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INTERACTION BETWEEN THE PYRUVATE DEHYDROGENASE COMPLEX AND CITRATE SYNTHASE

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Summary

Kinetic studies of the individual reaction of pig heart pyruvate dehydrogenase complex (pyruvate dehydrogenase (pyruvate:lipoamide oxidoreductase (decarboxylating and acceptor-acetylating), EC 1.2.4.1); dihydrolipoamide reductase(NAD^+) ($NADH$:lipoamide oxidoreductase, EC 1.6.4.3); dihydrolipoamide acetyltransferase (acetyl-CoA:dihydrolipoamide *S*-acetyltransferase, EC 2.3.1.12)), citrate synthase (citrate oxaloacetate-lyase (*pro*-3*S*-CH_2COO^- → acetyl-CoA), EC 4.1.3.7) and the pyruvate dehydrogenase complex-citrate synthase coupled system show that the K_m^{CoA} value of pyruvate dehydrogenase complex and K_m^{CoASAc} value of citrate synthase decrease in the coupled system when compared to those in the individual enzyme reactions. The explanation for this interaction may be an association between the two enzymes.

When it was centrifuged with 150 000 × *g* for 140 min, 30% of the citrate synthase sedimented in the presence of the pyruvate dehydrogenase complex, while no sedimentation was observed in the absence of the pyruvate dehydrogenase complex. Sedimentation of cytoplasmic malate dehydrogenase, phosphotransacetylase, hemoglobin and Blue albumin were negligible under the same condition.

In gel chromatography experiments a significant peak of citrate synthase activity co-migrated with the pyruvate dehydrogenase complex peak. This observation also suggests the possible association of two enzymes.

Introduction

Recently it has turned out that a lot of enzymes previously regarded as soluble are really aggregated to particular components of cell [1—4], while

other enzymes catalyzing consecutive reactions are frequently associated with each other [5—9].

The pyruvate dehydrogenase complex (pyruvate dehydrogenase (pyruvate: lipoamide oxidoreductase (decarboxylating and acceptor-acetylating), EC 1.2.4.1); dihydrolipoamide reductase(NAD^+) ($NADH$:lipoamide oxidoreductase, EC 1.6.4.3); dihydrolipoamide acetyltransferase (acetyl-CoA:dihydrolipoamide *S*-acetyltransferase, EC 2.3.1.12)) catalyzes the formation of CoASAc from pyruvate. Being regulated by many metabolites [10—16] and phosphorylation-dephosphorylation [17,18], the pyruvate dehydrogenase complex can reasonably be considered a regulatory key enzyme complex (for a review see Refs. 10, 19). The product of the pyruvate dehydrogenase complex is CoASAc, of which the most important metabolic route is that of forming citrate in the reaction catalyzed by citrate synthase (citrate oxaloacetate-lyase (*pro*-3*S*-CH_2COO^- → acetyl-CoA), EC 4.1.3.7). For the close functional relationship between the pyruvate dehydrogenase complex and citrate synthase, it seemed reasonable to study kinetic parameters of the coupled enzyme system. In the course of this study we found that the K_m^{CoA} value of the pyruvate dehydrogenase complex and K_m^{CoASAc} of citrate synthase, markedly decreased in the coupled systems when compared to those in the individual enzyme reaction. This finding led us to carry out sedimentation and gel chromatography experiments on the two enzymes, which are also presented in this paper.

Materials and Methods

Enzymes, reagents and chemicals. The commercially available enzymes, that is, carnitine acetyltransferase (acetyl-CoA:carnitine *O*-acetyltransferase, EC 2.3.1.7) from pigeon breast muscle, phosphotransacetylase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) from *Clostridium kluyveri* and citrate synthase from pig heart were purchased from Boehringer Mannheim GmbH, Mannheim (F.R.G.). Hemoglobin and Blue albumin were obtained from Serva, Heidelberg (F.R.G.). Sephadex G-200 was from Pharmacia, Bromma (Sweden). NAD^+, $NADH$, thiamine pyrophosphate, pyruvate, 5,5′-dithiobis(2-nitrobenzoic acid) and oxaloacetate were obtained from Sigma Chemical Company, St. Louis (MO, U.S.A.). Coenzyme A and acetyl-coenzyme A were purchased from PL Biochemicals, Inc., Milwaukee (WI, U.S.A.). L-Carnitine was a kind gift from Sigma-τ, Rome (Italy). *p*-Nitroaniline was obtained from Merck AG. Darmstadt (F.R.G.). All other materials were of the highest purity commercially available.

