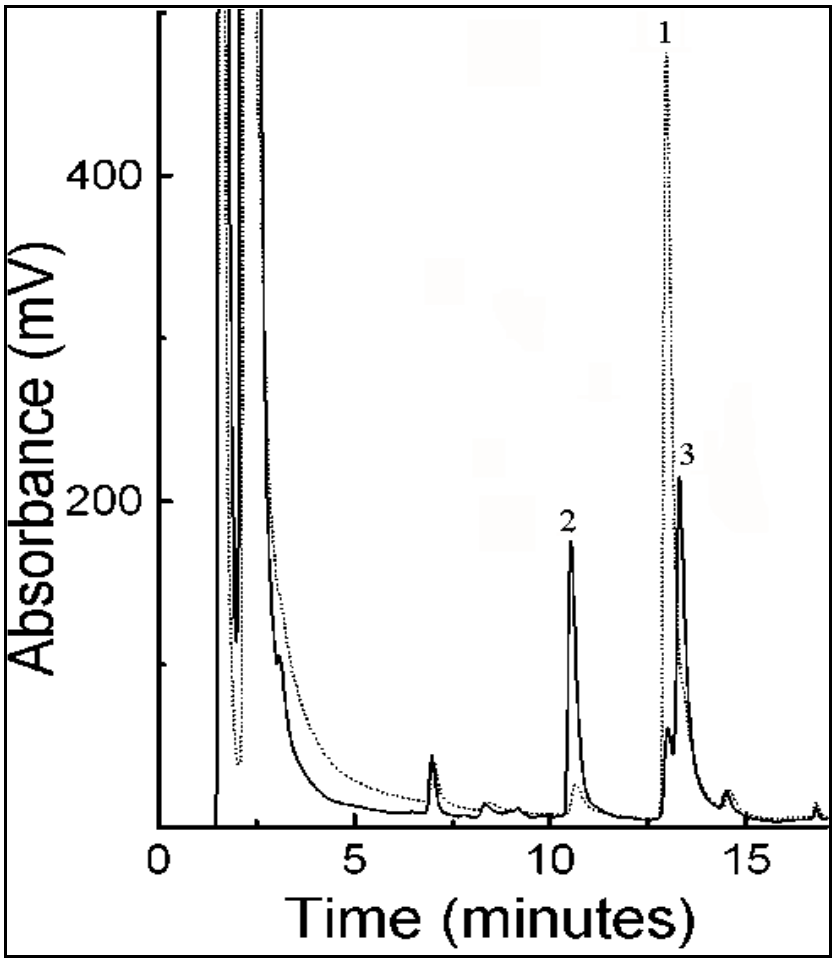


Figure 16. HPLC traces of a benzoyl-CoA reductase assay using benzoyl-CoA as the substrate and titanium(III) citrate as the electron donor. The lighter, dotted line (.....) represents the assay after one minute of incubation with the enzyme. The darker, solid line ())))) represents the assay after fifteen minutes of incubation with the enzyme. **1** - benzoyl-CoA (substrate); **2**,**3** - enzymatic products. The peak at seven minutes was present in the starting reaction and remained constant during the reaction and is most likely, a contaminant.

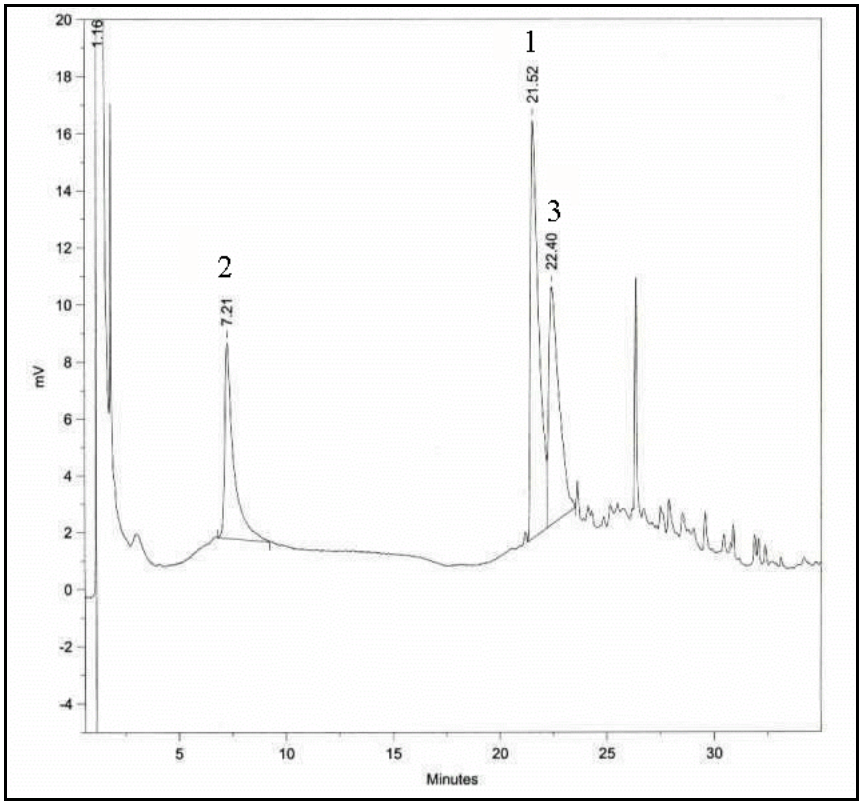


Figure 17. LC/MS chromatogram of a benzoyl-CoA reductase assay using benzoyl-CoA as the substrate and titanium(III) citrate as the electron donor. The assay was applied to a Zorbax C-18 reverse-phase column after fifteen minutes of incubation with the enzyme. **1** - benzoyl-CoA (substrate); **2**,**3** - enzymatic products.

the other peak was slightly less polar than the substrate peak, eluting just after the substrate peak at 13.3 minutes. The appearance of these two new peaks is dependent on both the presence of ATP and the electron donor, titanium (III) citrate in the enzyme assay. The identity of these new peaks was determined by LC/MS (Fig. 17). The assay was passed over a Zorbax C-18SB reverse phase HPLC column and the eluent of the column was analyzed by a Thermo-Finnigan

LCQ with a quadrupole ion trap. The atomic masses of any ions that formed were detected by monitoring the ion channel that yielded the best signal to noise ratio, which in this case, was the negative ion channel. The substrate, benzoyl-CoA, peak 1 in Fig. 17, had an atomic mass of 870 (Fig. 18A). This mass corresponds to the atomic mass of a negative ion of benzoyl-CoA, whose mass is equivalent to the calculated mass of benzoyl-CoA, 871, minus one proton. The less polar product, peak 2 in Fig. 17, had an atomic mass of 872 (Fig. 18B). The mass of this product is two mass units higher than that of the substrate, benzoyl-CoA, identifying this molecule as a likely reduction product of benzoyl-CoA and will be further referred to as the reduction product. The more polar product, peak 3 in Fig. 17, had an atomic mass of 890 (Fig. 19). The mass of this product is twenty mass units higher than that of benzoyl-CoA and eighteen mass units higher than that of the reduction product. The mass of this molecule could correspond to the hydration of the reduction product. This molecule will be further referred to as the hydration product. This hydration is probably catalyzed by 1,5-dienoyl-CoA hydratase which copurifies with benzoyl-CoA reductase (Boll et. al., 2000). These results correlate well with the previously identified products of the benzoyl-CoA reductase and the 1,5-dienoyl-CoA hydratase reactions, which are shown in Fig. 20 (Boll et. al., 2000). Thus, upon incubation with the enzyme solution, benzoyl-CoA reductase reduces benzoyl CoA (**1**) to the reduction product, which is likely cyclohexa-1,5-diene-1-carbonyl-CoA (**3**), and then hydrated to form the hydration product, which

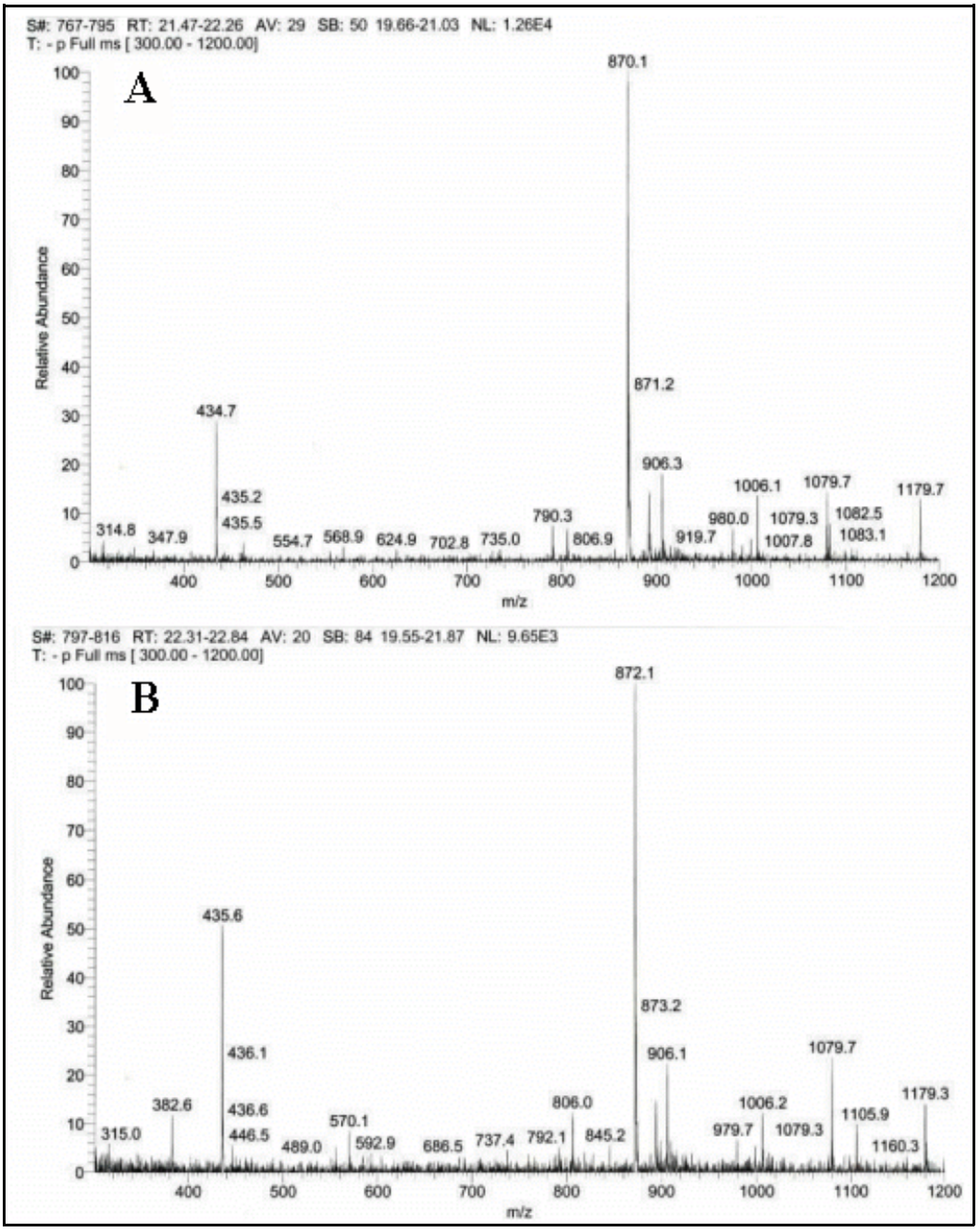


Figure 18. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on benzoyl-CoA (Peaks 1 & 3). (**A**) A time-slice of the ions formed by the molecules contained in peak 1, benzoyl-CoA (Fig. 17). The primary ion formed had a mass of 870. (**B**) A time-slice of the ions formed by the molecules contained in peak 3 (Fig. 17). The primary ion formed had a mass of 872. Ions were observed using the negative ion channel.

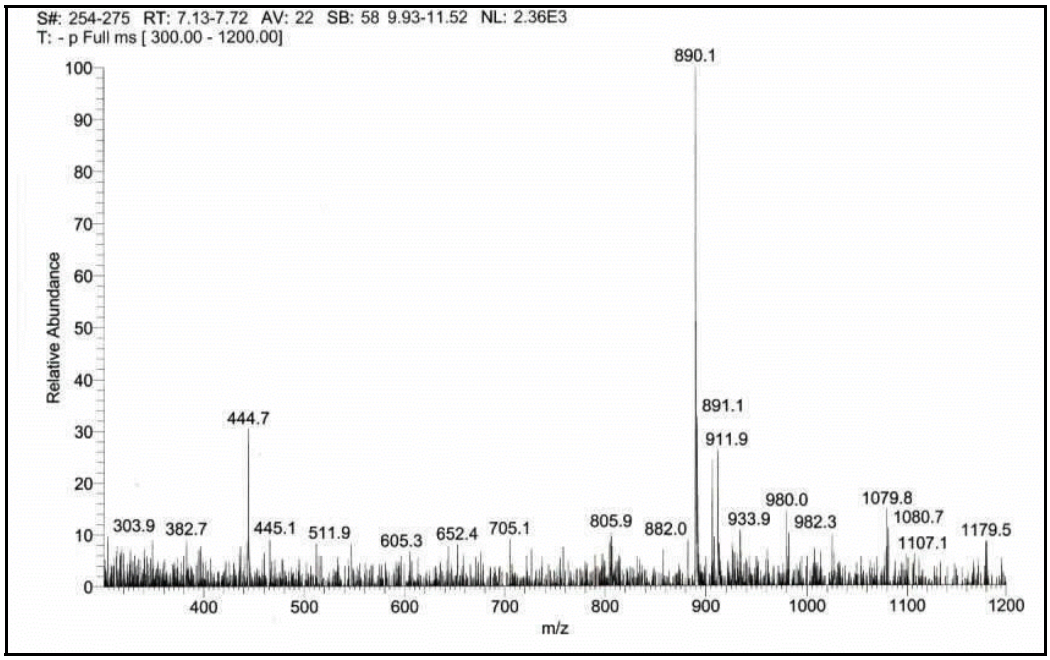


Figure 19. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on benzoyl-CoA (Peak 2). A time-slice of the ions formed by the molecules contained in peak 2 (Fig 17). The primary ion formed had a mass of 890. Ions formed were observed using the negative ion channel.

is likely 6-hydroxycyclohex-2-ene-1 carbonyl-CoA (**2**), by the 1,5-dienoyl-CoA hydratase (Fig. 20). This illustrated that the LC/MS assay method could be used to observe and identify the enzymatic products of the benzoyl-CoA reductase assay.

Kinetic isotope studies on benzoyl-CoA reductase. To study whether a proton transfer might be involved in the rate-determining step of the enzymatic reaction, which might indicate that hydride chemistry is occurring, the catalytic properties of benzoyl-CoA reductase were studied with the spectrophotometric assay, using methyl viologen as the reducing agent and varying concentrations of benzoyl-CoA as the substrate, in the presence of H_2O and in the presence of D_2O. Methyl

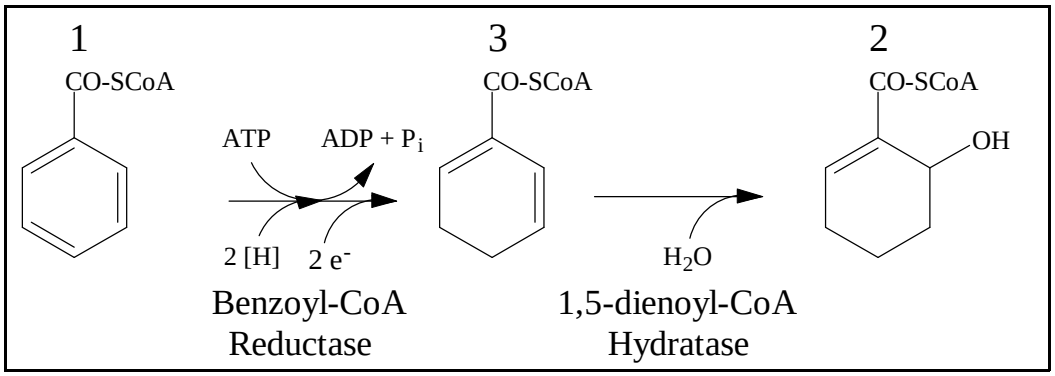


Figure 20. Products formed from benzoyl-CoA by benzoyl-CoA reductase and 1,5-dienoyl-CoA hydratase in the presence of titanium(III) citrate and ATP.

viologen was used as the reducing agent, instead of titanium(III) citrate, because it had a smaller background oxidation rate, providing a better signal to noise ratio. The K_m and V_{max} values for the enzyme in H_2O and in D_2O were determined by fitting the kinetic data with a hyperbolic curve fit (Fig. 21). The apparent K_m of the enzyme for benzoyl-CoA in H_2O was 33 µM and in D_2O, 18 µM benzoyl-CoA. The V_{max} values for the enzyme in H_2O and D_2O were 5.0 nmol min^{-1} and 2.8 nmol min^{-1} respectively. Given that the same enzyme solution and the same amount of enzyme was used in each reaction, the decrease in the V_{max} of the benzoyl-CoA reductase reaction in D_2O results in a deuterium isotope effect of 1.8 ± 1 ($V_{max}(H_2O)/V_{max}(D_2O)$). The atomic masses of the products formed from benzoyl-CoA by the reductase/hydratase activities in D_2O were determined by LC/MS as was done before for the same reaction carried out in H_2O (Figs. 22-24). The mass of the benzoyl-CoA substrate remained unchanged at 870, while the mass of the reduction product ranged from 872 to 874 (Fig 23). The masses correspond to a

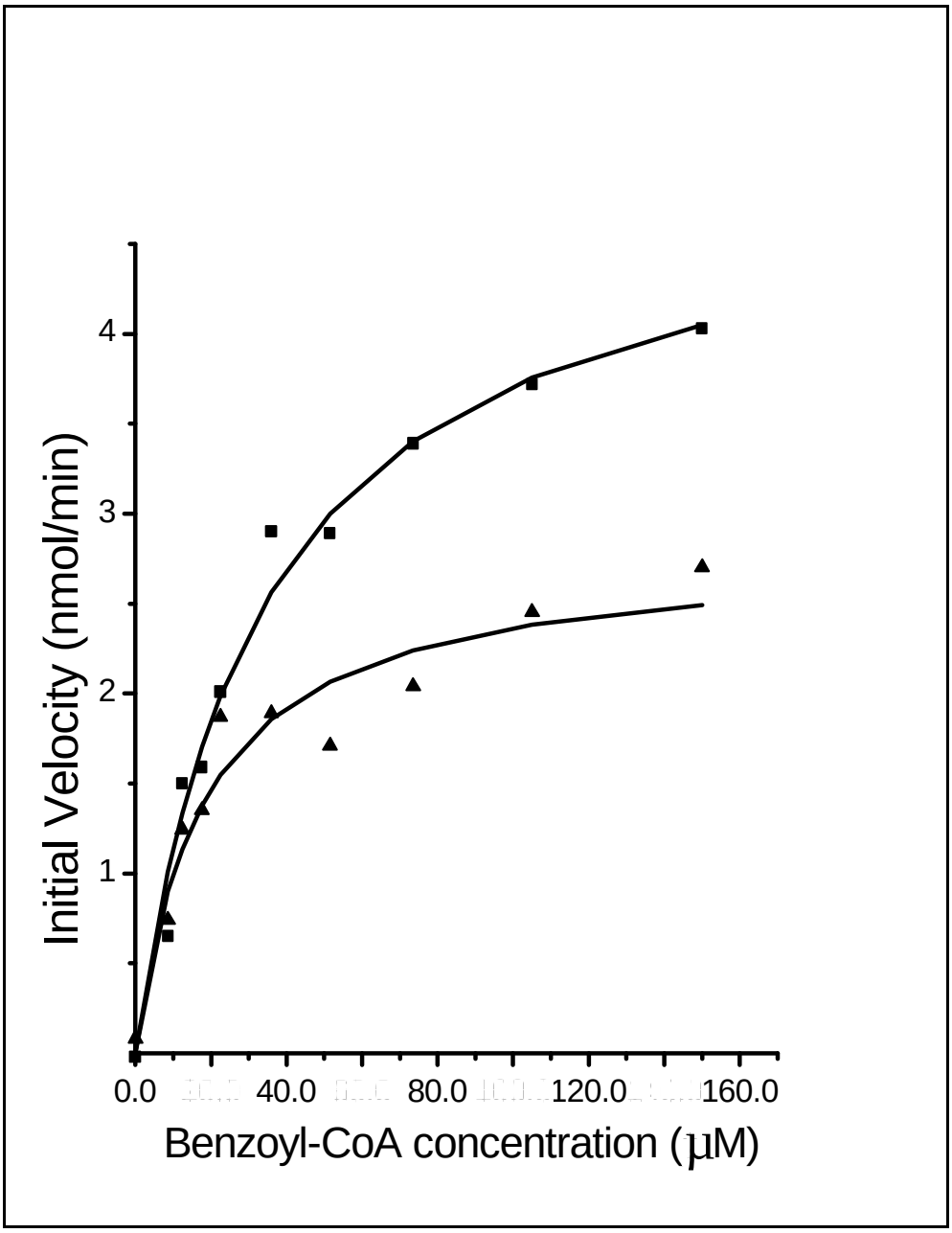


Figure 21. The effect of D_2O on K_m and V_{max} of benzoyl-CoA reductase. The initial velocity of the enzymatic reaction was plotted as a function of substrate concentration for the reactions carried out in 150 mM MOPS (square(■)) and the reactions carried out in D_2O (triangle(▲)). Assuming that two mole of electrons are required to reduce one mole of benzoyl-CoA, the rate of benzoyl-CoA consumption was calculated as being equal to twice the rate of the oxidation of dithionite-reduced methyl viologen.

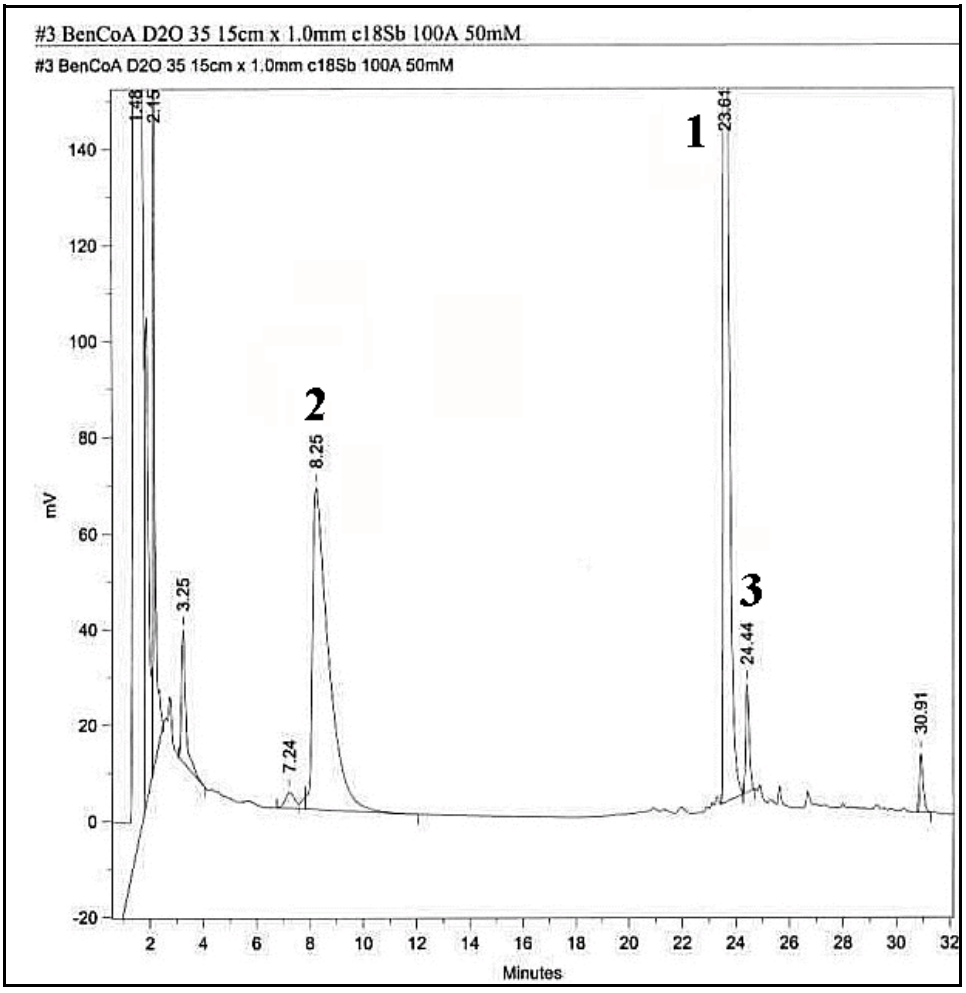


Figure 22. LC/MS chromatogram of a benzoyl-CoA reductase assay in D_2O using benzoyl-CoA as the substrate and titanium(III) citrate as the electron donor. The assay was applied to a Zorbax C-18 reverse-phase column after fifteen minutes of incubation with the enzyme. **1** - benzoyl-CoA (substrate); **2** - hydrated, reduced product. **3** - reduced product.

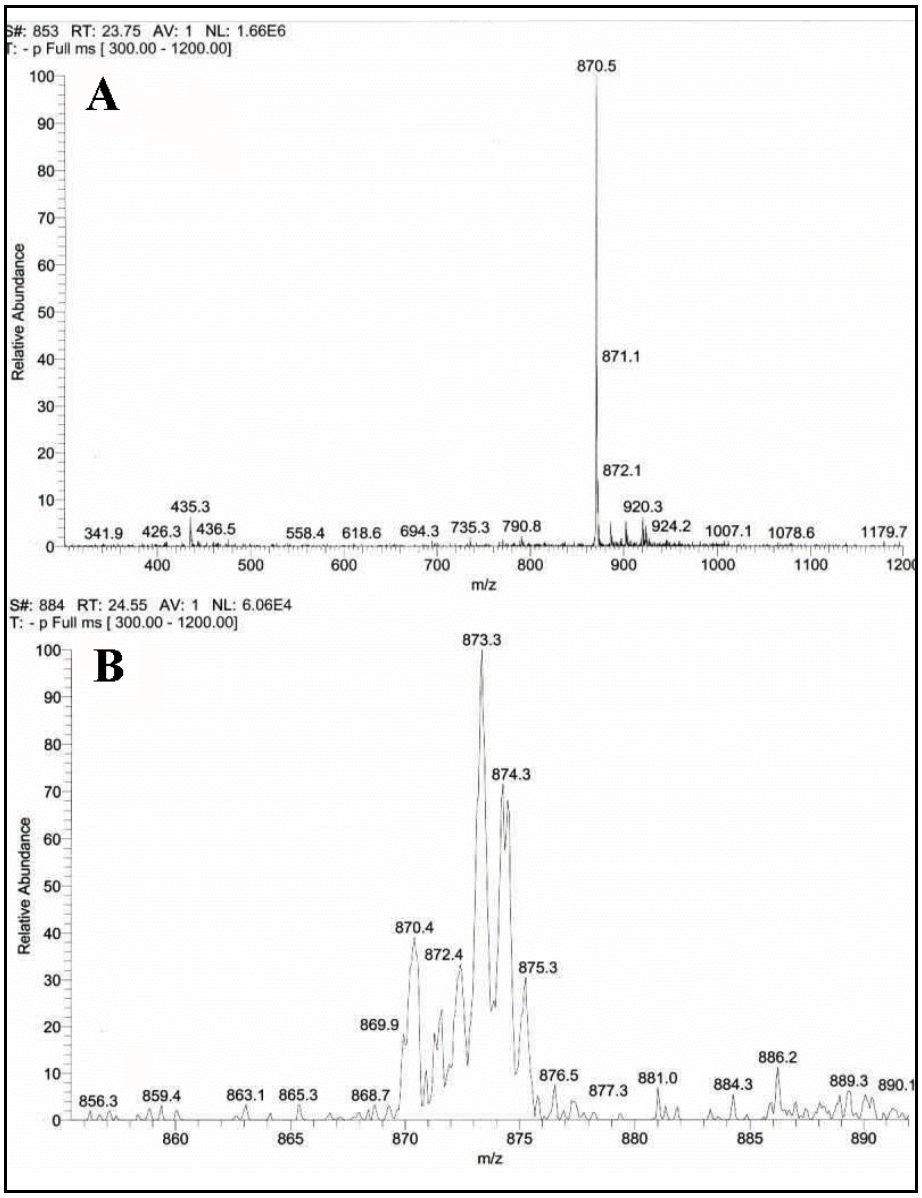


Figure 23. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on benzoyl-CoA in D_2O (Peaks 1 & 3). (**A**) A time-slice of the ions formed by the molecules of peak 1 (Fig. 22). The primary ion formed had a mass of 870. (**B**) A time-slice of the ions formed by the molecules of peak 3 (Fig.22). The primary ions that formed had a mass of 873 and 874. Ions were observed using the negative ion channel. The range of masses observed in the reduction product were caused by a varying incorporation of protons and deuterons into the reduction product during the enzymatic reduction of the substrate.

