Characterization of the reaction mechanism for the XL-I form of bovine liver xenobiotic/medium-chain fatty acid:CoA ligase

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The XL-I form of xenobiotic/medium-chain fatty acid:CoA ligase was purified to apparent homogeneity from bovine liver mitochondria and used to determine the reaction mechanism. A tersubstrate kinetic analysis was conducted by varying the concentrations of ATP, benzoate and CoA in turn. Both ATP and benzoate gave parallel double-reciprocal plots against CoA, which indicates a Ping Pong mechanism, with either pyrophosphate or AMP leaving before the binding of CoA. Addition of pyrophosphate to the assays changed the plots from parallel to intersecting; addition of AMP did not. This indicates that pyrophosphate is the product that leaves before binding of CoA. Based on end-product inhibition studies, it was concluded that the reaction follows a Bi Uni Uni Bi Ping Pong mechanism, with ATP binding first, followed in order by benzoate binding, pyrophosphate release, CoA binding, benzoyl-CoA release and AMP release. A similar mechanism was obtained when the ligase was examined with butyrate as substrate. However, butyrate activation was characterized by a much higher affinity for CoA. This is attributed to steric factors resulting from the bulkier nature of the benzoate molecule. Also, with butyrate there is a bivalent cation activation distinct from that associated with binding to ATP. This activation by excess Mg^{2+} results in non-linear plots of $1/v$ against $1/[ATP]$ for butyrate unless the concentrations of Mg^{2+} and ATP are varied together.

Key words: ATP, benzoate, butyrate, carboxylic acid, CoA.

INTRODUCTION

The xenobiotic/medium-chain fatty acid:CoA ligases (XM-ligases) catalyse the formation of the carboxylic acid-CoA thioesters of xenobiotic carboxylic acids and medium-chain fatty acids [1]. The ligases represent the rate-limiting step in the conjugation of carboxylic acid xenobiotics with amino acids [2–4], and the point of entry of medium-chain fatty acids into oxidation and esterification pathways [5]. We have isolated three forms of XM-ligase from bovine liver mitochondria [4].

Studies of the reaction [6] have indicated that it is ATP-dependent, with ATP being cleaved to AMP and pyrophosphate (PP_i):

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

It has been found that bivalent cations are essential, with Mg^{2+}, Mn^{2+} and Rb^{2+} being the most efficient [7].

Previous investigations of the reaction mechanism are inconclusive. Bar-Tana and Rose [8,9] found two forms of the enzyme butyrate:CoA ligase in an acetone powder preparation of a bovine liver particulate fraction. Fraction I [8] followed a Bi Uni Uni Bi Ping Pong mechanism, with ATP and butyrate binding first, while Fraction II [9] followed an ordered Ter Ter mechanism, with substrates binding in the order ATP, CoA, butyrate. Fraction I was also reported to exhibit co-operativity in the binding of ATP and CoA [8]. Graham and Park [10], using a similar preparation of the enzyme and octanoate as substrate, also found a Bi Uni Uni Bi Ping Pong mechanism, but with CoA binding first followed by octanoate; in addition, octanoyl-CoA was released before ATP binding. This lack of agreement prompted us to re-examine the mechanism of reaction for the bovine liver XM-ligases using much more stable preparations obtained by avoiding the use of organic solvents during their isolation. Further, we examined the enzyme mechanism both with a short-chain fatty acid (butyrate) and with a xenobiotic (benzoate).

MATERIALS AND METHODS

[1-^{14}C]Benzoic acid and [1-^{14}C]butyric acid were obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). AMP, ATP, benzoyl-CoA, butyryl-CoA, CoA, dithiothreitol, tetrasodium EDTA, Trizma, leupeptin, chymostatin and pepstatin A were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium pyrophosphate was obtained from Matheson, Coleman and Bell (Norwood, OH, U.S.A.), and contained less than 0.001 % heavy metals.

The XL-I ligase was isolated from bovine liver mitochondria as described previously [11,12]. The purified enzyme gave a single band on SDS/PAGE. The enzyme was stored at −80 °C and lost approx. 10 % activity per month, but more if repeatedly thawed and refrozen. The loss in activity was not accompanied by any change in kinetic behaviour, and thus appeared to be an all-or-none inactivation of the enzyme.