Enzyme preparations. Highly purified pyruvate dehydrogenase complex was prepared from pig heart mitochondria as described by Cooper et al. [20] and was stored at a concentration of 50—70 units/ml in small batches at —20°C. The specific activity was 9 units/mg protein.

The arylamine acetyltransferase (acetyl-CoA:arylamine *N*-acetyltransferase, EC 2.3.1.5) was prepared from pigeon liver according to Tabor et al. [21].

The cytoplasmic malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) was prepared from pig heart as described by Thorne and Cooper [22].

Enzyme assays. The reaction of the pyruvate dehydrogenase complex was

assayed spectrophotometrically in a 20 mm path length cuvette, by monitoring the NADH formation at 340 nm. The enzyme activities were calculated from the initial rates. To determine the K_m^{CoA} value of the pyruvate dehydrogenase complex the NADH formation was monitored spectrophotometrically at 340 nm and spectrofluorimetrically at 470 nm (with 360 nm exciting light). The reaction mixture contained 50 mM potassium phosphate buffer, pH 8.0/2 mM pyruvate/2 mM $MgCl_2$/0.2 mM thiamine pyrophosphate/1 mM NAD^+ in a final volume of 1 ml. Unless otherwise stated, the CoA concentration was 0.1 mM.

The activity of citrate synthase was measured according to Srere et al. [23]. When the K_m^{CoASAc} value of citrate synthase was measured in the individual reaction, the mixture was completed with the materials of the coupled enzyme system (see below) except for the pyruvate dehydrogenase complex. The completion was necessary, so that the K_m^{CoASAc} values in the citrate synthase reaction and in the coupled enzyme system should be comparable.

The activities of carnitine acetyltransferase, cytoplasmic malate dehydrogenase and phosphotransacetylase were determined as described [24]. Hemoglobin and Blue albumin were assayed spectrophotometrically at 408 and 605 nm, respectively. Protein was determined according to Lowry et al. [32].

Coupled systems. In the pyruvate dehydrogenase complex-citrate synthase coupled system, the mixture for the pyruvate dehydrogenase complex activity measurement was supplemented with citrate synthase and 1 mM oxaloacetate. In all cases the NADH formation was monitored at 340 nm. When the K_m^{CoA} value of the pyruvate dehydrogenase complex was to be measured, the ratio of enzyme concentrations was [citrate synthase] >> [pyruvate dehydrogenase complex] so that citrate synthase could regenerate CoA in this case [CoASAc]/[CoA] << 1. For the measurement of the K_m^{CoASAc} value of citrate synthase in the coupled system, CoASAc was used instead of CoA and the ratio of enzyme concentrations was [pyruvate dehydrogenase complex] >> [citrate synthase], and hence the pyruvate dehydrogenase complex could regenerate CoASAc for citrate synthase. The ratio of [CoA]/[CoASAc] was lower than 0.03 as determined according to Bergmeyer [24].

The K_m^{CoA} value of the pyruvate dehydrogenase complex was also determined in other coupled systems in which citrate synthase was replaced by other CoASAc-consuming enzymes, such as arylamine acetyltransferase, carnitine acetyltransferase and phosphotransacetylase. The reaction was followed by NADH formation in the pyruvate dehydrogenase complex-carnitine acetyltransferase and the pyruvate dehydrogenase complex-phosphotransacetylase systems. The reaction rate of the pyruvate dehydrogenase complex-arylamine acetyltransferase system was monitored at 405 nm, on the basis of the difference in the light absorption at 405 nm between *p*-nitroaniline and *N*-acetyl-*p*-nitroaniline.

Enzyme co-sedimentation. The investigated 'enzyme-pairs' were placed in a polyethylene tube under conditions identical to those for the pyruvate dehydrogenase complex activity measurement. After precincubation for 30 min at 4°C the tube was centrifuged for 140 min at 150 000 × *g*. After removing the supernatant solution the pellet was resuspended in the original sample volume. The enzyme activities and the amount of proteins in the supernatants and in the resuspended pellets were determined.

