Palmitoyl-Coenzyme A Synthetase

ISOLATION OF AN ENZYME-BOUND INTERMEDIATE

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An enzyme-bound intermediate of the overall reaction catalysed by rat liver microsomal long-chain fatty acyl-CoA synthetase is described. It was found to contain equimolar amounts of adenylate and fatty acid moieties bound to protein, and was stabilized by ATP. The intermediate reacted with CoA to give palmitoyl-CoA.

The overall reaction catalysed by purified rat liver palmitoyl-CoA synthetase (EC 6.2.1.3) apparently proceeds via an enzyme-bound intermediate that may be either enzyme-fatty acyl-AMP (Berg, 1956), palmitate-enzyme-AMP, palmitate-enzyme-PP_i or enzyme-palmitate. This conclusion is consistent with the following observations (Bar-Tana *et al.*, 1973): (a) the initial velocity pattern of the overall forward reaction conforms to either the Bi Uni Uni Bi Ping Pong (Cleland, 1963) or the Bi Bi Uni Uni Ping Pong mechanism, in which both ATP and palmitate have to combine with the enzyme before any product is released; (b) ¹⁸O is transferred from [*carboxy*-¹⁸O]-palmitate to AMP and palmitoyl-CoA and not to pyrophosphate; (c) 'pulse'-labelling of the enzyme with radioactive palmitate occurs only in the presence of ATP.

The distinction between the alternatives presented seemed to be of interest, since no partial reactions using synthetic palmitoyl-AMP as substrate, to yield ATP or palmitoyl-CoA in the presence of PP_i or CoA respectively, were found to be catalysed by the purified enzyme (Bar-Tana *et al.*, 1972). In the present paper the isolation of the enzyme-bound intermediate is described and its nature is discussed.

Materials and Methods

Inorganic pyrophosphatase (EC 3.6.1.1), hexokinase (EC 2.7.1.1) and potato apyrase (adenosine triphosphatase, EC 3.6.1.5) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sodium deoxycholate was obtained from Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A. Triton X-100 was from Serva, Heidelberg, Germany. Sephadex G-50 (fine grade) was from Pharmacia, Uppsala, Sweden. Bio-Gel P-30 was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. All other reagents were of A.R. grade. [9,10-³H]Palmitic acid, [1-¹⁴C]palmitic acid, [U-¹⁴C]ATP and [γ -³²P]ATP

were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. [1-¹⁴C]Palmitoyl-AMP was synthesized as described previously (Bar-Tana *et al.*, 1972). The purification of palmitoyl-CoA synthetase and materials used were as described previously (Bar-Tana *et al.*, 1971, 1972). 'Pulse'-labelling was carried out as described previously (Bar-Tana *et al.*, 1973).

Results

Attempts to isolate an enzyme-bound intermediate by labelling the enzyme in the presence of radioactive palmitate and ATP followed by isolation of the radioactive protein by gel filtration yielded only negligible amounts of bound intermediate. As shown in Table 1, [³H]palmitate was found to interact with the enzyme in the presence of ATP to form an enzyme-bound intermediate capable of reacting with CoA in a subsequent step. However, gel filtration of the treated enzyme with 0.05M-Tris-HCl buffer, pH 7.8, resulted in an almost complete loss of the specifically bound radioactivity of the recovered enzyme protein, as determined by treating the eluted enzyme with CoA to yield radioactive palmitoyl-CoA (Bar-Tana *et al.*, 1972). The apparent lability of the intermediate was investigated by subjecting the labelled protein obtained by a radioactive palmitate 'pulse-chase' experiment (Bar-Tana *et al.*, 1973) to various treatments such as dilution, aging and change of pH or concentration of substrates. It was found that the pH curve for optimum yield of the enzyme intermediate followed the pH curve for the overall reaction (Bar-Tana *et al.*, 1971). Further, the presence of ATP at sufficiently high concentration during the 'pulse' and 'chase' periods appeared to be of critical importance both for the formation and subsequent stabilization of the bound intermediate. Removal of the 'pulse' ATP by the addition of adenosine triphosphatase (or hexokinase and glucose) during the 'chase'

