# Determination of the mechanism of reaction for bile acid:CoA ligase

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The reaction of cholic acid, CoA and ATP to yield cholyl-CoA was investigated by kinetic analysis of the reaction as catalysed by guinea pig liver microsomes. The enzyme has an absolute requirement for divalent cation for activity so all kinetic analyses were carried out in excess $Mn^{2+}$. A trisubstrate kinetic analysis was conducted by varying, one at a time ATP cholate and CoA. Both ATP and cholate gave parallel double reciprocal plots versus CoA, which indicates a ping-pong mechanism with either pyrophosphate or AMP leaving prior to the binding of CoA. Addition of pyrophosphate to the assays changed the parallel plots to intersecting ones; addition of AMP did not. This indicates that pyrophosphate is the first product. The end-product, AMP, was a competitive inhibitor versus ATP, as was cholyl-CoA at saturating concentrations of cholate. Both AMP and cholyl-CoA were uncompetitive inhibitors versus CoA. Based on this information, it was concluded that the reaction follows a bi uni uni bi ping-pong mechanism with ATP binding first, and with the release of the final products, AMP and cholyl-CoA, being random. CoA showed substrate inhibition at high but non-saturating concentrations and this inhibition was competitive versus ATP, which is consistent with the predicted ping-pong mechanism. The ability of cholyl-CoA, but not cholate or CoA, to bind with high affinity to the free enzyme was suggestive of a high affinity of the enzyme for the thioester link.

## INTRODUCTION

Bile acids are synthesized in the liver and then conjugated to either glycine or taurine before secretion into bile. The liver is also responsible for the conjugation of bile acids which are deconjugated in the gut and then returned to the liver via the enterohepatic circulation. Conjugation has been shown to be important for both the excretion of bile acids into bile and also for the role of bile acids in aiding in the intestinal absorption of lipid [1–5].

Bile acid conjugates are formed by the reaction of bile acid-CoA thioesters with glycine or taurine in a reaction catalysed by a cytoplasmic *N*-acyltransferase, a well-characterized enzyme specific for bile acids [6,7]. The formation of the bile acid-CoA thioesters is the rate-limiting step in conjugation [8] and occurs in an ATP/$Mg^{2+}$-dependent reaction [Reaction (1)]

$$\text{Bile acid} + \text{CoA} + \text{ATP} \xrightarrow{Mg^{2+}} \text{Bile acid-CoA} + PP_i + \text{AMP} \quad (1)$$

where $PP_i$ stands for pyrophosphate. Reaction [1] is catalysed by the enzyme bile acid:CoA ligase. This is a dimeric enzyme firmly attached to the membranes of the endoplasmic reticulum [9,10]. Studies of the reaction indicated that ATP is cleaved to AMP and pyrophosphate [11], but the mechanism for the reaction is unknown. Also, it has been found that $Mn^{2+}$ is more efficient than $Mg^{2+}$ while several other divalent cations are less efficient but still functional in the reaction [9]. Preliminary kinetic characterization of the enzyme defined its substrate specificity toward a limited number of bile acids, and revealed its sensitivity to inhibition by conjugated bile acids [9,10]. In the present study we have used kinetic analyses of the enzyme in its native membrane environment to define the reaction mechanism.

## MATERIALS AND METHODS

Radiolabelled cholate was obtained from American Radiolabeled Chemicals, St. Louis, MO, U.S.A. ($^{14}C$) or from NEN Research Products, Wilmington, DE, U.S.A. ($^{3}H$). CoA, dithiothreitol (DTT), Trizma Preset, leupeptin, chymostatin and pepstatin A were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. ATP was obtained from Calbiochem, LaJolla, CA, U.S.A. and was 98.25% pure by h.p.l.c. and contained less than 10 p.p.m. of $Mg^{2+}$ and less than 30 p.p.m. $Ca^{2+}$. Sodium pyrophosphate was obtained from Matheson, Coleman and Bell, Norwood OH, U.S.A. and contained less than 0.001% heavy metals. Cholyl-CoA was synthesized and purified as described previously [7], and then further purified to remove all traces of cholate by chromatography on Sephadex LH-20 as described by Killenberg and Dukes [12].