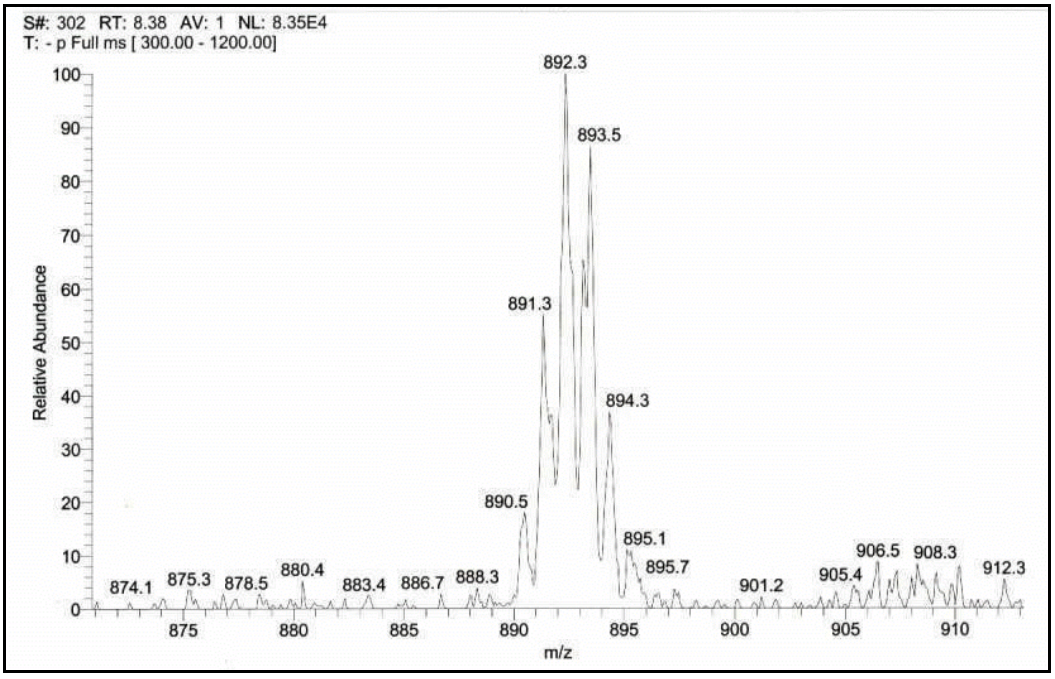


Figure 24. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on benzoyl-CoA in D_2O (Peak 2). (**A**) A time-slice of the ions formed by the molecules of peak 2 (Fig. 22). The primary ions formed had a mass of 892 and 893. Ions were observed using the negative ion channel.

varying incorporation of deuterium atoms into the reduction product. The main reduction product formed had a mass of 873, which suggests that one deuterium and one hydrogen atom were incorporated into the reduction product during the reduction of benzoyl-CoA by benzoyl-CoA reductase. The atomic mass of the hydrated increased from 890, its atomic mass in the reaction carried out in H_2O, to a mass of 892 and 893 (Fig. 24). This confirms the incorporation of deuterium in hydration product and suggests that deuterium may have been incorporated into the molecule during the hydration of the reduction product by the 1,5-dienoyl hydratase. The observed solvent kinetic isotope effect of 1.8 for benzoyl-CoA

reductase is relatively low for a primary kinetic isotope effect (Xiaoqing and Bisswanger, 2003). Xanthine oxidase and trimethylamine dehydrogenase are two enzyme systems that employ electron and proton transfer in order to catalyze their respective reactions. Proton transfer is involved in the rate-limiting step in both these enzyme systems and a large solvent isotope effect, between 7 and 10, is observed in both cases (Hille and Anderson, 2001). The low primary kinetic isotope effect observed for benzoyl-CoA reductase suggests that proton transfer is only slightly rate-limiting. Interestingly, while the V_{max} of the benzoyl-CoA reductase reaction carried out in D_2O decreased by a factor of 1.8, the K_m of the reaction also decreased by a factor of 1.8, resulting in the same V_{max}/K_m ratio as the reaction carried out in H_2O. As stated previously, the same enzyme solution was used and the same amount of enzyme was used in both sets of reactions. Therefore it can be said that k_{cat}/K_m of the benzoyl-CoA reductase reaction remained unchanged when the benzoyl-CoA reductase reaction took place in D_2O. The term k_{cat}/K_m is an apparent second order rate constant that controls the rate at which the enzyme binds to the substrate and the rate at which it turns the substrate over (Fersht, 1985). Therefore, if it is unchanged, then the rate of substrate binding is unchanged by the presence of D_2O. The reduction in the observed k_{cat} in D_2O must be the result of an slight isotope effect occurring in a reaction step after the enzyme-substrate complex forms, such as the protonation of an intermediate.

Reversibility of the 1-5-dienoyl hydratase and the oxidation of the reduction product by oxygen-exposed benzoyl-CoA reductase. While studying the benzoyl-CoA reductase reaction using benzoyl-CoA, it was observed that the hydration product and the reduction product appear simultaneously. Also, in several HPLC chromatograms, the two products had about the same relative peak height to peak height ratio, suggesting that they are in equilibrium with each other and that the hydratase activity is reversible. In order to test this hypothesis, benzoyl-CoA reductase was allowed to react with benzoyl-CoA in the presence of ATP and titanium(III) citrate. The hydration product was then separated from the other components in the reaction using a Platinum C-18 reverse-phase HPLC column. The hydration product, which, as shown by HPLC, was stable in the absence of the enzyme (Fig. 25), was then reintroduced to the enzyme in a 150 mM MOPS/KOH buffer at pH 7.3 containing dissolved oxygen for fifteen minutes. This assay was then passed over the reverse-phase column again. Benzoyl-CoA reductase requires ATP to carry out its reaction. Benzoyl-CoA reductase is also rapidly degraded and inactivated by oxygen. For this reason, the assay was carried out in the presence of oxygen so that only the activity of the 1-5-dienoyl hydratase would be observed. Of course, it was assumed that the hydratase activity would not be oxygen sensitive and that the hydratase activity would not require ATP, as the free energy of the reaction should be near zero and thus would not need the input of energy. As shown in Fig. 25, the hydration product (**1**) decreased after 15

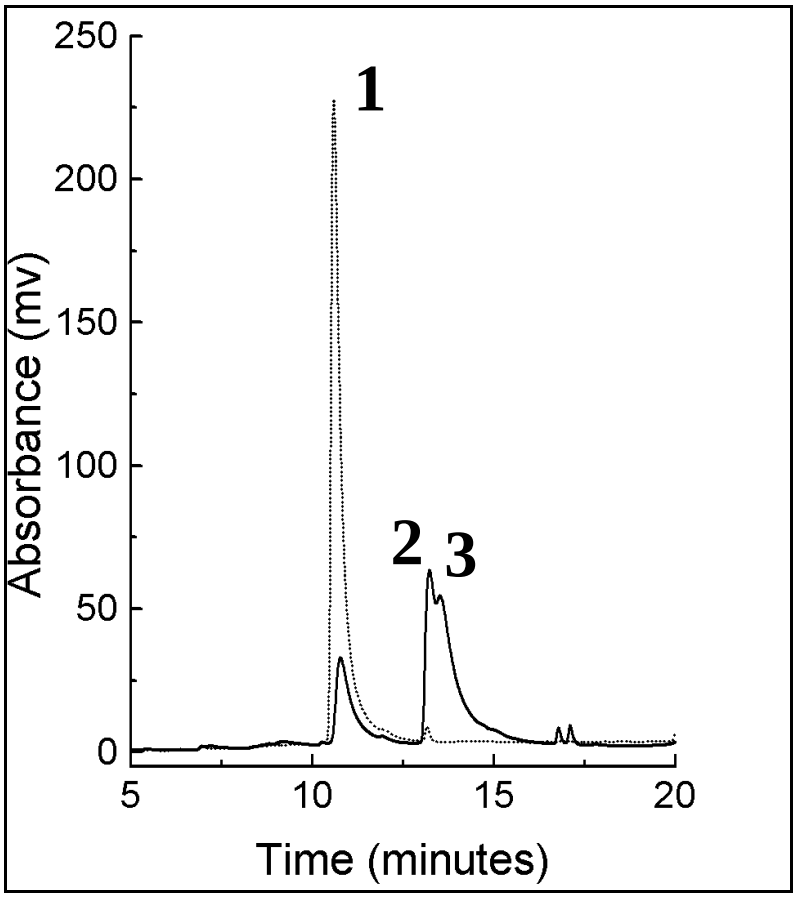


Figure 25. HPLC traces of an assay containing the reduced, hydrated benzoyl-CoA product and benzoyl-CoA reductase. The lighter, dotted line (.....) represents the assay after before the addition of enzyme. The darker, solid line ())))) represents the assay after fifteen minutes of incubation with the enzyme. The assay was carried out in 150 mM Mops/KOH at pH 7.3 containing dissolved oxygen. The assay did not contain ATP or a reducing agent. **1** - hydration product; **2** - suspected benzoyl-CoA peak; **3** - reduction product.

minutes of incubation with the enzyme and two new peaks (**2** and **3**) were observed. The retention times and elution pattern of peaks 2 and 3 identified them as benzoyl-CoA and the reduction product respectively. Since it was quite

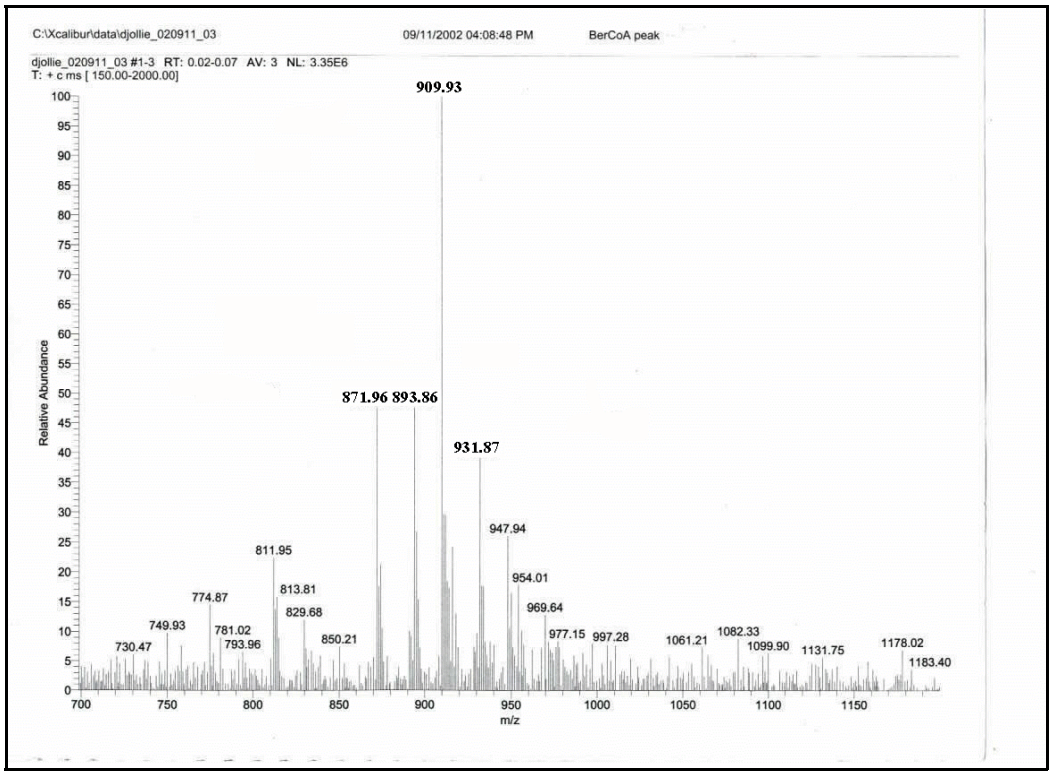


Figure 26. Mass determination of the suspected benzoyl-CoA peak (Fig. 25: 2) from the reversibility reaction by electrospray mass spectrometry. The primary ions formed had a mass of 872, 894, 910, and 932. The atomic mass of 872 corresponds to the positive ion of benzoyl-CoA. The 894, 910, and 932 atomic mass ions are likely K and Na complexed to benzoyl-CoA. Ions were observed using the positive ion channel.

surprising to see the reappearance of benzoyl-CoA, the substrate of the benzoyl-CoA reductase reaction, it was necessary to reconfirm its identity. To verify the identity of the reappearing substrate peak, it was separated from the other components of the assay as it exited the HPLC column. These fractions were then pooled, freeze-dried, and then re-dissolved in a small amount of 50 mM ammonium acetate at pH 5.5. The resulting solution was analyzed by electrospray mass spectrometry using a Thermo-Finnigan LCQ equipped with a quadrupole ion

trap (Fig. 26). Ions were monitored using the positive channel because it provided a better signal than the negative channel. A positive ion with an atomic mass of 872 was identified in the solution. This mass corresponds to the atomic mass of a positive ion of benzoyl-CoA, which is the mass of benzoyl-CoA, 871, plus the mass of a single proton. The other ions in the solution had an atomic mass of 894, 910, and 932. These masses are 23, 38, and 51 atomic mass units higher than that of benzoyl-CoA. These can be attributed to one or more sodium, 23 atomic mass units, and potassium, 38 atomic mass units, salt ions associated to the benzoyl-CoA ion. The formation of these ions formed as a result of an increased concentration of salt ions in the final solution, which was caused by the removal of water during the freeze-drying process. The formation of benzoyl-CoA from the hydration product in the presence of the oxygen-exposed enzyme shows that the 1,5-dienoyl-CoA hydratase reaction is readily reversible and does not require ATP. More importantly, it appears that benzoyl-CoA reductase is able to re-oxidize the reduction product back to its substrate, benzoyl-CoA, even though its normal enzymatic activity had been inactivated by the presence of oxygen in the solution. This was quite a surprise since the reoxidation of the reduction product to benzoyl-CoA does not require ATP and does not require that benzoyl-CoA reductase possess its normal "reductase" activity. In fact, the only apparent requirement for this reversibility is that the assay solution contain oxygen and the enzyme solution. To explore this further, a benzoyl-CoA reductase reaction, using titanium (III)

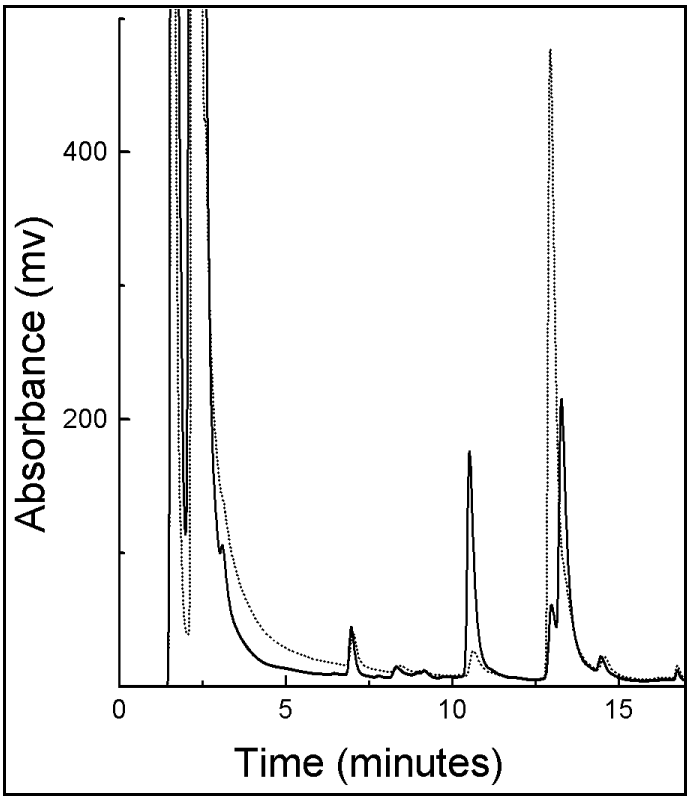


Figure 27. HPLC traces of a benzoyl-CoA reductase assay using benzoyl-CoA as the substrate and titanium(III) citrate as the electron donor before oxygen exposure. The lighter, dotted line (.....) represents the assay after one minute of incubation with the enzyme. The darker, solid line ())))) represents the assay after fifteen minutes of incubation with the enzyme.

citrate as the reducing agent, was allowed to react for fifteen minutes before being exposed to oxygen for forty minutes in order to allow for the oxidation of any unreacted titanium (III) citrate and for the dissolution of oxygen back into the solution. As seen in Fig. 27, before exposure to oxygen, the assay behaves normally, with the benzoyl-CoA peak decreasing and the reduced product peak and the reduced, hydrated product peak increasing after 15 minutes. After exposing the assay to oxygen for 40 minutes, the height of the benzoyl-CoA substrate peak, eluting oat 13 minutes, increased (Fig. 28). There was also a corresponding decrease in the height of the hydration product peak, eluting at 10.5 minutes, and in the height reduction product peak, eluting off at 13.3 minutes (Fig.

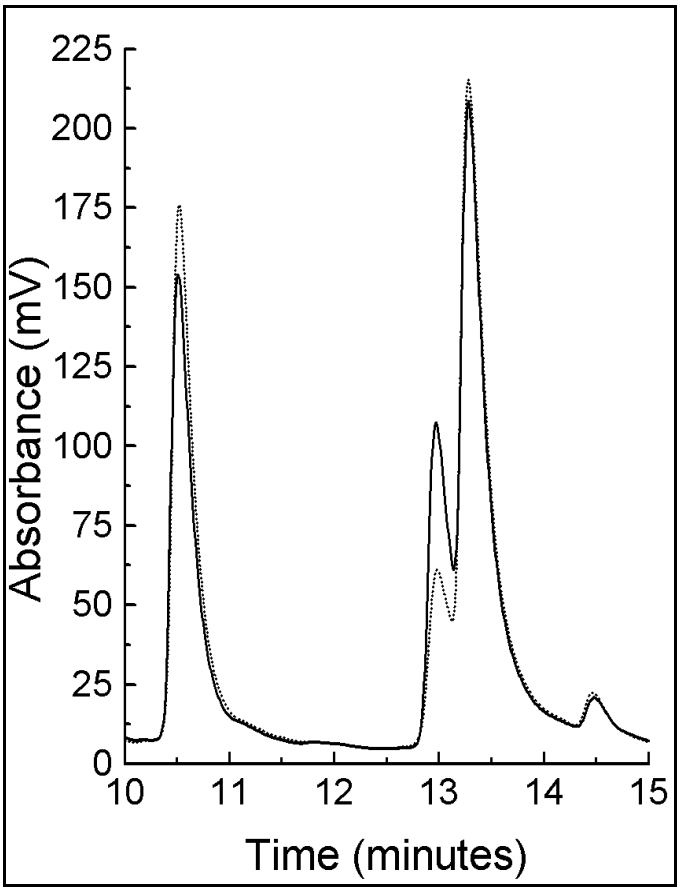


Figure 28. HPLC traces of a benzoyl-CoA reductase assay using benzoyl-CoA as the substrate and titanium(III) citrate as the electron donor after 40 minutes of oxygen exposure. The lighter, dotted line (.....) represents the assay after fifteen minutes of incubation with enzyme before oxygen exposure. The darker, solid line ())))) represents the same assay after forty minutes of oxygen exposure.

28). The reformation of the substrate in the assay after forty minutes of oxygen exposure supports the previous result that in the presence of oxygen, benzoyl-CoA reductase can catalyze the oxidation of its reduction product back to its substrate.

Synthesis of benzoyl-CoA analogues. In order to obtain a better understanding of how benzoyl-CoA reductase carries out the reduction of benzoyl-CoA, analogues of benzoyl-CoA were synthesized. Nitrogen-containing heterocyclic analogues were synthesized to study whether having a nitrogen atom in the aromatic ring might facilitate catalysis by benzoyl-CoA reductase. The nitrogen atom provides a site to stabilize a negative charge and thus would help to stabilize the radical anion

intermediate formed by the addition of the first electron (Birch and Slobbe, 1976). Also, the stabilization of negative charge by the nitrogen atom might cause the first radical anion intermediate to rearrange so that the nitrogen could stabilize the negative charge. This could alter the site of reduction and cause the formation of a different reduced product than that obtained from the reduction of benzoyl-CoA. Fluorinated analogues of benzoyl-CoA were synthesized to study whether benzoyl-CoA reductase would behave similarly to the Birch reduction and cause the reductive defluorination of p-fluorobenzoyl-CoA, but leave the fluorine on m-fluorobenzoyl-CoA. Analogues of benzoyl-CoA were synthesized by reacting the acid chloride, formed from their respective carboxylic acid (isonicotinic, nicotinic, picolinic, m-fluorobenzoic, or p-fluorobenzoic acid), with free CoA. After purification of the thioesters using a Sephadex G-10 gel filtration column, the synthesized analogues were identified by electrospray mass spectrometry using a Thermo-Finnigan LCQ equipped with a quadrupole ion trap. The ions were detected using the positive ion channel. Nicotinoyl-CoA, isonicotinoyl-CoA, and picolinoyl-CoA all have a calculated atomic mass of 872. The positive ion of these thioesters has an atomic mass of 873, which accounts for the addition of a proton to the neutral molecule. As shown in Figs. 29-31, a positive ion with an atomic mass of 873 was detected in each solution, confirming that nicotinoyl-CoA, isonicotinoyl-CoA, and picolinoyl-CoA were present in their respective solutions. The fluorinated analogues m-fluorobenzoyl-CoA and p-fluorobenzoyl-CoA both

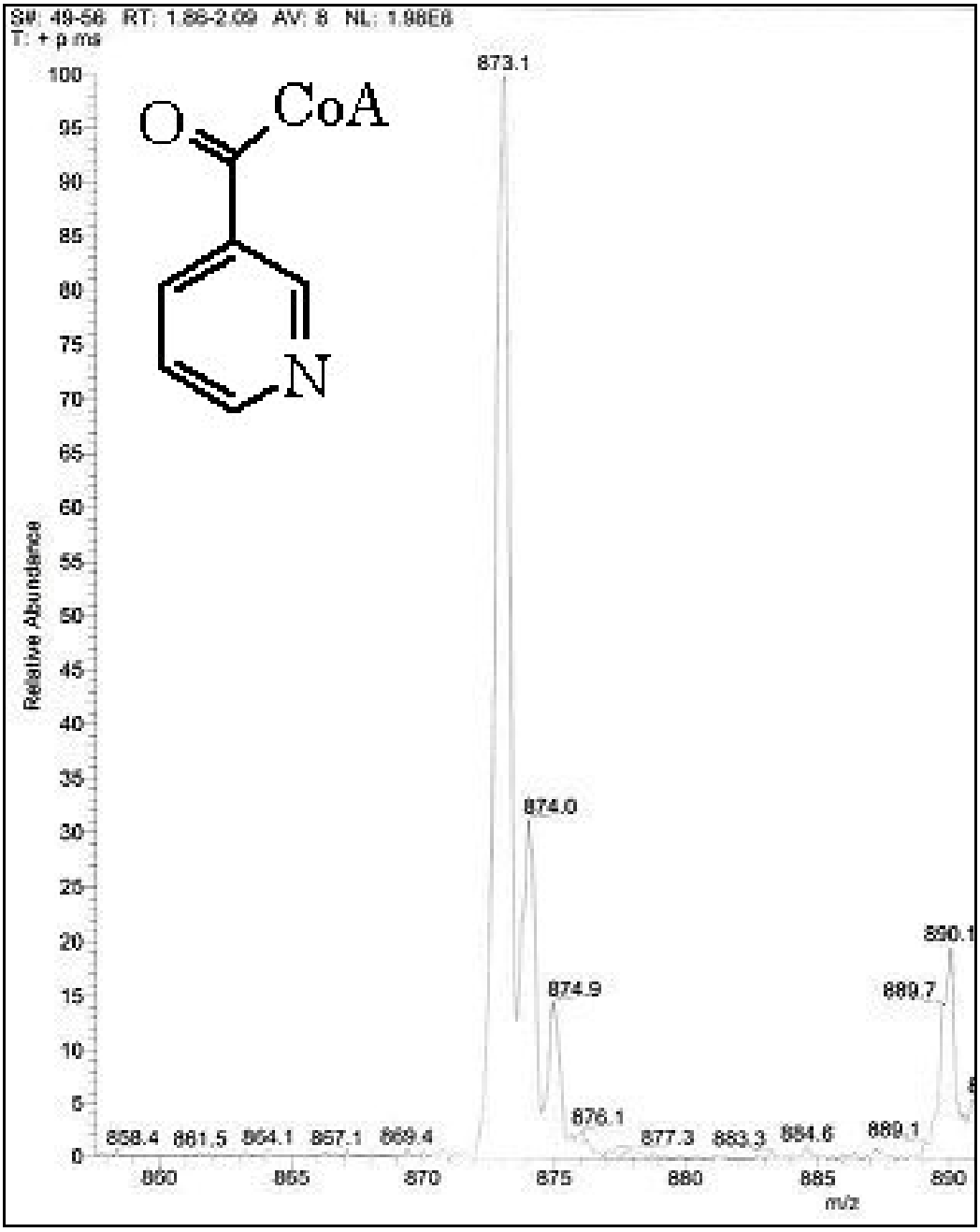


Figure 29. Mass determination of synthesized nicotinoyl-CoA by electrospray mass spectrometry. The primary ion formed had a mass of 873, which corresponds to the positive ion of nicotinoyl-CoA. Ions were observed using the positive ion channel.

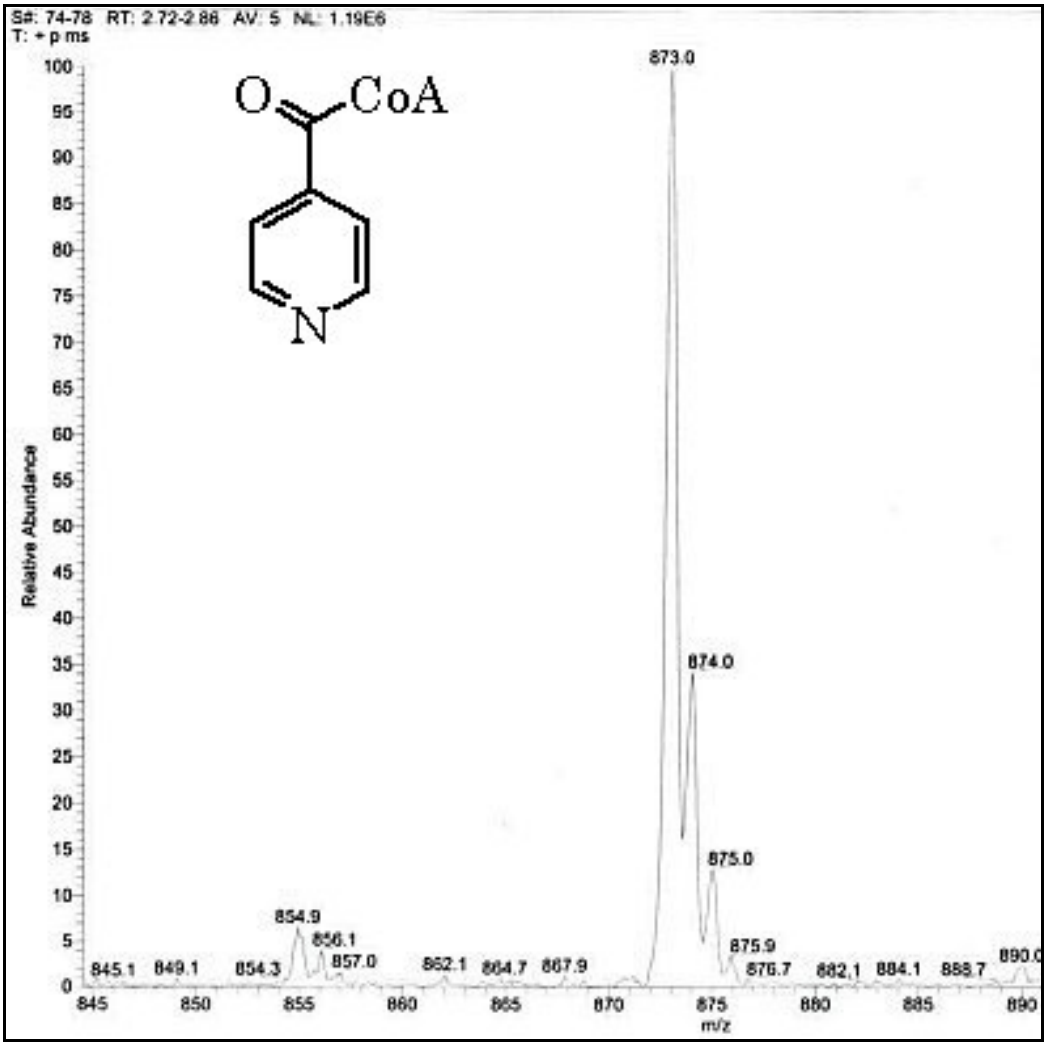


Figure 30. Mass determination of synthesized isonicotinoyl-CoA by electrospray mass spectrometry. The primary ion formed had a mass of 873, which corresponds to the positive ion of isonicotinoyl-CoA. Ions were observed using the positive ion channel.