Gel chromatography. The permeation of the pyruvate dehydrogenase complex-citrate synthase coupled system was investigated using a Sephadex G-200 column (diameter, 1 cm; height, 20 cm). The column was equilibrated and eluted with 50 mM potassium phosphate buffer, pH 8.0, containing 1.0 mM $MgCl_2$ and the specified concentration of citrate synthase at 4°C. Before passing the pyruvate dehydrogenase complex through the column, it was preincubated with the column-saturating concentration of citrate synthase at 4°C for 20 min. The condition of preincubation was the same as that for the pyruvate dehydrogenase complex activity measurement. The flow rate was 25 ml/h. Fractions of 0.5 ml were collected and the protein concentration was monitored at 280 nm by a model UA-5 absorbance monitor (Instrumentation Specialities Company). The fractions were assayed for the pyruvate dehydrogenase complex and citrate synthase activities.

Results

Kinetic experiments

The progress curve of the individual reaction of the pyruvate dehydrogenase complex was measured at a non-saturating, 8 μM CoA concentration and it was compared with that of the pyruvate dehydrogenase complex-citrate synthase coupled system. Fig. 1 shows that the pyruvate dehydrogenase complex activity in the coupled system is higher by about 70% over that in the individual pyruvate dehydrogenase complex reaction. Denatured citrate synthase did not effect any change in the reaction rate. The lower rate of individual pyru-

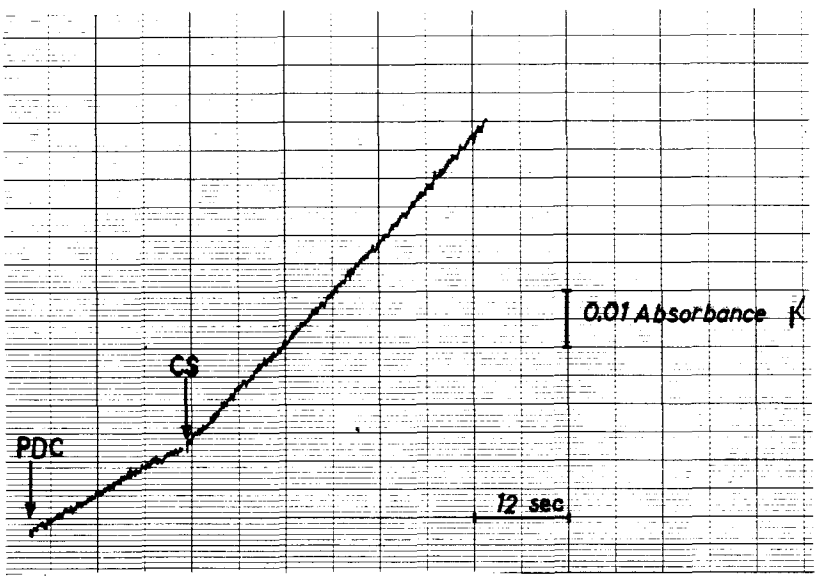

Fig. 1. Record of the NAD^+ reduction in the individual reaction of the pyruvate dehydrogenase complex and in the pyruvate dehydrogenase complex-citrate synthase coupled system at 8 μM concentration of CoA. The reaction mixture contained 50 mM potassium phosphate buffer, pH 8.0/2 mM pyruvate/1 mM oxaloacetate/0.2 mM thiamine pyrophosphate/2 mM $MgCl_2$/8 μM CoA/1 mM NAD^+ in a total volume of 1 ml (in a 20-mm path length cuvette). The individual reaction of the pyruvate dehydrogenase complex was started with 8 mU of enzyme complex at (↓PDC). The pyruvate dehydrogenase complex-citrate synthase coupled system was completed by adding 1.5 U of citrate synthase at (↓CS).

