Microsomal Palmitoyl Coenzyme A Synthetase from Rat Liver

PARTIAL AND EXCHANGE REACTIONS

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The partial and exchange reactions of long-chain fatty acid activation were determined by using purified microsomal long-chain fatty acyl-CoA synthetase (EC 6.2.1.3). No significant ATP formation from palmitoyl-AMP and PP_i, nor palmitoyl-AMP-dependent CoA disappearance could be demonstrated. Similarly, no palmitate-dependent [³²P]₂PP_i–ATP exchange was catalysed by the pure enzyme. The above partial and exchange reactions were, however, catalysed by the parent microsomal fraction at a rate similar to that of the overall reaction. The implications of these results are discussed.

Fatty acid activation catalysed by long-chain fatty acyl-CoA synthetase (EC 6.2.1.3) is believed to involve fatty acyl-AMP as an intermediate of the overall activation reaction. In support of this mechanism, 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 (Vignais & Zabin, 1958; Bar-Tana & Shapiro, 1964). However, by using an enzyme preparation solubilized with Triton X-100, it was observed that the increase in specific activity of the overall reaction obtained after partial purification was followed by a concomitant decrease of the ATP-forming activity (Bar-Tana & Shapiro, 1964). Similarly, palmitoyl-CoA formation from palmitoyl-AMP and CoA was found to decrease in Triton X-100 extracts of microsomal fractions, although the overall reaction activity was maintained (R. Brandes & R. Arad, personal communication). These findings, together with the reported activation of the ATP-forming activity by preincubation of the partially purified enzyme with CoA and ATP, led to the assumption that two forms of enzyme exist, an active one containing bound CoA and an inactive one lacking CoA. Solubilization and subsequent purification were therefore assumed to convert the active form into the inactive one, causing the decrease of the two partial activities, which are determined respectively in the absence of ATP or CoA (Bar-Tana & Shapiro, 1964). Enzyme-bound CoA was also claimed by Rao & Johnston (1967) to be part of the enzyme complex catalysing the esterification of free fatty acid to glyceride. In the light of these findings it was of interest to study the partial reactions of fatty acid activation with the recently purified long-chain fatty acyl-CoA synthetase (Bar-Tana *et al.*, 1971).

Materials and Methods

ATP (disodium salt), AMP, NADP, CoA, dithiothreitol (Cleland, 1964), hexokinase and glucose

6-phosphate dehydrogenase were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A., or from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Sodium deoxycholate and dephospho-CoA were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. 5,5'-Dithiobis-(2-nitrobenzoic acid) was from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Triton X-100 was from Serva, Heidelberg, Germany. Sepharose 6-B and Sephadex G-50 (fine) were from Pharmacia, Uppsala, Sweden. Norit A was obtained from Amend Drug and Chemical Co., New York, N.Y., U.S.A. All other reagents were of A.R. Grade. [9,10-³H₂]Palmitic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and [³²P]phosphoric acid from Nuclear Research Centre, Beer-Sheva, Israel. [³²P]₂PP_i was prepared by heating [³²P]phosphate (sodium salt) at 450°C for 6h, and was isolated by the method of Bergmann (1962). Palmitoyl-AMP was prepared by the method of Vignais & Zabin (1958). The ether-dried material was treated with Dole mixture (Dole, 1956) to remove contaminating free fatty acid. It was weighed before use and dissolved in 0.5M-tris–HCl buffer, pH 7.9. Desulpho-CoA was prepared by the method of Chase *et al.* (1966) and phosphopantetheine by that of Larrabee *et al.* (1965).

Preparation of enzyme

Palmitoyl-CoA synthetase was isolated from freeze-dried rat liver microsomal fractions by using the procedure of Bar-Tana *et al.* (1971). The final gel-filtration step was carried out with Sepharose 6-B instead of Sephadex G-200, since better resolution and recovery was achieved. The purified enzyme fraction was extensively dialysed against '20% (v/v) glycerol buffer' composed of 20% (v/v) glycerol, 50mM-tris–HCl buffer, pH 7.9 (at 4°C), 1mM-EDTA and 0.25mM-dithiothreitol and was divided into small portions which were stored at –15°C.