Table 1. *Attempted isolation of enzyme-bound intermediate*

(a) The reaction mixture during 'pulse'-labelling contained 150 mM-Tris-HCl buffer, pH 7.8, 4 mM-EDTA, 50 mM-MgCl₂, 16 mM-GSH, 50 mM-ATP (where stated), 0.1 mM-[9,10-³H]palmitate (50 μ Ci/ μ mol), 200 μ g of Triton X-100, 1 unit of inorganic pyrophosphatase and 98 μ g of enzyme protein in a total volume of 0.1 ml. 'Pulse'-labelling was carried out for 1 min followed by mixing with excess of unlabelled palmitate for 30 s. The 'chase' mixture was further incubated for 15 s in the presence of 1 mM-CoA (where stated) and 25 mM-ATP (where stated) in a final volume of 0.4 ml (final concentration of palmitate was 5 mM). The incubation was terminated by adding 1.0 ml of Dole's (1956) reagent, 0.2 ml of water and 0.6 ml of heptane. The lower phase was washed six times with 0.6 ml of heptane and the radioactivity of a sample was counted. (b) Conditions for 'pulse'-labelling were as described for (a)(2). After being mixed with an excess of unlabelled palmitate the 'chase' mixture was subjected to gel filtration on a Sephadex G-50 (fine grade) column (20 cm $\times$ 1 cm) and the protein was eluted with 0.1 M-Tris-HCl buffer, pH 7.8. The eluted enzyme was treated with 4 mM-EDTA, 50 mM-MgCl₂ and 1 mM-CoA (where stated). The radioactivity in the lower phase was determined as described above.

			Palmitoyl-CoA formed (d.p.m./0.1 mg of enzyme protein)
(a)	'Pulse' conditions	'Chase' conditions	
(1)	Enzyme + [³ H]palmitate	Palmitate + ATP + CoA	54000
(2)	Enzyme + [³ H]palmitate + ATP	Palmitate + CoA	96000
(3)	Enzyme + [³ H]palmitate	Palmitate	0
(b)	Eluted enzyme + CoA		2000
	Eluted enzyme - CoA		1900

caused complete breakdown of the enzyme intermediate. Also, the concentration of ATP (50 mM) required for maximal formation of the enzyme intermediate was much higher than that required for the overall activation reaction measured in the presence of CoA (Fig. 1) (K_m for ATP 4.65 mM; Bar-Tana *et al.*, 1971).

Unfortunately, the necessary presence of ATP during gel filtration (and hence also during the subsequent CoA reaction with the eluted enzyme intermediate) prevented distinction between the relevant and irrelevant palmitate bound to the eluted enzyme fraction. Further, since irrelevantly adsorbed radioactive palmitic acid equilibrates only very slowly with unlabelled palmitic acid carrier (Bar-Tana *et al.*, 1973), the overall activity obtained by reaction with CoA might still be high enough to mask activity due to the labelled intermediate concerned, even in the presence of excess of palmitic acid carrier present either during gel filtration or during CoA reaction with the protein fraction eluted from the gel.

To distinguish between relevantly and irrelevantly bound palmitate in the same eluted enzyme sample, the time-course of reaction with CoA of the intermediate formed during the routine 'pulse-chase' experiment was determined. As shown in Fig. 2(a), the reaction proceeded in two phases, an initial one in which CoA presumably reacted with the enzyme-bound intermediate and a second one where reaction with unspecifically bound and free palmitate took place to give a steady-state accumulation of

palmitoyl-CoA. Incubation of the enzyme with [³H]palmitate but without ATP during the 'pulse' resulted in a linear course of reaction with CoA (Fig. 2b). The amount of relevantly bound [³H]-palmitate bound to enzyme could thus be determined either by comparing the two time-courses (Fig. 2c) or by extrapolating the linear section of Fig. 2(a) to zero time.

