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Palmitoyl-Coenzyme A Synthetase

MECHANISM OF REACTION

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The mechanism of long-chain fatty acid activation catalysed by highly purified microsomal palmitoyl-CoA synthetase was investigated. The kinetics of the overall reaction were found to conform to the Bi Uni Uni Bi Ping Pong mechanism. ^{18}O was transferred from [^{18}O]palmitate to AMP and palmitoyl-CoA exclusively. The enzyme intermediate formed appeared to consist of enzyme-bound palmitate; this formation occurred only in the presence of ATP. However, the involvement of palmitoyl-AMP in the reaction catalysed by the purified enzyme has proved difficult to establish.

Fatty acid activation catalysed by microsomal long-chain fatty acyl-CoA synthetase (EC 6.2.1.3) is generally assumed to involve fatty acyl-AMP as an intermediate of the overall reaction. In support of this hypothesis, rat liver microsomal fractions were shown to catalyse the formation of ATP or palmitoyl-CoA from palmitoyl-AMP in the presence of PP_i or CoA respectively, as well as a [$^{32}P_2$]PP_i–ATP exchange reaction. However, by using a purified long-chain fatty acyl-CoA synthetase (Bar-Tana *et al.*, 1971) the activity of these partial reactions and the palmitate-dependent [$^{32}P_2$]PP_i–ATP exchange reaction were found to be nearly negligible (Bar-Tana *et al.*, 1972*b*).

To explain this observed discrepancy, it may be argued that two separate enzyme activities capable of catalysing fatty acyl-CoA synthesis coexist in rat liver microsomal fractions: one following a Bi Uni Uni Bi Ping Pong mechanism involving the postulated palmitoyl-AMP intermediate, and the other, isolated as the pure enzyme fraction, conforming to a different mode of action. A relative lack of partial activities with palmitoyl-AMP as substrate has also been observed by using rat liver mitochondria, adipose-tissue microsomal material and mitochondria, as well as with muscle sarcosomes and mitochondria (R. Brandes & R. Arad, unpublished work). Because of these observations it was of interest to further investigate the mechanism of the activation process catalysed by the pure enzyme fraction. In the present paper we describe a study of the kinetic analysis of the Ter Ter reaction, ^{18}O transfer from ^{18}O-labelled palmitic acid to AMP, and 'pulse-labelling' experiments with stoicheiometric amounts of enzyme, and the results are discussed in relation to the mechanism postulated for fatty acid activation.

Experimental

Materials

These were as described by Bar-Tana *et al.* (1972*b*) except for inorganic pyrophosphatase, obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., bis-(trimethylsilyl)acetamide, obtained from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A., and Dowex 1 (X2; Cl^- form; 200–400 mesh) from Dow Chemicals Co. (through Fluka A.G., Buchs SG, Switzerland). [9,10-^{3}H]Palmitic acid, [1-^{14}C]palmitic acid and [U-^{14}C]ATP were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Palmitoyl-CoA and [γ-^{32}P]ATP were prepared as described previously (Bar-Tana *et al.*, 1971). $H_2{}^{18}O$, deuterium normalized, 97.6 ^{18}O atom % excess and 0.517 ^{17}O atom % excess was kindly donated by Professor D. Wolf from the Weizmann Institute, Rehovot, Israel.

[^{18}O]Palmitic acid was prepared by incubating 36mg of methyl palmitate dissolved in 2.0ml of tetrahydrofuran with 0.36ml of $H_2{}^{18}O$ (97.6 ^{18}O atom % excess) and 36mg of KOH in a sealed tube for 48h at 150°C. Non-labelled palmitic acid, used as reference in the following studies, was obtained by using a similar mixture with non-labelled water. The incubation was terminated by evaporating the tetrahydrofuran under reduced pressure and the precipitate was treated with 1.6ml of Dole's (1956) reagent, 1.0ml of water, 1.0ml of heptane, acidified to pH4.0 and mixed well. The heptane layer was separated and the free palmitic acid was recovered by treating the heptane layer with alkaline ethanol (0.1M-NaOH in 50% ethanol) followed by extraction into heptane under acidic conditions as above. The yield of palmitic acid was nearly 100%. The ^{18}O content was deter-

mined by treating a sample with 100 mole excess of bis(trimethylsilyl)acetamide, dissolved in 3vol. of acetonitrile. The sample tube was sealed and incubated at room temperature for 1h, then evaporated to dryness under a stream of N_2. The residue was subjected directly to mass spectrometry and the proportion of the relative intensities at *m/e* 317:315:313 was determined. The preparation used in this study was found to contain 55.2 ^{18}O atom % excess. Analysis of the different molecular species present showed 10.4% of $CH_3[CH_2]_{14}C^{16}O^{16}O$, 68.8% of $CH_3[CH_2]_{14}C^{16}O^{18}O$ and 20.8% of $CH_3[CH_2]_{14}C^{18}O^{18}O$.

Methods

Palmitoyl-CoA formation from [1-^{14}C]*palmitate.* The reaction mixture contained 83.5mM-Tris–HCl buffer, pH7.4, 0.6mg of Triton X-100, 1.66mM-EDTA, 8.35mM-$MgCl_2$, 2.5μg of enzyme protein, [1-^{14}C]palmitate (1μCi/μmol), and CoA and ATP as stated, in a total volume of 0.6ml. Incubation was carried out at 30°C and terminated by the addition of 1.0ml of Dole's (1956) reagent as described by Bar-Tana *et al.* (1971). In blank experiments the enzyme was added at the end of incubation.

Palmitate formation from [1-^{14}C]*palmitoyl-CoA.* The reaction mixture contained 150mM-Tris–HCl buffer, pH7.4, 0.25mg of Triton X-100, 2mM-EDTA, 20mM-$MgCl_2$, 40μM-5,5'-dithiobis-(2-nitrobenzoic acid), 12.5μg of enzyme protein, [1-^{14}C]palmitoyl-CoA (1μCi/μmol), AMP and PP_i as stated in a total volume of 0.25ml. Incubation was carried out at 30°C and was terminated by the addition of 1.0ml of Dole's (1956) reagent as described by Bar-Tana *et al.* (1971). AMP was omitted in all blank experiments.