The microsomal cell fraction of liver cells was isolated from guinea pig (Simonsen Laboratories, Gilroy, CA, U.S.A.). Liver was excised and homogenized into approximately four volumes of buffer (0.15 M KCl, 10 mM Trizma 7.0 (pH = 7.55 at 5 °C), 2 mM DTT, and 250 $\mu$g/l of each of the protease inhibitors leupeptin, chymostatin and pepstatin A). The homogenate was centrifuged for 20 min at 7000 *g* and the resulting supernatant was ultracentrifuged for 45 min at 12000 *g*. The resulting microsomal pellet was resuspended in half the original volume of buffer and ultracentrifuged; the final pellet was resuspended in buffer to a protein concentration of 40 mg/ml and stored in aliquots at −80 °C.

Bile acid:CoA ligase activity was assayed radiochemically by following the formation of radiolabelled cholyl-CoA from either [$^{3}H$]- or [$^{14}C$]-cholate. A standard reaction tube contained 100 mM Tris/HCl, pH 7.4, at 30 °C (Sigma Pre-set Trizma, pH 7.5), 10 $\mu$M CoA, 10 $\mu$M cholate, 15 mM $Mn^{2+}$, 1 mM ATP

Abbreviation used: DTT, dithiothreitol.

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and ~ 0.05 mg of microsomal protein in a volume of 0.5 ml. The [$^{14}$C]- or [$^{3}$H]-cholate and CoA were added first to the water and buffer in a 30 °C water-bath followed by ATP and microsomes; the reaction was initiated by the addition of $MnCl_2$. Initial rates of reaction were determined by removing aliquots of 100 $\mu$l at timed intervals. The reaction was terminated by adding each aliquot to a tube containing 200 $\mu$l of 25 mM EDTA. This was followed by the addition of 200 $\mu$l of 60 mM succinic acid, and the tubes were then extracted with 1 ml of water-saturated butanol to remove the unreacted labelled cholate. This extraction was repeated once. The cholyl-CoA formed at each time-point was measured by removing 250 $\mu$l of the remaining aqueous layer for determination of radioactivity by liquid scintillation counting. Initial rates of reaction were proportional to the concentration of microsomal protein in the assay for concentrations up to 1 mg/ml.

## RESULTS

The microsomal fraction of guinea pig liver was used as the source of bile acid:CoA ligase for all kinetic analyses. This was necessitated by the fact that purification of the enzyme requires the use of detergents [10] and detergents form mixed micelles with the bile-acid substrates and introduce an artifact into the kinetic analysis. The ligase catalyses a ter-reactant reaction which has an additional divalent cation requirement. When the enzyme is assayed in a mixture containing 1 mM EDTA and without added divalent cation, there is no measurable reaction. Thus, the activity is completely dependent upon a divalent cation. Furthermore, we have found that $Mn^{2+}$ gives slightly higher rates than $Mg^{2+}$ [9]. Therefore, in the studies that follow, the divalent cation requirement was satisfied by employing in the assays a concentration of $Mn^{2+}$ which was always a minimum of 5-fold in excess of the concentration of ATP.

A ter-reactant kinetic analysis of the reaction was conducted by varying the concentration of the three substrates (ATP, cholate and CoA) one at a time. The concentrations of cholate employed were > 100-fold lower than its critical micelle concentration. When the concentration of cholate was held constant and the concentration of CoA varied at each of several different fixed concentrations of ATP, the plot shown in Figure 1 was obtained. The result was a series of apparently parallel lines. Replotting the data to show ATP as the variable substrate also gave a series of parallel lines. This is indicative of a ping-pong

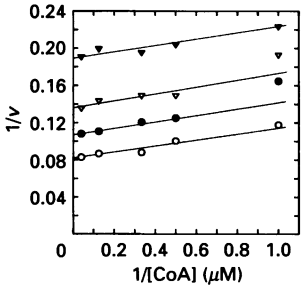



**Figure 1 Double reciprocal plot of reaction rates for variable concentrations of CoA versus variable concentrations of ATP at a fixed concentration of cholate**

CoA was varied from 1 to 25 $\mu$M as indicated for each of four different concentrations of ATP (mM): 0.15 (▼), 0.25 (▽), 0.4 (●) and 1.0 (○). The concentration of cholate was fixed at 10 $\mu$M. Reaction velocities are expressed as c.p.m. of cholyl-CoA formed per minute. Straight lines were drawn by hand.

