Studies on Medium-Chain Fatty Acyl-Coenzyme A Synthetase

PURIFICATION AND PROPERTIES

BY J. BAR-TANA, G. ROSE AND B. SHAPIRO

*Department of Biochemistry, The Hebrew University-Hadassah Medical School,
 Jerusalem, Israel*

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1. Medium-chain fatty acyl-CoA synthetase (EC 6.2.1.2) was isolated by the method of Mahler, Wakil & Bock (1953) and the enzyme activity determined by the disappearance of CoA in the presence either of butyrate and ATP or of butyryl-AMP, as well as by ATP formation from butyryl-AMP and PP_i. 2. Preincubation of the enzyme with CoA and ATP alone or together, followed by the removal of these substrates by gel filtration, caused a marked inhibition of ATP formation, contrary to results previously obtained with palmitoyl-CoA synthetase. 3. The effect of ATP on butyryl-AMP-dependent CoA disappearance was inconsistent. Low concentrations of ATP (0.1–0.5 mM) always caused inhibition, whereas higher concentrations (5–10 mM) activated in some enzyme preparations and inhibited in others. 4. This inconsistency was shown to be due to the presence of two enzyme fractions. Both fractions had similar activities when assayed by the butyryl-AMP- or butyrate-plus-ATP-dependent CoA disappearance. However, fraction I was activated by ATP as measured by butyryl-AMP-dependent CoA disappearance whereas fraction II was inhibited by it. Fraction I also catalysed ATP formation from butyryl-AMP and PP_i whereas fraction II was lacking in such activity. 5. The relationship of these observations with respect to other known mechanisms of fatty acid-activating systems is discussed.

The synthesis of fatty acyl-CoA by the overall reaction:

proceeds according to Berg (1956) by the following two reaction steps:

In studies on the palmitoyl-CoA synthetase (Bar-Tana & Shapiro, 1964) it was found that the enzyme was activated by preincubation with ATP and CoA. On the other hand, preincubation with palmitoyl-AMP, or with palmitate and ATP, in the absence of free CoA caused inactivation. Two forms of the enzyme were assumed to exist, an active one involving enzyme-bound CoA, and an inactive one devoid of CoA. In support of this view Rao & Johnston (1966, 1967) reported activation by the simultaneous presence of CoA and ATP of the glyceride synthetase complex isolated from hamster intestine. The enzyme thus activated catalysed the

transfer of fatty acid to fatty acid triglyceride in the absence of added CoA, and combination of CoA with the enzyme was suggested. In studies on succinyl-CoA synthetase (Cha, Cha & Parks, 1967; Upper, 1964; Moyer & Smith, 1966) formation of enzyme-bound CoA has also been shown.

For further elucidation of the nature of the effects of CoA and ATP on the palmitoyl-CoA synthetase, purified enzyme preparations were required. However, the activity of microsomal palmitoyl-CoA synthetase was found to be highly unstable after purification (Bar-Tana & Shapiro, 1964). Medium-chain fatty acyl-CoA synthetase purified by the method of Mahler, Wakil & Bock (1953), and subsequently further by gel filtration and DEAE-Sephadex ion-exchange chromatography, was therefore selected for further investigations, to serve as a possible model for the study of the mechanism involved in long-chain fatty acid activation.

The present paper deals with the behaviour of the enzyme towards ATP and fatty acid substrates in the presence and absence of CoA, and its significance with regard to the mechanism of reaction of the acyl-CoA synthetases.

EXPERIMENTAL

Source of enzyme. Butyryl-CoA synthetase was isolated from ox liver particle acetone-dried powder by the method of Mahler *et al.* (1953). The $(\text{NH}_4)_2\text{SO}_4$ 'fraction C' described by these authors was used in the present study since treatment with alumina or calcium phosphate gel resulted in no further purification in our hands. Since the stability of the enzyme was found to improve in a reducing environment, mercaptoethanol at a final concentration of 2.8 mM was added before the $(\text{NH}_4)_2\text{SO}_4$ fractionation. The purified enzyme fraction was stored at 0° in concentrated form in 0.02 M- KHCO_3 solution containing 2.8 mM-mercaptoethanol.