Kinetic studies. Initial velocities were measured by choosing enzyme concentrations ensuring a linear response during measurement. Kinetic results were analysed by using double-reciprocal plots followed by replots of slopes and intercepts as a function of the reciprocal concentration of the changing fixed substrate (Cleland, 1963*a*). Single points that deviated widely from the general pattern observed were discarded before calculation of the linear regression.

^{18}O*-transfer measurements.* The reaction mixture contained 41.5mM-Tris–HCl buffer, pH7.4, 5.0mg of Triton X-100, 0.9mM-EDTA, 47.5mM-$MgCl_2$, 9mM-ATP, 2.3mM-CoA, 47.5mM-NaF, 1.25mM-[^{18}O]-palmitate and 10mg of freeze-dried microsomal material or 1.0mg of purified palmitoyl-CoA synthetase in a final volume of 5.25ml. A similar incubation mixture with non-^{18}O-enriched palmitate served as reference. Incubation was carried out for 60min at 37°C and the extent of the overall reaction was followed by measuring the CoA disappearance on 0.03ml samples (Bar-Tana *et al.*, 1971). ^{18}O transfer to AMP or PP_i was determined as described by Bar-Tana *et al.* (1972*a*). ^{18}O transfer to palmitoyl-CoA was determined as follows: the incubation was terminated by Dole's (1956) reagent, acidified to pH2.0 with HCl and extracted five times with heptane. The heptane washings were discarded and palmitoyl-CoA in the lower phase was hydrolysed at 100°C for 15min under alkaline conditions. The free fatty acid derived was extracted into acidic heptane, silylated and analysed for ^{18}O content as described as above under 'Materials'. Mass spectrometry was carried out in a Varian MAT CH5 mass spectrometer, by using direct inlet, 70eV and 240°C ion-source temperature.

Results

Kinetic studies

Initial-velocity studies of the overall forward reaction were carried out at different concentrations of two of the three substrates, with the third one being kept constant. By using palmitate as the variable substrate at several fixed concentrations of CoA with ATP at saturating concentrations, the double-reciprocal plots were linear and constituted a family of parallel lines (Fig. 1). A replot of intercepts against reciprocal CoA concentrations was also linear. The apparent K_m values for CoA and palmitate were 20μM and 7μM respectively, under the incubation conditions specified. A similar parallel pattern was obtained maintaining the constant ATP concentration far below the saturation value. Since different incubation conditions were used in the present study, the apparent kinetic constants obtained differ from those published previously (Bar-Tana *et al.*, 1971).

With ATP as the variable substrate, at several fixed concentrations of CoA with palmitate at saturating concentrations, the double-reciprocal plots were again linear, constituting a family of parallel lines (Fig. 2). Replots of intercepts as a function of the reciprocal CoA concentrations were also linear. The apparent K_m values for ATP and CoA at the palmitate concentration specified were 300μM and 20μM respectively. The same pattern was obtained with a palmitate concentration of about 2.4 times its K_m value.

With ATP as the variable substrate, at several fixed concentrations of palmitate with [CoA] being kept constant at 34μM, the double-reciprocal plots intersected to the left of the ordinate (Fig. 3). Replots of slopes and intercepts against the reciprocal palmitate concentration were also linear. The apparent K_m values for ATP and palmitate were 300μM and 7μM respectively.

The pattern observed in Figs. 1–3 conforms to the initial-velocity rate law for the Bi Uni Uni Bi Ping Pong mechanism (Cleland, 1963*b*).

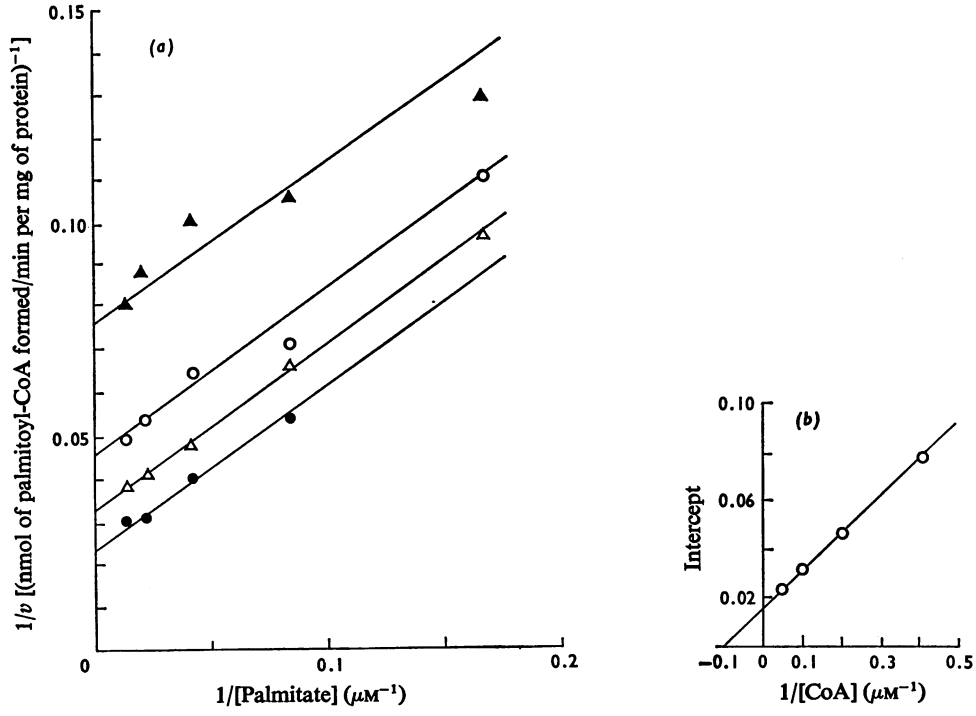
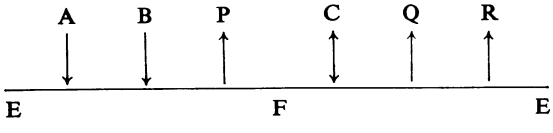
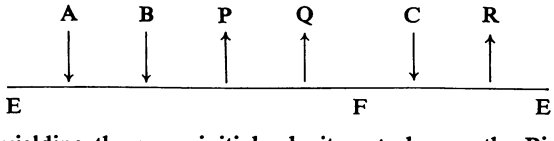


Fig. 1. *Initial-velocity studies of the overall forward reaction: titration of palmitate and CoA*

(*a*) Double-reciprocal plots of initial velocity against variable palmitate concentration at several fixed CoA concentrations are shown. [ATP] was kept constant at 5mM. CoA concentrations were: ▲, 2.5μM; o, 5.0μM; △, 10.0μM; ●, 20.0μM. Activity was measured as described in the Experimental section. (*b*) Replot of intercepts against reciprocal CoA concentrations.