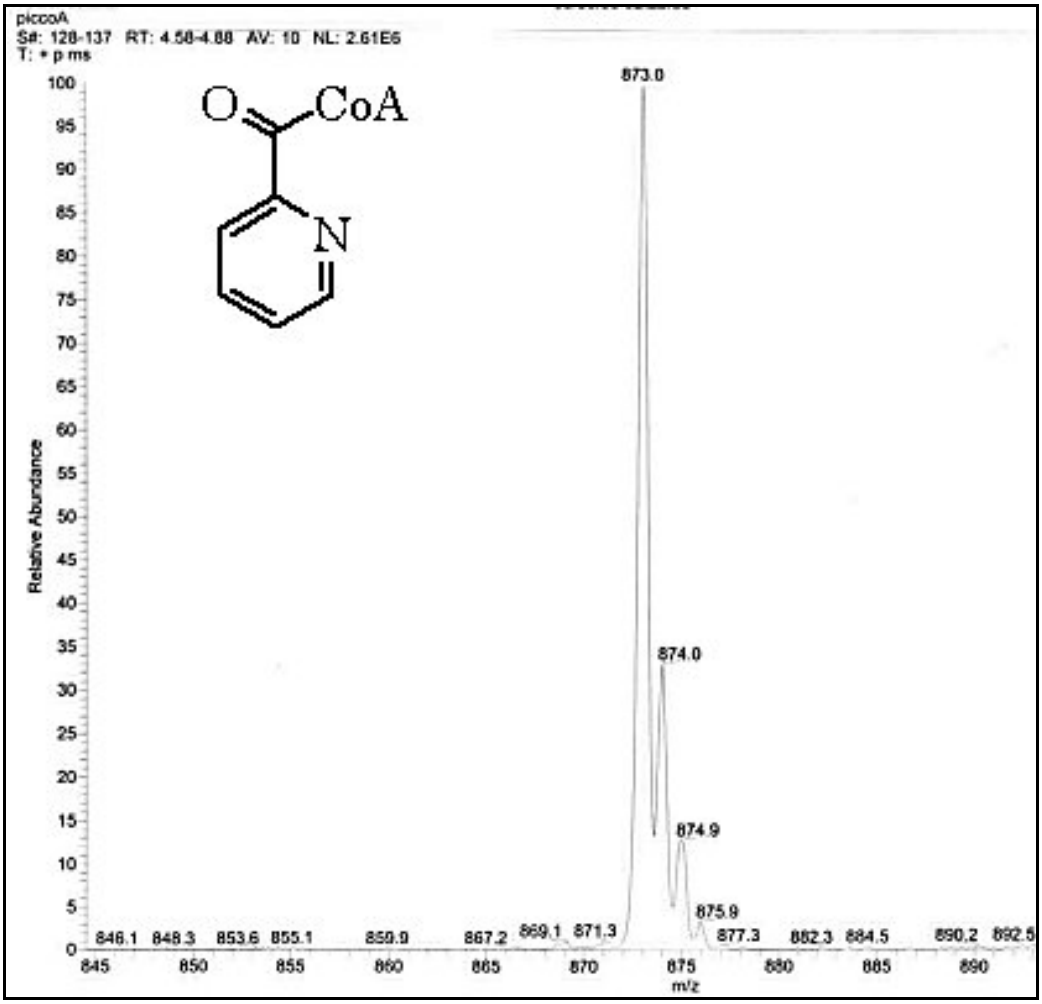


Figure 31. Mass determination of synthesized picolinoyl-CoA by electrospray mass spectrometry. The primary ion formed had a mass of 873, which corresponds to the positive ion of picolinoyl-CoA. Ions were observed using the positive ion channel.

have a calculated atomic mass of 889 and thus the positive ion of these two molecules has an atomic mass of 890. As shown in Figs. 32 and 33, a positive ion with an atomic mass of 890 was detected in each solution, confirming that m-fluorobenzoyl-CoA and p-fluorobenzoyl-CoA were present in their respective solutions.

Nitrogen-containing heterocyclic analogues of benzoyl-CoA. The reactivity of benzoyl-CoA reductase on the nitrogen-containing heterocyles nicotinoyl-CoA, isonicotinoyl-CoA, and picolinoyl-CoA were studied using either dithionite-reduced methyl viologen, or titanium (III) citrate as the electron donor. The results of these assays are summarized in Table 1.

Kinetic assays. Nicotinoyl-CoA did not show any apparent activity with benzoyl-CoA reductase, as the rate of oxidation of methyl viologen did not significantly increase after the addition of nicotinoyl-CoA to an assay containing the enzyme and ATP (Fig. 34). The background rate of methyl viologen oxidation by the enzyme alone was 0.056 nmol sec^{-1} and the rate of methyl viologen oxidation after the addition of 0.7 mM nicotinoyl-CoA was 0.057 nmol sec^{-1}. Both rates can be considered to be the same given an error of 0.003 nmol sec^{-1} in the least-squares fit of the kinetic data by the UV/Vis software. No enzymatic reaction was observed using nicotinoyl-CoA at concentrations of up to 2 mM, showing that nicotinoyl-CoA is not a substrate for benzoyl-CoA reductase. Higher concentrations were harder to obtain due to the limited amount of synthesized

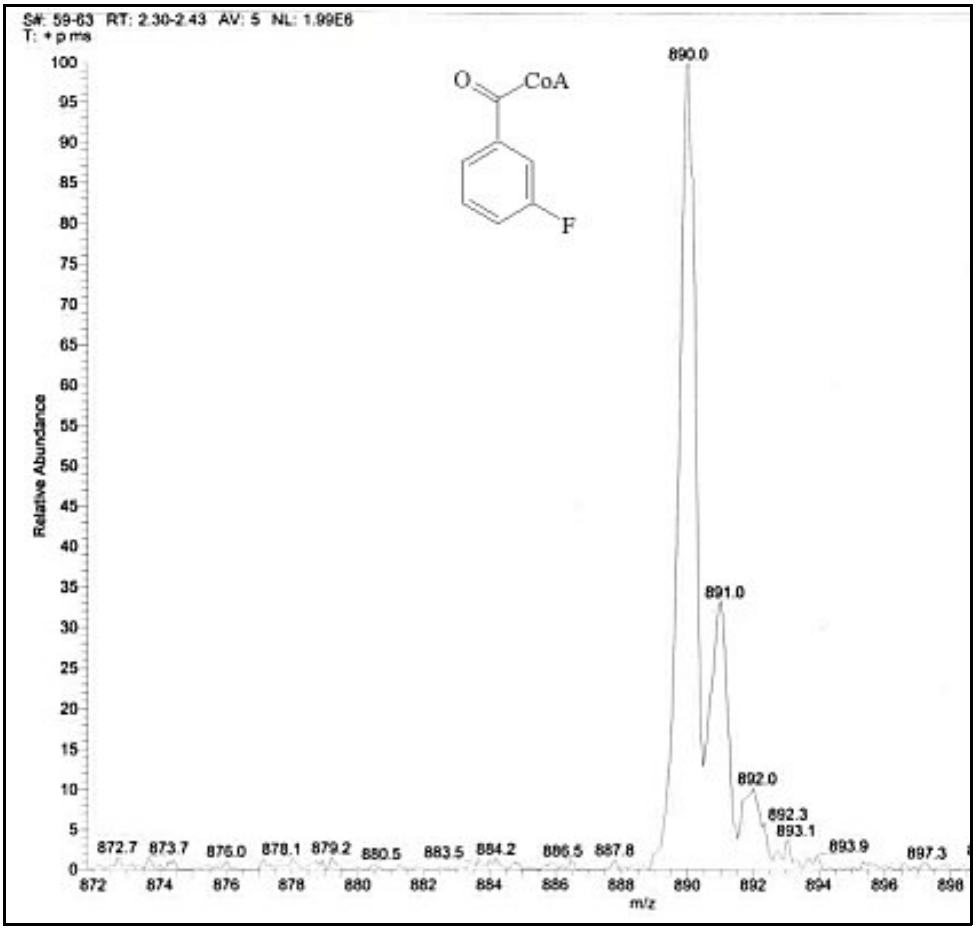


Figure 32. Mass determination of synthesized m-fluorobenzoyl-CoA by electrospray mass spectrometry. The primary ion formed had a mass of 890, which corresponds to the positive ion of m-fluorobenzoyl-CoA. Ions were observed using the positive ion channel.

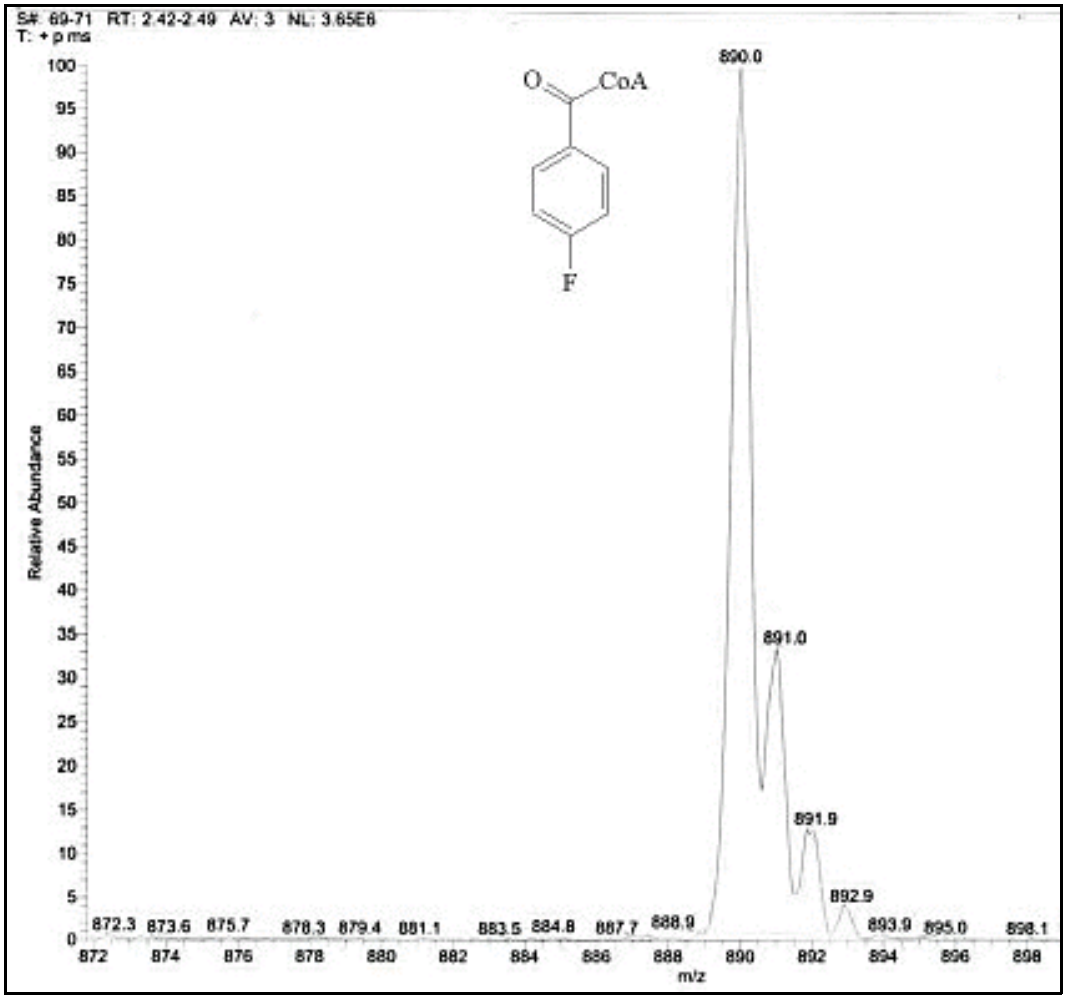
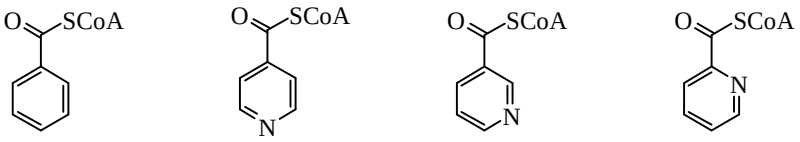


Figure 33. Mass determination of synthesized p-fluorobenzoyl-CoA by electrospray mass spectrometry. The primary ion formed had a mass of 890, which corresponds to the positive ion of p-fluorobenzoyl-CoA. Ions were observed using the positive ion channel.



Substrate	Benzoyl-CoA	Isonicotinoyl-CoA	Nicotinoyl-CoA	Picolinoyl-CoA
K_m	18 μM	C.N.D.	N.R.	0.4 mM
V_{max}	5.0 nmol/min	C.N.D.	N.R.	4.6 nmol/min

Table 1. K_m and V_{max} values for benzoyl-CoA and the nitrogen-containing heterocyclic analogues of benzoyl-CoA. C.N.D. - Could not determine (reduced non-enzymatically). N.R. - No Reaction

nicotinoyl-CoA. Isonicotinoyl-CoA apparently increased the rate of oxidation of dithionite-reduced methyl viologen in the absence of benzoyl-CoA reductase (Fig. 35). ATP was also present in this assay, but it was also found that isonicotinoyl-CoA oxidized reduced methyl-viologen in the absence of both the enzyme and ATP. Isonicotinoyl-CoA oxidized methyl viologen at a rate of 0.25 nmol sec^{-1}. Methyl viologen was oxidized by the buffer at a rate of 0.02 nmol sec^{-1} in the absence of isonicotinoyl-CoA. An assay containing isonicotinoyl-CoA and benzoyl-CoA reductase oxidized methyl viologen at a slightly higher rate, 0.30 nmol sec^{-1} (Fig. 35). Isonicotinoyl-CoA is reduced directly by dithionite-reduced methyl viologen. Adding benzoyl-CoA reductase did not increase the rate of this oxidation significantly, suggestingthat either the enzyme did not react on isonicotinoyl-CoA, or that it did so at a rate insufficient enough to observe over the high non-enzyme-dependent oxidation of methyl viologen.

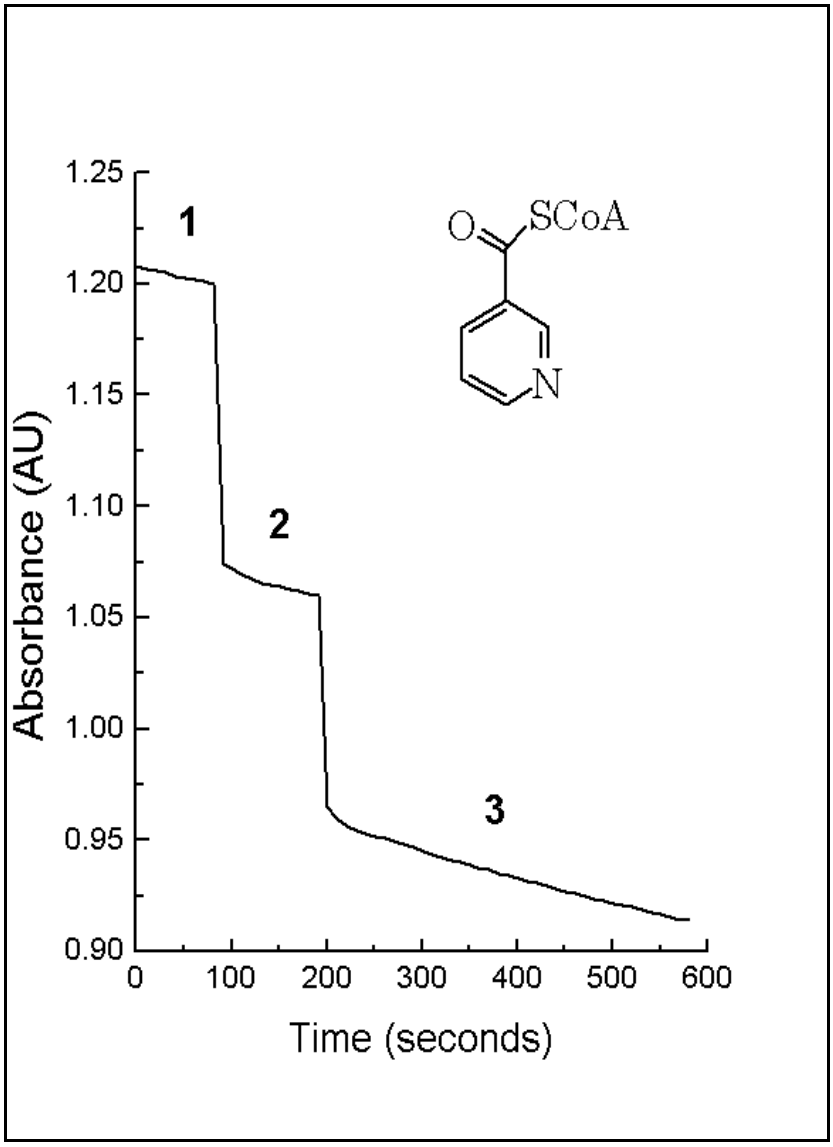


Figure 34. Methyl viologen oxidation by benzoyl-CoA reductase in the presence of nicotinoyl-CoA and ATP. The oxidation of methyl viologen was observed at 730 nm. After 40 µl of enzyme is incubated with 1 mM dithionite-reduced methyl viologen (1), 5 mM ATP is added (2), followed by the addition of 0.7 mM nicotinoyl-CoA (3). Note: The large observed drop in absorbance at the beginning of each addition is caused by the presence of trace amounts of oxygen in the stock solutions and by dilution.

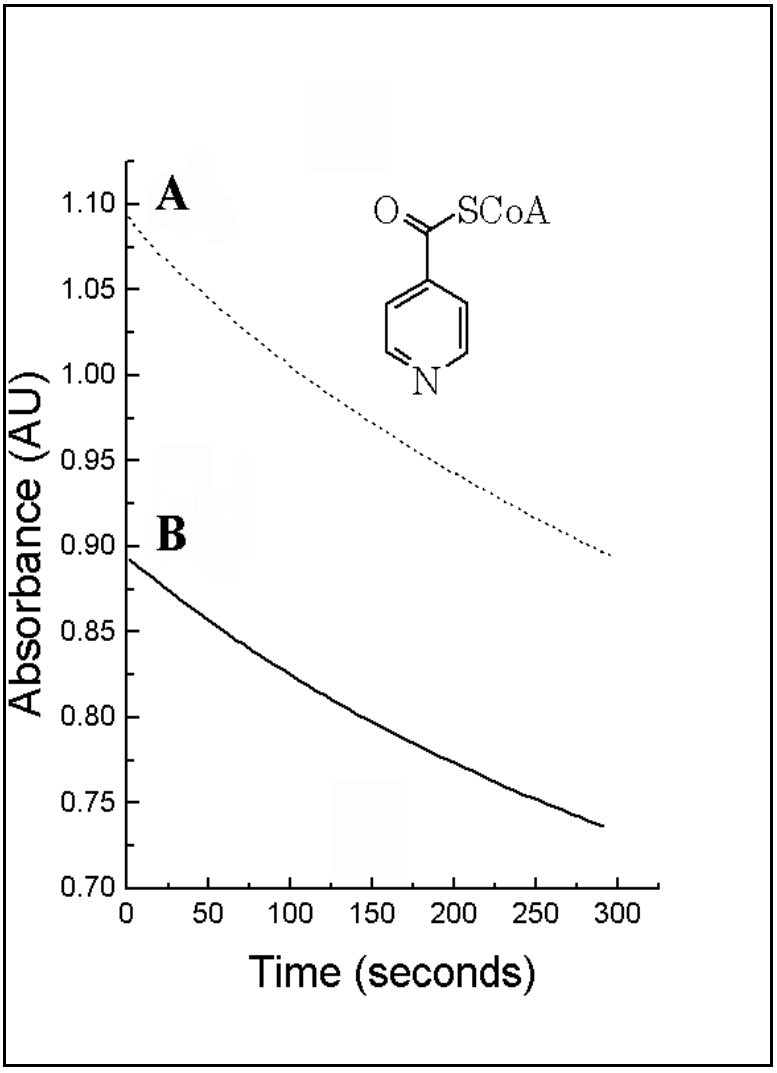


Figure 35. Methyl viologen oxidation by benzoyl-CoA reductase in the presence of isonicotinoyl-CoA and ATP. The oxidation of methyl viologen was observed at 730 nm. (**A**) 10 µl of enzyme solution is added to an assay solution containing 1 mM dithionite-reduced methyl viologen, 0.4 mM isonicotinoyl-CoA, and 5 mM ATP. (**B**) 0.4 mM isonicotinoyl-CoA is added to an assay solution containing 5 mM ATP and no enzyme.

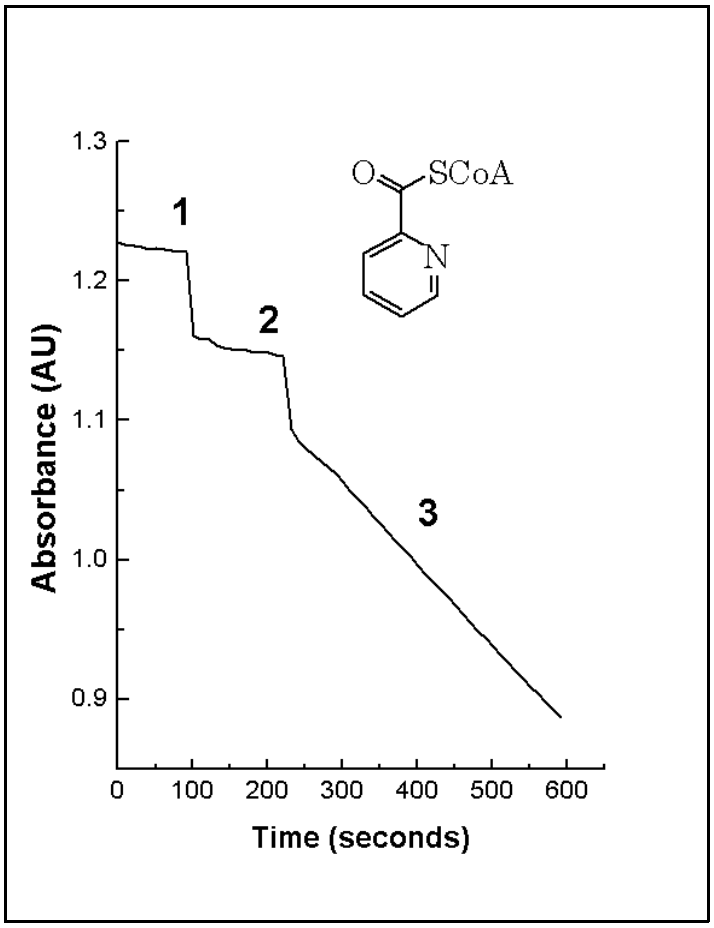


Figure 36. Methyl viologen oxidation by benzoyl-CoA reductase in the presence of picolinoyl-CoA and ATP. The oxidation of methyl viologen was observed at 730 nm. After 40 µl of enzyme is incubated with 1 mM dithionite-reduced methyl viologen (1), 0.7 mM picolinoyl-CoA is added (2), followed by the addition of 5 mM ATP (3). Note: The large observed drop in absorbance at the beginning of each addition is caused by the presence of trace amounts of oxygen in the stock solutions and by dilution.

Unlike the other nitrogen-containing heterocyclic analogues of benzoyl-CoA, picolinoyl-CoA appeared to serve as a substrate for benzoyl-CoA reductase. In an assay containing picolinoyl-CoA, benzoyl-CoA reductase catalyzed the ATP-dependent oxidation of dithionite-reduced methyl viologen (Fig. 36). Oxidation of the reduced methyl viologen only occured after ATP was added to the assay and did not occur when only picolinoyl-CoA was present. The catalytic properties of benzoyl-CoA reductase were studied with the spectrophotometric assay, using methyl viologen as the

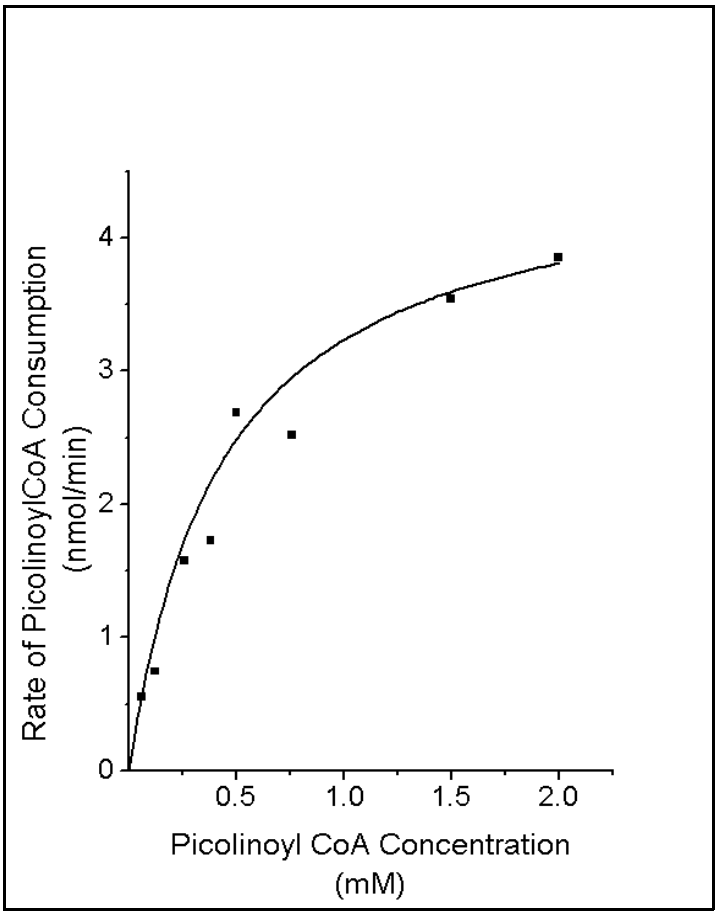


Figure 37. The determination of K_m and V_{max} of benzoyl-CoA reductase for picolinoyl-CoA. The initial velocity of the enzymatic reaction was plotted as a function of substrate concentration. Assuming that two mole of electrons are required to reduce one mole of picolinoyl-CoA, the rate of picolinoyl-CoA consumption was calculated as being equal to twice the rate of the oxidation of dithionite-reduced methyl viologen. Benzoyl-CoA reductase had a K_m of 0.4 mM and a V_{max} of 4.6 nmol min^{-1} using picolinoyl-CoA as a substrate.

reducing agent and varying concentrations of picolinoyl-CoA as the substrate (Fig. 37). The K_m and V_{max} values were determined by fitting the kinetic data with a hyperbolic curve fit (Fig. 37). The apparent K_m of benzoyl-CoA reductase for picolinoyl-CoA was 0.4 mM and the enzyme had a V_{max} of 4.6 nmol picolinoyl-CoA min^{-1}. When the V_{max} for picolinoyl-CoA was compared to the value of V_{max} found for benzoyl-CoA reductase, 5.0 nmol benzoyl-CoA min^{-1}, using the same enzyme solution and the same amount of enzyme, it was found that the enzyme catalyzes the reduction of the both benzoyl-

CoA and picolinoyl-CoA at nearly the same rate. However, as will be explained later, picolinoyl-CoA may be undergoing multiple reductions, which may not be enzymatically catalyzed. Therefore, the V_{max} found for the enzyme, using picolinoyl-CoA as the substrate, may be higher than the actual enzyme-catalyzed reduction rate. The K_m of the enzyme for picolinoyl-CoA was 0.4 mM, which is over a factor of twenty higher than the K_m value for benzoyl-CoA, which was 18 µM. The V_{max}/K_m value for nicotinoyl-CoA was 1.15×10^{-2} min^{-1} and the V_{max}/K_m value for benzoyl-CoA was 0.278 min^{-1}. This yields a V_{max}/K_m ratio of 24 when comparing benzoyl-CoA to nicotinoyl-CoA. Thus, having the higher V_{max}/K_m value, benzoyl-CoA is a better substrate for the enzyme than nicotinoyl-CoA.