Further purification of enzyme. A 10 ml. portion of $(\text{NH}_4)_2\text{SO}_4$ fraction (protein concentration about 100 mg./ml.) was subjected to gel filtration on a Bio-Gel P-300 column (1.8 cm. $\times$ 80 cm.) equilibrated with a buffer containing tris-HCl buffer, pH 7.6 (5 mM), KHCO_3 (50 mM) and mercaptoethanol (0.04 mM). The enzyme was eluted with the same buffer, the activity emerging in a single peak (300 mg./45 ml.) following a large peak of inactive protein. Samples (about 10 ml.) of the active fraction were then applied to a DEAE-Sephadex column (1.8 cm. $\times$ 60 cm.) equilibrated with the same buffer. Two protein peaks were eluted with this buffer, and a third peak emerged at 0.1 M-KCl on stepwise elution with increasing amount of KCl in the buffer. All three peaks showed enzyme activity. Other protein peaks that emerged when the KCl concentration was further increased were devoid of enzyme activity.

Determination of enzyme activity. Enzyme activity was determined by measuring the substrate-dependent disappearance of CoA with Ellman (1958) reagent [5,5'-dithiobis-(2-nitrobenzoic acid)]. The reaction mixture contained in a final volume of 0.5 ml.: 50 μ moles of tris-HCl buffer, pH 8.0, 7 μ moles of MgCl_2 , 0.1 μ mole of CoA, between 50 and 300 μ g. of enzyme, and either 1 μ mole of butyryl-AMP or 5 μ moles of sodium butyrate plus 3–5 μ moles of ATP. After incubation at 37° for 10 min., 0.5 ml. of 0.2 M-sodium phosphate buffer, pH 8.0, was added, followed by 0.03 ml. of Ellman reagent (4 mg./ml. in 0.2 M-sodium phosphate buffer, pH 7.4). Reaction mixtures without the fatty acid substrates (butyryl-AMP or butyrate) served as controls. As ATP caused a certain decrease in colour intensity, care was taken to add ATP at similar concentrations in control experiments.

The decrease in colour due to disappearance of CoA was measured at 413 m μ in a Beckman DU spectrophotometer.

The molar extinction coefficient given by CoA under these conditions was $1.36 \times 10^4 \text{ cm}^{-1}$. Enzyme specific activity was expressed in μ moles of CoA disappeared/mg. of enzyme protein/10 min. Enzyme activity was also measured by the formation of ATP from butyryl-AMP and pyrophosphate, as described for palmitoyl-CoA synthetase by Bar-Tana & Shapiro (1964). The incubation mixture contained in a final volume of 1 ml.: 50 μ moles of tris-HCl buffer, pH 7.5, 4.2 μ moles of MgCl_2 , 20 μ moles of NaF, 2 μ moles of PP_i , 2 μ moles of butyryl-AMP, 15 μ moles of glucose, 0.25 unit of hexokinase and 100–300 μ g. of enzyme. After incubation for 15 min. at 30° the reaction was terminated by addition of 0.15 ml. of 5 M-acetic acid, and the mixture was boiled for 1 min., neutralized with KOH and centrifuged. The amount of glucose 6-phosphate formed was determined by treating a sample of the supernatant with 1 μ mole of NADP⁺ and 2 units of glucose 6-phosphate dehydrogenase and measuring the increase in extinction at 340 m μ . Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with albumin as a standard, or in the more purified fractions by the method of Warburg & Christian (1941).