Hence A, B and C will represent ATP, palmitate and CoA respectively, or palmitate, ATP and CoA respectively; P will represent either PP_i or AMP and Q and R will represent either AMP and palmitoyl-CoA or PP_i and palmitoyl-CoA respectively. An alternative mechanism conforming to the initial-velocity pattern obtained is the Bi Bi Uni Uni Ping Pong mechanism:



yielding the same initial-velocity rate law as the Bi Uni Uni Bi Ping Pong mechanism suggested above. In this case, A, B and C will represent ATP, palmitate and CoA respectively or palmitate, ATP and CoA respectively. P and Q will represent either AMP or PP_i and R will stand for palmitoyl-CoA. The two Ping Pong alternatives considered here differ with respect to the enzyme-bound intermediate assumed to participate in the overall activation of fatty acid. Whereas enzyme-bound palmitate is the intermediate of the Bi Bi Uni Uni Ping Pong mechanism, the ternary complex consisting of AMP, palmitate and enzyme is the intermediate of the Bi Uni Uni Bi Ping Pong alternative. As pointed out by Bar-Tana *et al.* (1972*b*), no partial reactions with exogenous palmitoyl-AMP as substrate could be shown with the pure enzyme fraction, indicating perhaps that the enzyme-bound palmitate alternative is a better description of the intermediate than is enzyme-bound palmitoyl-AMP.

To distinguish kinetically between the two mechanisms a study of the initial velocity of the overall reverse reaction was undertaken. With palmitoyl-CoA as the variable substrate at several fixed concentrations of AMP and maintaining [PP_i] at the saturation value, the double-reciprocal plots were

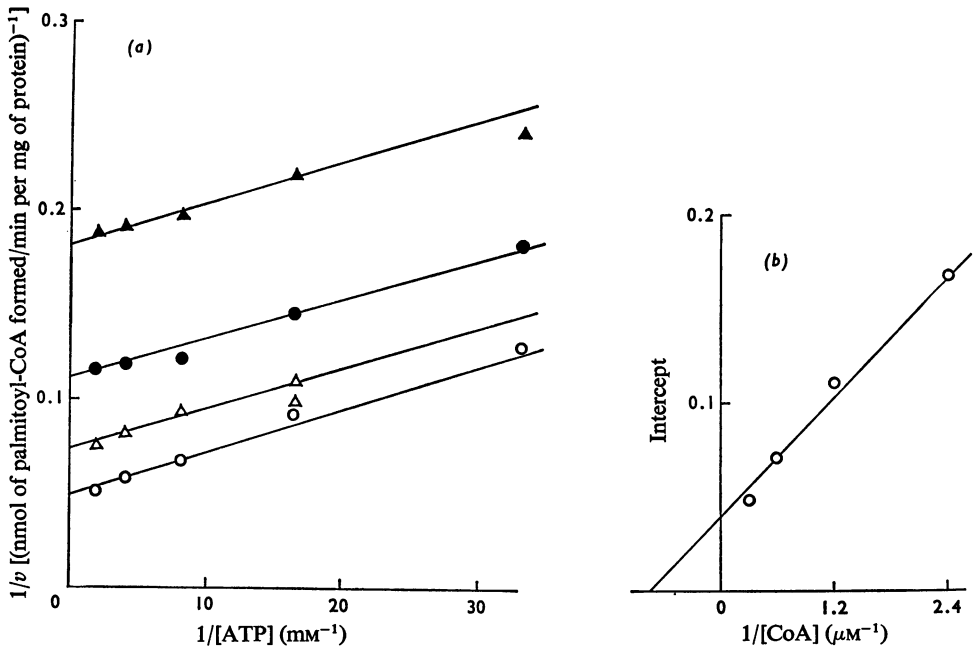


Fig. 2. *Initial-velocity studies of the overall forward reaction: titration of ATP and CoA*

(*a*) Double-reciprocal plots of initial velocity against variable ATP concentrations at several fixed CoA concentrations are shown. [Palmitate] was kept constant at 33μM. CoA concentrations were: ▲, 0.415μM; ●, 0.83μM; △, 1.65μM; ○, 3.30μM. Activity was measured as described in the Experimental section. (*b*) Replot of intercepts against reciprocal CoA concentrations.

linear and constituted a family of intersecting lines (Fig. 4). Replots of intercepts and slopes against the reciprocal AMP concentrations were also linear. The same pattern was observed also with PP_i concentrations far below the saturation value. The pattern observed excludes the Bi Bi Uni Uni Ping Pong mechanism, since according to the latter CoA combines with the stable enzyme form F and under initial-velocity conditions a non-reversible link would occur between the palmitoyl-CoA and AMP combination points, leading to a pattern of parallel lines (Cleland, 1963*b*). The initial study of the reverse overall reaction was done in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid), thus ensuring the absence of free CoA. Similarly, with PP_i as the variable substrate, at several fixed concentrations of AMP with [palmitoyl-CoA] maintained at 600μM (2.4$\times K_m$ value), the double-reciprocal plots were linear and constituted a family of parallel lines (Fig. 5). A replot of intercepts against the reciprocal concentrations of AMP was also linear. This pattern, also in agreement with the Bi Uni Uni Bi Ping Pong mechanism, excludes the other alternative considered, a Bi Bi Uni Uni Ping Pong mechanism. The latter would have been expected to yield a family of intersecting lines as combination of PP_i and AMP occurs here along reversibly connected combination points.