Ligase activity was assayed radiochemically by measuring the formation of radiolabelled [^{14}C]carboxyl-CoA from the respective [^{14}C]carboxylic acid. A standard reaction tube contained 100 mM Tris/HCl, pH 8.0 at 30 °C, 50 mM KCl, the indicated concentrations of CoA, $MgCl_2$, carboxylic acid, and ATP, and enzyme protein in a volume of 0.5 ml. For benzoate:CoA ligase activity, aliquots of 0.1 ml were removed and added to 0.4 ml of 12 mM tetrasodium EDTA/30 mM succinic acid (pH 4.5), extracted twice with butanol, and counted for radioactivity as described previously [4]. For butyrate:CoA ligase activity, the assay was the same as above, except that the aliquots removed at timed intervals were added to 0.4 ml of 12 mM tetrasodium EDTA/200 mM succinic acid (pH 3.4) and then extracted as above.

Abbreviation used: XM-ligase, xenobiotic/medium-chain fatty acid:CoA ligase.

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RESULTS

Benzoate:CoA ligase activity

The XL-I form of bovine liver XM-ligase was used for all kinetic analyses. The ligase catalyses a ter-reactant reaction with an additional bivalent cation requirement for ATP binding. Accordingly, the concentration of Mg^{2+} used in assays was always a minimum of 2-fold in excess of the concentration of ATP.

Analysis of the reaction mechanism was first conducted with benzoate as the carboxylic acid substrate. A tersubstrate kinetic analysis of the reaction was conducted by varying the concentration of ATP, benzoate and CoA in turn. The concentration of CoA was varied at each of several different fixed concentrations of ATP, with benzoate constant. The data were plotted in double-reciprocal form (Lineweaver–Burk plots; Figure 1), which provided a series of apparently parallel lines. Re-plots of the data with ATP as the variable substrate also gave a series of parallel lines (results not shown). This is indicative of a Ping Pong 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 before binding of the next substrate. When the concentration of ATP was held constant and CoA was varied at several different fixed concentrations of benzoate (Figure 2), a similar set of parallel plots was obtained. This indicates that an irreversible step also occurs between the binding of benzoate and that of CoA. Lastly, when CoA was held constant and ATP and benzoate 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 benzoate and ATP binding steps.

The only substrate that 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 benzoate bind to the enzyme first, and then, before the binding of CoA, that 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 0.5 mM PP_i was added to the assays (Figure 4), the parallel plots, generated by varying CoA and benzoate in turn, became convergent (intersecting); 5 mM AMP did not have this effect. This clearly indicates a Ping Pong mechanism, and establishes PP_i as the first product that is released from the enzyme, and the sole product released before CoA binding. The same approach was tried with ATP versus CoA, and in the presence of 0.2 mM PP_i the 1/[ATP] plots at different concentrations of CoA became intersecting (results not shown). Since PP_i is the sole product released before CoA binding, the AMP must be released after CoA binds. The benzoyl-CoA necessarily must be released after CoA binds.

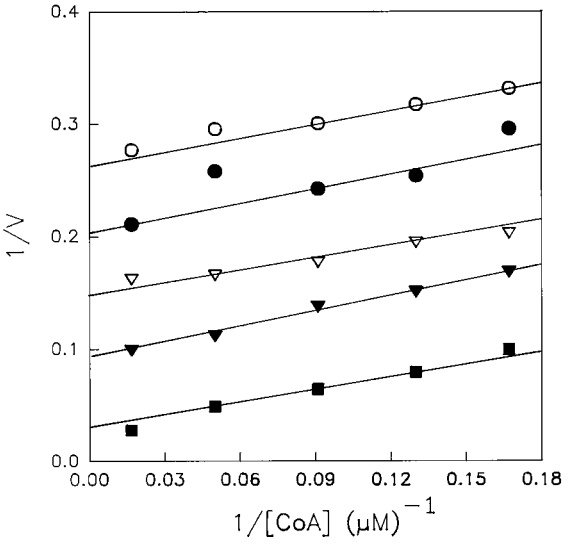


Figure 1 Double-reciprocal plot of reaction rates for variable concentrations of CoA against variable concentrations of ATP at a fixed concentration of benzoate

CoA was varied from 6 μM to 60 μM as indicated, for each of five different concentrations of ATP (μM): 5 (○), 6.7 (●), 10 (▽), 16.7 (▼) and 80 (■). The concentration of benzoate was fixed at 20 μM. Reaction velocities are expressed as nmol of benzoyl-CoA formed/min per ml of enzyme solution.

