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A bifunctional enzyme complex in coenzyme A biosynthesis: purification of pantetheine phosphate adenylyltransferase and dephospho-CoA kinase

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Pantetheine phosphate adenylyltransferase (EC 2.7.7.3) and dephospho-CoA kinase (EC 2.7.1.24) were purified to near homogeneity from pig liver. The purification steps included the use of Sepharose-linked triazine dyes and affinity elution by CoA. Both activities co-purified at every stage of the 18000-fold purification. An M_r of 115000 was obtained by gel filtration on Sephadex G-150, and the final preparation yielded one major band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, with a subunit M_r of 57000. It is concluded that pantetheine phosphate adenylyltransferase and dephospho-CoA kinase exist as a bifunctional dimeric protein, which could be designated CoA synthetase.

The enzymes pantetheine phosphate adenylyltransferase (EC 2.7.7.3) and dephospho-CoA kinase (EC 2.7.1.24) catalyse the final reactions of CoA biosynthesis (Hoagland & Novelli, 1954; Abiko, 1975):

$$\text{4'-Phosphopantetheine} + \text{ATP} \overset{Mg^{2+}}{\rightleftharpoons} \text{dephospho-CoA} + PP_i$$

$$\text{Dephospho-CoA} + \text{ATP} \xrightarrow{Mg^{2+}} \text{CoA} + \text{ADP}$$

Partial purification (250-fold) of the rat liver cytosolic enzymes was carried out by Suzuki *et al.* (1967), with the use of DEAE-cellulose and CM-cellulose chromatography and Sephadex G-200 gel filtration. The activities could not be separated by these procedures, and the possibility of a bifunctional enzyme complex was suggested. The presence of both enzymes in rat liver mitochondria has been demonstrated (Skrede & Halvorsen, 1979), and the intramitochondrial localization has been examined (Skrede & Halvorsen, 1983).

The purpose of the present study was to purify both activities from pig liver cytosol and so further investigate the existence of a bifunctional enzyme complex.

Experimental

Materials

CoA was purchased from P-L Biochemicals, Milwaukee, WI, U.S.A. Phosphate acetyltransferase (EC 2.3.1.8), malate dehydrogenase (EC 1.1.1.37), pyruvate kinase (EC 2.7.1.40), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 3'-nucleotidase (EC 3.1.3.6) were obtained from Boehringer, Mannheim, West Germany. Hexokinase (EC 2.7.1.1), lactate dehydrogenase (EC 1.1.1.27) and citrate synthase (EC 4.1.3.7) were supplied by Sigma Chemical Co., Poole, Dorset, U.K.

DEAE-cellulose (DE-52) was from Whatman Biochemicals, Maidstone, Kent, U.K. Sephadex G-25, Sephadex G-150, Sepharose 4B, Blue Sepharose CL-6B, PBE 94 and Polybuffer 74 were supplied by Pharmacia, Uppsala, Sweden. Procion Red HE-3B was obtained from BDH Chemicals Poole, Dorset, U.K., and was linked to Sepharose 4B by the method of Baird *et al.* (1976).

Dephospho-CoA was prepared from CoA by incubation with 3'-nucleotidase (Wang *et al.*, 1954); the release of phosphate was monitored by using the assay method of Heinonen & Lahti (1981). The dephospho-CoA was purified by chromatography on DEAE-cellulose, with the use of a 0–0.15 M-LiCl gradient in 3 mM-HCl (Moffatt & Khorana, 1961).

Enzyme assays

All enzyme assays were carried out at 30°C, and the change in absorbance at 340nm was monitored.

Units of activity correspond to μmol of product formed/min, and any blank rates without dephospho-CoA were subtracted. The units of coupling enzymes used refer to those specified under the conditions of the suppliers.

Pantetheine phosphate adenylyltransferase. This activity was assayed in the reverse direction by monitoring the production of ATP from dephospho-CoA and PP_i by using the method of Lamprecht & Trautschold (1974). The 1 ml assay system consisted of 0.1 mM-dephospho-CoA, 2 mM-PP_i, 2 mM-$MgCl_2$, 1 mM-$NADP^+$, 5 mM-glucose, 2 units of hexokinase and 3 units of glucose 6-phosphate dehydrogenase in 50 mM-Tris/HCl buffer, pH 8, containing 0.5 mM-dithiothreitol.