Materials. Butyryl-AMP was prepared by the method of Lee Peng (1956). ATP (disodium salt) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.: CoASH and NADP⁺ were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; hexokinase and glyceraldehyde phosphate dehydrogenase were from C. F. Boehringer und Soehne G.m.b.H. or Sigma Chemical Co. Pyrophosphatase was prepared by the method of Heppel & Hilmo (1955). Bovine albumin fraction V was obtained from Pentex Inc., Kankakee, Ill., U.S.A.; Sephadex G-50 and DEAE-Sephadex were from Pharmacia, Uppsala, Sweden; Bio-Gel was from Calbiochem, Los Angeles, Calif., U.S.A.; 5,5'-dithiobis-(2-nitrobenzoic acid) was from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. All reagents were of A.R. grade.

RESULTS

The enzymic activity of the ammonium sulphate fraction was determined by three methods (Table 1): (1) the disappearance of CoA dependent on butyryl-AMP (reaction *b* above); (2) the disappearance of CoA dependent on butyrate and ATP (overall reaction); (3) the formation of ATP from butyryl-AMP and PP_i (reversal of reaction *a*).

Table 1. Comparison of specific enzyme activities of partially purified acyl-CoA synthetase ('fraction C' described by Mahler *et al.* 1953)

Reaction mixtures were as described in the Experimental section. The results were obtained with three different enzyme preparations (1, 2 and 3).

Assay	Enzyme specific activity		
	Prep. 1	Prep. 2	Prep. 3
(1) CoA disappearance (μ mole/mg. of protein/10 min.)			
(a) Butyrate + ATP as substrate	0.22	0.07	0.07
(b) Butyryl-AMP as substrate	0.44	0.44	0.45
(2) ATP formation (μ mole/mg. of protein/10 min.)	0.58	0.20	0.26

The degree of purification obtained by the different fractionation steps was similar to that found by Mahler *et al.* (1953), though the actual specific activities of the overall reaction differed because of the different assay conditions. Enzyme specific activities as measured by reaction (b) tended to be constant in preparations derived from several acetone-dried powders, whereas specific activities as assayed by the overall reaction, or the reversal of reaction (a), showed considerable variations.

Effect of enzyme preincubation with CoA and ATP on ATP formation. Bar-Tana & Shapiro (1964) demonstrated an activation of palmitoyl-CoA synthetase, as measured by ATP formation, by preincubation of the enzyme in the presence of both CoA and ATP. A similar experiment was carried out on an enzyme fraction (equivalent to 'fraction C' described by Mahler *et al.* 1953) by preincubation of the enzyme in the presence of CoA or ATP, either alone or together. The enzyme was subsequently passed through a column of Sephadex G-50 to remove excess of CoA and ATP and assayed for ATP formation from butyryl-AMP and PP_i. As shown in Table 2, preincubation of enzyme with either CoA or ATP alone caused a substantial decrease in enzyme specific activity. When the enzyme was preincubated in the presence of both substrates no activation occurred, contrary to the findings with microsomal palmitoyl-CoA synthetase, and enzyme specific activities were even

lower than obtained after preincubation with each substrate alone.

Effect of ATP on butyryl-AMP-dependent CoA disappearance. Activation of the enzyme by the simultaneous presence of CoA and ATP was next attempted, by measuring the butyryl-AMP-dependent CoA disappearance in the presence of ATP. As ATP is neither substrate nor product in this reaction step, it did not have to be removed after preincubation. Addition of small amounts of ATP to the incubation mixture inhibited in all preparations. In some cases the inhibition could be reversed by further increasing the amount of ATP, until at high ATP concentrations CoA disappearance rose well above the value obtained in the absence of ATP. In other preparations the activating effect of ATP was absent and the latter continued to inhibit at all concentrations. These results are illustrated in Fig. 1.