Analysis of enzymatic products from the picolinoyl-CoA reaction by mass spectrometry. To observe the disappearance of picolinoyl-CoA and the appearance of any products during the benzoyl-CoA reductase reaction, two samples of an assay containing the enzyme solution, 5 mM ATP, 0.8 mM titanium(III) citrate, and 0.4 mM picolinoyl-CoA were analyzed using HPLC by being passed over a Platinum C-18 reverse-phase column. The two samples were taken, one at the start of the assay and the other was taken fifteen minutes after the start of the assay (Fig. 38). After fifteen minutes, the picolinoyl-CoA peak (1) decreased to almost a third of its original height, and several new product peaks appeared, which eluted off 6 minutes (2), 7.7 minutes (3), and 8.0 minutes (4) (Fig. 38). To identify these peaks, the sample taken fifteen minutes after the start

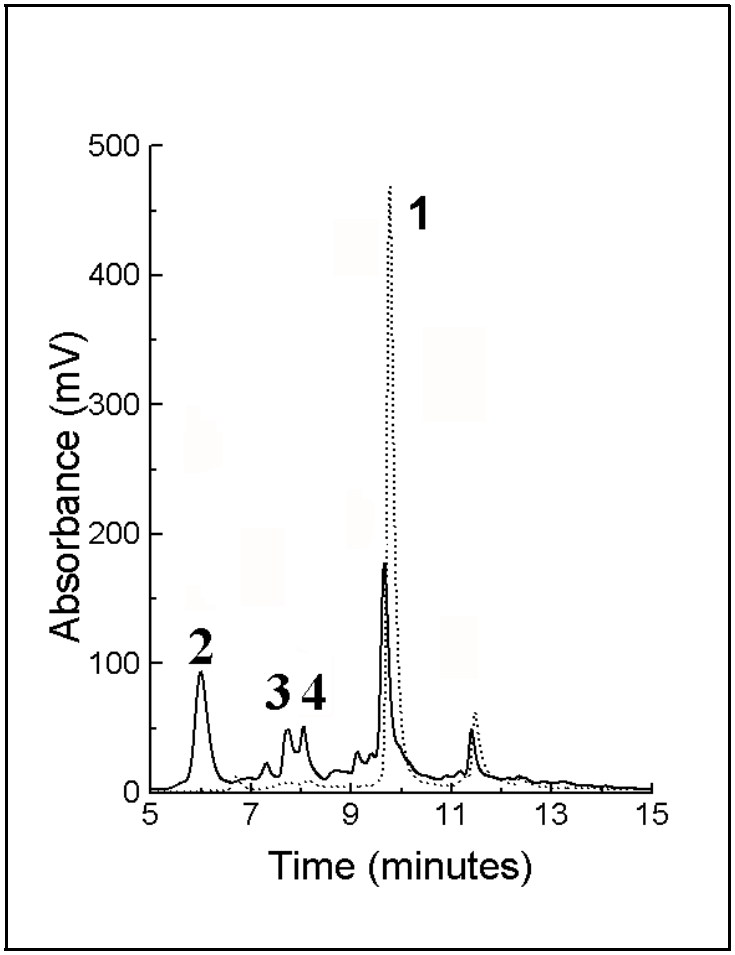


Figure 38. HPLC traces of a benzoyl-CoA reductase assay using picolinoyl-CoA as the substrate and titanium(III) citrate as the electron donor. The lighter, dotted line (.....) represents the assay just after the addition of enzyme. The darker, solid line ())))) represents the assay after fifteen minutes of incubation with the enzyme. **1** - picolinoyl-CoA (substrate); **2**,**3,4** - enzymatic products.

of the assay was analyzed by LC/MS. The sample was passed over a Platinum C-18 reverse-phase column and the eluent was then analyzed by mass spectrometery. The atomic masses of any ions that formed were detected by monitoring the positive ion channel. The chromatogram obtained during the LC/MS analysis is shown in Fig. 39. There was evidence of a shift in the relative amounts of the products from when the sample was originally analyzed by HPLC. Peak 2 had increased and peak 3 had decreased from its original height. This could be attributed to the degradation of enzymatic products.

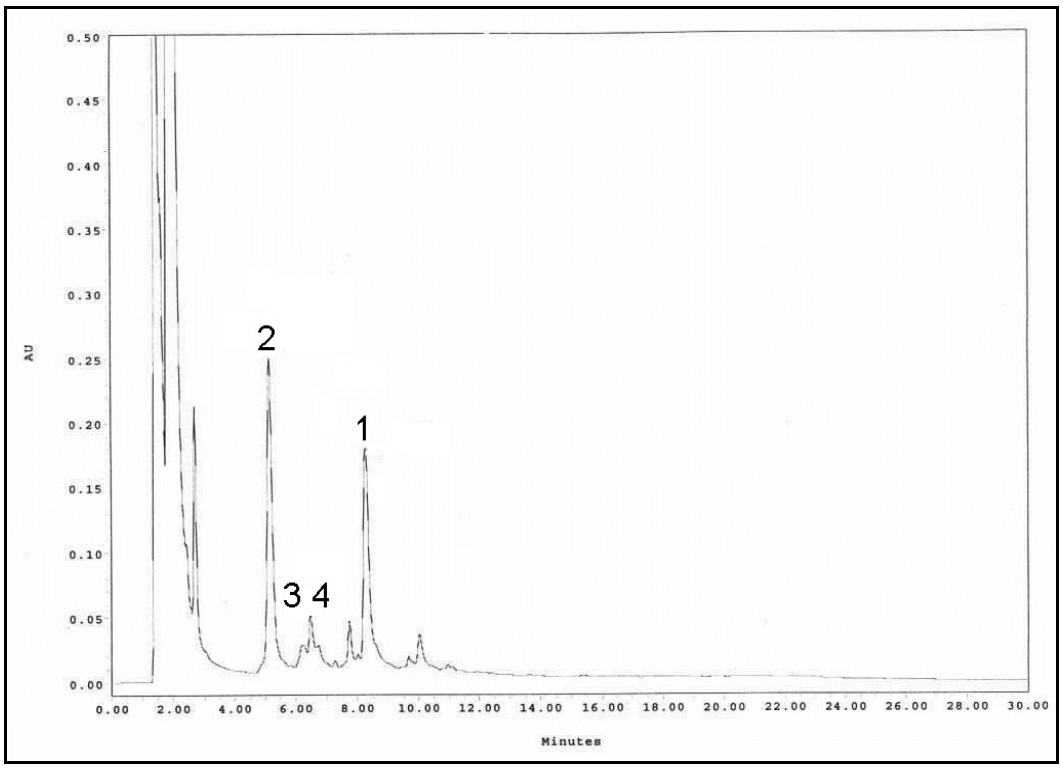


Figure 39. LC/MS trace of a benzoyl-CoA reductase assay using picolinoyl-CoA as the substrate and titanium(III) citrate as the electron donor. The assay was allowed to react with 20 µl of enzyme solution for 15 minutes. This sample was passed over a Platinum C-18 reverse-phase column. **1** - picolinoyl-CoA (substrate); **2,3,4** - enzymatic products.

A summary of the results obtained from mass spectrometry analysis of the picolinoyl-CoA assay is shown in Table 2. Mass spectrometry analysis found that peak 2 had an atomic mass of 768.0 amu, which corresponds to the atomic mass of a positive ion of a free CoA molecule (Fig. 40). The mass of peak 1 corresponded to that of the positive ion of picolinoyl-CoA, which has a mass of 873 atomic mass units, confirming the identity of the peak as the substrate of the enzymatic reaction, picolinoyl-CoA (Fig. 41). A time-slice showing the ions formed by the molecules

Peak #	Peak 1	Peak 2	Peak 3	Peak 4
Atomic mass	873	768	879	875
Identification	Picolinoyl-CoA	Free CoA	Complete reduction	Single reduction

Table 2. **A summary of the masses obtained from the LC/MS analysis of a benzoyl-CoA reductase assay using picolinoyl-CoA as the substrate**. The peak numbers correspond to those in Fig. 39. Peak 1 is the substrate peak and peaks 2, 3, and 4 are the product peaks. Identification of the product peaks were made from the atomic masses.

leaving the column at 6.4 minutes revealed the presence of a positive ion with an atomic mass of 879 (Fig. 42A). This time slice would correspond to peak 3 in Fig. 39. An atomic mass of 879 could be that of a positive ion of a molecule of picolinoyl-CoA that had been reduced three times, meaning six electrons and six protons were added to the aromatic ring system. The reduction of picolinoyl-CoA three times would result in the formation of a completely reduced ring system, or 2-carbonyl-CoA piperidine. The formation of such a product might provide an explanation as to why a large amount of free CoA was formed during the assay. Free CoA can form from the hydrolysis of the thioester of either picolinoyl-CoA, or an enzymatic product formed by the reaction of picolinoyl-CoA with benzoyl-CoA reductase. HPLC analysis of the picolinoyl-CoA stock solution found that it is stable in the absence of the enzyme and can be stored for several months. Therefore, it can be concluded that free CoA must have formed as a result of the degradation of one of the products formed from the reduction of picolinoyl-CoA by benzoyl-CoA reductase. The nitrogen in the piperidine product would be

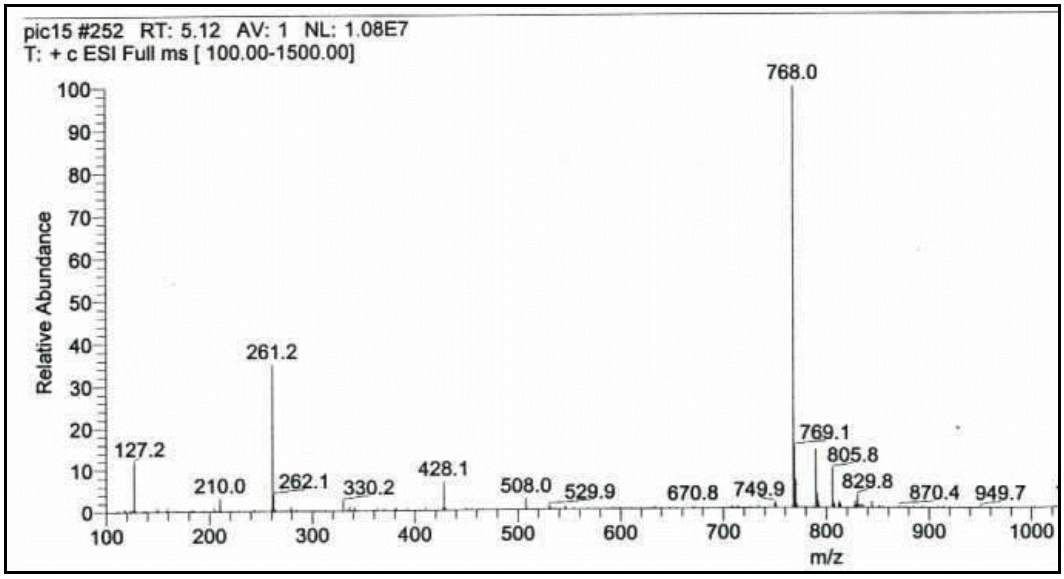


Figure 40. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on picolinoyl-CoA (Peak 2). A time-slice of the ions formed by the molecules of peak 2 (Fig. 39). The primary ion formed had an atomic mass of 768, corresponding to the mass of the positive ion of free CoA. Ions were observed using the positive ion channel.

moderately basic, having a pK_b around 2.8. A basic group ortho to a thioester could promote the hydrolysis of the thioester by causing the formation of hydroxide ions in close proximity to the thioester (Webster et. al., 1974). Multiple reductions of a substrate by this enzymatic assay are possible. Further reduction of the native product of benzoyl-CoA reductase, cyclohexa-1,5-diene-1-carbonyl-CoA, to cyclohex-1-ene-1-carbonyl-CoA by benzoyl-CoA reductase has been reported previously, but it is unclear whether this is caused by an in vitro effect, or is actually an activity of the enzyme (Boll et. al., 2000). As seen with isonicotinoyl-CoA, the reducing agent, titanium(III) citrate, might interact directly with the molecules in the assay. Although picolinoyl-CoA appears to be resistant to direct

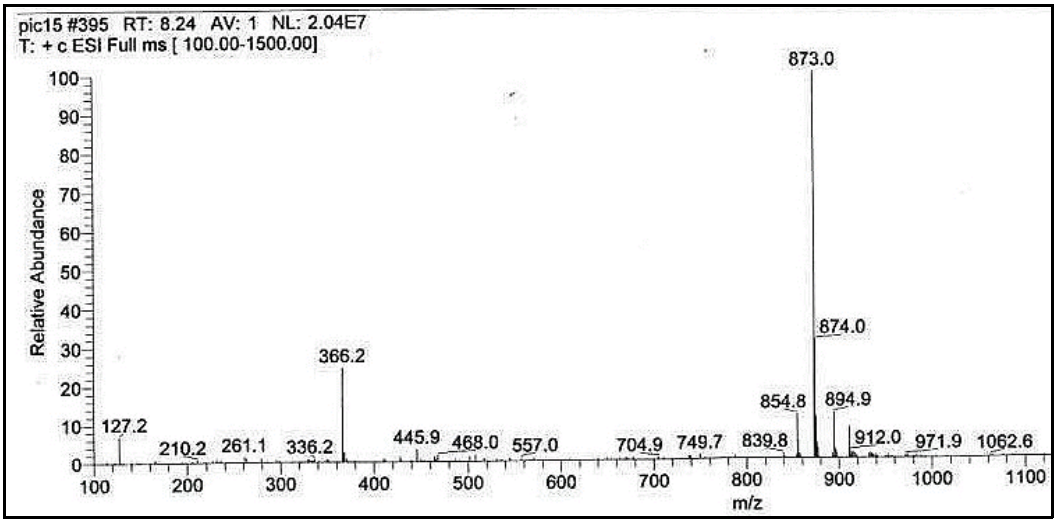


Figure 41. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on picolinoyl-CoA (Peak 1). A time-slice showing the ions formed by the molecules of peak 1 (Figure 39). The primary ion formed had an atomic mass of 873, corresponding to the mass of the positive ion of picolinoyl-CoA. Ions were observed on the positive ion channel.

reduction in the presence of titanium(III) citrate, the product, or products, formed by its reaction with benzoyl-CoA reductase may not be. A time-slice taken showing the ions formed by the molecules of peak 4 revealed the presence of a positive ion with an atomic mass of 875 (Fig. 42B). This ion was also present in the time-slice containing molecules of peak 4 (Fig. 42A), but in only a small abundance. This can be explained by the slight overlap in the elution of peaks 3 and 4 from the column. The atomic mass of 875 could represent the positive ion of a molecule of picolinoyl-CoA that had been reduced once, meaning two electrons and two protons were added to the aromatic ring system. A scan of the population of all of the ions that eluted off of the HPLC column failed to show the presence of

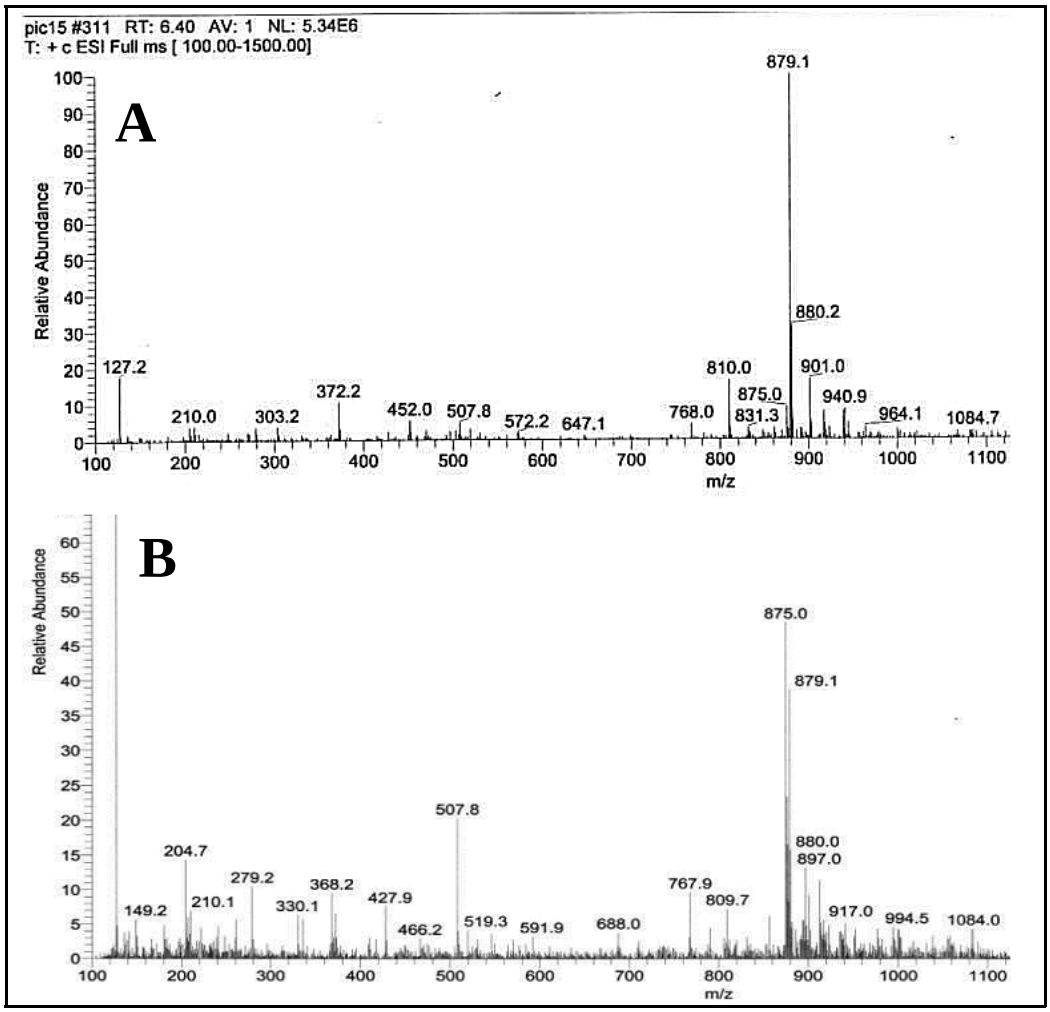


Figure 42. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on picolinoyl-CoA (Peaks 3 & 4). (A) A time-slice showing the ions formed by the molecules of peak 3 (Fig. 39). The primary ion formed had an atomic mass of 879, corresponding to the mass of the positive ion of picolinoyl-CoA that has been reduced twice. (B) A time-slice showing the ions formed by the molecules of peak 4 (Fig. 39). The primary ion formed had a mass of 875, corresponding to the mass of the positive ion of picolinoyl-CoA that had been reduced once. Ions were observed on the positive ion channel.

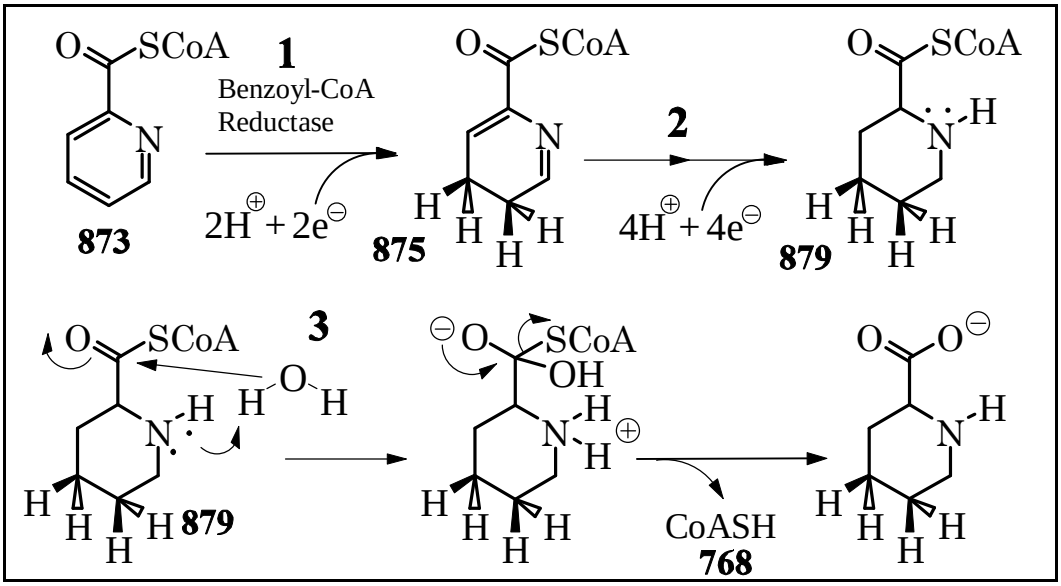


Figure 43. Hypothetical reaction of benzoyl-CoA reductase with picolinoyl-CoA. (1) Benzoyl-CoA reductase catalyzes the ATP-dependent, two-electron reduction of picolinoyl-CoA. The double bonds in this product are shown as being conjugated as one-electron reductions seen in the Birch reduction require conjugated double bonds. (2) The reduced product is reduced enzymatically, or non-enzymatically, to a fully reduced ring system. (3) The hydrolysis of the thioester bond is facilitated by the presence of a basic nitrogen atom nearby. The atomic masses shown are that of the positive ion of the respective molecule.

an ion with the atomic mass of 877, which would correspond to a positive ion of picolinoyl-CoA that had been reduced twice. Also, there was no evidence of any hydrated products formed from the reaction of the reduced picolinoyl-CoA products with the contaminant enzyme, 1,5-dienoyl-CoA hydratase. Apparently, benzoyl-CoA reductase reduces picolinoyl-CoA through the addition of two electron and two protons to the aromatic ring (Fig. 43). Then, either enzymatically, or chemically, the reduced picolinoyl-CoA product is reduced twice more, completely reducing the ring system (Fig. 43). The thioester in this product is

unstable due to the proximity of the basic nitrogen functionality to the thioester. The nitrogen atom in the ring can abstract a proton from a water molecule, resulting in the formation of a hydroxide ion which can then hydrolyze the thioester bond (Fig. 43).

Fluorinated analogues of benzoyl-CoA. The ATP-dependent reduction of m-fluorobenzoyl-CoA and p-fluorobenzoyl-CoA by benzoyl-CoA reductase was studied using either dithionite-reduced methyl viologen, or titanium(III) citrate as the electron donor.

As reported earlier by Boll and Fuchs, 1995, benzoyl-CoA reductase can catalyze the ATP-dependent oxidation of titanium(III) citrate using either m-fluorobenzoyl-CoA, or p-fluorobenzoyl-CoA as the substrate (Fig. 44). However, the enzymatic products of these reaction were not identified. To observe the disappearance of the fluorinated benzoyl-CoA substrates and the appearance of any products as a result of the benzoyl-CoA reductase reaction, samples were taken at various time points from an assay containing 20 µl of the enzyme solution, 5 mM ATP, 0.8 mM titanium(III) citrate, and either 0.5 mM m-fluorobenzoyl CoA, or 0.5 mM p-fluorobenzoyl CoA. The assay components in the samples were separated using a Platinum C-18 reverse-phase HPLC column. Thioesters eluting from the column were observed by monitoring 260 nm during the separation.

Analysis of enzymatic products from the m-fluorobenzoyl-CoA reaction by mass spectrometry. As shown in Fig. 45, when benzoyl-CoA reductase was

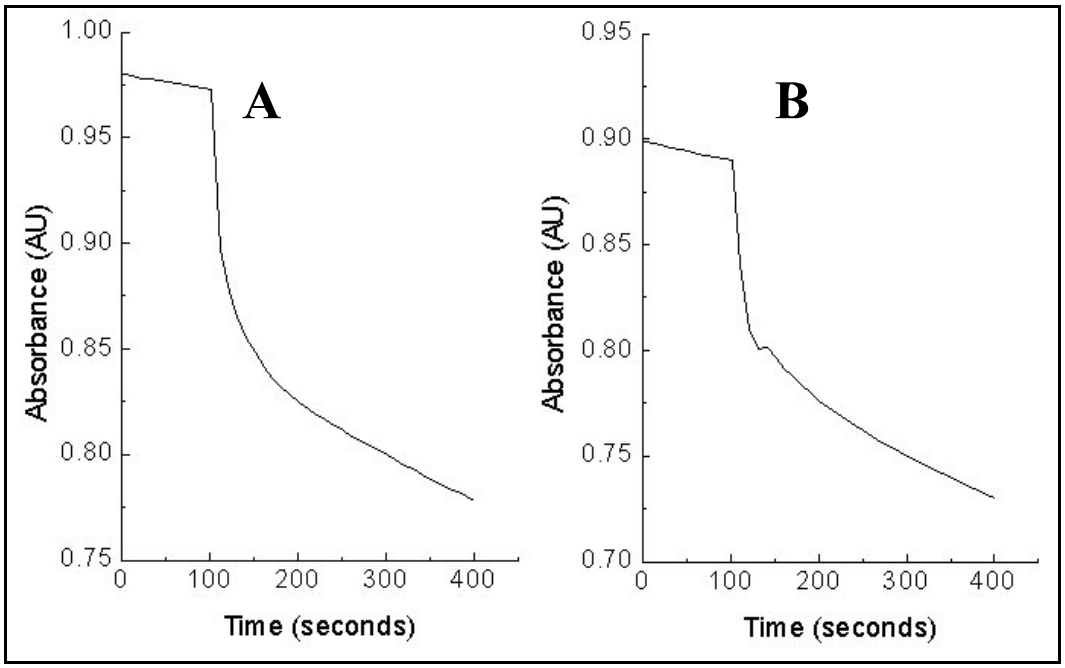


Figure 44. Titanium(III) citrate oxidation by benzoyl-CoA reductase in the presence of fluorinated benzoyl-CoA analogues and ATP. The oxidation of titanium(III) citrate was observed at 340 nm. (A) After 100 seconds, 20 µl of enzyme solution was added to a reaction assay containing 0.8 mM titanium(III) citrate, 5 mM ATP, and 0.5 mM m-fluorobenzoyl-CoA. (B) After 100 seconds, 20 µl of enzyme solution was added to a reaction assay containing 0.8 mM titanium(III) citrate, 5 mM ATP, and 0.5 mM p-fluorobenzoyl-CoA. Note: The large observed drop in absorbance at the beginning of each addition is caused by the presence of trace amounts of oxygen in the stock solutions and by dilution.