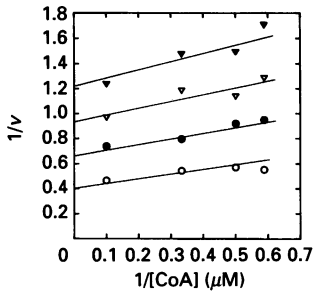



**Figure 2 Double reciprocal plot of reaction rates for variable concentrations of CoA versus variable concentrations of cholate at a fixed concentration of ATP**

CoA was varied from 1.7 $\mu$M to 10 $\mu$M as indicated for each of four different concentrations of cholate ($\mu$M): 1 (▼), 1.3 (▽), 2 (●) and 5 (○). The concentration of ATP was fixed at 1.0 mM. Reaction velocities are expressed as c.p.m. of cholyl-CoA formed per minute.

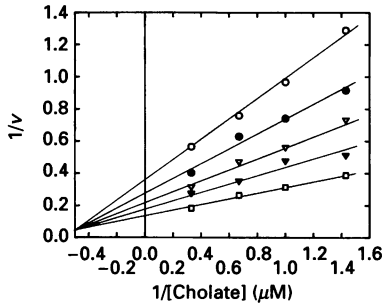



**Figure 3 Double reciprocal plot of reaction rates for variable concentrations of ATP versus variable concentrations of cholate at a fixed concentration of CoA**

Cholate was varied from 1.3 $\mu$M to 10 $\mu$M as indicated for each of five different concentrations of ATP (mM): 0.125 (○), 0.17 (●), 0.25 (▽), 0.4 (▼) and 1.0 (□). The concentration of CoA was fixed at 10 $\mu$M. Reaction velocities are expressed as c.p.m. of cholyl-CoA formed per minute.

type mechanism in which an irreversible step(s) exists between the binding of ATP and the binding of CoA to the enzyme [13], irrespective of the order in which the two bind. Irreversible steps are generated when a product is released from the enzyme prior to the binding of the next substrate [13]. When the concentration of ATP was held constant and CoA varied at several different fixed concentrations of cholate (Figure 2), a similar set of parallel plots was obtained. This indicates an irreversible step also occurs between cholate and CoA binding. Lastly, when CoA was held constant and ATP and cholate were varied, a set of converging lines was obtained (Figure 3). The fact that these lines are intersecting indicates that a reversible connection exists between the cholate and ATP binding steps.

The only substrate which generates a fragment other than $H^+$ or $OH^-$ in the course of the reaction is ATP, which is split into AMP and $PP_i$. Based on studies of other CoA-ligases, it seemed most likely that ATP and cholate bind to the enzyme first, and then before the binding of CoA, either AMP or $PP_i$ (or both) is released. To verify this mechanism one can remove the irreversible step by saturating the system with the released product [13]. When 0.2 mM $PP_i$ was added to the assays (Figure 4), the parallel plots, generated by varying CoA and cholate in turn, became convergent (intersecting); 5 mM AMP did not have this

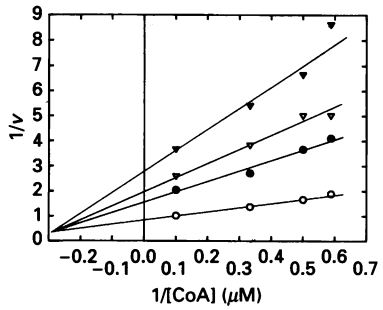



**Figure 4 Double reciprocal plot of reaction rates for variable concentrations of cholate versus variable concentrations of CoA at a fixed concentration of 1 mM ATP and 0.2 mM pyrophosphate**

Cholate was varied from 1.0 μM to 5.0 μM as indicated at each of four concentrations of CoA (μM): 1.8 (▼), 2.0 (▽), 3.0 (●) and 10 (○). ATP was fixed at 1 mM and pyrophosphate at 0.2 mM. Reaction velocities are expressed as c.p.m. of cholyl-CoA formed per minute.