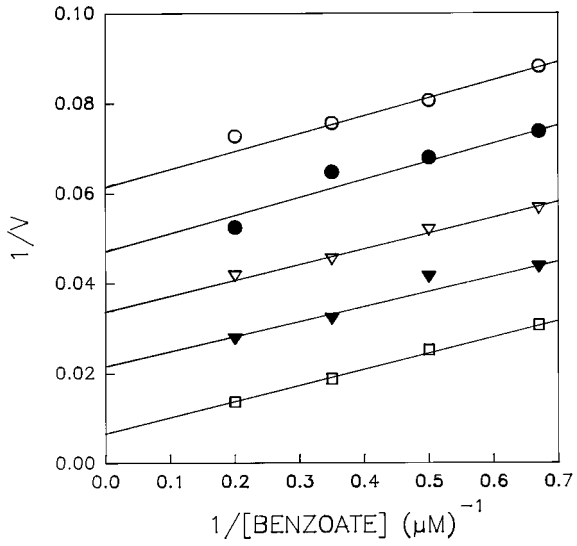


Figure 2 Double-reciprocal plot of reaction rates for variable concentrations of benzoate against variable concentrations of CoA at a fixed concentration of ATP

Benzoate was varied from 1.5 μM to 25 μM as indicated, for each of five different concentrations of CoA (μM): 15 (○), 20 (●), 28.6 (▽), 50 (▼) and 300 (□). The concentration of ATP was fixed at 3 mM. Reaction velocities are expressed as nmol of benzoyl-CoA formed/min per ml of enzyme solution.

The two products, AMP and benzoyl-CoA, were utilized as product inhibitors in order to discern the order of addition of ATP and benzoate. The results of the product inhibitions are shown in Table 1. Benzoyl-CoA was a competitive inhibitor with respect to CoA, which means that it binds to the same enzyme form as CoA, i.e. the enzyme·benzoate·AMP complex, giving rise to dead-end inhibition. Benzoyl-CoA was a mixed inhibitor with respect to benzoate at low concentrations of benzoate ($< 5\ \mu$M), but was an uncompetitive inhibitor at higher concentrations of benzoate. The uncompetitive inhibition is consistent with the existence of an irreversible connection between the binding of ATP and the binding of benzoyl-CoA to the inhibitory site. The mixed inhibition at low concentrations of benzoate is due to low-affinity binding at the benzoate site. Benzoyl-CoA was also an uncompetitive inhibitor with respect to ATP. Product inhibition by AMP yielded a competitive inhibition pattern with respect to ATP and mixed inhibition with respect to benzoate. However, AMP was an uncompetitive inhibitor with respect to CoA. PP_i was also a product inhibitor, although a relatively

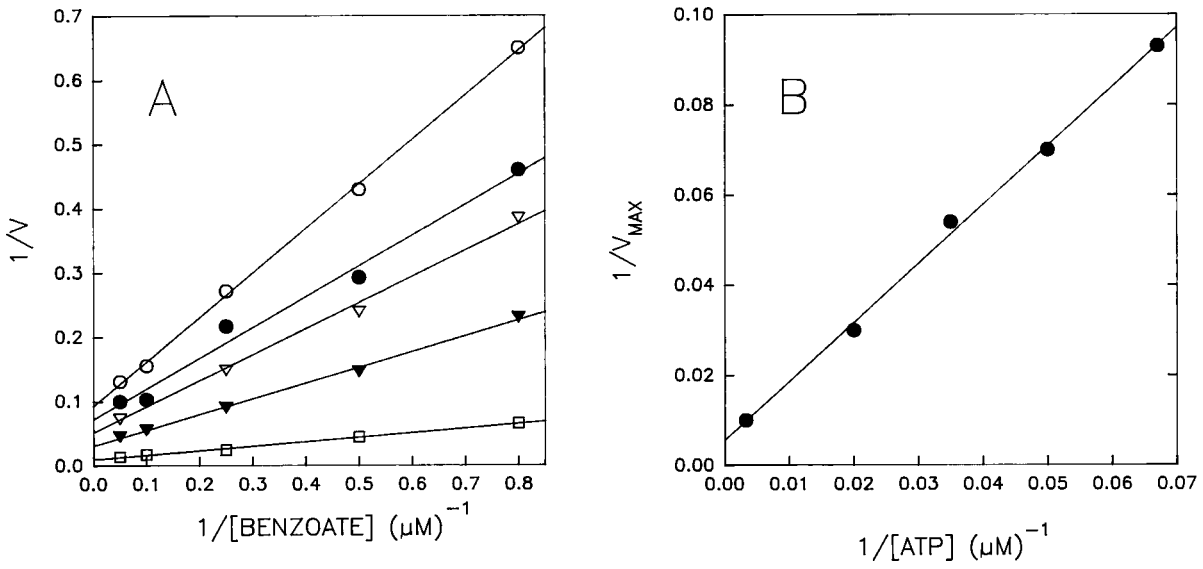


Figure 3 Double-reciprocal plots of reaction rates for variable concentrations of benzoate against variable concentrations of ATP at a fixed concentration of CoA