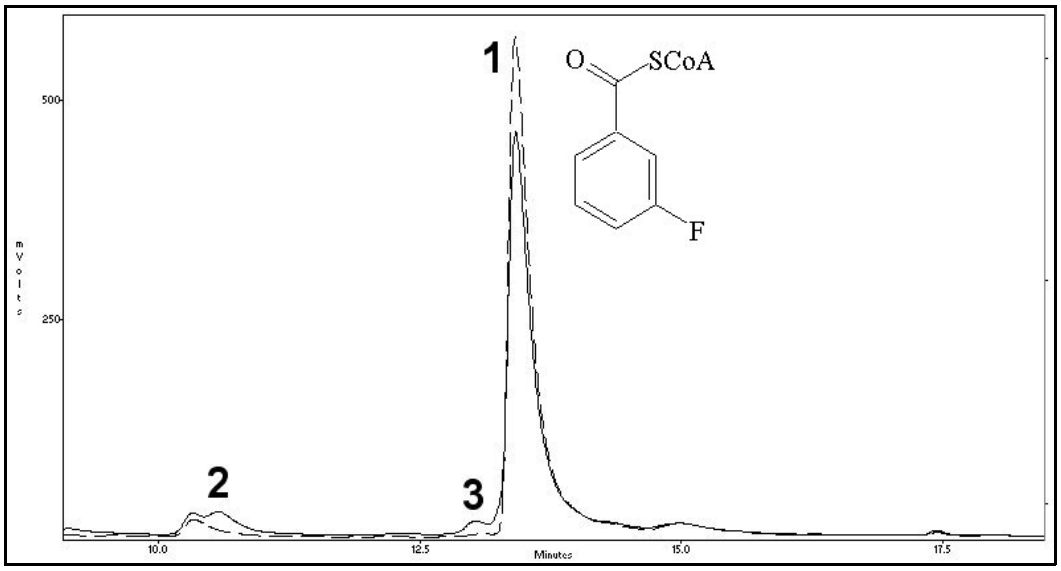


Figure 45. HPLC traces of a benzoyl-CoA reductase assay using m-fluorobenzoyl-CoA as the substrate and titanium(III) citrate as the electron donor. The dashed line (- - -) represents the assay just after the addition of enzyme. The solid line ())))) represents the assay after thirty minutes of incubation with the enzyme. **1** - m-fluorobenzoyl-CoA (substrate); **2** - hydrated, reduced benzoyl-CoA; **3** - benzoyl-CoA. There is also the appearance of a small shoulder on peak 1 just after peak 3, which may be reduced benzoyl-CoA, or a reduced m-fluorobenzoyl-CoA molecule.

allowed to react with m-fluorobenzoyl-CoA (1) for thirty minutes, two new product peaks were formed (2,3). Product peak 2 had a similar retention time to that of the hydration product from the benzoyl-CoA reductase reaction on benzoyl-CoA. Product peak 3had a similar retention time to that of benzoyl-CoA. Also, there is the appearance of a small shoulder on the front of m-fluorobenzoyl-CoA peak, peak 1. The retention time of this shoulder is similar to that of the reduction product seen in the benzoyl-CoA reductase reaction on benzoyl-CoA (Fig. 45). To confirm the identity of these peaks, a sample taken thirty minutes after the start of

Peak #	Peak 1	Peak 2	Peak 3
Atomic mass(es)	874, 890, 894	892, 910	872
Identification	1. reduced benzoyl-CoA 2. m-fluoro-benzoyl-CoA 3. double reduction product of m-fluoro-benzoyl-CoA	1. hydrated, reduced benzoyl-CoA 2. hydrated, reduced m-fluoro-benzoyl-CoA	Benzoyl-CoA

Table 3. **A summary of the masses obtained from the LC/MS analysis of a benzoyl-CoA reductase assay using m-fluorobenzoyl-CoA as the substrate**. The peak numbers correspond to those in Fig. 46. Peak 1 contains the substrate as well as products of the reaction. Peaks 2 and 3 are product peaks. Identification of the product peaks were made from the atomic masses. The retention times of benzoyl-CoA, reduced benzoyl-CoA (reduction product), and hydrated, reduced benzoyl-CoA (hydration product) were also helpful in identifying them.

a benzoyl-CoA reductase assay, using m-fluorobenzoyl-CoA as the substrate, was analyzed by LC/MS. A summary of the results from the LC/MS analysis of the m-fluorobenzoyl-CoA assay is shown in Table 3. The sample was passed over a Platinum C-18 reverse-phase column and the eluent was analyzed by a Thermo-Finnigan LCQ equipped with a quadrupole ion trap. The atomic masses of any ions that formed were detected by monitoring the positive ion channel. The chromatogram obtained during the LC/MS analysis is shown in Fig. 46. Mass spectrometry analysis found that the primary ion formed from molecules in peak 3 had an atomic mass of 872.0 amu, which corresponds to the atomic mass of a

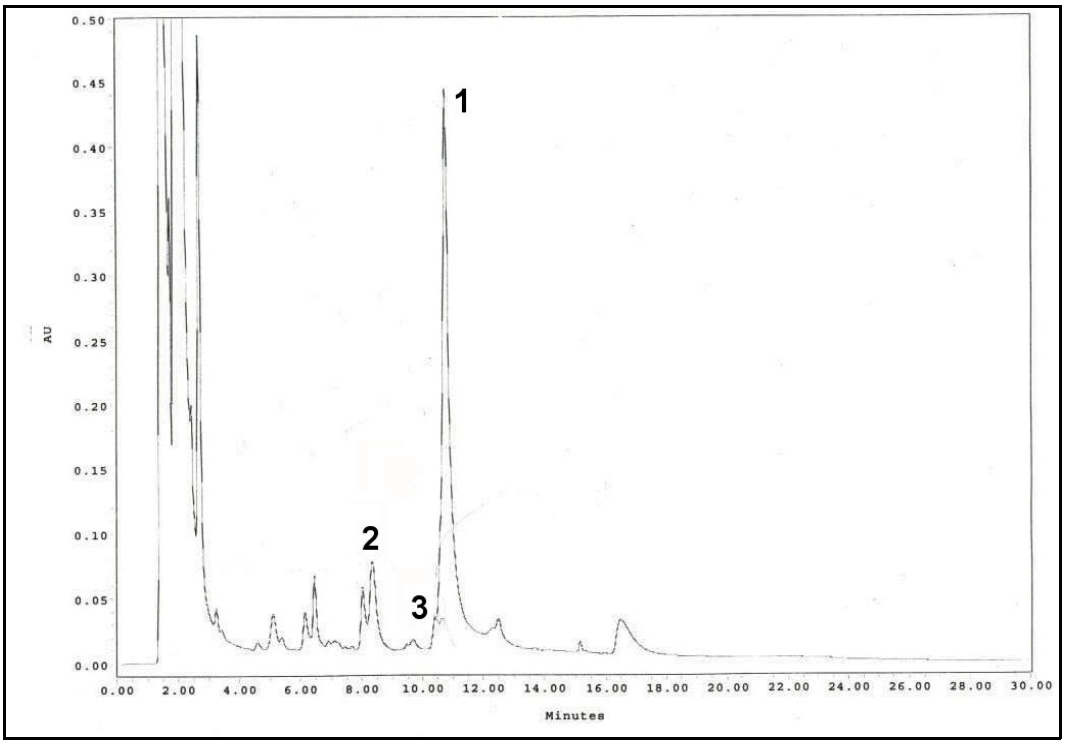


Figure 46. LC/MS trace of a benzoyl-CoA reductase assay using m-fluorobenzoyl-CoA as the substrate and titanium(III) citrate as the electron donor. The assay was allowed to react with 20 µl of enzyme solution for 30 minutes. This sample was passed over a Platinum C-18 reverse-phase column. **1** - m-fluorobenzoyl-CoA (substrate); **2,3 -** enzymatic products. Peaks not labeled in the chromatogram were present at the start of the assay and are not considered to be enzymatic products. A peak at 6.2 minutes did increase slightly from the amount present during assay. The retention time of this peak corresponds to that of free CoA, which could have accumulated from the slow hydrolysis of thioesters while the sample was being stored.

positive ion of benzoyl-CoA (Fig. 47A). Benzoyl-CoA could have only formed if benzoyl-CoA reductase catalyzed the reductive defluorination of the substrate, m-fluorobenzoyl-CoA. The primary ion formed from molecules in peak 2 had an atomic mass of 892.0, which is the same mass as the positive ion the hydration product from the benzoyl-CoA reductase reaction on benzoyl-CoA (Fig. 47B). The

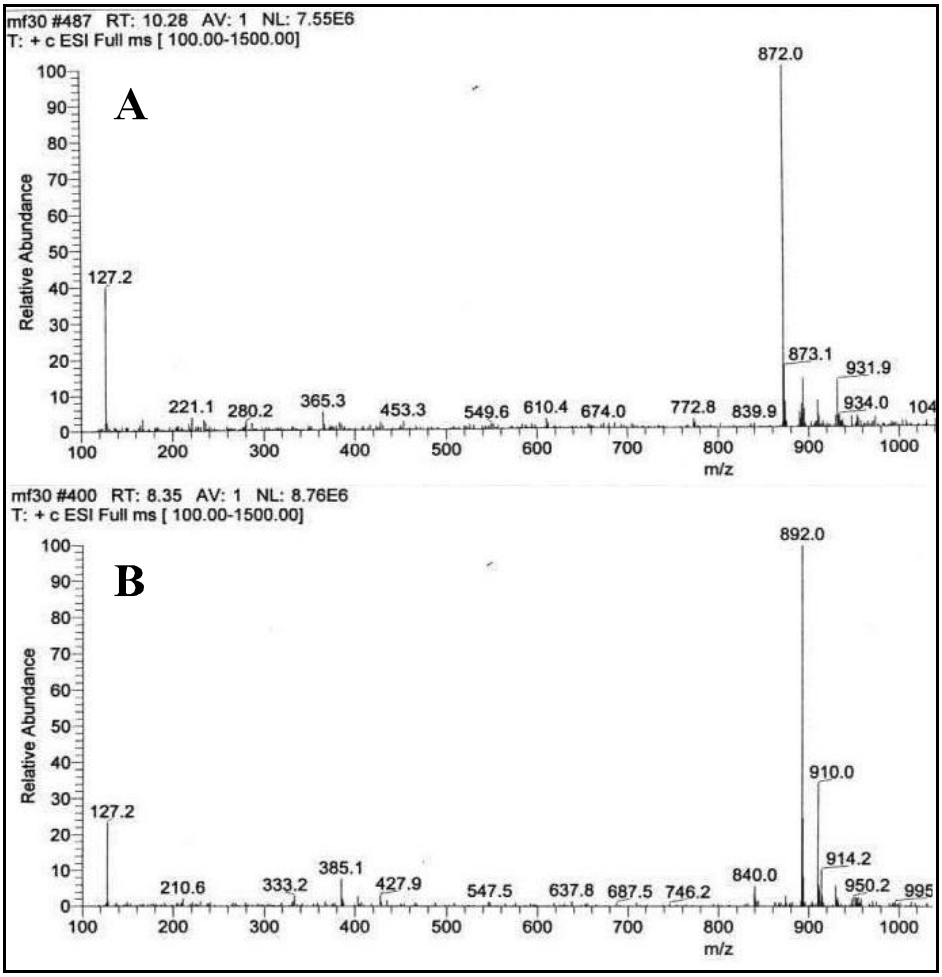


Figure 47. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on m-fluorobenzoyl-CoA (Peaks 3 & 2). (A) A time-slice showing the ions formed by the molecules of peak 3 (Fig. 46). The primary ion formed had an atomic mass of 872, corresponding to the mass of the positive ion of benzoyl-CoA. (B) A time-slice showing the ions formed by the molecules of peak 2 (Fig. 46). The primary ion formed had an atomic mass of 892, corresponding to the mass of the hydrated, reduced product (hydration product) of the benzoyl-CoA reductase reaction on benzoyl-CoA. Ions were observed on the positive ion channel

presence of the hydration product makes sense because if benzoyl-CoA is formed during the reaction, then it should be reduced further by the benzoyl-CoA reductase to form the reduction product and then hydrated by 1,5-dienoyl-CoA hydratase to form the hydration product. To determine whether other enzymatic products may be eluting out with the m-fluorobenzoyl-CoA substrate, a time-slice was taken of the front shoulder of the m-fluorobenzoyl-CoA peak (Fig. 46:**1**). This time-slice revealed the presence of a positive ion with an atomic mass of 894, which corresponds to the mass of a positive ion of m-fluorobenzoyl-CoA that had been reduced twice (Fig. 48A). An ion with an atomic mass of 874 was also present in this time-slice, which is the atomic mass of the reduction product of benzoyl-CoA (Fig. 48A). The presence of the reduction product makes sense because it would be expected that molecules of benzoyl-CoA formed as a result of the reductive defluorination of m-fluorobenzoyl-CoA by benzoyl-CoA reductase. The presence of a molecule of m-fluorobenzoyl-CoA that had undergone a double reduction was a little surprising, but as was observed with picolinoyl-CoA as the substrate, multiple reductions of a substrate can occur. A time-slice showing the ions formed by the molecules leaving the column at the very beginning of peak 2 (Fig 46:**2**) found the presence of an ion with an atomic mass of 910 (Fig. 48B). This mass is twenty atomic mass units higher than that of a positive ion of m-fluorobenzoyl-CoA. This ion could represent the atomic mass of a positive ion of m-fluorobenzoyl-CoA that had been reduced and then hydrated. This identification is

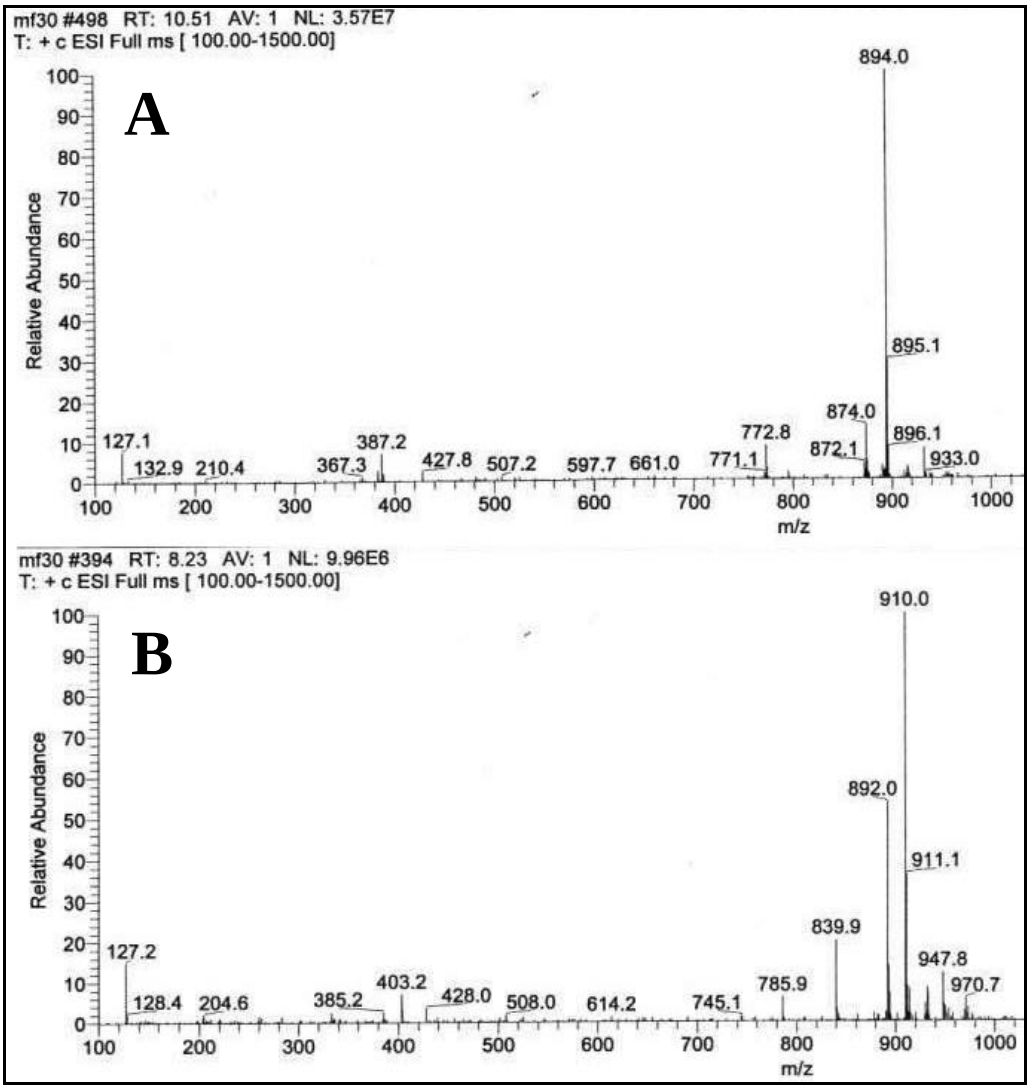


Figure 48. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on m-fluorobenzoyl-CoA (Peaks 1 & 2). (A) A time-slice showing the ions formed by the molecules at the very beginning of peak 1 (Fig. 46). The primary ion formed had an atomic mass of 894, corresponding to the mass of the positive ion of m-fluorobenzoyl-CoA that had been reduced twice. (B) A time-slice showing the ions formed by the molecules leaving the column at the very beginning of peak 2 (Fig 46). The primary ion formed had an atomic mass of 910, corresponding to the mass of m-fluorobenzoyl-CoA that had been reduced once and then hydrated. Ions were observed on the positive ion channel.

supported further by the retention time of the molecule, which is slightly earlier than the hydration product, the hydrated, reduced product from the benzoyl-CoA reductase reaction on benzoyl-CoA. In fact, in this time slice, the positive ion of the hydration product, which has an atomic mass of 892, is also present, but in a smaller relative abundance (Fig. 48B). A time-slice taken a moment later, showed an increasing amount of the hydration product ion and a decreasing amount of the hydrated, reduced m-fluorobenzoyl-CoA ion (Fig 47B). Apparently, the region corresponding to product peak 2 on the chromatogram may contain more than just one enzymatic product (Fig. 45). To confirm the identity of peak 1 in Fig. 46 as the substrate of the reaction, m-fluorobenzoyl-CoA, a time-slice was taken in the middle of the peak. The primary ion detected in this time-slice had an atomic mass of 890, which is the atomic mass of the positive ion of the m-fluorobenzoyl-CoA (Fig. 49). In this time-slice, there is also the presence of a small amount of a positive ion with an atomic mass of 874, which is the mass of the reduction product, the reduced form of benzoyl-CoA (Fig. 49). Interestingly, a positive ion of reduced m-fluorobenzoyl-CoA, having an atomic mass of 892, was not present in the region of the chromatogram that the ions of m-fluorobenzoyl-CoA and of the multiple reduction product m-fluorobenzoyl-CoA were found in. The only ion found with an atomic mass of 892 eluted in the same region that the hydrated, reduced products did and most likely corresponds to the hydration product, the hydrated form of reduced benzoyl CoA. There are two possibilities, either reduced

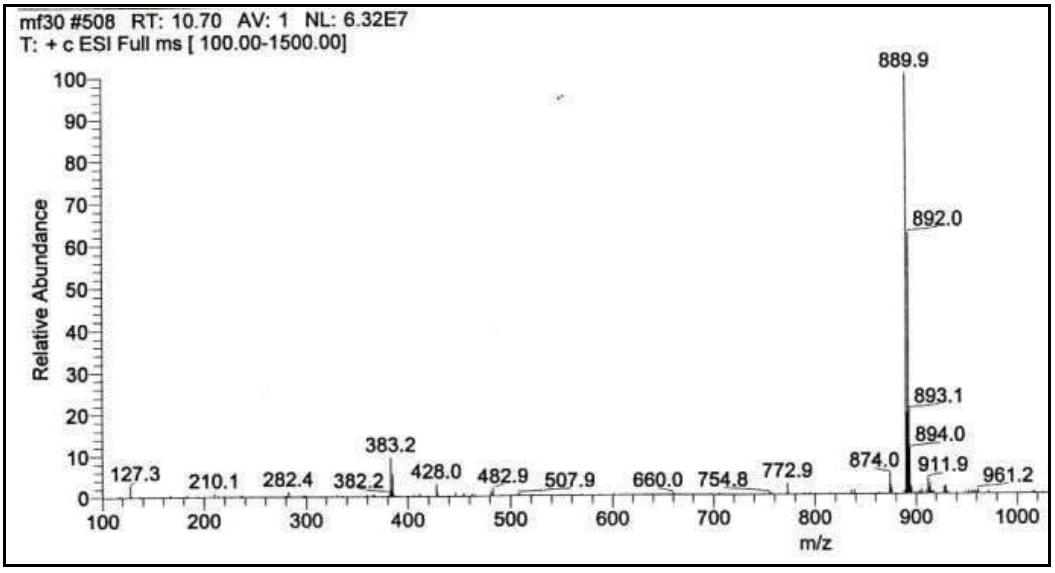


Figure 49. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on m-fluorobenzoyl-CoA (Peak 1). A time-slice showing the ions formed by the molecules in the middle of peak 1 (Fig. 46). The primary ion formed had an atomic mass of 890, corresponding to the mass of the positive ion of the substrate of the reaction, m-fluorobenzoyl-CoA Ions were observed on the positive ion channel.

m-fluorobenzoyl-CoA is not formed during its reaction with benzoyl-CoA reductase, or when it is formed, it is quickly hydrated by 1,5-dienoyl-CoA hydratase, or reduced again. The second reduction could occur enzymatically, or non-enzymatically. Given the presence of both a hydrated, reduced m-fluorobenzoyl-CoA molecule and a m-fluorobenzoyl-CoA molecule that had been reduced twice, it is most likely that a reduced m-fluorobenzoyl-CoA molecule is being formed. This means that when benzoyl-CoA reductase acts on m-fluorobenzoyl-CoA, it either reductively defluorinates m-fluorobenzoyl-CoA, forming benzoyl-CoA, or it reduces the aromatic ring, leaving the fluorine attached

(Fig. 50). Benzoyl-CoA reductase can then reduce benzoyl-CoA to form the reduction product (Fig. 50). The reduction product is then hydrated by the 1,5-dienoyl-CoA hydratase to form the hydration product. The reduced m-fluorobenzoyl-CoA molecule can either be hydrated, or reduced a second time (Fig. 50). It is unclear whether this second reduction is enzymatic or non-enzymatic. After being reduced the second time, the fluorinated ring is not hydrated by the 1,5-dienoyl-CoA hydratase, as the hydrated form of this double-reduced product, which would form a positive ion with the atomic mass of 912, was not found. This suggests that the fluorine atom might be interfering with the ability of the hydratase to hydrate the double bond.

Analysis of enzymatic products from the p-fluorobenzoyl-CoA reaction by mass spectrometry. As shown in Fig. 51, when benzoyl-CoA reductase was allowed to react with p-fluorobenzoyl-CoA (peak 1) for five minutes, two new product peaks, peaks 2 and 3, were formed. Product peak 2 had a similar retention time to that of the hydration product formed by the benzoyl-CoA reductase reaction on benzoyl-CoA. Product peak 3 had a similar retention time to that of benzoyl-CoA. After ten minutes of reaction, two new product peaks, peaks 4 and 5, appeared (Fig. 52). To identify these peaks, a sample, taken thirty minutes after the start of a benzoyl-CoA reductase assay using p-fluorobenzoyl-CoA as the substrate, was analyzed by LC/MS. The sample was passed over a Platinum C-18 reverse-phase column and the eluent was analyzed by a Thermo-Finnigan LCQ equipped

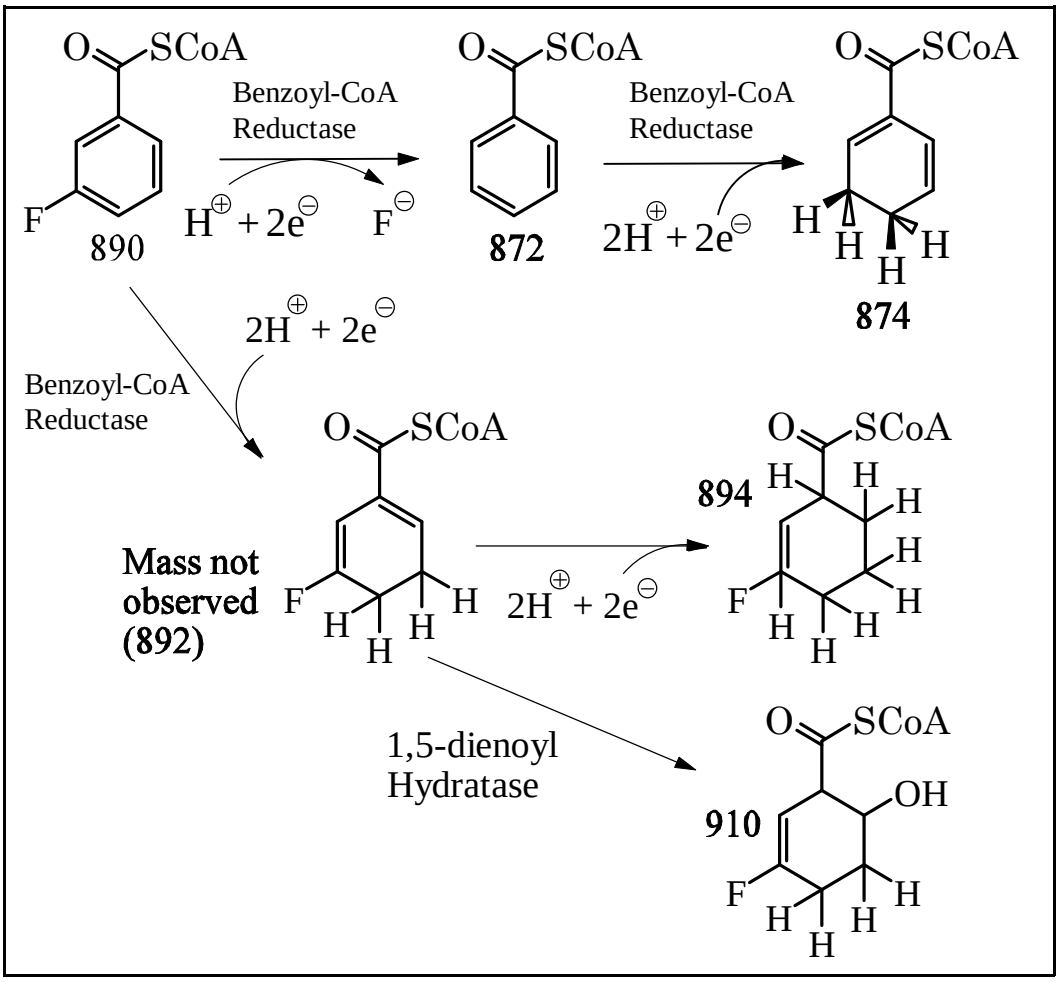


Figure 50. The reaction of benzoyl-CoA reductase with m-fluorobenzoyl-CoA. The reaction of benzoyl-CoA reductase with m-fluorobenzoyl-CoA may proceed by two different pathways. (1) Benzoyl-CoA reductase catalyzes the ATP-dependent, two-electron reduction of m-fluorobenzoyl-CoA, resulting in the defluorination of the compound and the formation of benzoyl-CoA. Benzoyl-CoA is then further reduced to the reduction product by the reductase. (2) Benzoyl-CoA reductase catalyzes the ATP-dependent, two-electron reduction of m-fluorobenzoyl-CoA. This product, whose presence wasn't detected in the assay, is then either reduced again, or hydrated. This second reduction may occur either enzymatically, or non-enzymatically. The atomic mass of each molecule is shown. The structures of the products shown are hypothetical. The actual orientation of the double bonds in the products cannot be determined from the masses of the molecules alone.

