A Product-Inhibition Study of the Mechanism of Mitochondrial Octanoyl-Coenzyme A Synthetase

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By a study of the product-inhibition kinetics of the octanoyl-CoA synthetase from ox liver mitochondria, evidence was obtained consistent with the hypothesis that the enzyme reacts by a Bi Uni Uni Bi Ping Pong type of mechanism in which the order of addition and evolution of substrates and products is CoA, octanoate, octanoyl-CoA, ATP, PP_i and AMP. There is also evidence that more than one molecule of CoA can add to the enzyme and that it may act as an allosteric activator.

During studies of the inhibition of fatty acid oxidation by salicylate, the mechanism of addition of reactants to octanoyl-CoA synthetase was investigated kinetically by using the product-inhibition technique (Cleland, 1963*a,b*).

Acyl-CoA synthetases (EC 6.2.1.x) catalyse the reaction:

and enzymes specific for short-, medium- and long-chain fatty acids have been isolated from a number of sources (Jencks, 1962).

The most widely accepted mechanism of fatty acid activation is that of Berg (1956), who, on the basis of studies with the enzyme from yeast, suggested that the formation of acetyl-CoA by acetyl-CoA synthetase occurs in two stages:

Evidence for this formulation arose from exchange studies of isotopically labelled reactants and products, from the ready enzymic synthesis from acetyl-AMP of ATP in the presence of PP_i and of acetyl-CoA in the presence of CoA, and from the formation of acetylhydroxamic acid from ATP, acetate and hydroxylamine in the absence of added CoA. Webster & Campagnari (1962) and Webster (1963) have also presented evidence for the presence of enzyme-bound acetyl-AMP in the reaction.

Evidence has been obtained that the medium-chain (Jencks & Lipmann, 1957) and long-chain (Vignais & Zabin, 1958) acyl-CoA synthetases follow a similar mechanism to the short-chain enzyme.

The results of the present investigation are not consistent with the mechanism of Berg (1956), and

a novel mechanism for this enzymic reaction is presented.

MATERIALS AND METHODS

Materials. ATP (99% pure), AMP (98% pure), bovine serum albumin (fraction V) and CoA were obtained from Sigma (London) Chemical Co. Ltd. (London, S.W. 6). The purity of the CoA preparation (85–90%) was assessed by using the nitroprusside procedure of Mahler, Wakil & Bock (1953), assuming the value of the molar extinction coefficient of CoA at 520 mμ given by them. Octanoic acid (British Drug Houses Ltd., Poole, Dorset) was redistilled at atmospheric pressure, the fraction boiling at 236–237° being used. All other reagents used in the assay procedures were of A.R. grade, if available. The pH of reagent solutions was adjusted with KOH or HCl.

Purification of octanoyl-CoA synthetase. The enzyme was purified from fresh ox liver essentially by the method of Mahler *et al.* (1953) to the stage before the treatment with alumina C₉. The enzyme fraction used thus corresponds to fractions C+C-1 of Mahler *et al.* (1953) after dialysis. The enzyme was kept as a concentrated solution in 0.02 M-KHCO₃ at 1°.

Determination of protein. The protein content of solutions was determined by the modified Folin method of Miller (1959), with bovine serum albumin as standard.

Determination of octanoyl-CoA synthetase activity. All kinetic measurements were carried out at 38 ± 0.1°.

Initially, during the purification of the enzyme, its activity was measured by the release of PP_i in the presence of ATP and octanoate, with hydroxylamine as acyl acceptor. However, the specific activity apparently decreased on purification when determined by this method, whereas the opposite was the case when the nitroprusside method (Grunert & Phillips, 1951; Mahler *et al.* 1953), in which CoA is the acyl acceptor, was used. A fivefold increase in activity was noted when 0.42 mM-CoA was included in the hydroxylamine assay medium to permit the intermediate formation of octanoyl-CoA. A similar dependence on the presence of CoA of the activity of a microsomal palmitoyl-CoA synthetase was reported by Bar-Tana & Shapiro (1964).

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Most of the determinations of enzymic activity were made by using the nitroprusside method (Mahler *et al.* 1953), in which CoA is the acyl acceptor, with a Unicam SP.600 spectrophotometer. The reaction medium, unless otherwise stated, was composed of CoA (0.85 mM), ATP (5.5 mM), octanoate (10 mM), $MgCl_2$ (12.5 mM), glycylglycine-KOH buffer, pH 8.0 (37.5 mM), KBH_4 (5.0 mM) and enzyme (0.02–0.05 ml. containing 0.42–0.63 mg. of protein), in a total volume of 0.2 ml.