Benzoate was varied from 1.25 μM to 20 μM as indicated, for each of five different concentrations of ATP (μM): 15 (○), 20 (●), 28.6 (∇), 50 (▼) and 300 (□). The concentration of CoA was fixed at 0.5 mM. Reaction velocities are expressed as nmol of benzoyl-CoA formed/min per ml of enzyme solution, and are plotted in double-reciprocal form in (**A**). A secondary plot of the activity at V_{max} against the concentration of ATP is shown in (**B**).

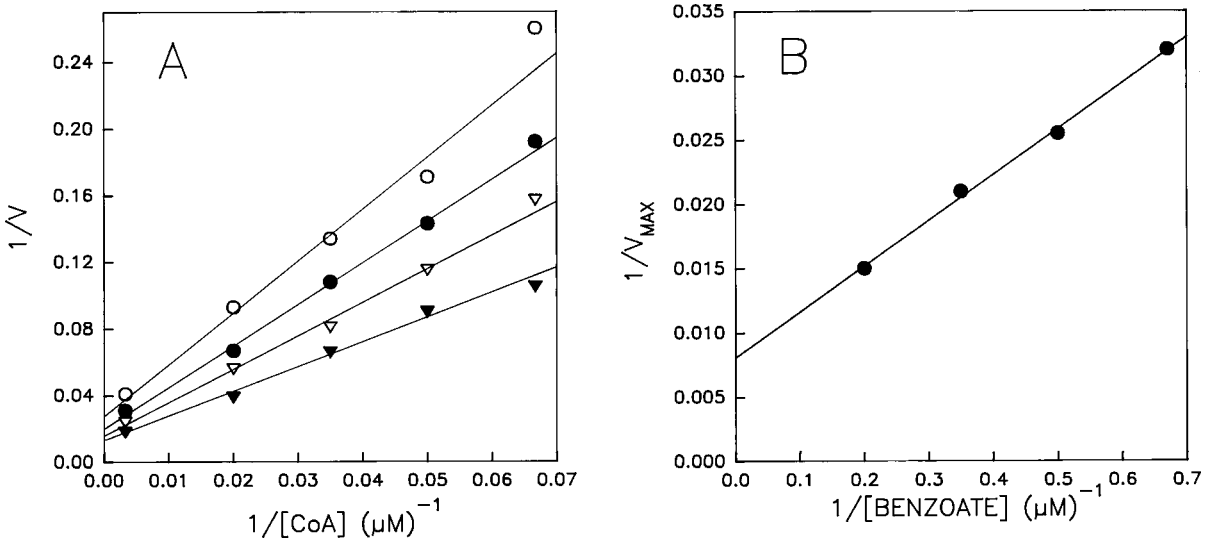


Figure 4 Effect of PP_i on double-reciprocal plots of reaction rates for variable concentrations of CoA against variable concentrations of benzoate at a fixed concentration of ATP

All assays contained 0.5 mM PP_i. CoA was varied from 15 μM to 300 μM as indicated, for each of four different concentrations of benzoate (μM): 5 (○), 6.7 (●), 10 (∇) and 16.7 (▼). The concentration of ATP was fixed at 3 mM. Reaction velocities are expressed as nmol of benzoyl-CoA formed/min per ml of enzyme solution, and are plotted in double-reciprocal form in (**A**). A secondary plot of the activity at V_{max} against the concentration of benzoate is shown in (**B**).

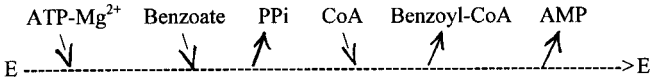
weak one, and acted as a competitive inhibitor with respect to CoA and a mixed inhibitor with respect to benzoate.

Since AMP is a competitive inhibitor with respect to ATP, a mixed inhibitor with respect to benzoate and an uncompetitive inhibitor with respect to CoA, this suggests the Bi Uni Uni Bi Ping Pong mechanism shown in Scheme 1. AMP is a competitive inhibitor with respect to ATP because both bind to the same enzyme form, i.e. free enzyme. AMP is a mixed inhibitor with respect to benzoate because there are reversible connections between their binding steps, but it is uncompetitive with respect to CoA because of the absence of a reversible connection between their binding steps. According to Scheme 1, benzoyl-CoA should not bind to the enzyme in the absence of AMP. The fact that binding does occur would suggest that benzoyl-CoA is binding abnormally. Indeed, the fact that it is a competitive inhibitor with respect to CoA indicates that it is binding as a

Table 1 End-product inhibition studies of the benzoate and butyrate reactions

The inhibition pattern was determined from double-reciprocal plots of data obtained in experiments in which reaction rates were measured at several concentrations of the indicated substrate both in the presence and in the absence of the inhibitor. The results were defined as competitive (C), uncompetitive (UC) or mixed (M).