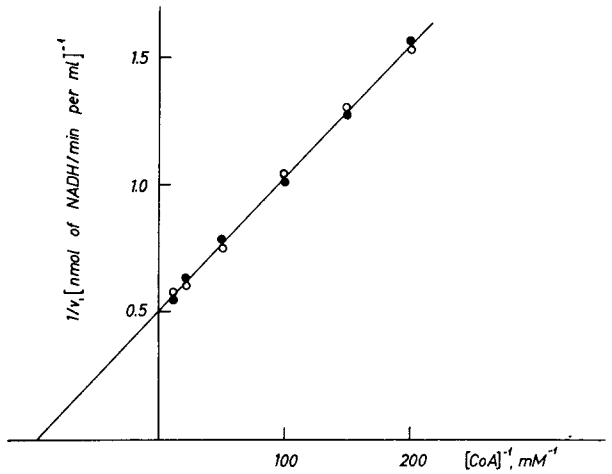

Fig. 2. Determination of the K_m^{CoA} value of the pyruvate dehydrogenase complex with fluorimetry (●———●) and photometry (○———○). Assay conditions were as described under Materials and Methods. The activity of the pyruvate dehydrogenase complex in the reaction mixture was 2 mU/ml.

vate dehydrogenase complex reaction could not be a consequence of product inhibition, because the initial rate was determined before the 15% decrease of the CoA concentration and the progress curve was linear in the studied time interval.

To explain this phenomenon we compared the K_m^{CoA} values of the pyruvate dehydrogenase complex in the individual reaction and in the coupled enzyme

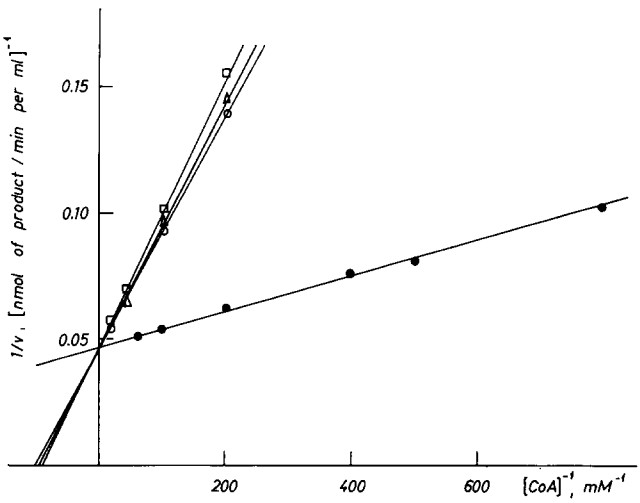

Fig. 3. Lineweaver-Burk plots with respect to CoA for the pyruvate dehydrogenase complex-citrate synthase (●———●), the pyruvate dehydrogenase complex-carnitine acetyltransferase (△———△), the pyruvate dehydrogenase complex-phosphotransacetylase (□———□) and the pyruvate dehydrogenase complex-arylamine acetyltransferase (○———○) coupled systems. The reaction mixture had the same composition as in the pyruvate dehydrogenase complex activity assay plus 1 mM oxaloacetate and 1.5 U citrate synthase (●), 2 mM carnitine and 2.0 U carnitine acetyltransferase (△), 6.0 U phosphotransacetylase (□), 1 mM *p*-nitroaniline and 1.5 U arylamine acetyltransferase (○). The activity of the pyruvate dehydrogenase complex was 20.8 mU/ml.

system. The dependence of the individual pyruvate dehydrogenase complex reaction on the concentration of CoA is shown in Fig. 2. The K_m^{CoA} value obtained by photometric and fluorimetric methods was 10.2 ± 1 μM. This K_m^{CoA} value was near to those reported by others [16,25,26], but was higher than that reported by Walsh et al. [27].

In a pyruvate dehydrogenase complex-citrate synthase coupled system ([citrate synthase] >> [pyruvate dehydrogenase]) almost all of the CoA is in the non-acetylated form. Therefore, such a system is appropriate to measure the K_m^{CoA} value of the pyruvate dehydrogenase complex. In this system the K_m^{CoA} value of the pyruvate dehydrogenase complex was found to be 1.5 μM (Fig. 3), which value is much lower than that found in the individual reaction. However, the $K_m^{NAD^+}$, $K_m^{pyruvate}$ and V values of the pyruvate dehydrogenase complex did not change in the coupled enzyme system (data not shown). In other systems the pyruvate dehydrogenase complex was coupled with acetyl-CoA-consuming enzymes, which were also in great excess to the pyruvate dehydrogenase complex. It is also seen from Fig. 3, that the marked decrease of the K_m^{CoA} value of the pyruvate dehydrogenase complex does not occur, when citrate synthase is replaced by other enzymes. In all experiments the ratio of [CoASAc]/[CoA] was lower than 0.04. The K_m^{CoA} value of the pyruvate dehydrogenase complex in the pyruvate dehydrogenase complex-carnitine acetyltransferase, pyruvate dehydrogenase complex-phosphotransacetylase and pyruvate dehydrogenase complex-arylamine acetyltransferase coupled systems were 9.8, 11.0 and 10.6 μM, respectively. From the above, the decrease of the K_m^{CoA} value of the pyruvate dehydrogenase complex seems to be specific for citrate synthase, rather than a general phenomenon.