^{18}O-transfer studies

Whatever the nature of the true intermediate, enzyme-bound palmitate or enzyme-bound palmitoyl-AMP, interaction between palmitic acid and the nucleotide is implied from the above kinetic data. To establish the interaction of ATP and palmitate upon combination with the enzyme, ^{18}O transfer from [^{18}O]palmitic acid to AMP was investigated. As shown in Table 1, displacement of the pyrophosphate phosphorus of ATP by the carboxylic acid oxygen occurred with the liberation of [^{18}O]AMP and [*carboxyl*-^{18}O]palmitoyl-CoA. No [^{18}O]PP_i was liberated, thus indicating that whatever the mechanism, the P–O–P bridge of the nucleotide was affected on its α-phosphorus side. Essentially similar results were obtained by using the microsomal fraction instead of the pure enzyme fraction. No discrimination between the two enzyme intermediate alternatives suggested above is afforded by this result. However,

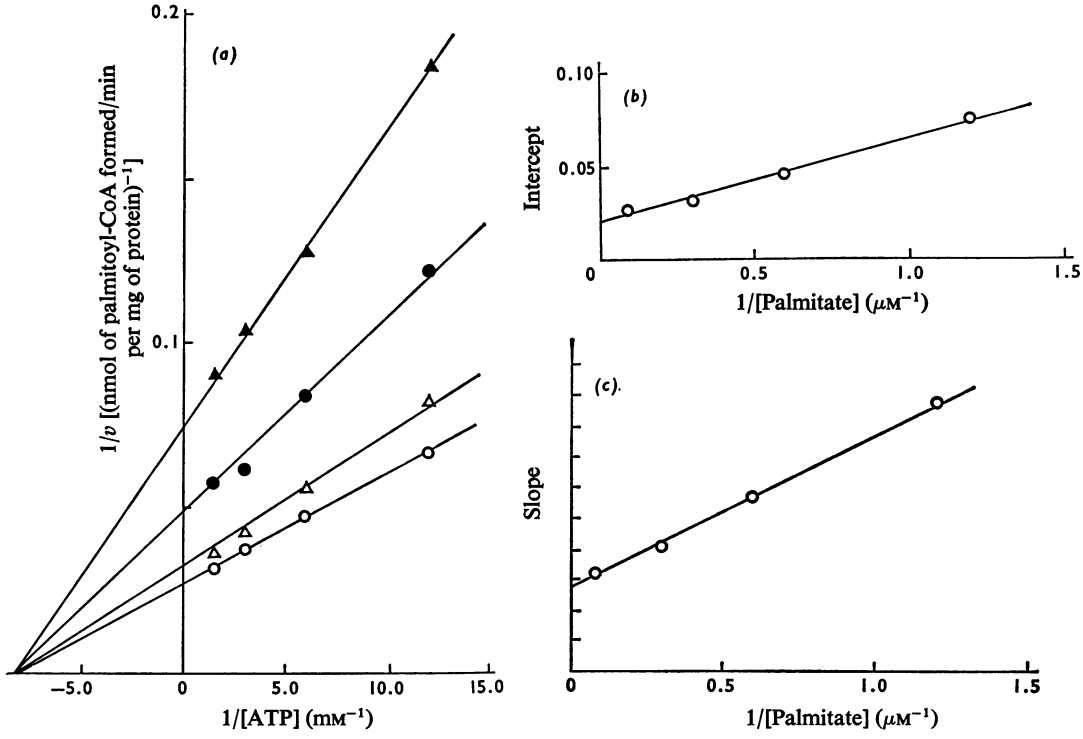


Fig. 3. *Initial-velocity studies of the overall forward reaction: titration of ATP and palmitate*

(*a*) Double-reciprocal plots of initial velocity against variable ATP concentrations at several fixed palmitate concentrations are shown. [CoA] was kept constant at 34 μM. Palmitate concentrations were: ▲, 0.83 μM; ●, 1.66 μM; △, 3.35 μM; ○, 11.6 μM. Activity was measured as described in the Experimental section. (*b*) Replot of intercepts against reciprocal palmitate concentrations. (*c*) Replot of slopes against reciprocal palmitate concentrations. The scales are the ratios of the ordinates to the abscissa in (*a*).

mechanisms involving ATP splitting into AMP and PP_i not dependent upon palmitate may be excluded, confirming the results of the kinetic analysis.

'Pulse labelling' of enzyme

The interaction between palmitate and ATP was further investigated by using [^{3}H]palmitate 'pulse labelling' of the pure enzyme fraction (Meister *et al.*, 1962). This method consists in general of incubating the enzyme in stoicheiometric amounts with one or two of the three substrates necessary for the overall reaction (the 'pulse'). The substrate to be tested for binding is radioactively labelled and of high specific radioactivity. After the 'pulse', the free labelled substrate is substantially diluted by adding a large amount of the same non-radioactive substrate and after subsequent reaction with the remaining substrate(s) (the 'chase') the radioactive product is isolated and counted. It is assumed that the highly labelled enzyme-bound substrate is not diluted by the carrier substrate giving rise to a labelled product of the original specific radioactivity. In the control experiment, the highly labelled substrate and the carrier are added simultaneously. As shown in Table 2, palmitate combined with the enzyme in a manner suitable for subsequent reaction with CoA. ATP was obligatory for this palmitate binding and in its absence non-specific palmitate adsorption occurred which could generally be diluted with non-radioactive palmitate introduced subsequently. However, palmitate of high specific radioactivity introduced during the 'pulse' period was not always fully diluted by palmitate introduced subsequently in the 'chase' period. Thus, the net overall radioactivity obtained from the (enzyme+[^{3}H]palmitate) 'pulse' was greater than the overall radioactivity obtained on mixing the high-specific-radioactivity 'pulse' palmitate with the cold 'chase' palmitate, before reaction with CoA and ATP (Table 2, tubes 2, 3). Allowing sufficient time for dilution by non-radioactive 'chase' palmitate before adding (CoA+ATP) decreased the