	Type of inhibition					
	Benzoate			Butyrate		
Inhibitor	ATP	Benzoate	CoA	ATP	Butyrate	CoA
AMP	C	M	UC	C	M	UC
Benzoyl-CoA	UC	UC	C	–	–	–
Butyryl-CoA	–	–	–	C	M	UC
PP_i	M	M	C	C	M	M



Scheme 1 Suggested mechanism of action of XM-ligase

dead-end inhibitor to the same enzyme form as CoA, i.e. to the enzyme·AMP·benzoate complex. This dead-end complex has no reversible connection to the benzoate binding step, and thus benzoyl-CoA is an uncompetitive inhibitor of the binding of both ATP and benzoate. PP_i is a competitive inhibitor with respect to CoA because they bind to the same enzyme form.

An ordered addition of substrates in which benzoate binds first is precluded by the product inhibition pattern. If benzoate bound first, followed by ATP, then either benzoyl-CoA would have been a competitive inhibitor with respect to benzoate (if benzoyl-CoA were the last product released) or AMP would have been a mixed inhibitor with respect to ATP (if AMP were the last product released). Product inhibition studies with AMP revealed that it is an uncompetitive inhibitor with respect to CoA, an indication that there is no reversible connection between AMP binding and CoA binding. In the absence of benzoyl-CoA, the reaction cannot run backwards, and thus the binding of AMP cannot be reversibly connected to the binding of CoA. The fact that AMP can bind in the absence of benzoyl-CoA means that AMP can bind to free enzyme. The inability of benzoyl-CoA to compete with either ATP or benzoate suggests that it cannot bind to free enzyme in a functional way. This provides evidence that the release of these products from the enzyme is ordered, with AMP released last. As noted above, when AMP and benzoyl-CoA were added simultaneously, the inhibition with respect to CoA became mixed, which is consistent with the sequential order of release of these two products.

Also consistent with an ordered addition of substrates with ATP binding first is the fact that AMP is a mixed inhibitor with respect to benzoate. This suggests that benzoate and AMP do not bind to the same enzyme form, and therefore that benzoate does not bind to free enzyme. The reaction is thus viewed as having the mechanism shown in Scheme 1.

Substrate inhibition by CoA was noted for concentrations of CoA in excess of four times the concentration of ATP. The CoA inhibition pattern (results not shown) was of a mixed type with respect to benzoate, but competitive with respect to ATP. This is characteristic of a Ping Pong mechanism and suggests that, if CoA binds out of sequence, a portion of the molecule overlaps into the ATP site and prevents binding of ATP. Thus the overlapping fragment has to be discharged from the enzyme-bound ATP before CoA can bind with high affinity.

To get an idea of the affinities of end products for the enzyme, we analysed the inhibition data according to the method of Dixon [14] (results not shown). The K_i value for benzoyl-CoA competing with CoA (at a saturating benzoate concentration) was 500 μM. A Dixon plot of AMP competing with ATP yields a K_i value of 235 μM. A Dixon plot of AMP inhibition with respect to benzoate gave a pattern consistent with mixed inhibition, and yielded a K_i value of 2.0 mM.

The data in Figures 1–3 were analysed to determine the apparent K_m [K_m (app)] values for ATP, benzoate and CoA. Replots of the intercepts against the concentration of variable substrate gave a K_m (app) for ATP of 200 μM, for benzoate of 7 μM and for CoA of 60 μM.

Butyrate:CoA ligase activity

Several key features of the kinetics with butyrate as substrate were noticeably different from those obtained with benzoate. First, with butyrate as substrate, the K_m (app) for CoA was approx. 10-fold lower. Secondly, under conditions similar to those used for benzoate, specifically at a fixed concentration of Mg^{2+} of 5 mM, double-reciprocal plots of $1/v$ against 1/[ATP] are non-linear. This non-linearity disappears if the concentration of Mg^{2+} is not fixed at 5 mM, but instead varies in proportion to the ATP concentration. Thus it does not appear to be due to allosteric effects in ATP binding, but rather is related to the fact that the butyrate reaction is stimulated by Mg^{2+} in excess of that needed to complex with ATP (D. A. Vessey and M. Kelley, unpublished work), which is not the case for the benzoate reaction [7]. Thus, in experiments with a fixed concentration of Mg^{2+}, as the concentration of ATP is increased the concentration of unbound Mg^{2+} is decreased; hence the activating effect of Mg^{2+} is lost and 1/[ATP] plots are non-linear.