To gain further information regarding the mutual effect of both enzymes we also determined in the K_m^{CoASAc} value of citrate synthase in the pyruvate dehydrogenase complex-citrate synthase system. In the coupled system the pyruvate

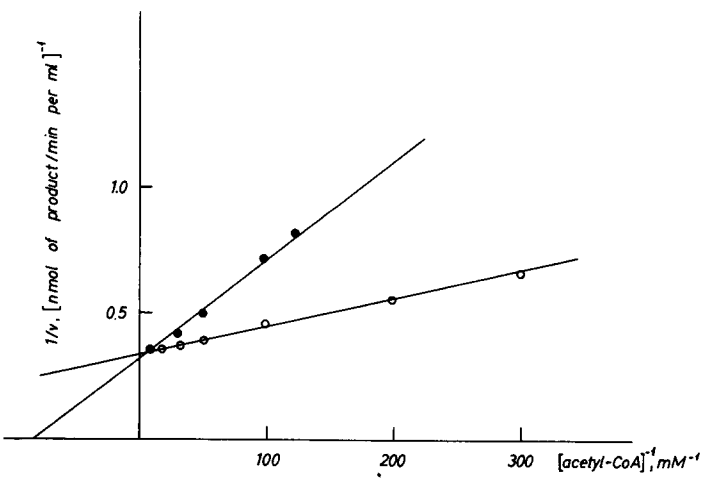

Fig. 4. Lineweaver-Burk plots with respect to CoASAc for the individual citrate synthase reaction (●——●) and the pyruvate dehydrogenase complex-citrate synthase coupled system (○——○). The activity of the pyruvate dehydrogenase complex was 1.6 U/ml in the coupled system, while the activity of citrate synthase was 3.1 mU/ml in both cases. Assay conditions were as described under Materials and Methods.

dehydrogenase complex was in excess so that CoA was mainly in CoASAc form ([CoA]/[CoASAc] < 0.04). The K_m^{CoASAc} values in citrate synthase reaction and in the pyruvate dehydrogenase complex-citrate synthase coupled system were found to be 12 and 3.1 μM, respectively (Fig. 4). That is, the coupling of pyruvate dehydrogenase complex caused a marked decrease of the K_m^{CoASAc} value of citrate synthase.

The kinetic interaction outlined above raises the possibility that the two enzymes contact each other forming a loose complex. Therefore, we studied the behaviour of the two enzymes in their mixed solution during sedimentation and gel permeation.

Ultracentrifugation experiments

The sedimentation technique is a known method to detect association between macromolecules [28—30]. Citrate synthase, cytoplasmic malate dehydrogenase, phosphotransacetylase, hemoglobin and Blue albumin were preincubated alone, and in combination with the pyruvate dehydrogenase complex, and centrifuged. Being an enzyme complex with an extremely large molecular weight, the pyruvate dehydrogenase complex sediments under the ultracentrifugation. It is seen in Table I that the pyruvate dehydrogenase complex carries down citrate synthase with itself. The sedimentation of cytoplasmic malate dehydrogenase, phosphotransacetylase, hemoglobin and Blue albumin was negligible under the same conditions.