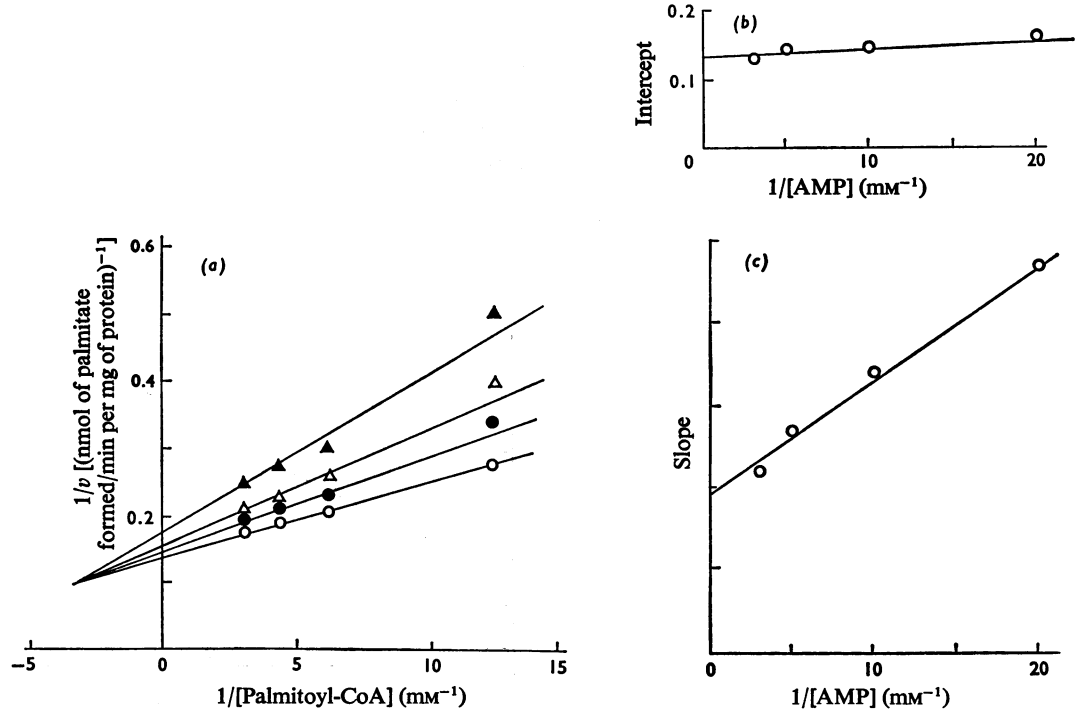


Fig. 4. *Initial-velocity studies of the overall reverse reaction: titration of palmitoyl-CoA and AMP*

(*a*) Double-reciprocal plots of initial velocity against variable palmitoyl-CoA concentrations at several fixed AMP concentrations are shown. [PP_i] was kept constant at 4.0mM. AMP concentrations were: ▲, 0.05mM; △, 0.10mM; ●, 0.20mM; ○, 0.30mM. (*b*) Replot of intercepts against reciprocal AMP concentrations. (*c*) Replot of slopes against reciprocal AMP concentrations. The ordinate scales are the ratios of the ordinates to abscissa in (*a*).

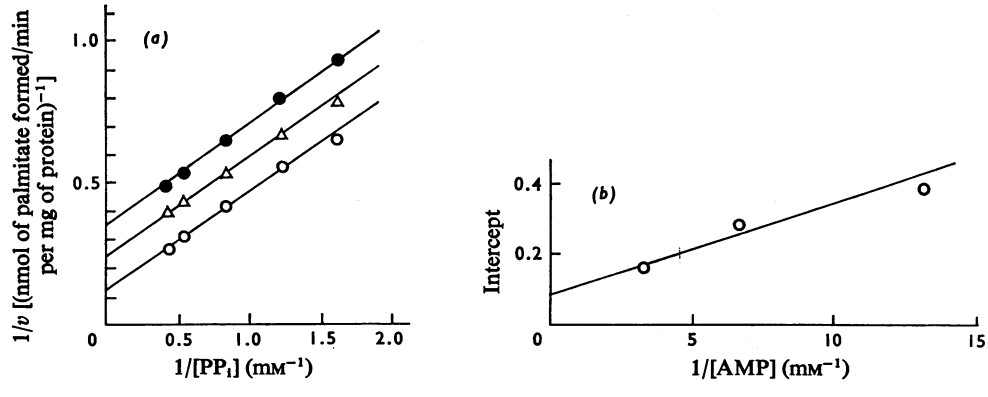


Fig. 5. *Initial-velocity studies of the overall reverse reaction: titration of PP_i and AMP*

(*a*) Double-reciprocal plots of initial velocity against variable PP_i concentrations at several fixed AMP concentrations are shown. [Palmitoyl-CoA] was kept constant at 0.6mM. AMP concentrations were: ●, 0.075mM; △, 0.150mM; ○, 0.300mM. (*b*) Replot of intercepts against reciprocal AMP concentrations.

Table 1. *^{18}O transfer catalysed by palmitoyl-CoA synthetase*

Details are as described in the Experimental section. TMS represents the trimethylsilyl group.

Enzyme fraction	^{18}O atom % excess: Initial palmitic acid	[^{18}O]PP_i $(TMS)_3$*	[^{18}O]AMP $(TMS)_5$*	[^{18}O]Palmitoyl-CoA (TMS)†
Purified enzyme fraction	55.2	0.0	55.5	55.2
Microsomal enzyme fraction	55.2	0.1	55.0	55.2

* For calculation of values see Bar-Tana *et al.* (1972*a*).

† Values are calculated as described in the Experimental section; the value represents the % of [^{18}O]palmitoyl-CoA out of the total palmitoyl-CoA formed.

Table 2. *'Pulse-labelling' experiments with [^3H] palmitate*

The reaction mixture during 'pulse labelling' contained 150μM-Tris–HCl buffer, pH7.6, 5mM-EDTA, 0.25mg of Triton X-100, 50mM-$MgCl_2$, 10mM-reduced glutathione, 1 unit of pyrophosphatase, 50mM-ATP (where stated), 4mM-CoA (where stated), 0.1mM-[^{3}H]palmitate (0.1mCi/μmol) and 200μg of purified enzyme fraction in a total volume of 0.1ml. 'Pulse labelling' was carried out for 1min followed by mixing with 'chase' reactants for 15s. For the 'chase' reaction non-radioactive palmitate, ATP (where stated) and CoA (where stated) were added to give a final concentration of 5mM-palmitate, 12.5mM-ATP and 1mM-CoA. The incubation was terminated by adding 1.0ml of Dole's (1956) reagent, and the [^{3}H]palmitoyl-CoA was extracted six times with 0.6ml of heptane. A sample of the washed lower phase (palmitoyl-CoA) was counted for radioactivity and the results were expressed relative to a palmitate blank ['palmitoyl-CoA (c.p.m.)']. The 'relative net pulse' and the 'net pulse' are calculated as described in the text.