A further contribution to the non-linearity of 1/[ATP] plots was found with butyrate as substrate. Thus an unidentified water-soluble butyryl adduct, which is not butyryl-CoA, can be formed in low amounts. This adduct has similar extraction properties to butyryl-CoA and thus cannot be distinguished from butyryl-CoA in our assay. The formation of this adduct is apparent in butyrate reactions conducted in the presence of ATP but in the absence of CoA. In the presence of CoA, its contribution to overall rates of formation of radioactive adducts from [^{14}C]butyrate is minimized. However, due to the low K_m (app) for ATP (approx. 10 μM) for this side-reaction, at low concentrations of ATP (< 100 μM) this reaction may also contribute to the rates of reaction measured by our radioassay technique, provided that the reaction contains excess Mg^{2+}, since this reaction is stimulated several-fold by Mg^{2+} in excess of that necessary for chelating with ATP. Thus, at low ATP and high Mg^{2+}, this minor reaction may contribute to the non-linearity of the 1/[ATP] plots for the butyrate reaction. Benzoate does not give rise to an equivalent side-reaction.

The reaction mechanism with butyrate as substrate was studied in the same manner as with benzoate, with the exception that, in experiments in which the concentration of ATP was varied, the Mg^{2+}/ATP ratio was maintained constant at 1:1. Lineweaver–Burk plots for CoA against ATP (Figure 5) and CoA against butyrate (Figure 6) gave parallel lines, which could be converted into intersecting lines in the presence of PP_i. A Lineweaver–Burk plot of variable ATP against variable butyrate was intersecting. This, in conjunction with the product inhibition patterns (Table

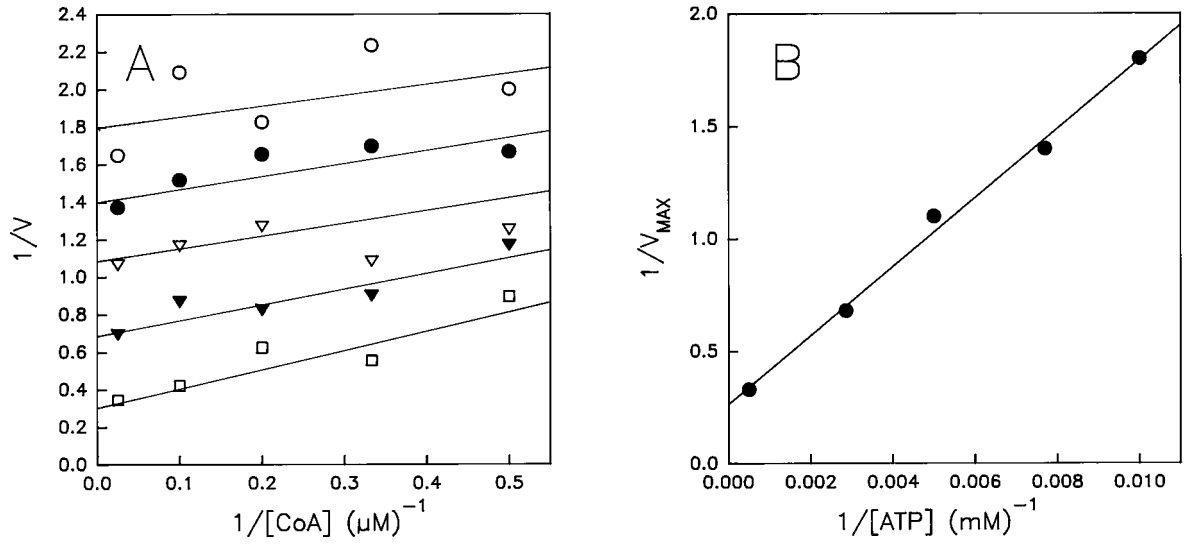


Figure 5 Double-reciprocal plot of reaction rates for variable concentrations of CoA against variable concentrations of ATP at a fixed concentration of butyrate

CoA was varied from 1.5 μM to 40 μM. The concentrations of ATP and Mg^{2+} were varied equivalently, and were (mM): 0.10 (○), 0.13 (●), 0.20 (▽), 0.35 (▼) and 2.00 (□). The concentration of butyrate was fixed at 100 μM. Reaction velocities are expressed as nmol of butyryl-CoA formed/min per ml of enzyme solution, and are plotted in double-reciprocal form in (**A**). A secondary plot of the activity at V_{max} against the concentration of ATP is shown in (**B**).