**Table 1 The results of product and substrate inhibition studies**

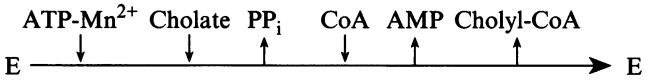
AMP and cholyl-CoA were tested as end-product inhibitors. The inhibition patterns were determined from double reciprocal plots of data obtained in experiments in which reaction rates were determined at several fixed concentrations of the indicated substrate both in the presence and absence of the inhibitor. Also, the substrate inhibition produced by high concentrations of CoA was analysed from double reciprocal plots obtained by varying the concentration of the test substrate at either 1 mM CoA (the optimum concentration) or 15 mM CoA (an inhibitory concentration).

| | Variable Substrate | | |
|---|---|---|---|
| Inhibitor | ATP | Cholate | CoA |
| AMP | C | M | UC |
| Cholyl-CoA | C* | C† | UC |
| Pyrophosphate | – | – | M |
| 15 mM CoA | C | M | – |

* At saturating concentrations of cholate.
† At saturating concentrations of ATP.

effect. This clearly indicates a ping-pong mechanism and establishes $PP_i$ as the first product released from the enzyme, and the sole product released before CoA binding. The same approach was tried with ATP versus CoA. However, in the presence of 0.2 mM $PP_i$ the 1/[ATP] plots were non-linear. This was thought to be due to the fact that $PP_i$ binds divalent cations as efficiently as ATP [14] and is thus competing with the ATP for $Mn^{2+}$. This is a possibility in the $PP_i$ experiments because it was necessary to keep the concentration of $Mn^{2+}$ relatively low (2–3 mM) to avoid the precipitation of $Mn^{2+}$–$PP_i$. Thus, as the concentration of ATP is varied there is a constantly shifting equilibrium of $Mn^{2+}$ between $Mn^{2+}$–$PP_i$, $Mn^{2+}$–ATP and $Mn^{2+}$–enzyme.

Since $PP_i$ is the sole product released before CoA binding, the AMP must be released after CoA binds. The cholyl-CoA necessarily must be released after CoA binding. The two products AMP and cholyl-CoA were utilized to probe the mechanism further by using them as product inhibitors. Product inhibition by pyrophosphate was also studied but its results are interpreted in the Discussion section. The results of the product inhibitions are shown in Table 1. Cholyl-CoA was an uncompetitive inhibitor versus CoA, which is consistent with a ping-pong mechanism. Product inhibition by cholyl-CoA yielded a mixed inhibition pattern versus both ATP and cholate in assays at non-saturating concentrations of alternate substrates. However, cholyl-CoA was a competitive inhibitor versus ATP in assays conducted with saturating concentrations of cholate ( > 10 μM) and CoA ( > 75 μM), and was also a competitive inhibitor versus cholate in the presence of saturating concentrations of ATP (4 mM) and CoA ( > 75 μM). This indicates that cholyl-CoA can bind at two different steps in the sequence. For cholyl-CoA to be competitive versus both ATP and cholate is suggestive of the following mechanism (Scheme 1).




**Scheme 1**

Cholyl-CoA is competitive versus ATP because they are both able to bind to the same enzyme form, free enzyme. The fact that ATP and cholyl-CoA do not form a dead-end ternary complex with the enzyme, which would give rise to a mixed inhibition pattern, is probably due to the fact that it is a ping-pong mechanism and thus the pyrophosphate leaving group is located in the ATP site in a way which overlaps the CoA site and thus CoA cannot bind properly until pyrophosphate has left. In this view, the binding of cholyl-CoA to free enzyme would sterically hinder ATP binding. It may in addition cause a conformational change that further interferes with ATP binding.