Methods of enzyme determination

1. CoA disappearance in the forward overall reaction (palmitoyl-CoA synthesis) was determined as described by Bar-Tana *et al.* (1971). 2. Palmitoyl-AMP-dependent CoA disappearance was determined similarly by substituting palmitoyl-AMP (0.25–1.10mM) for palmitate and ATP. 3. Palmitoyl-CoA formation from [^3H]palmitate was determined as described previously (Bar-Tana *et al.*, 1971). 4. Palmitate formation from [^3H]palmitoyl-CoA was determined as described by Bar-Tana *et al.* (1971). 5. ATP formation from palmitoyl-AMP and PP_i was measured by one of the following procedures. (A) The reaction mixture contained 100mM-tris-HCl buffer, pH 7.4, 1.0mg of Triton X-100, 1.87mM-EDTA, 18.7mM-MgCl₂, 1mM- PP_i , 0.36mM-palmitoyl-AMP, 20mM-glucose, 0.26mM-NADP⁺, 0.525 unit of hexokinase, 0.7 unit of glucose 6-phosphate dehydrogenase and 50–200 μg of freeze-dried microsomal fractions or 400–800 μg of purified palmitoyl-CoA synthetase in a total volume of 1.0ml. Control experiments were carried out in the absence of palmitoyl-AMP. Incubations were done at 32°–37°C and activity was followed at 340nm. Under these conditions, 0.1 μmol of NADPH formed gave an extinction change of 0.610 (1cm light-path).

(B) The reaction mixture contained 140mM-tris-HCl buffer, pH 7.4, 0.25mg of Triton X-100, 2mM-EDTA, 20mM-MgCl₂, 1mM- $[\text{P}_2]\text{PP}_i$ (1 $\mu\text{Ci}/\mu\text{mol}$), 10mM-palmitoyl-AMP and 50 μg of purified palmitoyl-CoA synthetase in a total volume of 0.25ml. Incubations were carried out for 60min at 37°C and terminated by the addition of 1.0ml of 5% (w/v) cold trichloroacetic acid and 0.25ml of water. The mixture was centrifuged, and 1ml of the supernatant was adsorbed on to 200mg of acid-washed Norit A. The

Norit was then washed by the method of Stulberg & Novelli (1962) and the adsorbed [^{32}P]ATP hydrolysed and analysed for radioactivity.

6. The rate of [$^{32}\text{P}_2$]PP_i-ATP exchange was measured by the method of Cole & Schimmel (1970). The reaction mixture contained 200mM-tris-HCl buffer, pH 7.4, 0.5mg of Triton X-100, 2mM-EDTA, 5mM-MgCl₂, 2mM- $[\text{P}_2]\text{PP}_i$ (1–3 $\mu\text{Ci}/\mu\text{mol}$), 2mM-ATP and 50–200 μg of freeze-dried microsomal fractions or 25–100 μg of purified palmitoyl-CoA synthetase in a total volume of 0.25ml. Portions were taken for adsorption on to activated Norit A and treated as described under method 5(B).

Results

The rates of the two partial reactions catalysed by freeze-dried microsomal fractions with palmitoyl-AMP as substrate to yield ATP or palmitoyl-CoA in the presence of PP_i and CoA respectively are compared with the rates obtained with the purified enzyme (Table 1). Whereas the rate of both partial reactions catalysed by microsomal fractions was similar to that of the overall reaction, almost no partial activity was observed with the purified enzyme fraction. Variation of the concentration of substrates over a wide range or the addition of heated microsomal fractions (70°C for 15min) to the purified enzyme did not improve the partial activities. These findings agree with the observation that on treatment of rat liver microsomal fractions with Triton X-100 the increase in the specific activity of the overall reaction does not show a concomitant increase of the ATP-forming step (Bar-Tana & Shapiro, 1964) nor of palmitoyl-AMP-dependent CoA disappearance. The role played by the detergent regarding the lack of the

Table 1. Overall reaction and partial reaction rates catalysed by palmitoyl-CoA synthetase

V_{max} values in nmol of the respective reactant/min per mg of protein are given. Numbers in parentheses indicate the method of determination as described in the Materials and Methods section.