The isolation and characterization of the enzyme-bound intermediate consisted of incubating substrate amounts of the enzyme in the presence of [¹⁴C]ATP and [³H]palmitate of known specific radioactivities under the 'pulse' conditions. After incubation the mixture was diluted with carrier palmitate and subjected to gel filtration in the presence of all the other constituents of the 'pulse' reaction mixture. The eluted protein fraction was treated with CoA and the time-course of the reaction followed for about 120 s. The value obtained by extrapolation of the time-curve to zero time was taken to represent the fatty acid content of the enzyme-bound intermediate. The amount of nucleotide bound to the enzyme fraction was determined by measuring the ¹⁴C radioactivity of the eluted enzyme. As a control, an equivalent experiment was carried out in which reaction with CoA or PP_i preceded the gel-filtration step. The fatty acid and nucleotide radioactivities in the eluted enzyme fraction were determined as described. As shown in Fig. 3, incubation of the enzyme in the presence of [¹⁴C]ATP and [³H]palmitate followed by gel filtration under the conditions specified did yield relevantly bound [³H]palmitate in the amount

Fig. 1. ATP titration curve in the 'pulse'-labelling experiment

The reaction mixture for 'pulse'-labelling contained: 137.5mM-Tris-HCl buffer, pH7.8, 3.3mM-EDTA, 41mM-MgCl₂, 11.5mM-GSH, ATP as stated, 385 μ M-[9,10-³H]palmitate (45.5 μ Ci/ μ mol), 65 μ g of Triton X-100, 0.6 unit of inorganic pyrophosphatase and 136 μ g of enzyme protein in a final volume of 0.04ml. After a 5 min 'pulse', unlabelled palmitate was added in excess followed by ATP, to yield a final concentration of 2.1mM-palmitate and 57mM-ATP in a total volume of 0.42ml. The 'chase' mixtures were allowed to react for 1 min in the presence of 0.6mM-CoA, and the radioactive palmitoyl-CoA was isolated and determined as described in Table 1. In the control experiment ATP was omitted during 'pulse' labelling but was added together with CoA in a final concentration of 57mM.

represented by the intercept value obtained on extrapolation of the activity curve to zero time. On the other hand, treatment of the 'pulse'-labelled enzyme with CoA before gel filtration caused the disappearance of the relevantly enzyme-bound palmitate and a single-phase time-plot was obtained. The quantitative relationship of the fatty acid to nucleotide moieties in the enzyme-bound intermediate is illustrated in Table 2; the molar ratio of fatty acid to nucleotide is about 1:1.

In a similar experiment where [U-¹⁴C]ATP was replaced by [γ -³²P]ATP, but otherwise under strictly similar conditions, no ³²P could be recovered in the eluted enzyme fraction although the relevantly bound [³H]palmitate remained the same. This observation eliminates the possibility of ATP-enzyme-palmitate as the intermediate. Thus the only two possibilities that remain to be considered are that the inter-

mediate is either enzyme-palmitoyl-AMP or AMP-enzyme-palmitate (see the Discussion section).

Discussion

In the light of the evidence presented here it appears that purified palmitoyl-CoA synthetase catalyses the activation of fatty acid through an enzyme-bound intermediate, which consists of nucleotide and fatty acid in a molar ratio of 1:1. The intermediate formed then reacts with CoA to yield AMP and palmitoyl-CoA in a subsequent step.

The stabilization of the enzyme-bound intermediate by ATP is of interest. Stabilization by ATP described here may resemble a similar case where a membrane-bound phosphate intermediate formed by ATP phosphorylation could be isolated in a sucrose gradient only in the presence of ATP (McFarland & Inesi, 1971). Whether ATP is a specific stabilizer in the instance of palmitoyl-CoA synthetase or may be replaced by other nucleotides has yet to be investigated. However, it should be pointed out that the concentration of ATP required for maximal formation is far higher than that required for the maximal overall activation reaction in the presence of CoA. Whatever the nature of the enzyme-palmitate bond of the intermediate, release of free palmitate in the absence of ATP may indicate some conformational transition caused by ATP favouring tight binding of palmitate, or rendering the enzyme-palmitate or the palmitoyl-AMP bond inaccessible to hydrolysis. It is noteworthy that luciferyl-AMP bound to luciferase is hydrolysed in the presence of ATP (DeLuca & McElroy, 1965), in contrast with the stabilizing effect of ATP described here.