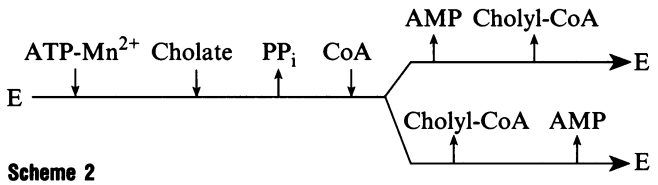
An ordered addition of substrates in which cholate binds first is precluded by the competitive inhibition by cholyl-CoA versus ATP, since if ATP did not bind until cholate had bound first, the bound cholate would block cholyl-CoA binding. Thus, an ordered ping-pong reaction with cholate binding first would be expected to give rise to a mixed inhibition by cholyl-CoA with respect to ATP, rather than a competitive inhibition pattern. Finally, if cholate bound first, cholyl-CoA would be expected to be a competitive inhibitor versus cholate even in the absence of saturating ATP, reflecting the fact that once cholate is bound there is no site available for cholyl-CoA binding. This is not the case and thus cholate must bind subsequent to ATP.

Consistent with the mechanism shown in Scheme 1, cholyl-CoA is an uncompetitive inhibitor versus CoA (Table 1). This indicates either that there is a need for AMP to reverse the sequence or that the sequence is irreversible. When AMP and cholyl-CoA were added simultaneously, the inhibition versus CoA appeared to become mixed although not decidedly so. This suggests that the step may be weakly reversible, but only in the presence of both products.

Product inhibition studies with AMP revealed that it is also an uncompetitive inhibitor with respect to CoA, which indicates that there is no reversible connection between AMP binding and CoA binding. In the absence of the other product the reaction cannot run backwards and thus neither product alone can be reversibly connected to CoA. The fact that AMP can bind in the absence of cholyl-CoA means that both AMP and cholyl-CoA can bind to free enzyme and this suggests that the release of these products from the enzyme is random. As noted above, when AMP and cholyl-CoA were added simultaneously, the inhibition versus CoA became mixed which is consistent with the sequential order of release of these two products.

Inhibition by AMP is competitive versus ATP (data not shown). Thus AMP can bind to the enzyme at the ATP site, even in the absence of cholyl-CoA. This also suggests that the binding and release of products is random and that AMP as well as cholyl-CoA can bind to free enzyme. AMP is a mixed inhibitor versus cholate which means that cholate and AMP do not bind

to the same enzyme form, and thus cholate does not bind to free enzyme [13]. This further indicates that the addition of substrates is not random, but is ordered with ATP binding first. The reaction is thus viewed as having the following mechanism:




**Scheme 2**

This mechanism can be termed: bi uni uni bi ping-pong with random product release.

As the concentration of CoA was increased above 1 mM, substrate inhibition was noted. This inhibition (result not shown, see summary in Table 1) was mixed versus cholate but was found to be competitive versus ATP. This is characteristic of a ping-pong mechanism and suggests that if CoA binds out of sequence, a portion of it overlaps the ATP site and prevents ATP binding. Thus, some fragment must be removed from the enzyme-bound ATP before CoA can bind with high affinity. When the concentration of ATP was increased above 5 mM in the presence of excess $Mn^{2+}$, it also became a substrate inhibitor. However, accurate data could not be obtained at these very high concentrations of ATP and $Mn^{2+}$ and thus no kinetic analysis of this inhibition was done.

To get an idea of the affinity of the end products for the enzyme, we analysed the inhibitions following the format of Dixon [15]. Dixon plots of cholyl-CoA inhibition versus ATP (at saturating cholate) and versus cholate (at saturating ATP) are shown in Figures 5 and 6 respectively. Both inhibition patterns are consistent with competitive inhibition. The $K_I$ value for cholyl-CoA competing with ATP (at saturating cholate) was 1 $\mu$M while the $K_I$ value for cholyl-CoA competing with cholate (at saturating ATP) was 2 $\mu$M. A Dixon plot of AMP competing with ATP is shown in Figure 7 and yields a $K_I$ value of 1.7 mM. A Dixon plot (data not shown) of AMP inhibition versus cholate gave a pattern consistent with a mixed inhibition, and yielded a $K_I$ value which was 2.0 mM. Dixon plots of $PP_i$ inhibition were non-linear.