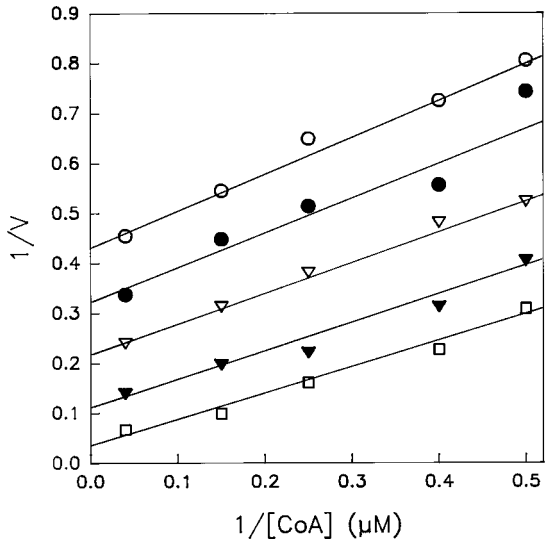


Figure 6 Double-reciprocal plot of reaction rates for variable concentrations of CoA against variable concentrations of butyrate at a fixed concentration of ATP

CoA was varied from 2 μM to 25 μM as indicated, for each of five different concentrations of butyrate (μM): 30 (○), 40 (●), 56 (▽), 100 (▼) and 300 (□). The concentration of ATP was fixed at 3 mM and that of Mg^{2+} at 5 mM. Reaction velocities are expressed as nmol of butyryl-CoA formed/min per ml of enzyme solution.

1), suggests the same Bi Uni Uni Bi Ping Pong mechanism as seen with benzoate. What was different from the benzoate mechanism was that butyryl-CoA bound at the butyrate binding site (a competitive inhibitor with respect to butyrate), as opposed to the CoA site. Also, PP_i was a mixed inhibitor with respect to CoA, rather than competitive as found for benzoate. This may be due to the smaller butyrate molecule allowing the binding of both CoA and PP_i as a dead-end complex.

Re-plots of the kinetic data for the butyrate reaction revealed that K_m (app) values for CoA were 4–13 μM. These are significantly lower than those with benzoate [K_m (app) values for CoA of 60–250 μM]. Also, CoA was found to cause substrate inhibition in the butyrate reaction. This occurred for concentrations of CoA above approx. 0.3 mM at standard concentrations of ATP (< 0.3 mM). At higher concentrations of ATP, higher concentrations of CoA were required for substrate inhibition.

We investigated the alternative substrate inhibition patterns for butyrate and benzoate. Benzoate was a competitive inhibitor of butyrate activation at both low and high ATP concentrations. Likewise, butyrate was a competitive inhibitor of benzoate at both low and high concentrations of ATP. This suggests a common binding site. Further, for benzoate and butyrate activation alike, benzoyl-CoA was a competitive inhibitor with respect to CoA, but a mixed inhibitor with respect to both benzoate and butyrate. Butyryl-CoA was a competitive inhibitor with respect to both butyrate and benzoate, with the same K_i values.

DISCUSSION

We have used tersubstrate kinetic analyses to show that the XL-I form of XM-ligase follows a Bi Uni Uni Bi Ping Pong mechanism, regardless of whether benzoate or butyrate is used as the carboxylic acid substrate. Most ATP-dependent pyrophosphatolytic ligases follow a Ping Pong mechanism. The ordered addition of substrates occurs, with ATP binding first, then benzoate. This is followed by pyrophosphatolytic cleavage of ATP and the release of PP_i. PP_i must be released from the enzyme before CoA can bind. The product inhibition studies indicate that the release of the products is also ordered, with benzoyl-CoA being released before AMP can be released. Thus the mechanism for the reaction appears to be a Bi Uni Uni Bi Ping

Pong mechanism, with the order of addition being ATP, benzoate/butyrate and CoA, as illustrated in Scheme 1.