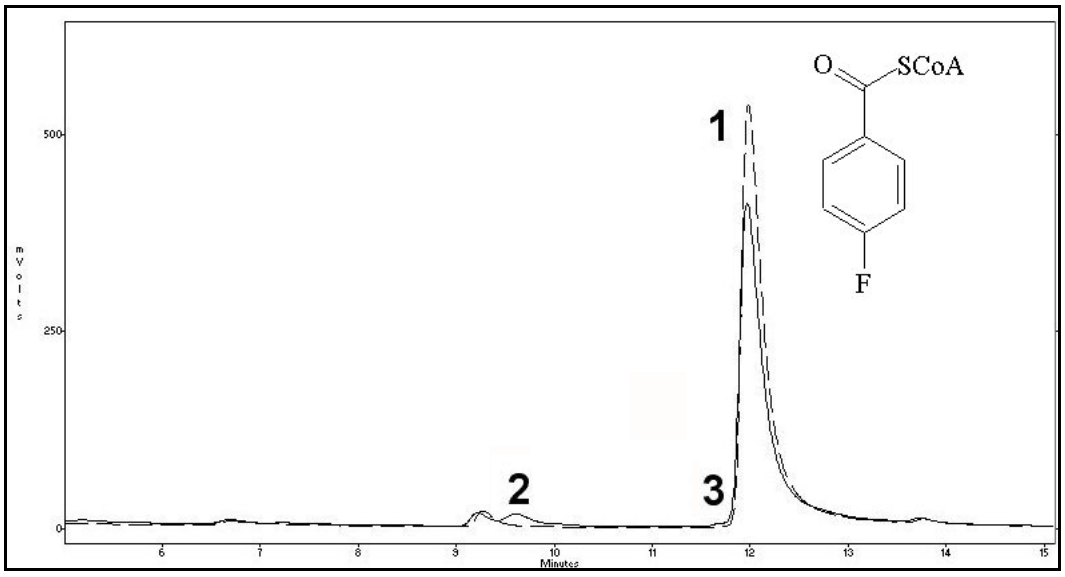


Figure 51. HPLC traces of a benzoyl-CoA reductase assay using p-fluorobenzoyl-CoA as the substrate and titanium(III) citrate as the electron donor (5 minutes). The lighter, dotted line (.....) represents the assay just before the addition of enzyme. The darker, solid line ())))) represents the assay after five minutes of incubation with the enzyme. **1** - p-fluorobenzoyl-CoA (substrate); **2**,**3** - enzymatic products.

with a quadrupole ion trap. The atomic masses of any ions that formed were detected by monitoring the positive ion channel. The chromatogram obtained during the LC/MS analysis is shown in Fig. 53 and the results of the analysis are summarized in Table 4. Mass spectrometry analysis found that ions formed from peak 3, had an atomic mass of 872.0, identifying it as a the positive ion of benzoyl-CoA (Fig. 54A). As stated before, benzoyl-CoA could only form if benzoyl-CoA reductase catalyzed the reductive defluorination of a fluorobenzoyl-CoA substrate, which in this case, was p-fluorobenzoyl-CoA. The primary ion formed from molecules in peak 2 had an atomic mass of 892.0 which is the same mass as the

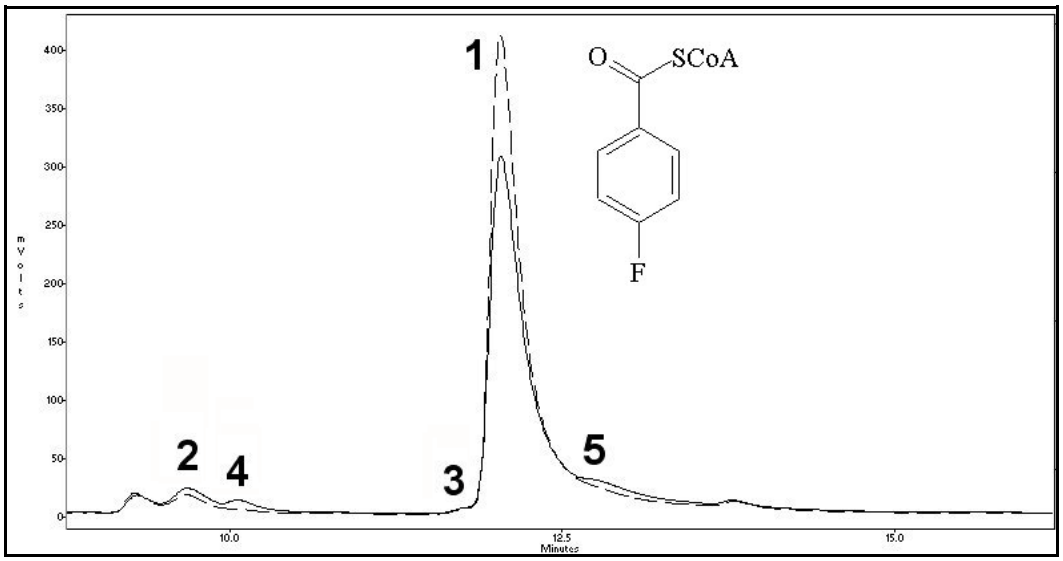


Figure 52. HPLC traces of a benzoyl-CoA reductase assay using p-fluorobenzoyl-CoA as the substrate and titanium(III) citrate as the electron donor (10 minutes). The lighter, dotted line (.....) represents the assay after five minutes of incubation with the enzyme. The darker, solid line ())))) represents the assay after ten minutes of incubation with the enzyme. **1** - p-fluorobenzoyl-CoA (substrate); **2**,**3,4,5** - enzymatic products.

hydration product, which was previously defined as the hydrated, reduced product formed from the benzoyl-CoA reductase reaction on benzoyl-CoA (Fig. 54B). The presence of both benzoyl-CoA and the hydration product in the assay implies that benzoyl-CoA reductase defluorinates p-fluorobenzoyl-CoA , forming benzoyl-CoA. Benzoyl-CoA is then reduced by the enzyme to form the reduction product, which is then hydrated by 1,5-dienoyl-CoA hydratase to form the hydration product. Peaks 4 and 5 form later in the reaction of p-fluorobenzoyl-CoA with benzoyl-CoA reductase and are visible after ten minutes of reaction (Fig. 52). Ions formed from peak 5 showed the presence of two ions, one with an atomic mass of 876 and the other with an atomic mass of 890 (Fig. 55A). The ion with the atomic mass of 876

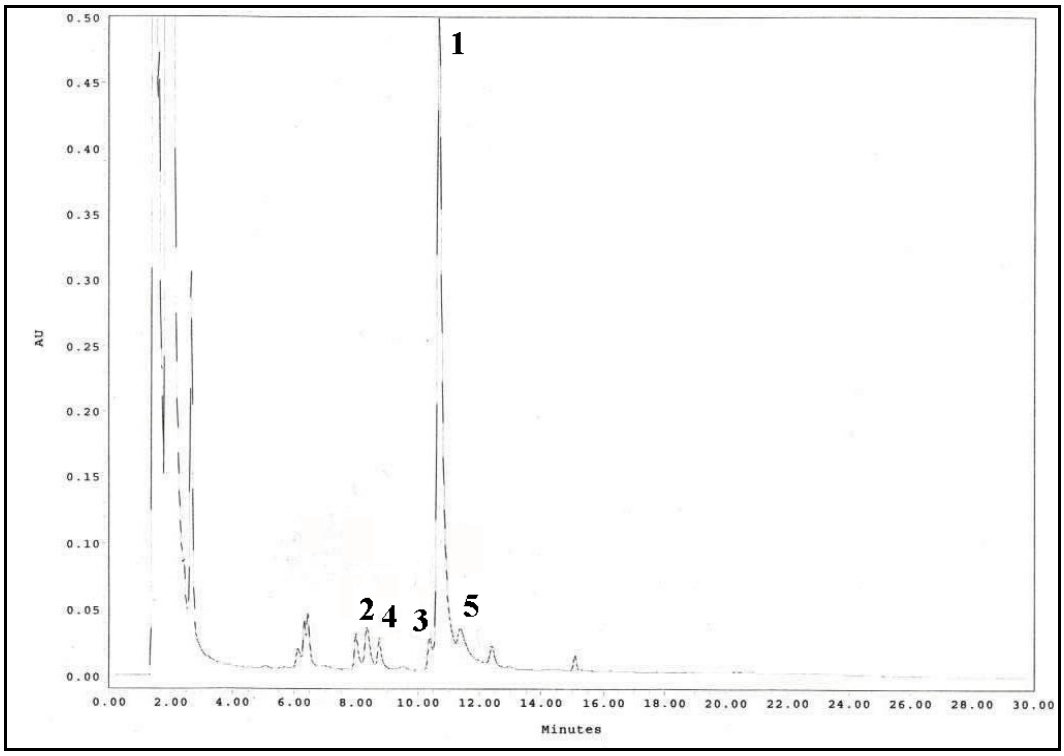


Figure 53. LC/MS trace of a benzoyl-CoA reductase assay using p-fluorobenzoyl-CoA as the substrate and titanium(III) citrate as the electron donor. The assay was allowed to react with 20 µl of enzyme solution for 30 minutes. This sample was passed over a Platinum C-18 reverse-phase column. **1** - p-fluorobenzoyl-CoA (substrate); **2,3 -** products observed after 5 minutes; **4,5 -** products observed after 10 minutes. Peaks not labeled in the chromatogram were present at the start of the assay and are not considered to be enzymatic products. A peak at 6.2 minutes did increase slightly from its original height observed when the sample was taken. The retention time of this peak corresponds to that of free CoA, which accumulated from the slow hydrolysis of thioesters while the sample was being stored awaiting LC/MS analysis.

Peak #	Peak 1	Peaks 2 & 4	Peak 3	Peak 5
Atomic mass(es)	874, 890	892, 894	872	894
Identification	1. reduced benzoyl-CoA (reduction product) 2. p-fluoro-benzoyl-CoA	(2) hydrated, reduced benzoyl-CoA (hydration product) (4) hydroxyhexane-1-carbonyl-CoA (hydroxyhexane)	benzoyl-CoA	cyclohexene-1-carbonyl-CoA (cyclohexene)

Table 4. **A summary of the masses obtained from the LC/MS analysis of a benzoyl-CoA reductase assay using p-fluorobenzoyl-CoA as the substrate**. The peak numbers correspond to those in Fig. 53. Peak 1 contains the substrate as well as a product of the reaction. Peaks 2, 3, 4, and 5 are product peaks. Identification of the product peaks were made from the atomic masses. The retention times of benzoyl-CoA, reduced benzoyl-CoA (reduction product), and hydrated, reduced benzoyl-CoA (hydration product) were also helpful in their identification.

could be the positive ion of cyclohexene-1-carbonyl-CoA, which would have a mass four atomic mass units higher than that of benzoyl-CoA . One possible origin of this molecule would be the two-electron reduction of reduced benzoyl-CoA by benzoyl-CoA reductase. Furthermore, the retention time of this molecule suggests that it is a molecule of benzoyl-CoA that has been reduced twice, as it is less polar than the reduction product, having a retention time that is slightly later than that of the reduction product (Boll, Laempe et. al., 2000). Cyclohexene-1-carbonyl-CoA will be further referred to as cyclohexene. The position of the carbon-carbon double bond in cyclohexene cannot be determined from the mass data alone. The ion with the atomic mass of 890, also present in this time slice,

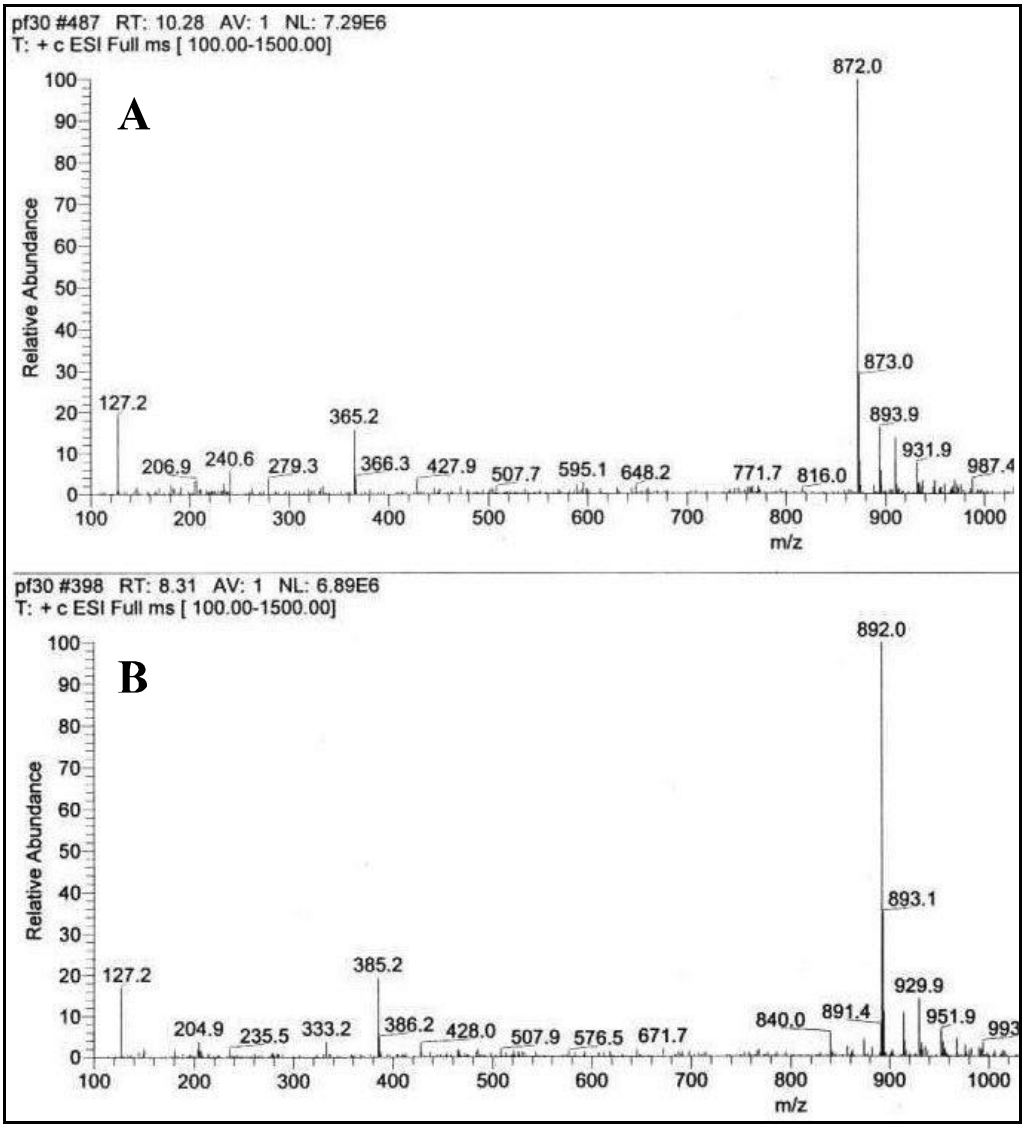


Figure 54. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on p-fluorobenzoyl-CoA (Peaks 3 & 2). (A) A time-slice showing the ions formed by the molecules of peak 3 (Fig. 53). The primary ion formed had an atomic mass of 872, corresponding to the mass of the positive ion of benzoyl-CoA. (B) A time-slice showing the ions formed by the molecules of peak 2 (Fig. 53). The primary ion formed had an atomic mass of 892, corresponding to the mass of the hydration product, the hydrated, reduced product of the benzoyl-CoA reductase reaction on benzoyl-CoA. Ions were observed on the positive ion channel

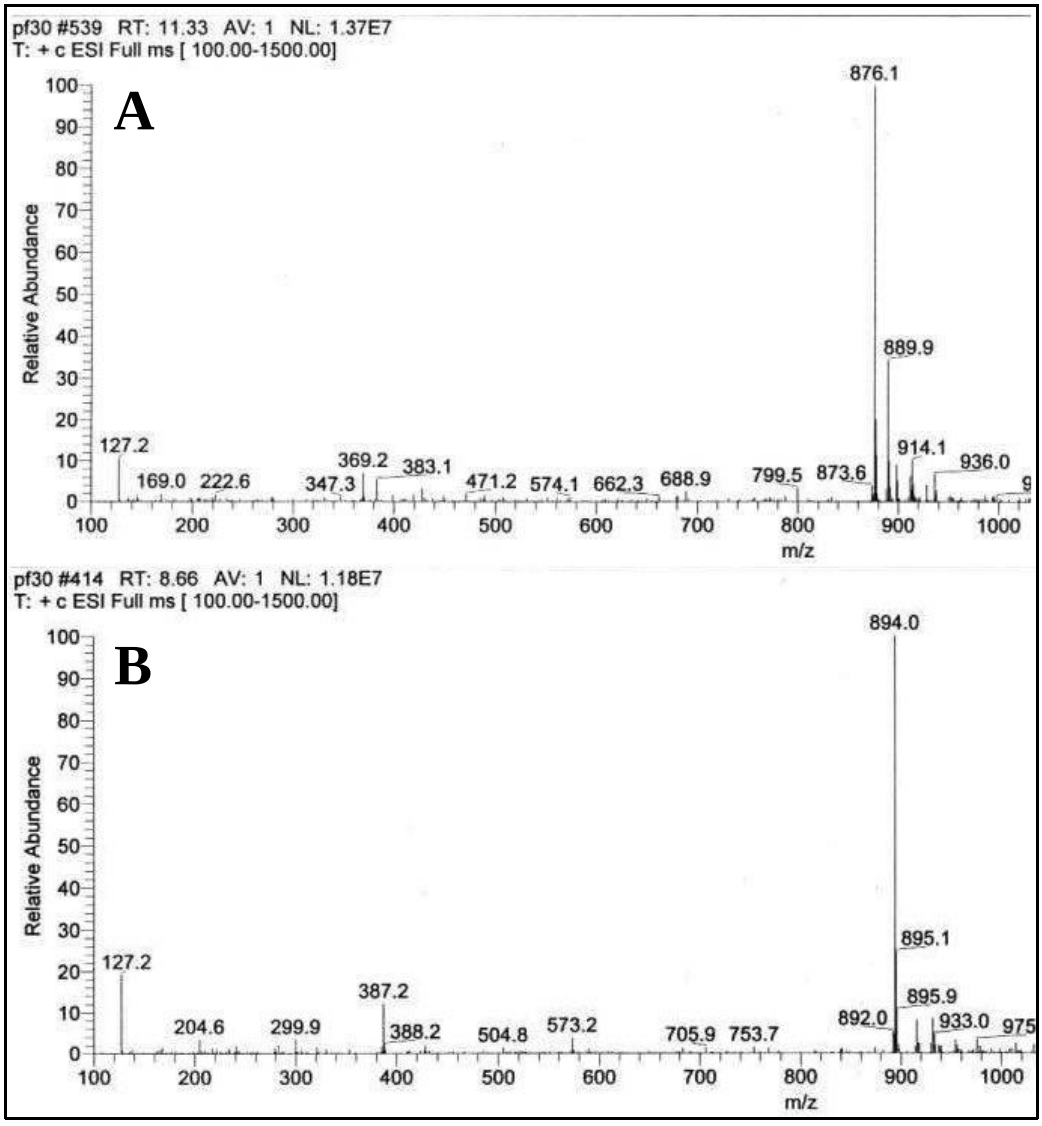


Figure 55. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on p-fluorobenzoyl-CoA (Peaks 5 & 4). (A) A time-slice showing the ions formed by the molecules of peak 5 (Fig. 53). The primary ion formed had an atomic mass of 876, corresponding to the mass of the positive ion of cyclohexene-1-carbonyl-CoA. (B) A time-slice showing the ions formed by the molecules of peak 4 (Fig. 53). The primary ion had an atomic mass of 894, corresponding to the mass of a hydroxyhexane-1-carbonyl-CoA. Ions were observed on the positive ion channel.

corresponds to the mass of a positive ion of p-fluorobenzoyl-CoA, which was still eluting from the column at that time (Fig. 55A). A time-slice of the population of ions formed from molecules of peak 4 showed the presence of an ion with an atomic mass of 894 (Fig. 55B). This ion could be the positive ion of hydroxycyclohexane-1-carbonyl-CoA, which is formed from the hydration of the cyclohexene product by 1,5-dienoyl-CoA hydratase (Boll et. al., 2000). For brevity, hydroxycyclohexane-1-carbonyl-CoA will be referred to as hydroxycyclohexane. Hydroxycyclohexane would have a mass 22 atomic mass units higher than that of benzoyl-CoA. The presence of hydroxycyclohexane suggests that the carbon-carbon double bond in the cyclohexene compound identified earlier is either located at the 1 position, or the 5 position of the ring because 1,5-dienoyl-CoA hydratase catalyzes the hydration of double bonds at those positions. To confirm the identity of peak 1 in Fig. 53 as the substrate of the reaction, p-fluorobenzoyl-CoA, a time-slice was taken at time peak 1 eluted from the column. The primary ion detected in this time-slice had an atomic mass of 890, which is the atomic mass of the positive ion of the p-fluorobenzoyl-CoA (Fig. 56). In this time-slice, there is also the presence of a small amount of a positive ion with an atomic mass of 874, which is the mass of the reduction product, the reduced form of benzoyl-CoA (Fig. 56). There wasn't any evidence of a positive ion of reduced p-fluorobenzoyl-CoA, which has an atomic mass of 892, in the region of the chromatogram that the ions of p-fluorobenzoyl-CoA and cyclohexene were present in. Also absent from this

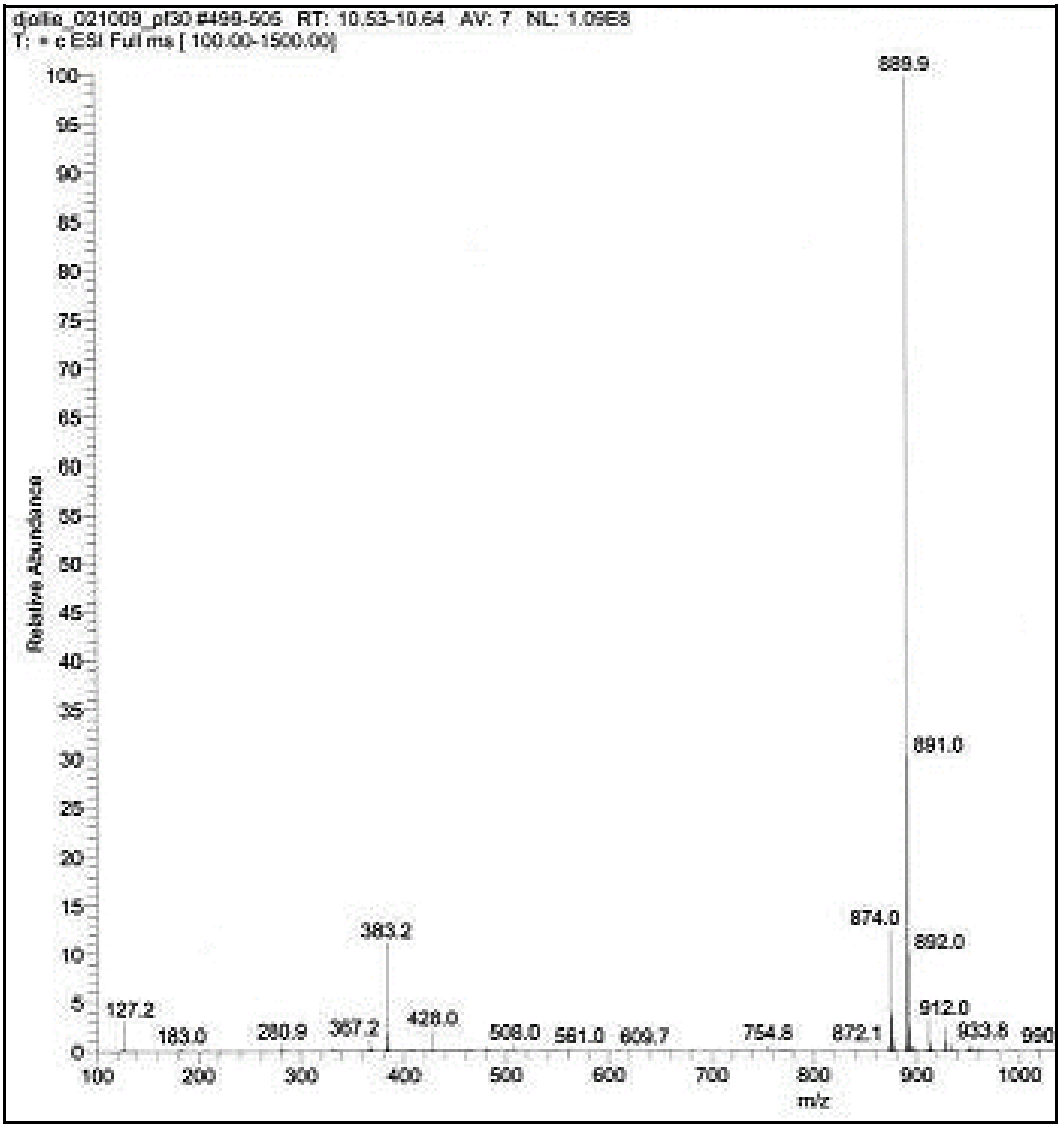


Figure 56. Mass determination of the enzymatic products from the benzoyl-CoA reductase reaction on p-fluorobenzoyl-CoA (Peak 1). A time-slice showing the ions formed by the molecules of peak 1 (Fig. 53). The primary ion formed had an atomic mass of 890, corresponding to the mass of the positive ion of p-fluorobenzoyl-CoA. A secondary ion with an atomic mass of 874 is present. This ion has the same mass as the reduction product produced from the benzoyl-CoA reductase reaction on benzoyl-CoA. Ions were observed on the positive ion channel.

region, was evidence of a p-fluorobenzoyl-CoA molecule that had been reduced twice, whose positive ion would have an atomic mass of 894. So, unlike m-fluorobenzoyl-CoA, no reduced, fluorinated compounds were observed when using p-fluorobenzoyl-CoA as a substrate. The only ions found with the atomic masses of 892 and 894 eluted in the same region that the hydrated, reduced products did and most likely corresponds to the hydration product (hydrated, reduced benzoyl-CoA) and hydroxyhexane product respectively. This might suggest that cyclohexene, is produced from the further reduction of the reduction product (reduced benzoyl-CoA). However, when considering the products formed from the reaction of benzoyl-CoA reductase on other substrates, this idea does not hold up. When benzoyl-CoA reductase is allowed to react with benzoyl-CoA for fifteen minutes under the same assay conditions, the cyclohexadiene product is produced, but further reduction of this compound to a cyclohexene does not occur. Not surprisingly, hydroxycyclohexane is not formed either. Also, neither of these two compounds are formed during the reaction of m-fluorobenzoyl-CoA with benzoyl-CoA reductase. This suggests that the formation of these two compounds might be produced from a different compound than cyclohexadienoyl-CoA, possibly from a reduced p-fluorobenzoyl-CoA molecule. The lack of any evidence of a reduced p-fluorobenzoyl-CoA molecule may be due its rapid consumption by the reaction, enzymatic, or non-enzymatic, that produces the double reduction product. A

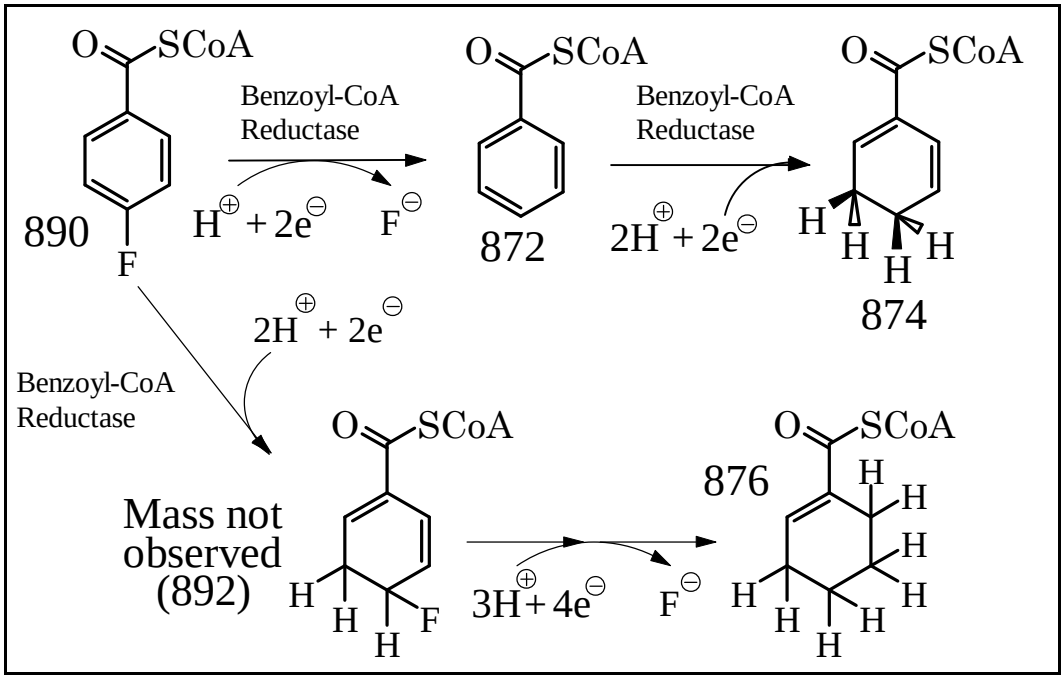


Figure 57. The reaction of benzoyl-CoA reductase with p-fluorobenzoyl-CoA. The reaction of benzoyl-CoA reductase with p-fluorobenzoyl-CoA may proceed by two different pathways. (1) Benzoyl-CoA reductase catalyzes the ATP-dependent, two-electron reduction of p-fluorobenzoyl-CoA, resulting in the defluorination of the compound and the formation of benzoyl-CoA. Benzoyl-CoA is then further reduced to the reduction product by the reductase. (2) Benzoyl-CoA reductase catalyzes the ATP-dependent, two-electron reduction of p-fluorobenzoyl-CoA. This results in the formation of a reduced, fluorinated thioester. This product is then reduced enzymatically, or non-enzymatically, twice to a cyclohexene-1-carbonyl-CoA compound that does not contain fluorine. The structures shown are hypothetical and their actual structures cannot be determined from the atomic mass data alone. The atomic masses of the positive ions of these compounds are also shown.

possible reaction scheme for benzoyl-CoA reductase on p-fluorobenzoyl-CoA is shown in Fig. 57.