Gel permeation experiment

For studying the possible association between the pyruvate dehydrogenase complex and citrate synthase the Hummel-Dreyer procedure [31] was also applied. The pyruvate dehydrogenase complex was chromatographed on a column of Sephadex G-200 equilibrated and developed with citrate synthase. The elution profile (Fig. 5) shows that the pyruvate dehydrogenase complex

TABLE I

CO-SEDIMENTATION OF PROTEINS UNDER INFLUENCE OF ULTRACENTRIFUGATION

Citrate synthase (5 nmol/ml), cytoplasmic malate dehydrogenase (5 nmol/ml), phosphotransacetylase (5 nmol/ml), Blue albumin (5 nmol/ml) and hemoglobin (5 nmol/ml) were preincubated (30 min at 4°C) in a mixture identical to that for pyruvate dehydrogenase complex activity measurement, but with (0.5 nmol/ml), or without, the pyruvate dehydrogenase complex. After centrifugation for 140 min at 150 000 × *g* the supernatant and the resuspended pellet was assayed for enzymes and proteins. The molecular weights of proteins: the pyruvate dehydrogenase complex, 7 000 000; citrate synthase, 100 000; cytoplasmic malate dehydrogenase, 52 000; phosphotransacetylase, 40 000; Blue albumin, 67 000; hemoglobin, 65 000.

Protein	Percentage of added proteins in the pellet	
	alone	with the pyruvate dehydrogenase complex
Pyruvate dehydrogenase complex	98.3	—
Citrate synthase	0	32.0
Cytoplasmic malate dehydrogenase	0	2.9
Phosphotransacetylase	0	2.3
Blue albumin	0	3.1
Hemoglobin	0	2.8

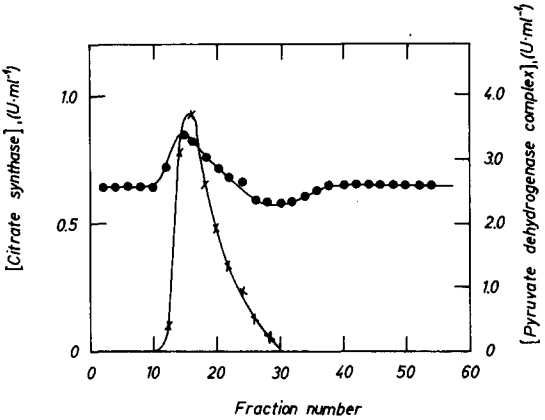

Fig. 5. Elution profile of the pyruvate dehydrogenase complex (X———X) and citrate synthase (•———•) on Sephadex G-200. 15 U pyruvate dehydrogenase complex were preincubated for 30 min at 4°C in an assay mixture of pyruvate dehydrogenase complex containing citrate synthase (0.7 U/ml) in a total volume of 0.5 ml. This solution was chromatographed on a column of Sephadex G-200 equilibrated and developed with 50 mM potassium phosphate buffer, pH 8.0, containing 1 mM $MgCl_2$ and citrate synthase (0.7 U/ml). The fractions were collected and tested for citrate synthase and the pyruvate dehydrogenase complex. The activities of enzymes were determined as under Materials and Methods.

peak coincides with the citrate synthase peak, which is followed by depletion in the citrate synthase activity. These results indicate the association of the two enzymes in gel filtration experiments.

Discussion

In this study we have got a very low K_m^{CoA} value (1.5 μM) for the pyruvate dehydrogenase complex in the coupled enzyme system, which is one-seventh of that in the individual pyruvate dehydrogenase complex reaction (Fig. 2) and in other acetyl-CoA-trapping systems (Fig. 3). The low K_m^{CoA} value of the pyruvate dehydrogenase complex has already been observed by Walsh et al. [27]. These authors also studied the pyruvate dehydrogenase complex reaction in the presence of oxaloacetate and citrate synthase to remove CoASAc. The K_m^{CoA} value of the pyruvate dehydrogenase complex decreases only in the coupled enzyme systems, which fact suggests that the interaction is specific for this system. Moreover, demonstrating a low K_m^{CoASAc} value for citrate synthase in the pyruvate dehydrogenase complex-citrate synthase coupled system, we have shown that the interaction between the two enzymes is mutual.

The plausible explanation for the low K_m^{CoA} value of the pyruvate dehydrogenase complex and K_m^{CoASAc} of citrate synthase is that there is an interaction between the two enzymes, which interaction probably results in a direct transfer of CoA and CoASAc between the two enzymes.

Gel permeation and sedimentation experiments indicate that a complex is formed between the pyruvate dehydrogenase complex and citrate synthase. Therefore, the active site of enzymes in the complex may be near to each other, so the direct transit of CoA and CoASAc between actives sites becomes more favourable than their diffusion into the solution.

As for cellular localization, both enzymes are located in the mitochondrial matrix. If their complex existed in the cell, it would bring about a compartmentation.

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