In the absence of AMP, benzoyl-CoA can only bind as a dead-end inhibitor at the CoA binding site; bound benzoate does not block benzoyl-CoA binding. The fact that ATP and benzoyl-CoA do not form a dead-end ternary complex with the enzyme (which would give rise to a mixed rather than an uncompetitive inhibition pattern) is probably related to the Ping Pong mechanism. Thus the PP_i leaving group is located in the ATP site, where it overlaps the CoA site. Hence CoA cannot bind properly until PP_i has been discharged. Since benzoate cannot bind until ATP has bound, and since benzoyl-CoA cannot occupy the benzoate site until AMP has bound, the binding of the adenine nucleotide to the enzyme must cause a conformational change that opens up the benzoate binding site.

Using partially purified preparations of enzyme extracted from acetone powders of ox liver, Bar-Tana and Rose [8,9] isolated two enzyme fractions with butyrate ligase activity, Fraction I and Fraction II. For their Fraction I, they reported the same Ping Pong mechanism for butyrate activation [8] that we obtained with XL-I. However, they also reported co-operativity in the binding of ATP to the ligase, based on non-linear (upward bending) double-reciprocal plots of $1/v$ against 1/[ATP]. However, their data were obtained over a very narrow and high range of ATP concentrations (6.7–12 mM). Our data clearly demonstrate linear plots in this concentration range. The non-linearity of their kinetics is likely to be due to Mg^{2+} depletion. In their experiments, the concentrations of Mg^{2+}-binding compounds (protein at up to 0.1 mg/ml, ATP at 6.7–12 mM and butyrate at 71 mM) greatly exceeded the concentration of Mg^{2+} (14 mM). Thus, in this range, increases in ATP concentration do not directly translate into increases in $ATP \cdot Mg^{2+}$ concentration, and the rates will be lower than expected at higher ATP concentrations (upward bending plots).

However, with butyrate as substrate, we did obtain non-linear but downward bending plots of $1/v$ against 1/[ATP] at low concentrations of ATP (< 0.06 mM), provided that the concentration of Mg^{2+} was held fixed at 3–5 mM. This same protocol gave rise to linear plots with benzoate as substrate. The basis for the non-linearity with butyrate as substrate is related to the excess Mg^{2+}. Thus if the concentrations of Mg^{2+} and ATP are varied together, either at equal concentrations or at a constant ratio of 2:1 (Mg^{2+}/ATP), then the 1/[ATP] plots are linear. Thus the non-linearity does not appear to represent an allosteric effect in the binding of ATP to the enzyme. This seems reasonable, because ATP binds to the enzyme first, and thus its binding is unaffected by whether the carboxylic acid substrate is to be benzoate or butyrate. Therefore the same ATP binding behaviour is expected for both substrates. It is possible, though, that excess Mg^{2+} opens up a second ATP site for butyrate activation. Alternatively, excess Mg^{2+} may enable the enzyme to catalyse the synthesis of an alternative water-soluble butyryl derivative, such as butyryl-AMP or butyryl phosphate, which would not be discriminated from butyryl-CoA in our radioassay. Indeed, the low-level formation of such an adduct was noted in butyrate reactions conducted in the absence of CoA.

Bar-Tana and Rose [8,9] also reported co-operativity in the binding of CoA, based on non-linear double-reciprocal plots. Again, using more sensitive assays, we obtained linear plots over a wide range of CoA concentrations, provided that the concentration of CoA did not exceed that of ATP. Substrate inhibition by CoA results when its concentration exceeds that of ATP. Thus the enzyme does not appear to show co-operativity in the binding of any substrate.

Bar-Tana and Rose reported that their Fraction II enzyme followed an ordered Ter mechanism, with ATP binding first [9]. It is possible that the ordered mechanism arose as a result of partial denaturation caused by the use of acetone to extract the enzyme. It would not have been due to the presence of XL-III, as we have done a preliminary kinetic analysis of XL-III and found that it also follows a Ping Pong mechanism.

Graham and Park [10] used octanoate as substrate in a kinetic characterization of the enzyme from an acetone preparation of ox liver. Using solely product inhibition patterns, they also reported a Bi Uni Uni Bi Ping Pong mechanism, but with CoA binding first followed by octanoate, and then the release of octanoyl-CoA prior to ATP binding. This is not in agreement with our more extensive characterization. However, their analysis was limited by their reliance solely on product inhibition studies. Further, their data were obtained using a very insensitive assay, with an impure preparation of enzyme.

The ligase-catalysed reaction was found to have another difference between butyrate and benzoate. Most notable was a > 10-fold lower K_m (app) for CoA in the butyrate reaction. For example, the K_m (app) values for CoA were in the range 4–13 μM for butyrate activation, compared with 60–250 μM for benzoate. This difference might be due to steric factors, with the larger benzoate molecule partially occluding the CoA binding site.

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