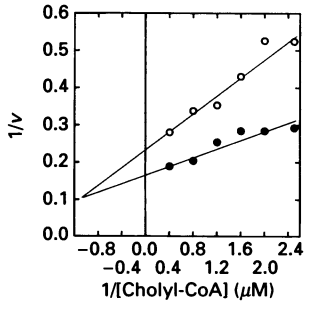



**Figure 5 Dixon plot of cholyl-CoA inhibition versus ATP in the presence of saturating cholate**

Cholyl-CoA was varied from 0.4 $\mu$M to 2.4 $\mu$M at each of two concentrations of ATP, 0.15 mM (○) and 0.4 mM (●), and in the presence of 10 $\mu$M cholate. The reciprocal of the reaction velocity (c.p.m. of cholyl-CoA formed per minute) is plotted versus the concentration of cholyl-CoA.

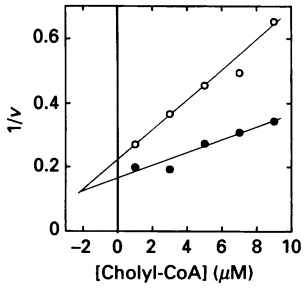



**Figure 6 Dixon plot of cholyl-CoA inhibition versus cholate in the presence of saturating ATP**

Cholyl-CoA was varied from 1.0 $\mu$M to 9.0 $\mu$M at each of two concentrations of cholate, 2 $\mu$M (○) and 6 $\mu$M (●), and in the presence of 4 mM ATP. The reciprocal of the reaction velocity (c.p.m. of cholyl-CoA formed per minute) is plotted versus the concentration of cholyl-CoA.

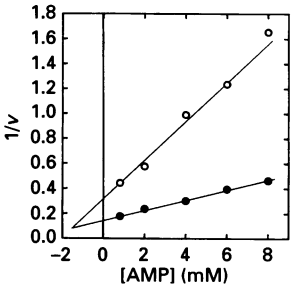



**Figure 7 Dixon plot of AMP inhibition versus ATP**

AMP was varied from 0.8 mM to 8.0 mM at each of two concentrations of ATP, 0.2 mM (○) and 1.0 mM (●), in assays containing 50 $\mu$M CoA and 5 $\mu$M cholate. The reciprocal of the reaction velocity (c.p.m. of cholyl-CoA formed per minute) is plotted versus the concentration of AMP.

## DISCUSSION

Most ATP-dependent pyrophosphorylytic ligases have a divalent cation requirement and follow a bi uni uni bi ping-pong mechanism. In this paper, kinetic analyses have been used to show that the microsomal bile acid:CoA ligase follows this mechanism. The ordered addition of substrates requires that ATP bind first, followed by cholate. The data indicate that at this point, $PP_i$ must be split off and released from the enzyme before CoA can be bound. The product inhibition studies indicate that, in contrast to the addition of substrates, which is ordered, the release of the products AMP and cholyl-CoA is random. Thus, the mechanism for the reaction appears to be a bi uni uni bi ping-pong with random release of products, as illustrated in Scheme 2.

There was one piece of data which appeared inconsistent with this mechanism. $PP_i$ should be a competitive end-product inhibitor versus CoA as both bind to the same enzyme form. But, as noted in Table 1, $PP_i$ is a mixed inhibitor versus CoA. This mixed inhibition could be the result of dead-end inhibition in which the CoA-saturated enzyme is still able to bind pyrophosphate and thus the inhibition could not be competed out. Alternatively, $PP_i$ may have a dual effect as suggested by the Dixon plots of $PP_i$ inhibition which are non-linear. This could result from $PP_i$ binding at two sites on the enzyme or it could be related to the divalent ion-binding capacity of $PP_i$.

Cholyl-CoA appears to bind to free enzyme with high affinity. This is based on the finding that the inhibition of ATP-binding by cholyl-CoA at saturating cholate is characterized by a low $K_I$ of 1 $\mu$M. This is very interesting since neither cholate nor CoA binds to free enzyme with high affinity. This implies a high affinity of the enzyme for the thioester linkage. The fact that cholyl-CoA binds at saturating cholate implies that this binding is probably via the CoA. Alternatively, at saturating ATP, cholyl-CoA binds to the enzyme–ATP complex and this binding must be at a different site which is not blocked by ATP. Presumably this site is the cholate site. The competitive inhibition versus cholate also shows a high binding affinity ($K_I = 2\ \mu$M) which is consistent with binding at the cholate or CoA site (see below).