	V_{max} (nmol of reactant/min per mg of protein)	
	Microsomal fractions	Pure enzyme
Overall forward reaction (1, 3)	15.0	300.0
Overall reverse reaction (4)		40.0
ATP formation (5A)	6.5–10.0	1.7
ATP formation (5B)	9.0	1.0
Palmitoyl-AMP-dependent CoA disappearance (2)	9.0	3.0
Palmitoyl-AMP-dependent CoA disappearance in the presence of ATP (12mM) (2)	10.0–14.0	155.0

partial activities with the pure enzyme fraction is worth noting, since examination of the various enzyme fractions obtained in the course of the purification revealed that loss of the partial activities occurred after the deoxycholate-solubilization step: 'delipidated' rat liver microsomal fractions before solubilization by deoxycholate were still able to catalyse both partial reactions. A specific inhibitory effect of the detergent may be ruled out, since removal of traces of the detergent bound to the pure enzyme fraction by zone electrophoresis on Sephadex G-50 (fine) as the supporting medium did not restore the partial activities concerned. Similarly, a low affinity of the pure enzyme fraction for exogenous palmitoyl-AMP probably cannot account for the observed lack of the partial activities, since the K_m value for the palmitoyl-AMP substrate in the formation of ATP is far below the K_m value for either palmitoyl-CoA or AMP in the reverse overall reaction (Table 2). The possibility that the microsomal partial activities are not relevant to the overall acyl-CoA formation will be discussed below. However, it is worth noting that the K_m value for PP_i of the observed (albeit very small) ATP formation catalysed by the purified palmitoyl-CoA synthetase is identical with the K_m value for PP_i of the reverse overall reaction (Table 2). Further, CoA inhibited almost completely the ATP-forming activity of the pure enzyme, indicating that palmitoyl-AMP may be involved as an intermediate of the overall activation reaction.

In view of the absence of the two partial reactions expected to be catalysed by the purified enzyme fraction, the previously assumed activation of palmitoyl-CoA synthetase by CoA and ATP was attempted. For this purpose, the second partial-reaction step was carried out in the presence of ATP simulating the ATP-CoA activation of the ATP-forming step reported (Bar-Tana & Shapiro, 1964). As shown in Table 1, the presence of ATP raised the rate of

palmitoyl-CoA formation from palmitoyl-AMP and CoA to a value half that of the overall reaction rate. With higher palmitoyl-AMP concentrations a rate approaching that of the overall reaction rate was obtained. However, the possibility that the partial reaction activity obtained in the presence of ATP is due to overall reaction activity with free fatty acid which contaminates the palmitoyl-AMP substrate had to be considered. Although the purified enzyme fraction does not contain palmitoyl-AMP hydrolase, a spontaneous non-enzymic hydrolysis of palmitoyl-AMP occurs (0.5nmol/min with 0.25mM-palmitoyl-AMP), contributing minute amounts of fatty acid substrate which may cause the disappearance of CoA in the presence of ATP. Also, the apparent K_m value for ATP used in the activation of the second partial reaction was the same as that for the ATP substrate in the overall reaction (Table 3). To clarify

Table 2. Kinetic parameters of the overall reverse reaction and the ATP-formation step catalysed by purified palmitoyl-CoA synthetase

K_m values (μM) were derived from Lineweaver-Burk plots of the respective saturation curves. Activities of the ATP-formation step and of the overall reverse reaction were measured as described in the Materials and Methods section (method 5B and method 4 respectively).

Substrate	K_m (μM)	
	Overall reverse reaction	ATP formation
PP_i	890	800
Palmitoyl-CoA	2600	—
AMP	630	—
Palmitoyl-AMP	—	35

Table 3. Kinetic parameters of the overall forward reaction and palmitoyl-AMP-dependent CoA disappearance step catalysed by purified palmitoyl-CoA synthetase

K_m values (μM) were derived from Lineweaver-Burk plots of the respective saturation curves. Activities of the overall forward reaction and of the palmitoyl-AMP-dependent CoA disappearance step were measured as described in the Materials and Methods section (methods 1 and 3, and method 2 in the presence of 12mM-ATP, respectively).