For measurements of enzymic activity in the presence of various CoA concentrations, the formation of octanoyl-CoA was measured by the increase in ultraviolet absorption by the method given by Mahler *et al.* (1953). The optimum wavelength was 224.5 $m\mu$. Preliminary measurements showed that there was a linear relationship between the activity of the enzyme as measured by this procedure and the enzyme concentration. A similar reaction medium to that in the nitroprusside assay was used. Extinction measurements were made with a Unicam SP.800 spectrophotometer.

Data processing. Measurements of reaction velocities in the presence of various substrate concentrations were carried out in pairs, i.e. in the presence and absence of inhibitor. Data from these measurements were initially plotted as reciprocal plots (Lineweaver & Burk, 1934). When these

were linear, the data were fitted to eqn. (1) by using a least-squares method (Wilkinson, 1961) and on the assumption of equal variance for the experimental velocities:

$$v = Vs/(K+s) \quad (1)$$

The calculations were performed by the Univac 1108 computer of the National Engineering Laboratory, East Kilbride, by using a Fortran IV programme providing values of $1/V$, K/V , K , V and V/K and the standard errors (Cleland, 1967).

The effects of inhibitors on the slopes and intercepts of the reciprocal plots, i.e. on $1/V$ and K/V , were assessed separately by using Student's *t* test (Cleland, 1967), two values being considered to be significantly different when $P < 0.05$. In this way the most probable pattern of inhibition could be determined.

RESULTS

The calculated values of the slopes and intercepts of the reciprocal plots in the presence of various concentrations of ATP and octanoate and when inhibited by AMP and PP_i are given in Table 1. Also given in Table 1 are the standard errors of the

Table 1. *Kinetic results obtained with various ATP and octanoate concentrations in the presence and absence of PP_i or AMP*

Results were obtained over the concentration ranges 0.2–1.0 mM for ATP and 0.1–0.5 mM for octanoate. Further details are given in the text.

Varied substrate	Product inhibitor	Concn. of inhibitor (mM)	No. of points	$1/V \pm \text{s.e.}$ [($\Delta E/10 \text{ min.}$) ⁻¹]	$K/V \pm \text{s.e.}$ [mM($\Delta E/10 \text{ min.}$) ⁻¹]	Type of inhibition
ATP	AMP	0	4	9.1 ± 1.5	6.81 ± 0.88	Uncompetitive
		0.5	5	18.2 ± 2.0 ($P < 0.02$)	6.9 ± 1.2 ($P > 0.1$)	
ATP	AMP	0	5	6.17 ± 0.92	4.68 ± 0.65	
		0.5	4	17.0 ± 2.6 ($P < 0.02$)	6.7 ± 1.4 ($P > 0.1$)	
ATP	AMP	0	4	8.40 ± 0.54	2.59 ± 0.28	
		0.5	4	16.4 ± 1.9 ($P < 0.02$)	3.66 ± 0.96 ($P > 0.1$)	
ATP	PP_i	0	4	8.21 ± 0.34	3.40 ± 0.22	Non-competitive
		0.5	4	15.6 ± 0.87 ($P < 0.01$)	6.71 ± 0.47 ($P < 0.01$)	
ATP	PP_i	0	4	7.2 ± 1.1	4.40 ± 0.83	
		0.5	5	16.7 ± 1.2 ($P < 0.01$)	8.56 ± 0.80 ($P < 0.02$)	
Octanoate	AMP	0	4	12.1 ± 3.0	2.85 ± 0.85	Non-competitive
		0.5	4	22.5 ± 1.2 ($P < 0.05$)	3.20 ± 0.32 ($P > 0.1$)	
Octanoate	AMP	0	4	9.91 ± 0.54	2.35 ± 0.19	
		0.5	4	20.42 ± 0.97 ($P < 0.001$)	5.29 ± 0.27 ($P < 0.001$)	
Octanoate	PP_i	0	5	10.2 ± 1.1	1.41 ± 0.32	Uncompetitive
		0.5	4	27.5 ± 1.1 ($P < 0.001$)	1.80 ± 0.30 ($P > 0.1$)	
Octanoate	PP_i	0	4	8.76 ± 0.29	3.24 ± 0.10	
		0.5	4	12.80 ± 0.94 ($P < 0.02$)	3.25 ± 0.32 ($P > 0.1$)	

Table 2. *Kinetic results obtained with various CoA concentrations from apparently linear portions of the reciprocal plots in the presence or absence of AMP or PP_i*