The role of the isolated enzyme intermediate in the course of the overall activation reaction may be evaluated by comparing the steady-state overall activity under saturating conditions (the steady-state time-phase of Fig. 2a) with the initial rate of the reaction of the enzyme-bound intermediate with CoA (Fig. 2c), allowance being made for the 52-fold dilution of the palmitate specific radioactivity during the 'chase'. The steady-state overall rate was about 600nmol/min per mg of protein whereas the rate of the reaction of the enzyme intermediate (formed in the absence of CoA) with CoA was found to be only 80nmol/min per mg of protein. This observed difference in rates cannot be accounted for by assuming that ATP may be 'synergistic' in the second partial step (Bridger *et al.*, 1968), since the reaction of the enzyme-bound intermediate was measured in the presence of ATP (Fig. 2). It may be that in the course of the overall reaction, where all the three substrates are present, the enzyme-bound intermediate is sensitized for subsequent reaction with CoA by a CoA-induced conformational change. The discrepancy between the steady-state rate and the rate of reaction

Fig. 2. Reaction of the enzyme-bound intermediate with CoA

(a) Palmitoyl-CoA obtained from labelled enzyme intermediate. The reaction mixture for 'pulse'-labelling contained: 150 mM-Tris-HCl buffer, pH 7.8, 4 mM-EDTA, 50 mM-MgCl₂, 14 mM-GSH, 50 mM-ATP, 100 μM -[9,10-³H]-palmitate (117 $\mu\text{Ci}/\mu\text{mol}$), 0.4 mg of Triton X-100, 2 units of inorganic pyrophosphatase and 0.545 mg of enzyme protein in a final volume of 0.21 ml. After a 'pulse' of 1 min, an excess of unlabelled palmitate was added to a final concentration of 5.2 mM in a total volume of 0.71 ml. Samples (0.05 ml) of the 'chase' mixture were allowed to react with 0.6 mM-CoA for the time-periods stated, in a total volume of 0.45 ml (5.5 mM-ATP, 0.825 mM-palmitate; 0.57 $\mu\text{Ci}/\mu\text{mol}$). The radioactive palmitoyl-CoA was isolated and determined as described in Table 1. (b) Same as (a), except that ATP was omitted during 'pulse'-labelling but was added together with the CoA, to a final concentration of 5.5 mM. (c) Time-curve of net reaction of the enzyme-bound intermediate with CoA obtained by subtracting the (b) curve from the (a) curve.

of the pre-formed intermediate with CoA appears to resemble results obtained with various amino acid-activation systems, where esterification with tRNA fails by a factor of 10^2 – 10^3 to account for the overall activation rate (Loftfield, 1972). A tRNA-induced conformational transition occurring before tRNA substrate reaction is assumed to be the reason for the observed discrepancy (Mehler & Chakraborty, 1971).

In an attempt to distinguish between the two alternatives the behaviour of the enzyme-bound intermediate in Dole's (1956) medium (propan-2-ol-heptane–0.5 M-H₂SO₄, 40:10:1, by vol.) was compared with that of synthetic palmitoyl-AMP (Bar-Tana *et al.*, 1973). AMP-enzyme-[³H]palmitate would be expected to release its radioactivity into the heptane (upper) phase whereas enzyme-[³H]-palmitoyl-AMP, like liberated palmitoyl-CoA, would be expected to be present in the hydrophilic lower phase. No ³H radioactivity was detected in the lower phase, although synthetic [³H]palmitoyl-AMP could be recovered in a 90% yield under similar conditions.