CHAPTER III. Discussion and Summary

Discussion. There are three mechanistic possibilities for the reduction of the aromatic ring of benzoyl-CoA by benzoyl-CoA reductase, hydrogenation, hydride reduction, and a two one-electron addition mechanism analogous to that of the Birch reduction. Of the three mechanisms mentioned, only the two one-electron addition mechanism can explain the results presented in the thesis.

Hydrogenation involves the concerted addition of a molecule of hydrogen across a double bond. The hydrogenation of fluorobenzenes yields fluorocyclohexanes. Elimination of a fluoride only occurs after the hydrogenation of the ring and is eliminated by a non-catalytic process (Blum et al., 1999). Since it was found that benzoyl-CoA reductase catalyzed the reductive defluorination of both m-fluorobenzoyl-CoA and p-fluorobenzoyl-CoA, hydrogenation does not seem to be a plausible mechanism for benzoyl-CoA reductase. Also, the lack of a significant isotope effect argues against this mechanism, as it would require that solvent protons take a very active role, first in the formation of molecular hydrogen and then in the reduction of the aromatic ring.

The hydride reduction of the aromatic ring involves the simultaneous addition of two electrons to the ring system. In order to produce the conjugated

cyclohexa-1-5-diene, the hydride ion would have to attack the electrophilic carbon para to the thioester and the resulting anion would then be protonated at the meta position. The dehalogenation of aromatic rings can occur through hydride reduction. NADPH-dependent reductive dehalogenation of 2,4-dichlorobenzoyl-CoA to 4-chlorobenzoyl-CoA has been observed in *Corynebacterium sepedonicum* KZ-4 (Romanov and Hausinger, 1996). Also, the hydrolytic dehalogenation of 4-dichlorobenzoyl-CoA to 4-hydroxybenzoyl-CoA, which follows a mechanism similar to that of the reductive dehalogenation of 2,4-dichlorobenzoyl-CoA, has also been reported (Dong and Carey, 2002). The electrophilicity of the carbon atoms at the para and ortho positions is increased by the presence of the thioester bond, as the resulting negative charge formed by the attack of a nucleophile at either position can be delocalized into the carbonyl. This negatively charged intermediate then isomerizes, resulting in the elimination of the halogen (Fig. 58)(Romanov and Hausinger, 1996). While this mechanism seems plausible for the elimination of fluoride in p-fluorobenzoyl-CoA, it does not offer an explanation for the observed elimination of fluoride from m-fluorobenzoyl-CoA. Also, this mechanism relies on the electrophilicity of the substrate. Nicotinoyl-CoA, having a nitrogen atom meta to the thioester, would be expected to be a more electrophilic substrate than benzoyl-CoA, since the negatively charged intermediate formed after the attack of the hydride could be further stabilized by the nitrogen atom. However, nicotinoyl-CoA was not used by the enzyme, while picolinoyl-CoA,

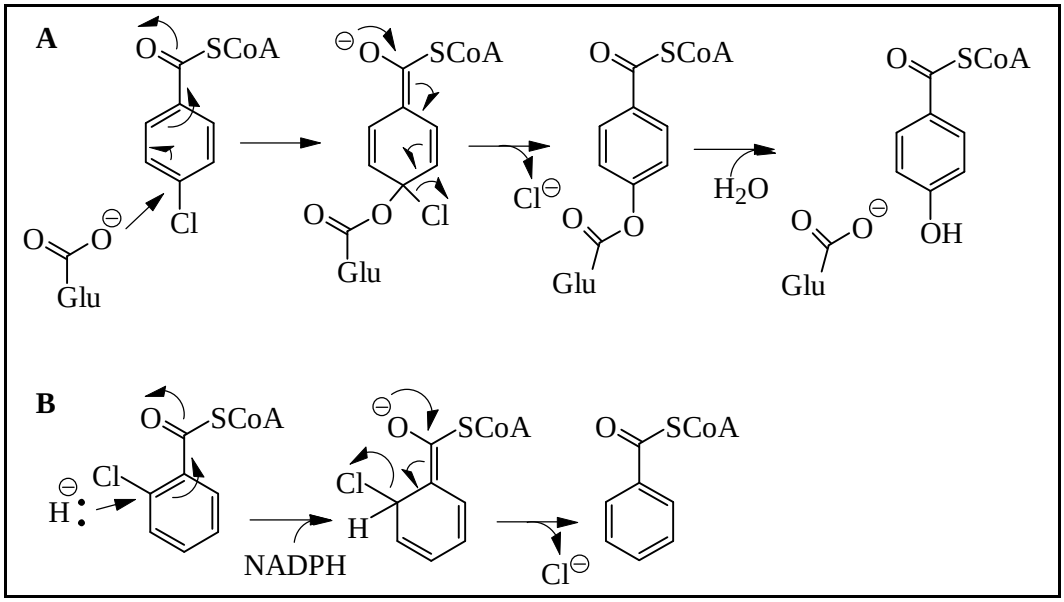


Figure 58. Dehalogenation of p-chlorobenzoyl-CoA and of o-chlorobenzoyl-CoA. (A) The hydrolytic dehalogenation of p-chlorobenzoyl-CoA by 4-chlorobenzoyl-CoA dehalogenase. (B) The reductive dehalogenation of o-chlorobenzoyl-CoA by *Corynebacterium sepedonicum* KZ-4.

which has a nitrogen atom ortho to the thioester, was found to be a substrate for the enzyme. Picolinoyl-CoA would be expected to be a poor substrate for hydride reduction, since the intermediate of such a reaction would place a negative charge next to the nitrogen atom, which is unfavorable (Birch and Slobb, 1976). Furthermore, it was found that benzoyl-CoA reductase exhibits only a small solvent kinetic isotope effect of 1.8 when using its normal substrate, benzoyl-CoA. This kinetic isotope effect is lower than the range of 3-5, observed for $NAD(P)^+$-dependent alcohol dehydrogenases where hydride transfer between the substrate and the cofactor is the rate-limiting step (Bridge et. al., 1995). This argues against a hydride transfer mechanism since the transfer of a proton and two electrons to the

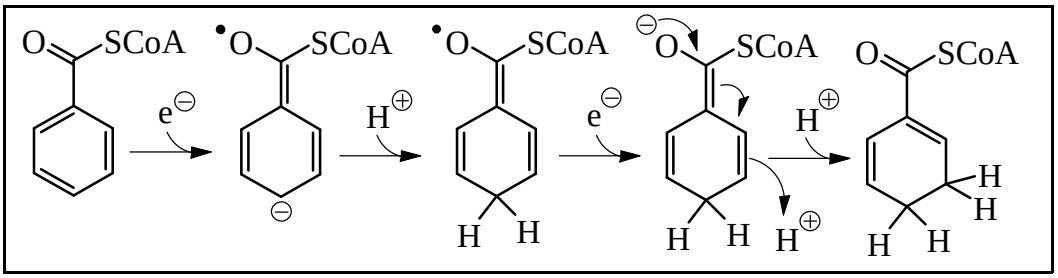


Figure 59. The currently proposed mechanism for benzoyl-CoA reductase.

substrate would occur simultaneously. If the enzyme did transfer a hydride to its substrate, then it would be logical to assume that this process would be rate-limiting, since the reduction of the stable aromatic ring is unfavorable thermodynamically. It is possible that another step in the reaction could be rate-limiting in such a situation. In such a case, the low isotope effect only shows that hydrogen transfer is not involved in the rate-limiting step of the benzoyl-CoA reductase reaction. However, given that both m-fluorobenzoyl-CoA and p-fluorobenzoyl-CoA both underwent reductive defluorination and that nicotinoyl-CoA was found not to be a substrate for the enzyme, it appears that benzoyl-CoA reductase does not reduce its substrate via a hydride reduction. This leaves the proposed mechanism of reduction for benzoyl-CoA reductase, a two one-electron transfer mechanism similar to that of the Birch reduction, as the likely mechanism.

The currently proposed mechanism for benzoyl-CoA reductase, shown in Fig. 59, is a "Birch-like" reduction, involving two one-electron additions to the aromatic ring system. The first electron is added to the oxygen of the carbonyl, generating an anion radical with a negative charge located para to the thioester.

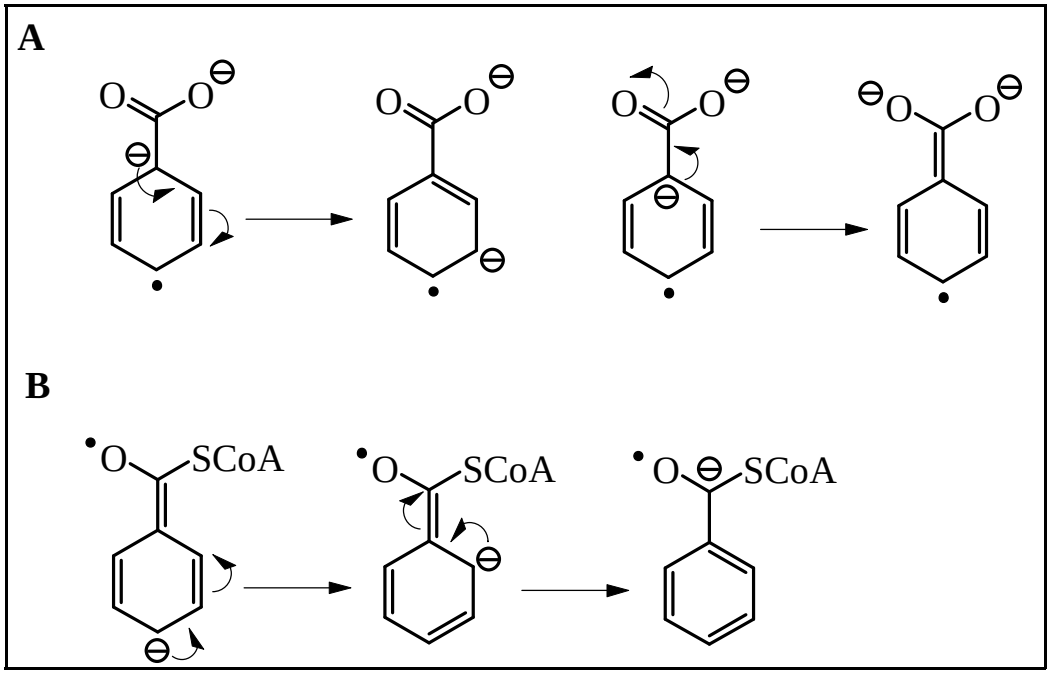


Figure 60. Resonance forms of the anion radical formed after the addition of the first electron to the aromatic ring. (A) Resonance forms of a benzoic acid anion radical, where the first electron is added para to the carboxylic acid. (B) Resonance forms of the proposed benzoyl-CoA anion radical, where the first electron is added to the oxygen of the carbonyl.

This negative charge is delocalized throughout the ring, but is primarily restricted to the para and ortho positions of the aromatic ring and also the carbon of the carbonyl (Fig. 60A). Note that this differs from that of an anion radical where the radical is added to the para position of the aromatic ring. The negative charge on this anion radical would resonate between carbons one and three of the aromatic ring and the oxygen of the carbonyl (Fig. 60B). Since nitrogen has a higher electron-affinity than carbon, when considering the nitrogen-containing, heterocyclic analogues of benzoyl-CoA, those analogues having a nitrogen in the proper position to stabilize the negative charge, are more easily reduced (Birch and

Slobbe, 1976). Thus, picolinoyl-CoA and isonicotinoyl-CoA, having a nitrogen atom in the ortho and para position respectively, should be more easily reduced than benzoyl-CoA. Picolinoyl-CoA was reduced by benzoyl-CoA reductase, but its reduction was catalyzed at a slightly slower rate than benzoyl-CoA. The lack of a higher catalytic rate for picolinoyl-CoA might be explained by a significant decrease in the rate of the second protonation step. This step would result in the formation of a carbanion at meta position, resulting in the unfavorable concentration of negative charge next to the ortho nitrogen of picolinoyl-CoA. This unfavorable interaction could slow down the rate of second protonation step enough so that an increase in the reduction rate of the first step might not be observed. It was difficult to determine whether isonicotinoyl-CoA was a substrate for the enzyme as it seemed to be reduced by methyl viologen and titanium (III) citrate alone in the control reactions. The rate of this apparent reduction was not increased significantly upon the addition of enzyme to this reaction. These observations would imply that the reduction of isonicotinoyl-CoA is favorable enough that it can be reduced by a strong reducing agent without the need of enzyme catalysis. Any increase in this rate by benzoyl-CoA reductase was unobservable over this high background reduction. Interestingly, nicotinoyl-CoA did not show any reaction with the enzyme. Nicotinoyl-CoA contains a nitrogen at the meta position of the aromatic ring. Instead of helping to stabilize the negative charge in the anion radical, the nitrogen would destabilize it, as having a negative

charge next to the nitrogen atom is unfavorable. This would make nicotinoyl-CoA harder to reduce than benzoyl-CoA. This offers a possible explanation as to why nicotinoyl-CoA is not a substrate for benzoyl-CoA reductase. These findings would suggest that nicotinoyl-CoA is harder to reduce than both picolinoyl-CoA and isonicotinoyl-CoA, and that isonicotinoyl-CoA is the easiest to reduce, being nonenzymatically reduced by the chemical reductant. This pattern is also seen in the reduction of the analogous 1-methylcarbomethoxypyridinium cations, where the carbomethoxy functionality serves as the electron withdrawing substituent. For these compounds, it was determined that the electron-withdrawing group stabilized the radical species in the following order: para > ortho > meta (Kashti-Kaplan et. al., 1981). It was thought that the presence of the nitrogen atom in the ring might alter the reduction pattern, but as seen with the Birch reductions of substituted benzoic acids, the impact of the carboxyl group dominates that of any other groups present (Camps et. al., 1967). These observations support the notion that the first step of the reduction by benzoyl-CoA reductase is the formation of an anion radical with the radical on the carbonyl oxygen and a negative charge concentrated para to the thioester. This reaction mechanism is supported further by earlier observations that p-fluorobenzoyl-CoA and m-fluorobenzoyl-CoA react more slowly with the enzyme than o-fluorobenzoyl-CoA (Boll and Fuchs, 1995). After the first electron addition to p-fluorobenzoyl-CoA, the negative charge would be located on the fluorine-bearing carbon. Fluorine has been known to stabilize pyramidal or

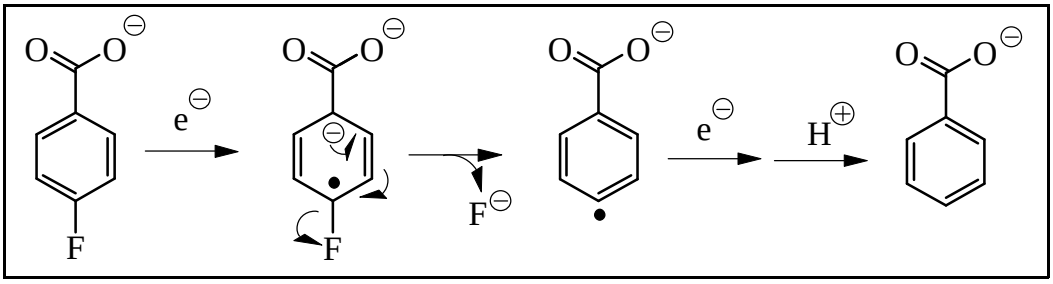


Figure 61. The reductive defluorination of p-fluorobenzoate by the Birch reduction.

nonconjugated carbanions, but it destabilizes carbanions that are directly conjugated (Jessup et. al., 1976). Thus, the formation of a carbanion on the fluorine-bearing carbon would be unfavorable, making p-fluorobenzoyl-CoA harder to reduce. While this is not the case with m-fluorobenzoyl-CoA, the presence of the fluorine might interfere with the protonation of the carbanion formed after the second electron addition, as the conjugated carbanion would be located on a fluorine-bearing carbon, destabilizing it.

The Birch reduction of p-fluorobenzoic acid results in the loss of fluorine, while fluorine is retained in the reduction of m-fluorobenzoic acid. The loss of fluorine during the Birch reduction of p-fluorobenzoic acid occurs upon the formation of the radical anion intermediate by the addition of the first electron. The defluorination of this intermediate results in the formation of an aryl radical, which can then accept another electron to form a carbanion (Jessup et. al., 1976). This carbanion can then be quickly protonated to form benzoic acid (Fig. 61). It was found that both m-fluorobenzoyl-CoA and p-fluorobenzoyl-CoA were slowly

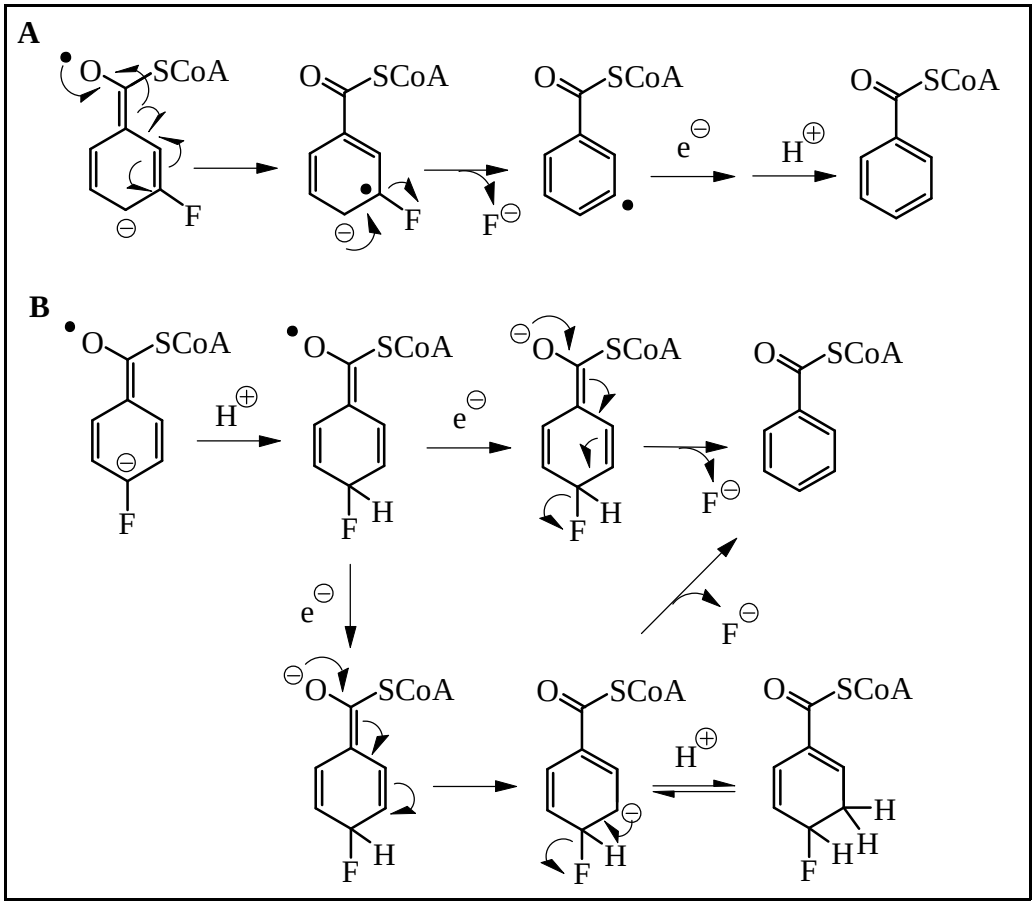


Figure 62. Possible reaction mechanisms for the defluorination of m-fluorobenzoyl-CoA (A) and p-fluorobenzoyl-CoA (B) by benzoyl-CoA reductase. Note: The mechanisms shown start from the anion radical formed by the addition of the first electron.

defluorinated by benzoyl-CoA reductase to form benzoyl-CoA. The defluorination of these two compounds most likely do not occur by the same mechanism. Given the proposed mechanism for benzoyl-CoA, it can be seen how m-fluorobenzoyl-CoA might defluorinate by the rearrangement of the radical anion formed after the first electron is added (Fig. 62A). However, p-fluorobenzoyl-CoA would not be able to follow such a mechanism and most likely is lost as a result of a

rearrangement of the anion formed after the second electron is added (Fig. 62B). This rearrangement could also occur as a result of the reversible protonation of the anion in the final reaction step. As seen in the Birch reduction, reversible protonation of the anion can lead to the more thermodynamically stable product, which would be the aromatic product, benzoyl-CoA (Birch and Slobbe, 1976). It has been suggested that the protonation of the radical anion formed from the first electron transfer is highly exergonic and that it may help to drive the first electron transfer (Boll and Fuchs, 1998). If the enzyme facilitates protonation of the anion radical and of the anion formed after the second electron transfer step, then these protonation events would likely compete with defluorination, resulting in the formation of reduced, fluorinated cyclohexenoyl-CoA compounds. While reduced, fluorinated compounds were not directly observed as a result of the reduction of m-fluorobenzoyl-CoA and p-fluorobenzoyl-CoA with benzoyl-CoA reductase, there was evidence that such compounds might have been formed and then either degraded or underwent further reaction. With m-fluorobenzoyl-CoA, there was evidence of the formation of a fluorinated cyclohexenoyl-CoA. This would require that m-fluorobenzoyl-CoA undergo two two-electron reductions. With p-fluorobenzoyl-CoA, a molecule with the same mass as cyclohexenoyl-CoA started to accumulate after about ten minutes of reaction with benzoyl-CoA reductase. The easiest explanation for the formation of this compound would be the further reduction of cyclohexa-1,5-dienoyl-CoA, which is formed as a result of the

reduction of defluorinated p-fluorobenzoyl-CoA, to the cyclohexene. In fact, further reduction of benzoyl-CoA by benzoyl-CoA reductase has been reported previously (Boll et. al., 2000). However, cyclohexenoyl-CoA was not formed during the reaction of benzoyl-CoA with benzoyl-CoA reductase, or with the reaction of m-fluorobenzoyl-CoA with the enzyme. For this reason, it is more likely that the cyclohexenoyl-CoA compound formed during the reaction of benzoyl-CoA reductase with the p-fluorobenzoyl-CoA substrate was a result of the further reduction of a reduced, fluorinated cyclohexadienoyl-CoA. This would require the defluorination of p-fluorobenzoyl-CoA after being reduced once by benzoyl-CoA reductase. From these experiments, it is difficult to tell whether these further reduction steps are enzymatic or chemical in nature. With m-fluorobenzoyl-CoA, when it is not defluorinated during its reduction by benzoyl-CoA reductase, it is reduced again to form a fluorinated, cyclohexenoyl-CoA molecule. In this case, the fluorine remains on the product. It has been shown that *Thauera aromatica* can grow using fluorobenzoates as a carbon source (Boll and Fuchs,1995). In order to use m-fluorobenzoyl-CoA as a metabolite, *Thauera aromatica* would require a later metabolic step to remove the fluorine from this fluorinated, cyclohexenoyl-CoA molecule. Otherwise, the fluorinated compound would build up to high concentrations, which could prove to be harmful to the bacterium.

The action of benzoyl-CoA reductase on p-fluorobenzoyl-CoA and m-fluorobenzoyl-CoA is interesting from an environmental standpoint because the enzyme cannot only remove a fluorine substituent from an aromatic ring system, it can then reduce the aromatic ring to produce a defluorinated, nonaromatic product. An avenue of future research would be to determine whether benzoyl-CoA reductase can reductively dehalogenate o-fluorobenzoyl-CoA and o-chlorobenzoyl-CoA, both of which have been shown to be substrates for the enzyme, but whose enzymatic products have yet to be identified (Boll and Fuchs, 1995). Regardless, the fact that benzoyl-CoA reductase can reduce halogenated aromatic thioester directly is valuable to the bioremediation of polychlorinated-biphenyl (PCB) contaminated sites, since chlorinated benzoates are intermediates in the degradation of PCB by microorganisms (Romanov and Hausinger, 1996). *Thauera aromatica* could conceivably take a chlorinated benzoate convert it to the respective thioester and then reduce it to a non-aromatic, halogenated hydrocarbon using benzoyl-CoA reductase. An enzyme that could dehalogenate these compounds, as well as dearomatize them, would make the metabolism of these halogenated compounds to carbon dioxide easier. Benzoyl-CoA reductase may very well be capable of performing such a function and since it does so without the use of oxygen, it could be a useful solution for bioremediation efforts in oxygen-deficient environments, such as in the sediment of swamps.

A rather unexpected result discovered while studying benzoyl-CoA reductase was that after being exposed to oxygen, the enzyme could catalyze the oxidation of its reduction product, the cyclohexadienoyl-CoA, forming its reduction substrate, benzoyl-CoA. The reduction of activity of benzoyl-CoA reductase activity is oxygen sensitive and the reductase activity is irreversibly lost after two minutes of oxygen exposure (Boll and Fuchs, 1995). It was surprising then to observe the enzyme-catalyzed oxidation of cyclohexadienoyl-CoA, as it has been assumed that one or more of the Fe-S clusters are degraded by oxygen exposure (Boll et. al., 2001). While it is conceivable that this oxidation could occur from the elimination of a hydride from the reduced product, however this does not offer an explanation for why molecular oxygen must be also be present for the enzyme-catalyzed production of benzoyl-CoA from the reduction product. Molecular oxygen cannot be a terminal electron acceptor for a hydride and can only accept one electron at a time. This supports a radical mechanism for the oxidation of cyclohexadienoyl-CoA to benzoyl-CoA. This suggests that benzoyl-CoA reductase is the likely enzymatic catalyst for this reaction. This was surprising since the reductase activity of this enzyme has been reported to be irreversibly inactivated by two minutes of oxygen exposure (Boll and Fuchs, 1995). 2-hydroxyglutaryl-CoA dehydratase, another oxygen-sensitive enzyme which has sequence similarities to benzoyl-CoA reductase and a similar enzymatic mechanism, is acapable of oxidizing a ketyl radical (Locher et. al., 2001). The dehydratase has two

components, CompA and CompD. CompA is a dimer containing a bridge [4Fe-4S] cluster and is responsible for binding and hydrolyzing ATP. CompA also transfers an electron to CompD in an ATP-dependent step. The substrate of the reaction, 2-hydroxyglutaryl-CoA, binds to CompD. After the transfer of the electron from CompA to CompD, CompD donates an electron to the substrate, generating a ketyl radical. CompD can then reaccept an electron from the ketyl radical on glutaconyl-CoA before product release (Locher et. al., 2001). CompA shares sequence similarity to the two subunits of benzoyl-CoA reductase where it is thought that ATP-dependent electron activation occurs. Like CompA, these two subunits of the reductase also contain a bridging [4Fe-4S] cluster (Unciuleac and Boll, 2001). In the case of 2-hydroxyglutaryl-CoA dehydratase, oxygen degrades the bridging [4Fe-4S] of the CompA dimer, causing the dissociation of the CompA dimer (Locher et. al., 2001). If a similar process happens when benzoyl-CoA reductase is exposed to oxygen, then the bridging [4Fe-4S] cluster of the two ATP-binding subunits would degrade and possibly cause the dissociation of these two subunits. This would destroy the enzyme's ability to reduce benzoyl CoA. However, if the remaining two subunits remain unperturbed, then benzoyl-CoA reductase could still bind to its substrate, or to its product. This provides an explanation of why benzoyl-CoA reductase is able to catalyze the oxidation of its reduction product in an oxygenated environment, but unable to catalyze the reduction of benzoyl-CoA. Also, if the two remaining [4Fe-4S] clusters are unaffected, then these Fe-S clusters may be able to

accept electrons from its reduction product in a manner similar to the way CompD can accept an electron from the ketyl radical of glutaconyl-CoA. Thermodynamically, the oxidation of 1,5-cyclohexadienoyl-CoA back to benzoyl-CoA is favorable and would not require a driving force. In such a situation, the electron transfer could only proceed from the reduction product to the [4Fe-4S] clusters of the enzyme, as transfer back to the reduction product would require an ATP-generated, low-potential electron. The enzyme might then transfer that electron to molecular oxygen, possibly generating superoxide.