Range of CoA concn. (mM)	Product inhibitor	Concn. of inhibitor (mM)	No. of points	$1/V \pm \text{s.e.}$ [[$\Delta E/10 \text{ min.}$] ⁻¹]	$K/V \pm \text{s.e.}$ [mM($\Delta E/10 \text{ min.}$) ⁻¹]
0.4-1.0	AMP	0	4	1.30 ± 0.12	1.01 ± 0.09
		0.5	3	1.12 ± 0.76 ($P > 0.1$)	1.73 ± 0.64 ($P > 0.1$)
0.6-1.2	AMP	0	4	-0.098 ± 0.227	1.85 ± 0.58
		1.0	4	-0.161 ± 0.367 ($P > 0.1$)	2.43 ± 0.67 ($P > 0.1$)
0.4-1.2	PP _i	0	4	0.60 ± 0.28	1.72 ± 0.27
		1.0	4	3.23 ± 0.39 ($P < 0.02$)	1.70 ± 0.28 ($P > 0.1$)

Fig. 1. Inhibition of octanoyl-CoA synthetase by AMP (1.0 mM) in the presence of various CoA concentrations. (a) Plot of $1/v$ against $1/[\text{CoA}]$; (b) plot of $1/v$ against $1/[\text{CoA}]^2$. ○, No AMP present; ●, AMP present.

estimates of each of the values and the value of P calculated by Student's t test for each pair of constants. In all cases where a significant difference is claimed between two values, in at least one of the duplicate or triplicate determinations $P < 0.02$. In one of the experiments in which AMP was the inhibitor in the presence of various octanoate concentrations, the error in the value of one of the slopes was too great to indicate that there was a significant difference between the two values. However, a duplicate experiment indicated very clearly that the difference was significant.

Experiments were carried out in which the concentration of CoA was varied. In this case plots of $1/v$ against $1/[\text{CoA}]$ consistently showed deviations from linearity towards higher values of $1/v$ at high values of $1/[\text{CoA}]$, i.e. low values of $[\text{CoA}]$. At low values of $1/[\text{CoA}]$ the plot was apparently linear, and these values were analysed as described above. The results given in Table 2 show that the differences between the values obtained in the presence

and absence of AMP are not significant, although it is noteworthy that the values of the intercepts in the presence of AMP are lower than those in its absence. This, together with the presence of a negative intercept in the plot obtained with 1 mM-AMP, indicates that the points in question are in fact part of a curve under the conditions of these experiments. The values obtained in the presence and absence of PP_i indicate uncompetitive inhibition.

The results obtained with various CoA concentrations in the presence and absence of AMP approximated more closely to linearity when $1/v$ was plotted against $1/[\text{CoA}]^2$. One of these plots, of the results in the presence and absence of 1 mM-AMP, is shown in Fig. 1(b), together with the reciprocal plot (Fig. 1a) for comparison. Both sets of experiments, with 0.5 mM- and 1.0 mM-AMP, when plotted in this way, indicated that the inhibition was competitive.

Variation of the octanoate concentration in the presence of two concentrations of ATP indicated a

Table 3. *Effect of different ATP concentrations in the presence of various octanoate concentrations on the kinetic parameters*

The concentration range for octanoate was 0.1–0.5 mM.

Concn. of ATP (mM)	No. of points	$1/V \pm \text{s.e.}$ $[(\Delta E/10 \text{ min.})^{-1}]$	$K/V \pm \text{s.e.}$ $[\text{mM}(\Delta E/10 \text{ min.})^{-1}]$
5.5	4	9.20 ± 0.66	1.25 ± 0.16
1.1	4	12.16 ± 0.75 ($P < 0.05$)	1.56 ± 0.20 ($P > 0.1$)

significant effect on the intercept of the reciprocal plot only (Table 3).

DISCUSSION

The linearity of the reciprocal plots, apart from those in the presence of various concentrations of CoA, is consistent with a non-random mechanism, at least over the concentration ranges considered in this work. The deviation from linearity of the reciprocal plot in the presence of various CoA concentrations indicates that CoA may act as an allosteric activator of the system. Thus effects arising from a possible allosteric activation of the enzyme by CoA appear to be superimposed on a basic mechanism.

From the types of product inhibition observed, this basic mechanism cannot be of the Ordered Ter Ter type (Cleland, 1963a,c). The observation that the slopes of plots of $1/v$ against $1/[\text{octanoate}]$ in the presence of two different concentrations of ATP are not significantly different (Table 3) is also evidence against this mechanism, but is consistent with a Ping Pong reaction (Cleland, 1963a).