The inhibitory effect of ATP was further investigated and found to be specific for ATP. ADP inhibited much less and AMP not at all when tested at concentrations similar to those used for ATP (Table 3). Hydrolysis of ATP abolished its inhibitory effect. Increasing concentrations of Mg²⁺ decreased somewhat the inhibitory effect of ATP. Thus 20mM-magnesium chloride caused only a 61% inhibition as compared with inhibitions of 78% and 74% in the presence of 10mM- and 14mM-magnesium chloride respectively. To find out whether the effect of ATP was due to appearance

Table 2. *Effect of preincubation of ammonium sulphate fraction with various amounts of CoA and ATP on ATP formation*

The preincubation mixture consisted of: tris-HCl buffer, pH 7.5, 100mM; MgCl₂, 3mM; NaF, 5mM; NaBH₄, 10mM; enzyme (see the text), 1.8mg.; CoA and ATP as stated; the total volume was 0.4ml. Preincubation was for 10min. at 30°. After passage of the enzyme through Sephadex G-50 to remove free CoA and ATP, the formation of ATP was measured as described in the Experimental section.

Expt. no.	Addition to preincubation mixture	ATP formation (μmole/mg. of protein/15 min.)
1	None	0.58
	CoA (0.1 μmole)	0.43
	CoA (0.2 μmole)	0.38
	CoA (0.4 μmole)	0.39
	CoA (0.6 μmole)	0.38
2	None	0.53
	ATP (5.0 μmoles)	0.33
	ATP (10.0 μmoles)	0.25
	ATP (15.0 μmoles)	0.22
	ATP (20.0 μmoles)	0.20
	CoA (0.12 μmole)	0.40
	CoA (0.12 μmole) + ATP (5.0 μmoles)	0.25
	CoA (0.12 μmole) + ATP (10.0 μmoles)	0.28
	CoA (0.12 μmole) + ATP (15.0 μmoles)	0.18
	CoA (0.12 μmole) + ATP (20.0 μmoles)	0.15

Fig. 1. Effect of ATP on butyryl-AMP-dependent disappearance of CoA catalysed by two different enzyme preparations (● and Δ) ('fraction C'; see the text). Conditions were as described in the Experimental section with additions of ATP as shown.

Table 3. *Specificity of the inhibitory effect of ATP on butyryl-AMP-dependent CoA disappearance*

Conditions were as described in the Experimental section.

Addition to incubation mixture	CoA disappearance (μmole/mg. of protein/10 min.)
None	0.405
ATP (0.4 μmole)	0.130
ATP (0.4 μmole) after acid hydrolysis (7 min. at 100° at pH 1-2) and reneutralization	0.408
ADP (0.4 μmole)	0.374
AMP (0.4 μmole)	0.405
ATP (0.4 μmole) added at end of incubation	0.398

of PP_i , which is a powerful inhibitor of reaction (b) (assuming that PP_i might have been formed by the overall reaction from ATP and small amounts of butyrate contaminating the substrate), purified pyrophosphatase was added to the reaction mixture. As illustrated in Table 4, pyrophosphatase only slightly affected the ATP inhibition, though it completely abolished inhibition by PP_i in reaction (b).

Studies with pure enzyme fractions. To resolve the dual effect of ATP on butyryl-AMP-dependent CoA disappearance (step b reaction), further purification of the enzyme was attempted. Gel filtration on Bio-Gel P-300 and subsequent DEAE-Sephadex chromatography resolved the enzyme into three distinct active fractions. The relative enzyme activities of the three fractions assayed by reaction (b) are shown in Table 5. The fractions

Table 4. *Effect of pyrophosphatase on inhibition of enzyme activity by ATP or PP_i*

Conditions were as described in the Experimental section.

Addition to incubation mixture	CoA disappearance (as measured by partial reaction b) (μmole/mg. of protein/10 min.)
None	0.57
PP_i (2 μmoles)	0.15
Pyrophosphatase (0.225 unit)	0.535
PP_i (2 μmoles) + pyrophosphatase (0.225 unit)	0.535
ATP (4 μmoles)	0.058
ATP (4 μmoles) + pyrophosphatase (0.225 unit)	0.083

could be distinguished by their different behaviour forwards ATP, which activated fraction I (as defined in Table 5) but completely inhibited fraction II (also defined in Table 5). Further assay by the two other methods, namely the overall reaction and ATP formation, showed other differences (Table 6). All three protein fractions showed maximal specific activities with butyrate as substrate, but fraction II was found to show similar activity with laurate. Most striking of all was the absence of ATP formation from butyryl-AMP and PP_i by fraction II. Neither the addition of CoA to the reaction mixture nor preincubation with CoA in the presence or absence of ATP initiated the formation of ATP from butyryl-AMP and PP_i .