Substrate	K_m (μM)	
	Overall forward reaction	Palmitoyl-AMP-dependent CoA disappearance
Palmitate	40	—
Palmitoyl-AMP	—	400
ATP	3250	3250
CoA	52	52

Table 4. *Stoichiometry of the overall forward reaction and palmitoyl-AMP-dependent CoA disappearance step determined in the presence of ATP catalysed by purified palmitoyl-CoA synthetase*

CoA disappearance, AMP appearance and PP_i appearance were measured as described previously (Bar-Tana *et al.*, 1971). The values for the AMP/CoA ratio were obtained at the stated incubation times.

Product appearance substrate disappearance	Overall forward reaction	Palmitoyl-AMP-dependent CoA disappearance in the presence of 12mM-ATP
PP _i /CoA	1.06	1.05
AMP/CoA 0–10min	1.05	1.06
20–30min	—	1.36
40–60min	0.92	2.1

Table 5. *Effect of enzyme preincubation in the presence of ATP and CoA on the ATP-forming activity catalysed by purified palmitoyl-CoA synthetase*

Preincubation was carried out with 965 µg of purified enzyme, 150mM-tris-HCl buffer, pH 7.4, 50mM-MgCl₂, 2mM-EDTA and 0.2mg of Triton X-100 in a total volume of 0.2ml. The reaction mixture was incubated at 37°C for 30min and followed by gel-filtration through Sephadex G-50 (fine). The enzyme was eluted at 4°C with '20% glycerol buffer' (see the text), and determined as described in the Materials and Methods section (method 5B).

Conditions	ATP formed (nmol/min per mg of protein)
Not preincubated	1.4
Preincubated	0.42
Preincubated with ATP (20mM)	1.41
Preincubated with CoA (3mM)	0.36
Preincubated with ATP (20mM) and CoA (3mM)	1.43

Table 6. *[³²P₂]PP_i-ATP exchange reaction in microsomal and pure-enzyme fractions catalysed by palmitoyl-CoA synthetases*

[³²P₂]PP_i-ATP exchange reaction rate was determined as described in the Materials and Methods section (method 6). Microsomal and pure-enzyme overall reaction rates under exchange conditions were 45.0 and 320.0nmol/min per mg of protein respectively.

Additions	[³² P ₂]PP _i -ATP exchange rate (nmol/min per mg of protein)	
	Microsomal fractions	Pure enzyme
None	29.0	21.0
Palmitate (0.2mM)	21.6	16.2
CoA (1.0mM)	3.5	1.3
Palmitate (0.2mM), CoA (1.0mM)	13.3	1.3

this point the stoichiometry of the second partial reaction in the presence of ATP was determined and compared with that of the overall reaction. Table 4 shows that ATP is cleaved in both cases, yielding a PP_i formed/CoA consumed ratio of 1.0, whether palmitate or palmitoyl-AMP serves as substrate. The AMP/CoA ratio, however, was observed to increase during incubation from 1.1 (close to the expected

ratio for the overall reaction of 1.0) to 2.0, indicating that during the second partial-reaction step in the presence of ATP, first the free palmitate contaminating palmitoyl-AMP is consumed as substrate and then the reaction becomes dependent on the slow, simultaneously occurring spontaneous hydrolysis of palmitoyl-AMP. In the latter case the expected AMP/CoA ratio would be 2.0, one AMP being obtained by

the spontaneous hydrolysis of palmitoyl-AMP and one by enzymic ATP cleavage. Thus, ATP does not activate the second partial reaction, leaving the absence of the latter reaction as yet unexplained.

The possible activation of purified enzyme by pre-incubation with ATP and CoA was now reinvestigated by using ATP formation as the measure of activity. The results shown in Table 5 indicate that ATP protects the enzyme against thermal inactivation during enzyme preincubation, but no activating effect could be demonstrated by the additional presence of CoA. These results are in agreement with the observed

Fig. 1. Effect of palmitate on $[^{32}P_2]PP_i$ -ATP exchange catalysed by purified palmitoyl-CoA synthetase

$[^{32}P_2]PP_i$ -ATP exchange reaction rate was determined as described in the Materials and Methods section (method 6), in the presence of 0.17mM-dithiothreitol. *V* is given in nmol exchanged/min per mg of protein.