The ability of benzoyl-CoA to reoxidize its reduction product, cyclohexadienoyl-CoA, in the presence of oxygen, suggests that benzoyl-CoA reductase can carry out two separate reactions, a reduction and an oxidation. The reduction reaction, which is anaerobic, is shown in Fig. 63. In this reaction, an electron donor donates one electron to the Fe-S cluster of the ATP-dependent electron activation module (subunits a and d) of benzoyl-CoA reductase by an electron donor, the hydrolysis of ATP alters the redox potential of the Fe-S clusters, generating a low-potential electron. This low-potential electron is then transferred to the Fe-S clusters of the substrate-binding module (subunits b and c) of the reductase and reduces the substrate (Fig 63). This cycle then repeats once more and results in the formation of the reduction product, cyclohexadienoyl-CoA. In this reaction, ATP is not directly involved in the transfer of electrons to the substrate, instead the hydrolysis of ATP drives the formation of the low-potential

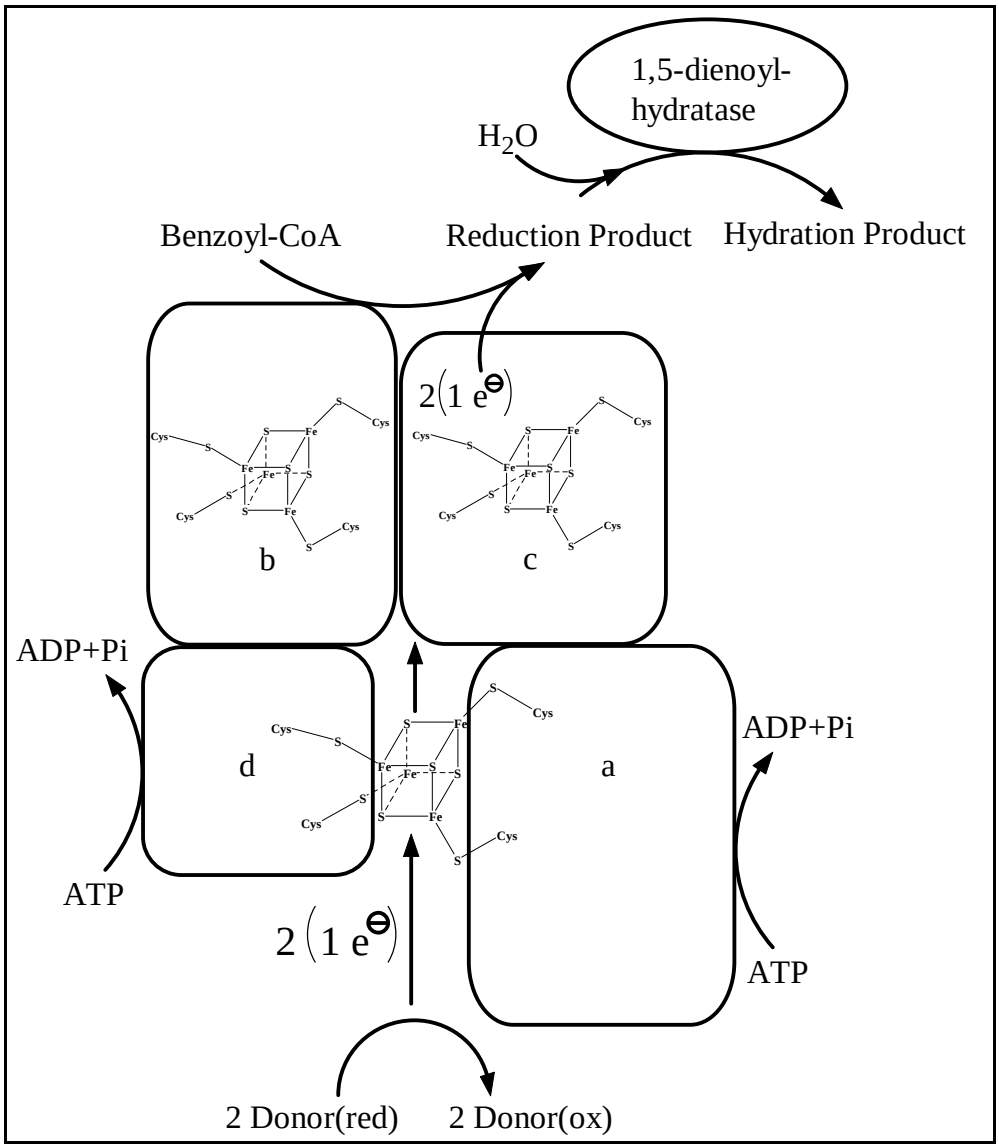


Figure 63. Possible reaction scheme for the reduction of benzoyl-CoA by benzoyl-CoA reductase. Two electrons are transferred from an electron donor to the substrate, benzoyl-CoA, one electron at a time. An electron is passed from an electron donor to the Fe-S cluster bridging subunits a and d. A molecule of ATP is then hydrolyzed, altering the redox potential of this Fe-S cluster. The electron is then passed to the Fe-S clusters of subunits b and c, where it is then used to reduce the substrate, benzoyl-CoA. These steps repeat once more, generating the reduction product, most likely 1,5-cyclohexadienoyl-CoA. This product is then hydrated by another enzyme, 1,5-dienoyl-hydratase, to form the hydration product.

electrons that will ultimately reduce the substrate. In the oxidation reaction, which is aerobic, the ability to generate these low-potential electrons is destroyed by the addition of oxygen to the system. In this case, the Fe-S clusters of the substrate-binding module (subunits b and c) two electrons are transferred from the reduction product, cyclohexadienoyl-CoA, one electron at a time. This results in the oxidation of cyclohexadienoyl-CoA to benzoyl-CoA (Fig. 64). It would be interesting to see whether oxygen-exposed benzoyl-CoA reductase is required for this process or whether thionine-oxidized benzoyl-CoA reductase could catalyze the oxidation of 1,5-cyclohexadienoyl-CoA in absence of oxygen.

Summary. In summary, we found evidence that benzoyl-CoA reductase uses a "Birch-like" reduction mechanism, consisting of two alternate one-electron and one-proton transfers steps, to reduce the aromatic ring of benzoyl-CoA. A hydrogenation mechanism was ruled out as a possible mechanism as we found that benzoyl-CoA reductase could reductively defluorinate both p-fluorobenzoyl-CoA and m-fluorobenzoyl-CoA, which is not likely to occur with a hydrogenation mechanism. We observed only a slight kinetic isotope effect for benzoyl-CoA reductase, suggesting that proton transfer is not directly involved in the rate-limiting step. The hydrogenation and hydride transfer mechanisms would require that proton transfer and electron transfer be coupled. If the reduction of the stable aromatic ring is considered to be the rate-limiting step of this reaction, then a larger kinetic isotope effect would have been observed if benzoyl-CoA reductase used

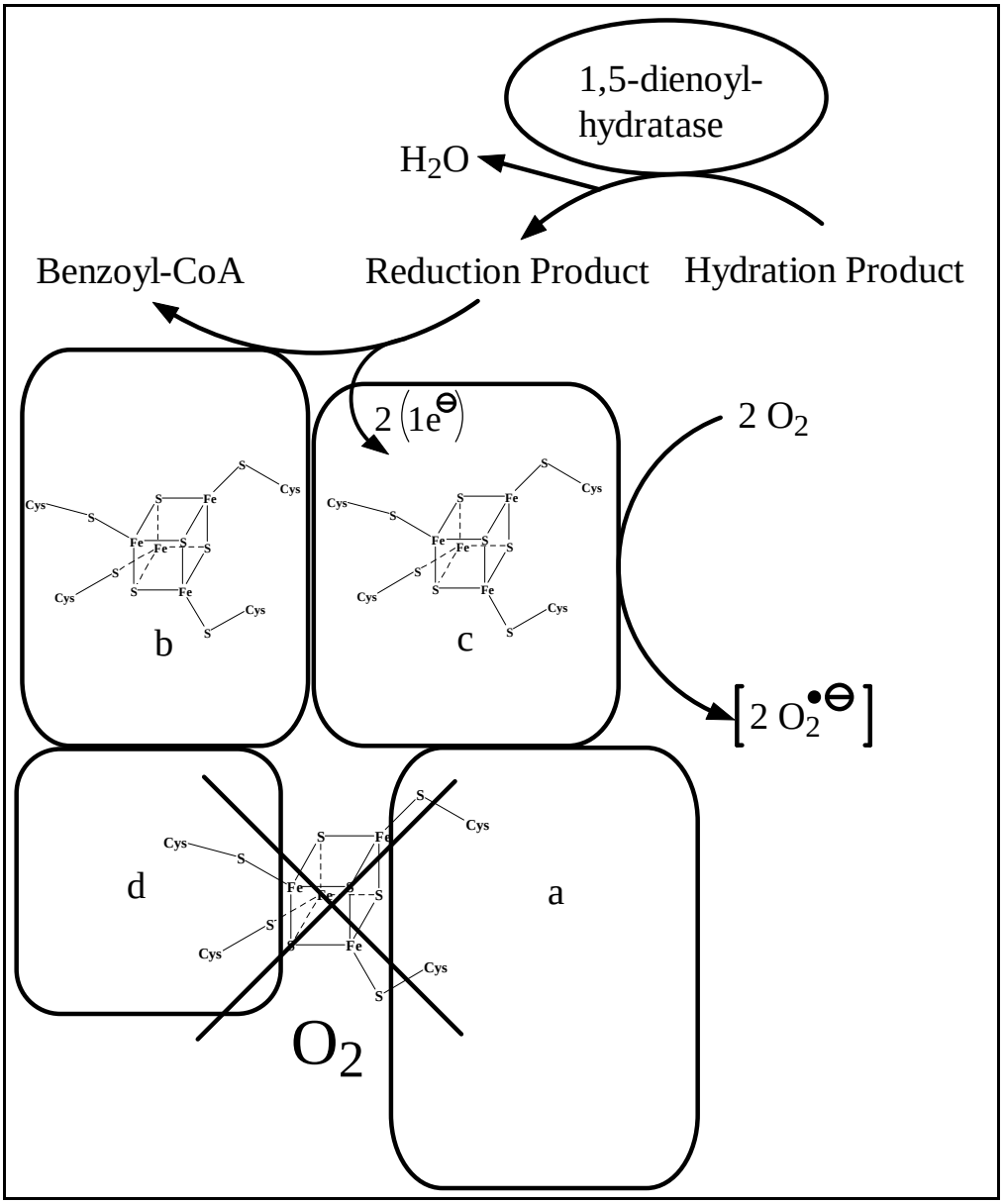


Figure 64. Possible reaction scheme for the oxidation of the reduction product by benzoyl-CoA in the presence of molecular oxygen by benzoyl-CoA reductase. 1,5-dienoyl hydratase catalyzes the dehydration of the hydration product, forming the reduction product. The reduction product then transfers two electrons, one at a time, to the Fe-S clusters of subunits b and c. The electron is then lost, possibly to molecular oxygen, resulting in the generation of superoxide. The Fe-S cluster bridging the a and d subunits has been inactivated (X) by oxygen exposure.

either a hydrogenation, or hydride transfer mechanism to catalyze the reduction of the substrate. We also found that only the nitrogen-containing heterocycle, picolinoyl-CoA was a substrate for the enzyme. Nicotinoyl-CoA was not reduced by the enzyme and isonicotinoyl-CoA was reduced nonenzymatically by the chemical reducing agent. Nicotinoyl-CoA would have been an ideal substrate for hydride attack at the para carbon, as the nitrogen would increase the electrophilicity of the para carbon. The fact that nicotinoyl-CoA was not a substrate for benzoyl-CoA reductase argues against a hydride transfer mechanism of reduction. We also found evidence supporting the formation of a ketyl radical, instead of a phenyl radical, after the addition of the first electron. Such a mechanism would support our findings that benzoyl-CoA reductase can reductively defluorinate m-fluorobenzoyl-CoA and p-fluorobenzoyl-CoA and that the enzyme can reduce picolinoyl-CoA, but not nicotinoyl-CoA.

The defluorination of p-fluorobenzoyl-CoA and m-fluorobenzoyl-CoA by benzoyl-CoA reductase had not been reported previously. Although, it had been determined earlier that benzoyl-CoA reductase can use these fluorinated benzoyl-CoA analogues as substrates, the product(s) formed from the enzymatic reduction of these compounds had not been identified (Boll and Fuchs, 1995). In fact, previous to this work, only the products of the benzoyl-CoA reductase reduction of benzoyl-CoA have been identified (Boll et. al. 2000). Our findings suggest that benzoyl-CoA reductase can reductively defluorinate these compounds before

reducing the aromatic ring. This brings up the interesting question as to whether benzoyl-CoA reductase can reductively eliminate substituents of other benzoyl-CoA analogues that it has been shown to have activity on, such as hydroxybenzoyl-CoA (Boll and Fuchs, 1995).

We also gained some insight as to how oxygen-exposure affects benzoyl-CoA reductase. It has been known that oxygen irreversibly inactivates the ability of benzoyl-CoA reductase to catalyze the reduction of benzoyl-CoA (Boll and Fuchs, 1995). However, we found that benzoyl-CoA reductase might still retain some activity after being exposed to oxygen. We found that, in the presence of oxygen, the reductase could oxidize its native reduction product, cyclohexadienoyl-CoA, to benzoyl-CoA. This finding suggests that the Fe-S clusters of the reductase's substrate binding subunits retain their activity upon oxygen exposure. It is then likely that the observed loss in the reduction capability of the enzyme upon oxygen exposure is a result of the degradation of the Fe-S cluster bridging the ATP-binding subunits of the reductase, which is thought to play a role in the ATP-dependent generation of low-potential electrons.

Appendix. Proton NMR Spectra of Benzoyl-CoA Analogues

The proton NMR spectra of nicotinoyl-CoA, picolinoyl-CoA, m-fluorobenzoyl-CoA and p-fluorobenzoyl-CoA are shown in Figs. 65-68.

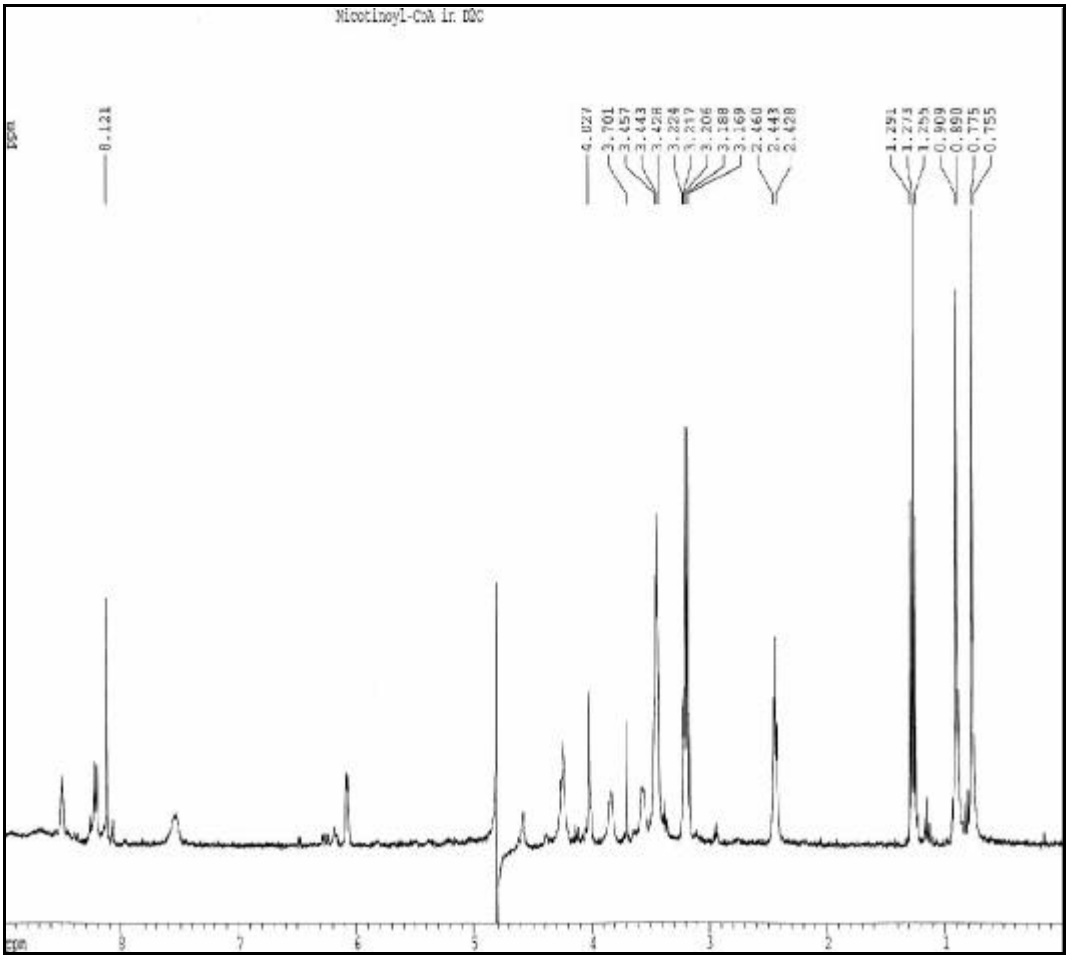


Figure 65. Proton NMR spectrum of nicotinoyl-CoA in D_2O.

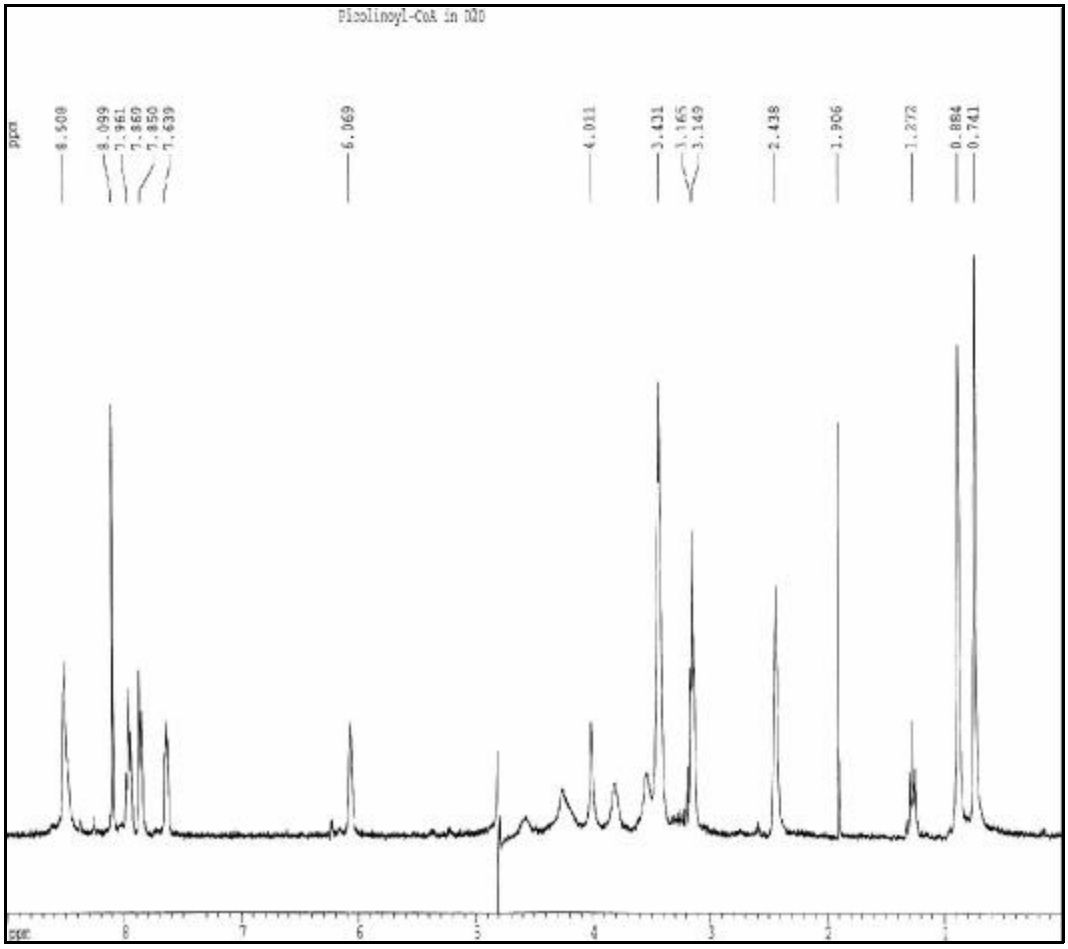


Figure 66. Proton NMR spectrum of picolinoyl-CoA in D_2O

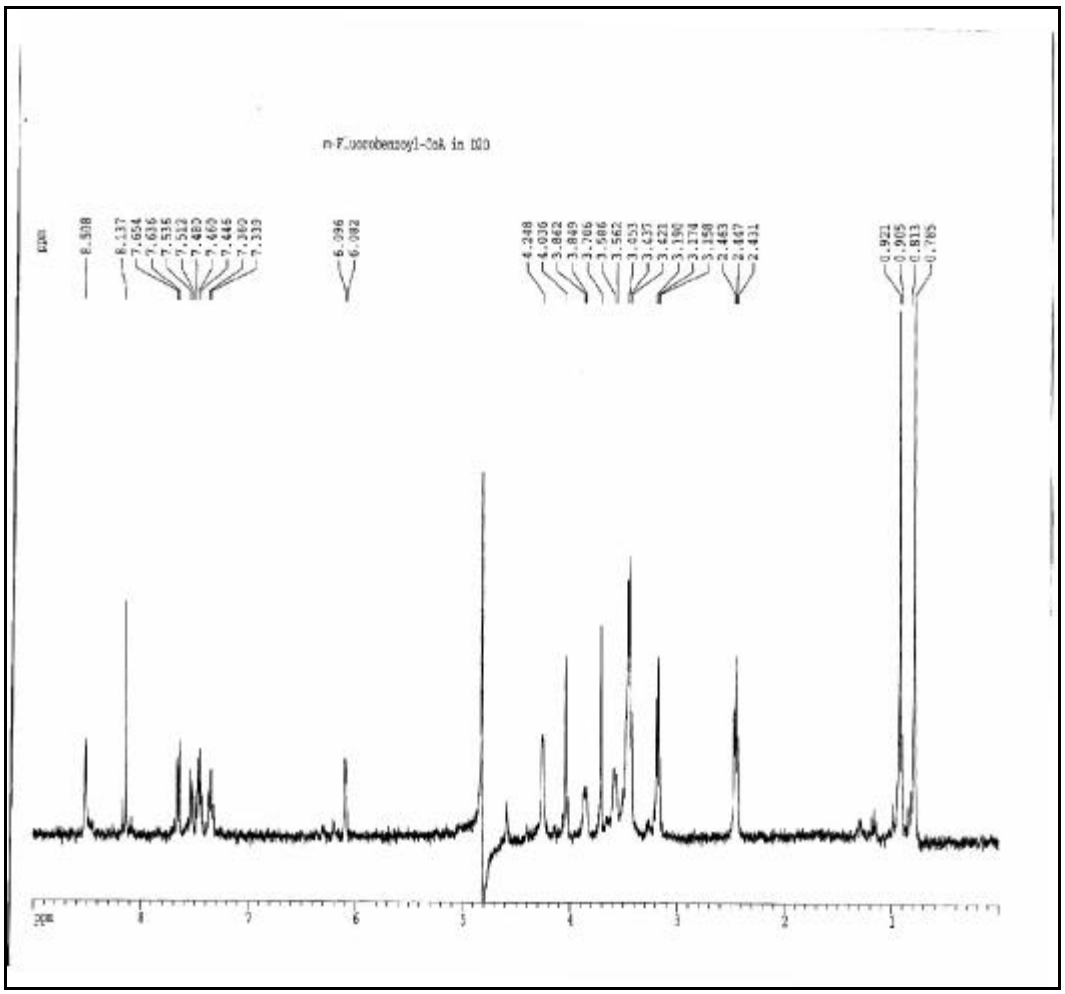


Figure 67. Proton NMR spectrum of m-fluorobenzoyl-CoA in D_2O.

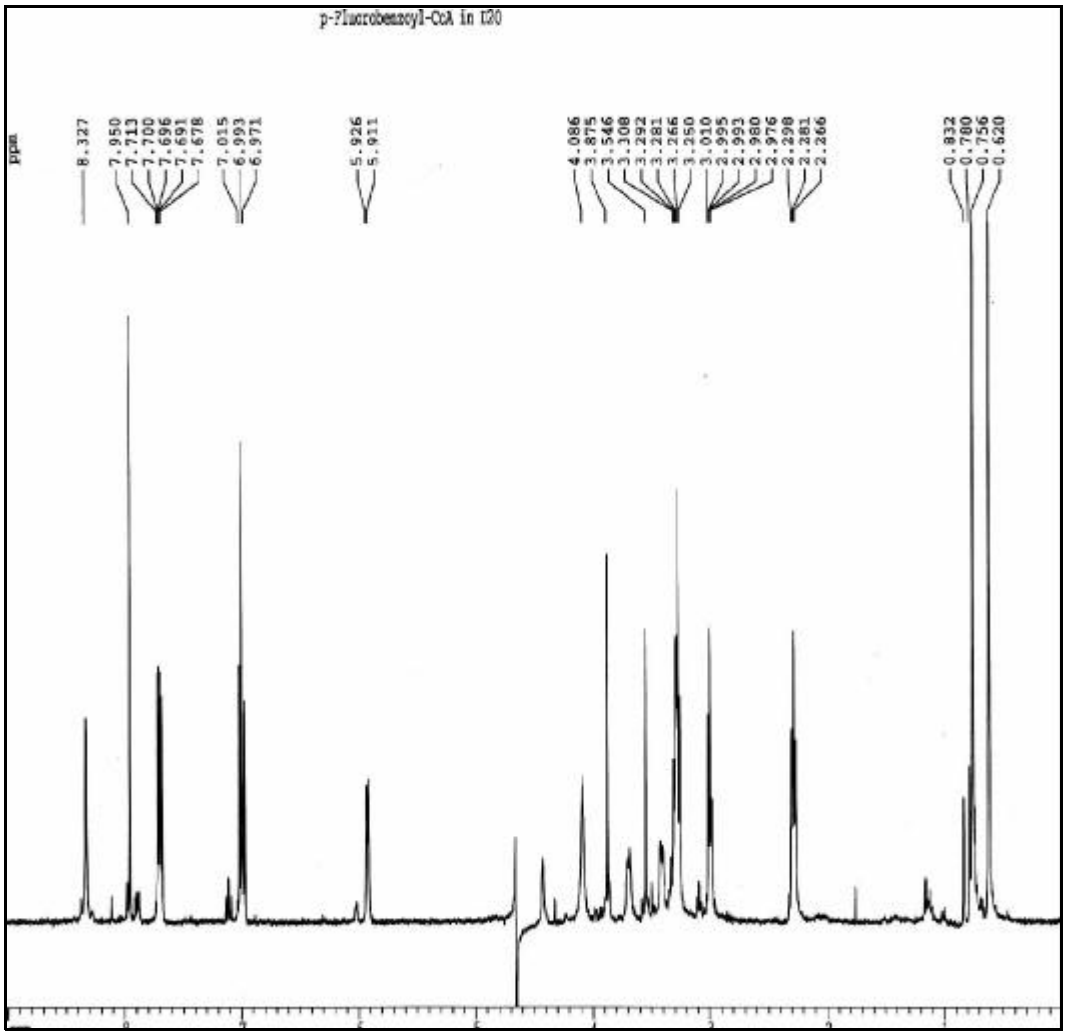


Figure 68. Proton NMR spectrum of p-fluorobenzoyl-CoA in D_2O

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