The mechanism of Berg (1956) can be considered to be of the Bi Uni Uni Bi Ping Pong type, which can be represented (Cleland, 1963a) as shown in Scheme 1, in which A and B represent ATP and acetate, Q and R represent AMP and acetyl-CoA, though not necessarily in that order, P represents pyrophosphate, and C represents CoA. However, the product-inhibition patterns obtained by a consideration of the rate equation for this type of mechanism (Cleland, 1963a), or alternatively by the inspection method given by Cleland (1963c), assuming Berg's (1956) mechanism, are not consistent with those observed in this work. Of the possible Bi Uni Uni Bi Ping Pong patterns, only that shown in Scheme 2 gives the inhibition observed (Table 4). The observed data do not allow a distinction to be made between this mechanism and the limiting case in which the concentration of free enzyme form E is zero, i.e. where there is a synchronous attack by CoA and departure of AMP from the active centre. This mechanism would, however, appear unlikely on steric grounds if it is of an S_N2 type.

Scheme 1.

The results given in Table 3 are also consistent with this mechanism.

The mechanism of Scheme 2 involves the reaction of a 'high-energy' form (E) of the enzyme with CoA and octanoate, resulting in their conversion into octanoyl-CoA and a 'low-energy' form (F) of the enzyme. Reaction of this latter form with ATP brings about the re-formation of the 'high-energy' form with hydrolysis of the ATP to AMP and PP_i. Studies on succinyl-CoA synthetase from ox liver mitochondria (Cha, Cha & Parks, 1965) and from *Escherichia coli* (Moyer, Ramaley, Butler & Boyer, 1967; Ramaley, Bridger, Moyer & Boyer, 1967) have indicated that these enzymes can be converted into 'high-energy' forms by exposure to succinyl-CoA or to CoA and the appropriate nucleotide triphosphate, and the mechanism of their action suggested (Upper, 1964; Cha *et al.* 1965) is similar in a number of respects to that proposed here.

The main evidence for Berg's (1956) mechanism has arisen from the reactivity of acyl-AMP compounds, isotope-exchange studies (Berg, 1956; Jencks & Lipman, 1957) and the isolation of an acyl-AMP from a mixture of enzyme and substrates in the absence of CoA (Webster & Campagnari, 1962; Webster, 1963).

Results obtained with acyl-AMP compounds as substrates must be interpreted with some caution. These compounds possess free energies of hydrolysis considerably higher than that of ATP (for acetyl-AMP at pH 8 some 7000–8000 cal./mole above that of ATP; Jencks, 1957), and are non-enzymic acylating reagents for a number of the groups present in proteins, including imidazole (Jencks, 1957) and amino groups (Castelfranco, Moldave & Meister, 1958). Severin & Scharkova (1967) noted that preincubation of acetyl-CoA synthetase with acetyl-AMP

Scheme 2.

Table 4. Comparison with that observed of product inhibition patterns expected from some Bi Uni Uni Bi Ping Pong mechanisms

Columns 3–6 are the patterns expected from various permutations of Berg’s (1956) mechanism applied to Scheme 1, with C=CoA and P=PP₁. Column 7 gives the pattern expected from Scheme 2. Abbreviations: Comp, competitive; UC, uncompetitive; NC, non-competitive.

Varied Substrate	Product inhibitor	Theoretical product-inhibition pattern				Proposed mechanism	Pattern observed
		A=ATP B=RCO ₂ H Q=RCOCOA R=AMP	A=ATP B=RCO ₂ H Q=AMP R=RCOCOA	A=RCO ₂ H B=ATP Q=RCOCOA R=AMP	A=RCO ₂ H B=ATP Q=AMP R=RCOCOA		
ATP	AMP	Comp	UC	NC	UC	UC	UC
ATP	PP ₁	NC	NC	NC	NC	NC	NC
Octanoate	AMP	NC	UC	Comp	UC	NC	NC
Octanoate	PP ₁	NC	NC	NC	NC	UC	UC
CoA	AMP	UC	NC	UC	NC	Comp	Comp
CoA	PP ₁	Comp	Comp	Comp	Comp	UC	UC

caused a considerable decrease in enzymic activity, and concluded that, in part at least, this was caused by acetylation of the enzyme. In view of the high reactivity of acyl-AMP compounds, and of their structural analogy to ATP, it seems reasonable to postulate that the formation of ATP on incubation of medium-chain acyl-AMP compounds with octanoyl-CoA synthetase arises from the acyl-AMP acting as an artificial substrate rather than as an enzyme-bound intermediate, the ATP being produced by the process (see Scheme 2):

The form E·AMP can then react with PP₁ as indicated in Scheme 2.