Fig. 2. Comparison of the rates of $[^{32}P_2]PP_i$ -ATP exchange and overall reaction catalysed by purified palmitoyl-CoA synthetase

The $[^{32}P_2]PP_i$ -ATP exchange rate (●) was determined as described in the Materials and Methods section (method 6) in the presence of 61.5 μg of purified enzyme fraction and 0.2mM-palmitate. The overall reaction rate (▲) was determined with the same reaction mixture after the addition of $[1-^{14}C]$ palmitate. $[1-^{14}C]$ Palmitoyl-CoA formation was determined as described in the Materials and Methods section (method 3).

Table 7. $[^{32}P_2]PP_i$ -ATP exchange reaction catalysed by purified palmitoyl-CoA synthetase in the presence of CoA analogues

$[^{32}P_2]PP_i$ -ATP exchange reaction rate was determined as described in the Materials and Methods section (method 6 in the presence of 0.2mM-palmitate). Overall reaction rate was determined by CoA disappearance or by the incorporation of $[1-C^{14}]$ palmitate in the presence of ATP and the respective analogue at saturation.

Additions	$[^{32}P_2]PP_i$ exchange (nmol/min per mg of protein)	Overall reaction (nmol/min per mg of protein)
None	21.4	0.0
Glutathione (2mM)	25.8	0.0
Glutathione (2mM)+pantetheine (0.91 mM)	22.2	0.7
Glutathione (2mM)+phosphopantetheine (0.58 mM)	21.0	40.0
Glutathione (2mM)+desulpho-CoA (0.5 mM)	20.6	0.0
Glutathione (2mM)+dephospho-CoA (0.91 mM)	16.8	—

absence of any activation by ATP of the second partial reaction as catalysed by the pure enzyme fraction.

To exclude the possibility of a specific inhibition exerted by exogenous palmitoyl-AMP, thus accounting for the absence of the two partial reactions with pure palmitoyl-CoA synthetase, the $[^{32}\text{P}_2]\text{PP}_i\text{-ATP}$ exchange was studied. As shown in Table 6, no palmitate-dependent $[^{32}\text{P}_2]\text{PP}_i\text{-ATP}$ exchange was catalysed by the pure enzyme, and the observed exchange reaction was still further inhibited by exogenous palmitate (Fig. 1). The rate of the overall reaction is compared with the rate of the $[^{32}\text{P}_2]\text{PP}_i\text{-ATP}$ exchange reaction in Fig. 2. Under the conditions described the overall activity approached equilibrium after about 90 min, whereas the fraction exchanged during that interval was about 10%. However, it is worth noting that presence of CoA completely inhibited the exchange, conforming with the inhibitory role of CoA in the ATP-formation step, which would be expected if enzyme-bound palmitoyl-AMP serves as an intermediate of the overall reaction. Addition of CoA analogues such as pantetheine, phosphopantetheine, desulpho-CoA or dephospho-CoA did not significantly affect the $[^{32}\text{P}_2]\text{PP}_i\text{-ATP}$ exchange reaction (Table 7). The small though significant overall activity observed with some of these analogues indicates that they are recognized by the purified enzyme.

The relatively low rate of the $[^{32}\text{P}_2]\text{PP}_i\text{-ATP}$ exchange catalysed by the purified enzyme fraction again contrasts with the rate of exchange obtained in microsomal fractions, which is similar to the overall reaction rate (Fig. 3). The presence of endogenous substrate probably accounts for the apparent non-dependence on exogenous palmitate of the $[^{32}\text{P}_2]\text{PP}_i\text{-ATP}$ exchange catalysed by microsomal fractions, and inhibition by CoA is expected, assuming the postulated two-step mechanism for microsomal palmitoyl-CoA synthetase (Table 6).

Since almost no partial reactions nor respective exchange reactions could be demonstrated by using the purified enzyme fraction, a study of other possible exchange reactions was undertaken. By using various concentrations of palmitate and palmitoyl-CoA, no palmitate-palmitoyl-CoA exchange appeared to be catalysed by the pure enzyme fraction in the absence of both PP_i and AMP. On the other hand, a residual AMP-ATP exchange existed (<1 nmol/min per mg of protein), but since it was not influenced by the various components of the system it does not appear to reflect any partial activity related to the overall activation reaction.