Tube no.	'Pulse' conditions	'Chase' conditions	Palmitoyl-CoA (c.p.m.)	Relative net pulse	Net pulse
1	(Enzyme+[^{3}H]palmitate)	+(Palmitate)	700		
2	(Enzyme+[^{3}H]palmitate)	+(Palmitate+ATP+CoA)	11800	6300	0
3	(Enzyme)	+([^{3}H]Palmitate+palmitate+ATP+CoA)	5500		
4	(Enzyme)	+([^{3}H]Palmitate+palmitate)	0		
5	(Enzyme+[^{3}H]palmitate+ATP)	+(Palmitate)	1700		
6	(Enzyme+[^{3}H]palmitate+ATP)	+(Palmitate+CoA)	21650	16450	9850
7	(Enzyme+ATP)	+([^{3}H]Palmitate+palmitate+CoA)	5200		
8	(Enzyme+ATP)	+([^{3}H]Palmitate+palmitate)	0		
9	(Enzyme+[^{3}H]palmitate+CoA)	+(Palmitate)	3600		
10	(Enzyme+[^{3}H]palmitate+CoA)	+(Palmitate+ATP)	13150	7150	1350
11	(Enzyme+CoA)	+([^{3}H]Palmitate+palmitate+ATP)	6000		
12	(Enzyme+CoA)	+([^{3}H]Palmitate+palmitate)	0		

surplus overall radioactivity towards control values. Because of this behaviour of palmitate, the net palmitate radioactivity (Table 2, 'net pulse') relevantly associated with the enzyme has to be calculated as the difference between the combinations in tube 6, Table 2, i.e. (enzyme+ATP+[^{3}H]palmitate)+(palmitate+CoA), and tube 2, Table 2, i.e. (enzyme+[^{3}H]-palmitate)+(palmitate+CoA+ATP), the latter representing adsorbed radioactive palmitate not fully diluted by non-radioactive palmitate during the 'chase' period. CoA did not replace ATP for the relevant binding of [^{3}H]palmitate to the enzyme (Table 2, tube 10). The high overall radioactivity obtained during the 'pulse' period reflects overall radioactivity for that time-interval which is probably due to ATP contaminating the CoA preparations (about 0.5% of ATP on a molar basis was present in CoA preparations).

Table 3. *'Pulse-labelling' experiments with [^{3}H]palmitate: effect of glutathione*

The conditions used were as described in Table 2.

'Pulse' conditions	'Chase' conditions	Palmitoyl-CoA (c.p.m.)	Relative net pulse
Glutathione present (10mM)			
(Enzyme+[^{3}H]palmitate+ATP)+(Palmitate)		1300	
(Enzyme+[^{3}H]palmitate+ATP)+(Palmitate+CoA)		9000	5250
(Enzyme+ATP)	+([^{3}H]Palmitate+palmitate+CoA)	3750	
(Enzyme+ATP)	+([^{3}H]Palmitate+palmitate)	0	
Glutathione absent			
(Enzyme+[^{3}H]palmitate+ATP)+(Palmitate)		340	
(Enzyme+[^{3}H]palmitate+ATP)+(Palmitate+CoA)		4400	500
(Enzyme+ATP)	+([^{3}H]Palmitate+palmitate+CoA)	3900	
(Enzyme+ATP)	+([^{3}H]Palmitate+palmitate)	0	

Table 4. *'Pulse-labelling' experiments with [U-^{14}C]ATP*

The reaction mixture during 'pulse labelling' contained 150mM-Tris–HCl buffer, pH7.6, 5mM-EDTA, 0.25mg of Triton X-100, 50mM-$MgCl_2$, 1 unit of inorganic pyrophosphatase, 10mM-reduced glutathione, 0.4mM-palmitate (where stated), 0.5mM-CoA (where stated), 0.58mM-[U-^{14}C]ATP (12.5μCi/μmol) and 200μg of purified enzyme fraction in a final volume of 0.1ml. 'Pulse labelling' was carried out for 1min followed by mixing with 'chase' reactants for 15s. For the 'chase' reaction non-radioactive ATP, palmitate (where stated) and CoA (where stated) were added to give a final concentration of 32mM-ATP, 0.5mM-CoA and 0.4mM-palmitate. The incubation was terminated by acidifying to pH2.0 and applying the reaction mixture to a column of Dowex-1 (Cl^- form) previously equilibrated with 0.02M-NaCl in 0.01M-HCl. The AMP fraction was eluted by the same buffer. A sample of the total AMP fraction was counted for radioactivity and results are expressed relative to the [U-^{14}C]ATP blank ['AMP (c.p.m.)'].

Tube no.	'Pulse' conditions	'Chase' conditions	AMP (c.p.m.)	Relative net pulse
1	(Enzyme+[U-^{14}C]ATP)	+(ATP+CoA)	3000	220
2	(Enzyme+[U-^{14}C]ATP)	+(ATP+CoA+palmitate)	3220	
3	(Enzyme+[U-^{14}C]ATP+palmitate)+(ATP)		1120	1380
4	(Enzyme+[U-^{14}C]ATP+palmitate)+(ATP+CoA)		2500	
5	(Enzyme+[U-^{14}C]ATP+CoA)	+(ATP)	13400	0
6	(Enzyme+[U-^{14}C]ATP+CoA)	+(ATP+palmitate)	10270	

The radioactive intermediate obtained during the 'pulse' period on incubation of the enzyme with ATP and high-specific-radioactivity palmitate is quite unstable in Dole's (1956) medium (Table 2, tube 5). Thus, in the absence of the reaction with CoA, hardly any radioactivity was recovered in Dole's (1956) lower phase. Synthetic palmitoyl-AMP could, however, be recovered in Dole's (1956) lower phase with a yield of 90–95%.