The second protein peak (Table 6) showed intermediate properties in all three assays and in its behaviour towards ATP and seemed likely to be a mixture of fraction I and fraction II. Repeated ion-exchange chromatography on DEAE-Sephadex resolved this peak almost completely into fractions I and II.

DISCUSSION

Medium-chain fatty acyl-CoA synthetase was chosen for this study as a possibly suitable model enzyme for the elucidation of the reaction mechanism suggested for long-chain fatty acyl-CoA synthetase (Bar-Tana & Shapiro, 1964). According to this mechanism, CoA is an obligatory substrate for the initiation of the overall reaction, or each of the two partial reactions (a) and (b). The mode of action of this enzyme was, however, found to be quite different from that of palmitoyl-CoA synthetase. In contrast with the activation of the latter (measured by ATP formation) obtained after preincubation with CoA and ATP, the enzyme used in the present study showed marked inhibition

Table 5. *Relative specific activities of enzyme after further purification, as assayed by butyryl-AMP-dependent CoA disappearance*

For details see the text.

Enzyme fraction	Total activity (μ moles of CoA disappeared/10 min.)	% of total activity	Relative specific activity
(NH ₄) ₂ SO ₄ fraction (24–30%, w/v)	535.0	(100.0)	(1.0)
After Bio-Gel P-300 filtration	245.0	46.0	1.6
DEAE-Sephadex fractions			
Peak 1 (fraction I)	25.9	4.85	3.4
Peak 2	19.7	3.7	2.7
Peak 3 (fraction II)	64.0	12.0	3.45

Table 6. *Substrate specificity and enzyme specific activity as assayed by the overall reaction and partial reactions (a) and (b)*

Conditions were as described in the Experimental section. The protein fractions are those referred to in Table 5.

Assay	Substrate	Peak 1 (enzyme fraction I)	Peak 3 (enzyme fraction II)	Peak 2
(1) Overall reaction (μ moles of CoA disappeared/mg. of protein/10 min.)	Acetate	1.3	0.27	0.45
	Butyrate	2.5	1.9	1.7
	Laurate	0.9	1.9	1.25
	Palmitate	0.55	0.27	0.09
(2) Partial reaction (b) (μ moles of CoA disappeared/mg. of protein/10 min.)	Butyryl-AMP alone	3.44	3.45	2.7
	Butyryl-AMP + ATP (20 mM)	6.35	0.00	1.3
(3) Partial reaction (a) (reversal) (μ moles of ATP formed/mg. of protein/10 min.)	Butyryl-AMP + PP _i (2 mM)	2.17	0.18	0.82

when treated in an identical manner. Inhibition was also caused by preincubation of the enzyme with CoA or ATP alone, and subsequently removal of these by gel filtration before the determination of ATP formation. The inhibitory effect of ATP could also be demonstrated in the partial reaction (b) (acyl-AMP-dependent CoA disappearance), whereas ATP activated reaction (b) catalysed by long-chain fatty acyl-CoA synthetase.

However, the fact that in some of our enzyme preparations higher concentrations of ATP overcame the ATP inhibition of reaction (b) and exerted a pronounced activating effect suggested that at least two different enzyme systems capable of butyrate activation and exhibiting contrasting ATP effects might be present. This was confirmed by further purification, when two different enzyme fractions catalysing butyrate activation, but differing in their response to ATP, were obtained.