Discussion

The evaluation of the results in the present paper regarding the difference in the partial reaction and exchange activities catalysed by the microsomal and

the purified enzyme respectively is open to speculation. Assuming a Bi Uni Uni Bi Ping Pong mechanism (Cleland, 1963) for the palmitoyl-CoA synthetase involving enzyme-bound palmitoyl-AMP as an intermediate, one is faced with the relative absence of the two partial-reaction steps when pure enzyme is used. Also, the observed lack of palmitate-dependent $[^{32}\text{P}_2]\text{PP}_i\text{-ATP}$ exchange activity is not what would be expected from the assumed Bi Uni Uni Bi Ping Pong mechanism. A specific inhibitory effect of detergent on the catalysis of the partial activities as well as substrate inhibition by exogenous palmitoyl-AMP could be ruled out.

Fig. 3. Comparison of the rates of $[^{32}\text{P}_2]\text{PP}_i\text{-ATP}$ exchange and overall reaction catalysed by microsomal palmitoyl-CoA synthetase

The $[^{32}\text{P}_2]\text{PP}_i\text{-ATP}$ exchange rate (●) was determined as described in the Materials and Methods section (method 6), in the presence of $216\mu\text{g}$ of 'delipidated' microsomal protein and 0.2mM -palmitate. The overall reaction rate (▲) was determined with the same reaction mixture after the addition of $[1\text{-}^{14}\text{C}]\text{palmitate}$. $[1\text{-}^{14}\text{C}]\text{Palmitoyl-CoA}$ formation was determined as described in the Materials and Methods section (method 3). The rates are given as percentage of the respective maximal activity.

'Substrate synergism' (Bridger *et al.*, 1968) seems an unlikely explanation of the results obtained, since neither CoA nor CoA analogues can evoke ATP formation from palmitoyl-AMP and PP_i, or palmitate-dependent [³²P]_i–ATP exchange. Similarly, ATP failed to induce a truly palmitoyl-AMP-dependent CoA disappearance. In this respect palmitic acid activation seems to differ from some of the amino acid activation systems, which show a synergistic effect of tRNA in the first partial-reaction step (Mehler, 1970).

In spite of the relative absence of the partial-reaction and exchange-reaction activities the possibility that palmitoyl-AMP still serves as an intermediate of the overall activation reaction cannot be ruled out. The assumption that an obligatory part is played by the microsomal membrane in the catalysis of the partial activities is supported by the coincidental close relationship between treatment of the microsomal fractions with detergent and the subsequent loss of the partial reaction activities. Thus, a conformational change induced by solubilization may be interfering with the catalysis of the partial activities without affecting the overall reaction catalysis. The residual partial activities observed may therefore be considered as true partial steps of the overall activation reaction and CoA inhibition of the respective partial activities supports this. Moreover, the absence of palmitate–palmitoyl-CoA and AMP–ATP exchange leaves these partial activities, however low they are, as the only ones that exist. Assuming a mechanism different from the Bi Uni Uni Bi Ping Pong mechanism for the purified enzyme and hence the original microsomal fraction (Cornforth, 1959; Boyer, 1960; Ingraham & Green, 1958), one is confronted with the presence of partial reaction activities in microsomal fractions. Since the rate of the microsomal partial reactions is in the range of the overall rate it seems unlikely that the former are not relevant to microsomal fatty acid activation.

To reconcile the findings obtained with the pure and the microsomal enzyme fractions respectively, it may be argued that two separate enzyme activities may catalyse microsomal fatty acyl-CoA synthesis. Thus the microsomal partial reactions may reflect

activity related to the enzyme involving bound palmitoyl-AMP as an intermediate, whereas the second activity, isolated as the pure enzyme fraction, may comprise activity catalysing fatty acid activation in the absence of partial reactions. A similar situation was encountered with medium-chain fatty acyl-CoA synthetase (Bar-Tana *et al.*, 1968; Bar-Tana & Rose, 1968*a,b*).

The results of further studies on purified palmitoyl-CoA synthetase may lead to further elucidation of the mechanism of long-chain fatty acid activation.

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