Jones, Lipmann, Hilz & Lynen (1953), by isotope-exchange studies with acetyl-CoA synthetase from yeast, suggested a mechanism for the action of the enzyme essentially similar to that presented here. Berg (1956), using a more highly purified enzyme preparation, was unable to confirm their observations, and from his own results concluded that the reaction course was via an acyl-AMP intermediate. Except for his observation that the incorporation of labelled acetate into acetyl-CoA by the enzyme required the presence of both AMP and PP₁, these

isotope-exchange results of Berg (1956) can be reconciled with the mechanism of Scheme 2 if it is assumed (a) that CoA is tightly bound to the enzyme and (b) that the enzyme used by him was composed mainly of the form E·CoA of Scheme 2.

Observations have been made by other workers in favour of these assumptions. Bar-Tana & Shapiro (1964) obtained evidence for the tight binding of CoA to their microsomal palmitoyl-CoA synthetase. Incubation of the enzyme with ATP and CoA, resulting in the conversion of the enzyme into the E·CoA form, with possibly a further molecule of CoA being bound at an allosteric site, and passage of the mixture through a Sephadex G-50 column, resulted in elution of the enzyme together with CoA but free of ATP. This would indicate that the position of the equilibrium between E and E·CoA is well over towards E·CoA.

These authors also noted that the acylation of hydroxylamine in the hydroxamate assay was dependent on the presence of CoA in the system. Observations indicating a similar conclusion were made by the present authors with octanoyl-CoA synthetase. Berg (1956), however, found that acetylhydroxamic acid was formed from ATP and acetate by acetyl-CoA synthetase in the apparent absence of CoA. Assuming that CoA is required for this reaction to take place, it would appear likely

that enzyme-bound CoA was present in Berg's (1956) preparation, although he could find no evidence for the presence of CoA in the solution phase after denaturing the enzyme.

The present authors are unable to reconcile the findings of Webster & Campagnari and of Webster (1963) who, however, used acetyl-CoA synthetase, with the mechanism presented in this paper.

With regard to the nature of the proposed allosteric activation, there is insufficient evidence to decide whether the allosterism is partial, with two alternative pathways, the more kinetically favoured one (which has been considered in this paper) being catalysed by enzyme allosterically activated by CoA, or total, in which adsorption of a molecule of CoA at the allosteric site is obligatory before reaction takes place at the active site (Mahler & Cordes, 1966). A further possibility is that the addition of CoA and fatty acid to the active site is a random process, the kinetically more favoured path adding CoA first (Ferdinand, 1966). To distinguish between these possibilities more extensive data are required.

Note added after submission. Since the submission of this paper, Bar-Tana, Rose & Shapiro (1968) and Bar-Tana & Rose (1968a,b) have published evidence indicating that mitochondrial medium-chain fatty acyl-CoA synthetase, prepared as in this paper, consists of two enzymes: a fraction I, apparently following Berg's (1956) mechanism and exhibiting allosteric properties, and a fraction II, with kinetics consistent with an ordered Ter Ter mechanism but lacking allosteric properties. Their results appear to contradict those presented here.

Had the enzyme fraction used in the present work contained significant amounts of a different enzyme catalysing the same reaction, non-linear reciprocal plots would have been expected. Apart from the results obtained with various concentrations of CoA, all the reciprocal plots appeared linear. The enzyme used in the present work appears to be identical with the fraction I described by Bar-Tana & Rose (1968a).

The present results were obtained at significantly higher CoA concentrations than those of Bar-Tana & Rose (1968a), and it is probable that different kinetic patterns would be observed at lower CoA concentrations. Indeed, the initial-velocity patterns with the natural substrates of the enzyme, namely fatty acid, CoA and ATP, presented by these authors for fraction I are consistent with the mechanism of the present paper if it is assumed that a modifier molecule, which can be either CoA or ATP, depending on their relative concentrations, is adsorbed at the allosteric site after addition of CoA to the E form of the enzyme but before the addition of a molecule of fatty acid. The present authors

consider that kinetic results obtained with acyl-AMP compounds must be treated with reserve in view of their doubtful role as intermediates in this reaction.

Since Bar-Tana *et al.* (1968) report that on standing at 0° fraction II can increase at the expense of fraction I, it seems probable that fraction II is a degraded form of fraction I, and thus it would be surprising if the mechanism of reaction of fraction II differed radically from that of fraction I.

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