A comparison of the characteristics of these two purified fractions with those of the original enzyme is presented in Table 7. The properties of the original enzyme with respect to ATP response and

ATP formation are clearly shown to be the combined effects of the isolated enzyme fractions I and II. This explains the finding that preparations of acyl-CoA synthetase, consisting probably of different proportions of fractions I and II, showed variable enzyme activities in assays of the separate partial reactions. Further, as ATP formation was absent from fraction II, it seems that the formation of ATP catalysed by unfractionated enzyme is due to the fraction I component, and hence the inhibition by ATP and CoA of this reaction step reflects the behaviour of fraction I.

The two opposite effects of ATP on enzyme fraction I, i.e. activation of step (b) and inhibition of the ATP-forming step, raises the question as to the possible mechanism of reaction of this enzyme. Though this enzyme follows the overall reaction and the two partial reactions according to Berg (Bar-Tana & Rose, 1968b), this mechanism does not explain the dual effect of ATP. Nor does the mechanism proposed for the long-chain fatty acyl-CoA synthetase seem to apply in this case, as ATP inhibits the ATP-forming step. It is difficult to

Table 7. *Comparison of the properties of 'fraction C' and fractions I and II*

For details of enzyme fractions see the text.

	Fraction I	Fraction II	'Fraction C'
Overall reaction	Present	Present	Present
Partial reaction (a) (reversal)	Present	Absent	Variable
Partial reaction (b)	Present	Present	Present
Effect of enzyme preincubation with ATP and CoA on partial reaction (a) (reversal)	Inhibitory	—	Inhibitory
ATP effect on partial reaction (b)	Activating	Inhibitory	Variable
Butyrate/Laurate activity ratio	2.8	1.0	—
Suggested mechanism	Bi Uni Uni Bi Ping Pong (Bar-Tana & Rose, 1968a)	Sequential (Bar-Tana & Rose, 1968b)	

believe that the two partial reactions (a) and (b) constituting the overall reaction are catalysed by two separate enzymes, each affected oppositely by ATP. As shown in a separate paper (Bar-Tana & Rose, 1968a), allosteric transformations initiated by various ligands including ATP and CoA are responsible for the effects of CoA and ATP in the ATP-forming system and partial reaction (b).

The complete absence of the ATP-forming system from fraction II raises the question as to the possible mechanism of action of this enzyme fraction. Absence of ATP formation, despite the occurrence of partial reaction (b), cannot be explained by Berg's (1956) scheme of reaction, according to which butyryl-AMP is cleaved by PP_i or CoA. Several alternative mechanisms to Berg's (1956) have been proposed, according to which fatty acyl-AMP is not a distinct intermediate of the overall reaction, but can act as a substrate analogue in the reaction system. In this case butyryl-AMP plus PP_i could give ATP with some enzyme species but not others. Further studies have confirmed a sequential mechanism for the overall reaction as catalysed by fraction II (Bar-Tana & Rose, 1968b). ATP inhibition of the butyryl-AMP-dependent CoA disappearance catalysed by this enzyme fraction is discussed in that paper.

The possible significance of the differences between the two enzyme fractions deserves comment. Though it is possible that the two systems represent two different enzymes activating butyrate, the fact that the two activities remain together until the last separation step in the isolation procedure indicates that their structures may be related to each other. In several enzyme preparations derived from different ox livers, the relative distribution of the two enzyme activities differed; some preparations contained more fraction I, whereas others, though treated in an identical manner, showed a preponderance of fraction II.

Reducing conditions, such as the presence of mercaptoethanol during the isolation procedure, seemed to influence the relative proportions of the two enzyme fractions, increasing the relative amount of fraction I. Also, it appeared that on storage at 0° of the ammonium sulphate fraction and subsequent further purification an increase of fraction II occurred at the expense of fraction I.

These preliminary findings, which may indicate that one of the fractions can change into the other, provide no information as to the native state of the enzyme in ox liver. Each of the two enzymes may occur naturally or could be an artifact as a result of purification. Further investigations with regard to the respective molecular weights and amino acid compositions of the two enzyme fractions are required to elucidate the exact relationship between the two enzyme fractions.

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