The presence of reduced glutathione during the 'pulse' period was obligatory for the ATP-dependent binding of palmitate (Table 3). However, no glutathione was required for the overall reaction, the presence of CoA possibly eliminating the need for an additional reducing agent.

'Pulse labelling' of the enzyme with high-specific-radioactivity [U-^{14}C]ATP followed by 'chase' non-radioactive ATP and the other substrates required for enzyme activity failed to further elucidate the nature of the enzyme–palmitate intermediate considered above. As shown in Table 4, no relevant nucleotide was bound to the enzyme whether incubated alone, in the presence of palmitate or in the presence of CoA during the radioactive-ATP 'pulse' interval. The specific radioactivity of ATP present during the 'pulse' was sufficiently high to allow detec-

Table 5. *'Pulse-labelling' experiments with [γ-^{32}P]ATP*

The reaction mixture during 'pulse labelling' contained 0.2mM-Tris–HCl buffer, pH7.6, 5mM-EDTA, 0.25mg of Triton X-100, 5mM-$MgCl_2$, 10mM-reduced glutathione, 1 unit of inorganic pyrophosphatase, 0.06mM-[γ-^{32}P]-ATP (14μCi/μmol), 0.2mM-palmitate (where stated), 0.66mM-CoA (where stated) and 114μg of enzyme fraction in a final volume of 0.1ml. 'Pulse labelling' was carried out for 1min followed by mixing with 'chase' reactants for 1min. For the 'chase' reaction non-radioactive ATP, palmitate (where stated) and CoA (where stated) were added to give a final concentration of 13.2mM-ATP, 0.66μM-CoA and 0.2mM-palmitate. The [^{32}P]P_i obtained was isolated and determined by the method of Pullman (1967). A sample of the phosphate fraction was counted for radioactivity and results are expressed relative to a [^{32}P]ATP blank ['P_i (c.p.m.)'].

Tube no.	'Pulse' conditions	'Chase' conditions	P_i (c.p.m.)	Apparent pulse	Overall reaction
1	(Enzyme+[γ-^{32}P]ATP)	+(—)	12000	5000	0
2	(Enzyme+[γ-^{32}P]ATP)	+(ATP+palmitate+CoA)	10000		
3	(Enzyme)	+([γ-^{32}P]ATP+ATP+palmitate+CoA)	5000		1500
4	(Enzyme)	+([γ-^{32}P]ATP+ATP+palmitate)	3500		
5	(Enzyme+[γ-^{32}P]ATP+palmitate)	+(—)	7000	4000	0
6	(Enzyme+[γ-^{32}P]ATP+palmitate)	+(ATP+CoA)	6000		
7	(Enzyme+palmitate)	+([γ-^{32}P]ATP+ATP+CoA)	2000		1000
8	(Enzyme+palmitate)	+([γ-^{32}P]ATP+ATP)	1000		
9	(Enzyme+[γ-^{32}P]ATP+CoA)	+(—)	51000	46700	0
10	(Enzyme+[γ-^{32}P]ATP+CoA)	+(ATP+palmitate)	50000		
11	(Enzyme+CoA)	+([γ-^{32}P]ATP+ATP+palmitate)	3300		1800
12	(Enzyme+CoA)	+([γ-^{32}P]ATP+ATP)	1500		

tion of enzyme-bound nucleotide had it occurred. The absence of ATP binding despite its obligatory part in palmitate binding is discussed below. Table 4 also shows that radioactive AMP is produced under conditions where overall reaction does not take place because of the absence of either CoA or palmitate. Further, in the presence of CoA, the amount of apparently irrelevant AMP formed was found to be still higher (Table 4, tube 5). This CoA-induced hydrolysis of ATP was further investigated by using [γ-^{32}P]ATP. As shown in Table 5, there was again no binding of nucleotide to the pure enzyme whether incubated alone, in the presence of palmitate or in the presence of CoA. Again the specific radioactivity of ATP was sufficiently high to allow detection of stable intermediate formation, had it occurred. The results similarly indicate that hydrolysis of ATP occurs during the 'pulse' period. The amount of radioactive phosphate liberated was proportional to the specific radioactivity of the ATP, the amount of enzyme and the time of incubation (not shown). Comparing the rate of ATP breakdown alone, in the presence of palmitate or in the presence of CoA (Table 5, tubes 1, 5, 9) it appears that CoA facilitates ATP breakdown whereas palmitate inhibits it somewhat. The simplest way to explain the effect of CoA would be to assume the presence of endogenous fatty acid substrate in the purified enzyme fraction. Indeed, incubating relatively large amounts of pure enzyme fraction with both ATP and CoA results in some CoA disappearance in the absence of exogenous fatty acid substrate. This time- as well as enzyme-dependent CoA disappearance may therefore reflect overall enzyme activity caused by endogenous substrate. It may be relevant that the first step of enzyme solubilization and purification consists of delipidation of microsomal fractions by dry butanol, acetone and diethyl ether. Incomplete delipidation might account for the presence of endogenous fatty acid substrate in the enzyme.

Another way to distinguish between the different enzyme-bound intermediates considered would be a 'pulse' experiment with radioactive palmitoyl-CoA. A positive 'pulse' dependent upon the presence of AMP would be expected to occur if AMP is part of the stable enzyme complex. As shown in Table 6, no 'pulse' was obtained, either in the presence or in the absence of AMP or PP_i respectively. None of the mechanisms previously considered are consistent with the non-occurrence of the expected palmitoyl-CoA 'pulse' and this observation is discussed below.

Table 6. *'Pulse-labelling' experiments with [^{3}H]palmitoyl-CoA*

The reaction mixture during the 'pulse labelling' contained 110mM-Tris–HCl buffer, pH7.6, 1.6mM-EDTA, 0.125mg of Triton X-100, 15.6mM-$MgCl_2$, 12.5mM-reduced glutathione, 0.069mM-[^{3}H]palmitoyl-CoA (8μCi/μmol), 2.5mM-AMP (where stated), 2.5mM-pyrophosphate (where stated) and 392μg of enzyme protein in a final volume of 0.16ml. 'Pulse labelling' was carried out for 1min followed by mixing with 'chase' reactants for 30s. For the 'chase' reaction non-radioactive palmitoyl-CoA was added to give a final concentration of 1.3mM. PP_i (where stated) and AMP (where stated) were also added to give a final concentration of 1.6mM for each substrate. The incubation was terminated by adding 1.0ml of Dole's (1956) reagent and the [^{3}H]palmitate was extracted as described by Bar-Tana *et al.* (1971). A sample of the upper phase (palmitate) was counted for radioactivity and results are expressed relative to a [^{3}H]palmitoyl-CoA blank ['Palmitate (c.p.m.)'].