The observed partition of the intermediate in Dole's medium (Bar-Tana *et al.*, 1973) compared with the partition of synthetic palmitoyl-AMP suggests that palmitate-enzyme-AMP may be the intermediate of the overall activation reaction rather than enzyme-palmitoyl-AMP. The absence of partial reactions with palmitoyl-AMP as substrate (Bar-Tana *et al.*, 1972), as well as the lack of acylhydroxamate formation in the presence of ATP, palmitate, enzyme and hydroxylamine (Bar-Tana & Shapiro, 1964), tend to support this hypothesis. If enzyme-palmitoyl-AMP is nevertheless the true intermediate of the reaction, one has to assume an enzyme-mediated hydrolysis of bound palmitoyl-AMP under Dole's conditions. Although enzyme-catalysed hydrolysis of palmitoyl-AMP was not observed under the standard experimental conditions it may possibly have still occurred at acidic pH. It should be remembered that enzyme intermediate formation was not observed at pH values below 6.5 and that the enzyme intermediate may be extremely labile under acidic conditions; one single turnover of hydrolysis would explain the partition observed. With

Fig. 3. Time-curve for reaction of the isolated enzyme-bound intermediate with CoA

(a) Palmitoyl-CoA obtained from isolated labelled enzyme intermediate. The reaction mixture for 'pulse' labelling contained: 500mM-Tris-HCl buffer, pH7.8, 13mM-EDTA, 165mM-MgCl₂, 46mM-GSH, 1.06mM-[9,10-³H]palmitate (160Ci/mol), 34mM-[U-¹⁴C]ATP (2.44Ci/mol), 66μg of Triton X-100, 2 units of pyrophosphatase and 1.36mg of enzyme protein in a total volume of 0.11 ml. After a 'pulse' of 3 min, unlabelled palmitate was added to a final concentration of 23.4mM in a total volume of 0.61ml and the mixture was subjected to gel filtration by using a Bio-Gel P-30 column (15cm×1 cm) equilibrated with a solution containing 0.1M-Tris-HCl buffer, pH7.9, 2mM-EDTA, 50mM-MgCl₂, 10mM-ATP, 10mM-GSH and 0.1 % Triton X-100. The protein was eluted with the same buffer and samples were treated for the times shown with 1mM-CoA in a total volume of 0.225 ml. The [³H]palmitoyl-CoA was isolated and determined in the lower phase as described in Table 1. (b) Same as (a), except that the mixture was treated with 0.3mM-CoA before gel filtration. Samples of the same specific radioactivity for the overall reaction were used for the ordinate values presented in both (a) and (b).

Table 2. Molar ratio of nucleotide to fatty acid constituting the isolated enzyme-bound intermediate

(a) Conditions were as given for Fig. 3. The relevant [³H]palmitic acid bound to the isolated protein was determined by extrapolation to zero of the steady-state time-phase in Fig. 3(a). The nucleotide bound to enzyme was determined by counting the ¹⁴C radioactivity remaining in the washed respective lower phase. See the text for details. (b) Conditions were as above except that [γ -³²P]ATP (1.75μCi/μmol) was used instead of [¹⁴C]ATP during 'pulse'-labelling. The nucleotide bound to enzyme was determined by counting the ³²P radioactivity of the respective lower phase.

Conditions	Relevant palmitate (nmol/mg of protein)	Radioactive nucleotide (nmol/mg of protein)	Nucleotide/fatty acid molar ratio
(a) [³ H]Palmitate, [U- ¹⁴ C]ATP			
(1) Absence of CoA before gel filtration (Fig. 3a)	2.4	2.2	0.92
(2) Presence of CoA before gel filtration (Fig. 3b)	0.0	0.0	
(b) [³ H]Palmitate, [γ - ³² P]ATP			
(1) Absence of CoA before gel filtration	2.1	0.0	0.0
(2) Presence of CoA before gel filtration	0.0	0.0	

regard to the absence of acylhydroxamate formation in the absence of CoA, it should be recalled that other known ligase-bound intermediates have shown similar behaviour towards hydroxylamine (Berg, 1956; Hirsch & Lipmann, 1968). Thus the exact nature of the intermediate in question still awaits final elucidation.

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