Secondary plots of slopes or intercepts can often be used to obtain $K_m$ values. In the case of the bi uni uni bi ping-pong mechanism this is not the case [16]. This can be seen by the fact that a replot of the intercepts from the cholate versus CoA experiments gives an apparent $K_m$ ($K_m^{App.}$) for cholate of 6 $\mu$M while a replot of the intercepts from the cholate versus ATP gives a $K_m^{App.}$ value for cholate of 0.4 $\mu$M. However, these values do provide an approximation of the concentration ranges over which the enzyme is responsive to changes in concentration of cholate. Since the total unconjugated bile-acid concentration in the liver is $\approx 300\ \mu$M [17], the enzyme would be expected to be saturated with bile acid under physiological conditions. For ATP and CoA, the apparent $K_m$ values are in better agreement and thus one obtains a better approximation of the concentration ranges over which the enzyme is responsive to changes in substrate concentration. The $K_m^{App.}$ value for CoA obtained from replots of the intercepts of both the cholate and ATP plots is $\approx 4\ \mu$M. Since the cytosolic concentration of CoA is $\approx 50\ \mu$M [18], the enzyme appears to also be saturated with CoA under physiological conditions. The value for the $K_m^{App.}$ for ATP–$Mn^{2+}$ obtained from both the CoA and cholate plots is $\approx 200\ \mu$M. Although the physiological substrate is predominantly ATP–$Mg^{2+}$ rather than ATP–$Mn^{2+}$, the $K_m^{App.}$ for ATP–$Mg^{2+}$ appears to be identical with that for ATP–$Mn^{2+}$ (unpublished observation). The whole cell concentration of ATP is generally around 2 mM [19], but the hepatic concentration of $Mg^{2+}$ is only 1 mM [20] and much of this is bound to various sites. This means that the level of ATP–$Mg^{2+}$ is far less than 1 mM and thus the ligase is probably not saturated with respect to ATP. Furthermore, the concentration of ATP declines markedly under different physiological conditions [19], and thus the enzyme would appear to be responsive to changes in the concentration of ATP.

The ligase-catalysed reaction has been shown to have an absolute requirement for divalent cation [21]. This absolute requirement reflects the fact that it is the ATP–divalent cation complex, and not free ATP, that is the substrate for enzyme [21]. This is consistent with other ATP-dependent ligases. In addition, it was found that increasing the concentration of ATP beyond that of divalent cation led to a decrease in the rate due to ATP sequestering $Mn^{2+}$ from a secondary site [21]. Although the identity of this secondary site is not known, it is clear that it has a non-essential but rate enhancing role. This activation could result from binding directly to the enzyme either at a site which leads to a conformational change in the enzyme, or at an alternative area of the active site such as at the CoA binding site. Alternatively, activation might be related to binding to CoA.

According to the proposed mechanism (Scheme 2), the enzyme should catalyse a couple of characteristic partial reactions. For example, in the presence of ATP and cholate the enzyme should catalyse both the formation of $PP_i$ and the exchange of radiolabelled $PP_i$ into ATP. However, hepatic microsomes contain enzymes that interfere with these partial reactions (e.g., ATPase, ADPase and inorganic pyrophosphatase). Furthermore, such interfering activities are even present in the ligase-containing fraction of detergent-solubilized microsomes. Cholyl-CoA thiolase activity is also found in both microsomes and detergent extracts, which therefore rules out study of partial reactions in the reverse direction. Studies of the partial reactions can only be done with a highly purified preparation of enzyme. However, with a purified membrane-bound enzyme one has to be concerned that the properties of the enzyme have become altered over the course of the detergent extraction or the subsequent purification procedure. With the data from the current study, it will now be possible to evaluate thoroughly the properties of a purified preparation of enzyme and determine its usefulness for further kinetic studies.

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