Tube no.	'Pulse' conditions	'Chase' conditions	Palmitate (c.p.m.)
1	(Enzyme+[^{3}H]palmitoyl-CoA)	+(—)	7686
2	(Enzyme+[^{3}H]palmitoyl-CoA)	+(Palmitoyl-CoA+AMP+PP_i)	8290
3	(Enzyme+[^{3}H]palmitoyl-CoA+AMP)	+(—)	7168
4	(Enzyme+[^{3}H]palmitoyl-CoA+AMP)	+(Palmitoyl-CoA+PP_i)	7727
5	(Enzyme+[^{3}H]palmitoyl-CoA+PP_i)	+(—)	7852
6	(Enzyme+[^{3}H]palmitoyl-CoA+PP_i)	+(Palmitoyl-CoA+AMP)	7746
7	(Enzyme)	+([^{3}H]Palmitoyl-CoA+palmitoyl-CoA+AMP+PP_i)	8800
8	(Enzyme)	+([^{3}H]Palmitoyl-CoA+palmitoyl-CoA)	7799

Discussion

The role played by palmitoyl-AMP as an intermediate of long-chain fatty acid activation catalysed by purified palmitoyl-CoA synthetase was questioned throughout the present study. Since the absence of the partial enzyme activities with palmitoyl-AMP as substrate (Bar-Tana *et al.*, 1972*b*) might imply a mechanism not involving enzyme-bound adenylate, the overall course of catalysis was subjected to kinetic analysis. The kinetics of the forward overall reaction (palmitoyl-CoA synthesis) indicate that release of products occurs before combination of all three substrates with the enzyme, thus excluding a sequential addition of substrates to form a quaternary central complex. Similarly, release of products of the reverse reaction (substrates of the forward overall reaction) takes place before combination of all the substrates of the reaction in that direction. However, the fatty acid substrates, palmitate and palmitoyl-CoA, probably exist as micelles in aqueous solution and also as mixed micelles in the presence of detergent (Triton X-100), so that their effective concentrations might differ appreciably from their actual concentrations in the reaction mixture. Thus the kinetic analysis which holds for water-soluble reactants may be questioned when water-insoluble substances serve as substrates.

The 'pulse-labelling' experiment, demonstrating ATP-dependent binding of palmitate, further indicates that a stable form of enzyme is produced, which is suitable for reaction with CoA in a subsequent step. In other words, a first partial reaction is implied here in accordance with a Ping Pong mechanism. It may therefore be assumed that in the course of the overall reaction, interaction of the nucleotide and the palmitate substrates yielding the stable enzyme intermediate precedes the combination of the enzyme with CoA, giving rise to one of the following intermediates: enzyme–palmitoyl-AMP, palmitate–enzyme–AMP, enzyme–palmitoyl-PP_i, palmitate–enzyme–PP_i or enzyme–palmitate, palmitate being covalently bound to the enzyme in the latter case. Studies with ^{18}O-labelled palmitic acid have shown, however, that ^{18}O is transferred to AMP and palmitoyl-CoA exclusively, ruling out the possibility of the palmitoyl-PP_i intermediates.

The remaining alternatives may be characterized by the kinetic analysis of the reverse overall reaction. The results indicate that PP_i combines only with the formed intermediate thus implicating enzyme-bound nucleotide, either as enzyme-bound palmitoyl-AMP or palmitate–enzyme–AMP, as the intermediate in question. To confirm this conclusion the ATP 'pulse-labelling' technique was attempted, as a palmitate-dependent ATP 'pulse' would imply an enzyme-bound nucleotide intermediate. The negative results obtained with both [U-^{14}C]ATP and [γ-^{32}P]ATP seem to be inconsistent with the kinetic analysis suggested. However, because of the nature of the 'pulse-labelling' technique, to get significant labelling the following conditions should be maintained: stoicheiometric amounts of enzyme, high specific radioactivity of the substrate in question, an appropriate concentration of this substrate, determined by

taking into account the high optimal concentration required during the 'pulse' and at the same time still allowing for sufficient dilution in the 'chase' reaction. Evaluation of the [^{3}H]palmitate 'pulse' experiments reveals that these conditions were satisfied because of the low K_m value for palmitate in the forward overall reaction. The apparent K_m value for ATP, however, was about 100 times higher, limiting the concentration of ATP present during the radioactive ATP 'pulse labelling' to only a fraction of its K_m value (about 100 times less than the concentration of non-radioactive ATP present during the [^{3}H]palmitate pulse). Thus, the negative radioactive ATP 'pulse' obtained may be explained by the insufficient amount of ATP used during the 'pulse' period. Higher concentrations could not be employed because of the difficulty of appropriate dilution with non-radioactive ATP during the 'chase' reaction.

The same reasoning may explain the absence of radioactive palmitoyl-CoA 'pulse labelling' under conditions of the reverse overall reaction. Here again, the apparent K_m value for palmitoyl-CoA is far higher than the concentration of this substrate maintained during the 'pulse labelling' in the absence or presence of AMP or PP_i. Thus, absence of the AMP-dependent radioactive palmitoyl-CoA 'pulse' as well as absence of the palmitate-dependent radioactive ATP 'pulse' do not altogether eliminate the possibility of an enzyme-bound nucleotide as intermediate of the overall reaction.

In summary, the overall activation reaction of long-chain fatty acid appears to proceed via an enzyme-bound palmitate intermediate formed in the course of a Ping Pong mechanism. The question whether this intermediate constitutes also an enzyme-bound nucleotide (either as AMP–enzyme–palmitate or as enzyme-bound palmitoyl-AMP) still remains open and awaits the actual isolation of the intermediate concerned.

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