Dephospho-CoA kinase. This enzyme was assayed by monitoring the production of ADP from dephospho-CoA and ATP by using the standard pyruvate kinase/lactate dehydrogenase coupling enzyme system (Jarowek *et al.*, 1974). The 1 ml assay incubation mixture consisted of 0.1 mM-dephospho-CoA, 2 mM-ATP, 2 mM-$MgCl_2$, 0.2 mM-NADH, 2.5 mM-phosphoenolpyruvate, 3 units of pyruvate kinase and 5 units of lactate dehydrogenase in 50 mM-Tris/HCl buffer, pH 8, containing 0.5 mM-dithiothreitol.

CoA formation. The above linear and continuous assays could not be used to determine activity in initial stages of purification, because of very low concentrations of enzyme and high interfering activities. Therefore the production of CoA from dephospho-CoA and ATP was examined in crude extracts by using the coupling enzyme system described by Michal & Bergmeyer (1974):

$$\text{CoA} + \text{acetyl phosphate} \rightleftharpoons \text{acetyl-CoA} + P_i$$

$$\text{Acetyl-CoA} + \text{oxaloacetate} \rightarrow \text{citrate} + \text{CoA}$$

$$\text{Malate} + NAD^+ \rightleftharpoons \text{oxaloacetate} + \text{NADH}$$

In this system acetyl-CoA is formed at a rate proportional to the CoA concentration, and regeneration of CoA by citrate synthase causes a displacement of the malate dehydrogenase equilibrium leading to the formation of NADH. The rate of reaction of phosphate acetyltransferase with dephospho-CoA is negligible in relation to CoA.

The following 1 ml assay mixture was used: 0.1 mM-dephospho-CoA, 2 mM-ATP, 2 mM-$MgCl_2$, 1 mM-NAD^+, 0.1 mM-NADH, 5 mM-acetyl phosphate, 5 mM-malate, 0.5 unit of phosphate acetyltransferase, 3 units of citrate synthase and 20 units of malate dehydrogenase in 50 mM-Tris/HCl buffer, pH 8, containing 0.5 mM-dithiothreitol.

Since CoA is recycled, the presence of dephospho-CoA kinase causes an acceleration in NADH production. Bordreaux *et al.* (1981) used a similar assay for CoA release, and suggested that a plot of A_{340} against $t^2/2$ would be linear, with a slope proportional to the rate of CoA production. However, this was found to be strictly true only if NADH was added initially to the assay, as expected from the arguments of Pearson (1965); this addition also increases the sensitivity of the assay.

To calibrate this assay for use with crude extracts, a standard line of slopes against kinase activity was produced with purified enzyme of known kinase activity (from the ADP-production assay).

Protein determination

Protein concentrations were determined by the method of Peterson (1977), with bovine serum albumin as a standard. Protein elution in column fractions was monitored by A_{280}.

Sodium dodecyl sulphate / polyacrylamide - gel electrophoresis

Electrophoresis in 12.5% slab gels was performed at pH 8.5 by using the method of Laemmli (1970). Gels were stained and fixed in 50% (v/v) methanol/10% (v/v) acetic acid containing 0.1% (w/v) Coomassie Brilliant Blue, and destained in 5% (v/v) methanol/10% (v/v) acetic acid. Bovine serum albumin, pyruvate kinase, lactate dehydrogenase, carbonic anhydrase and trypsinogen were used as molecular-mass standards.

Chromatofocusing

The isoelectric point of the enzyme was determined by using the chromatofocusing technique of Pharmacia, with a PBE 94 column and Polybuffer 74 (Richey & Beadling, 1982).

Results

Purification

All steps were carried out at 4°C, and pH values refer to this temperature. Fresh pig liver (600 g) was chopped up, homogenized in a blender for 30 s in 3 vol. of 0.25 M-sucrose/10 mM-Tris/HCl buffer, pH 8, and centrifuged at 17000 ***g*** for 20 min. The pellet was discarded, and 0.06 vol. of 2% (w/v) protamine sulphate solution (pH 8) was added dropwise to the stirred supernatant. The suspension was centrifuged at 17000 ***g*** for 15 min, and the pellet was discarded. $(NH_4)_2SO_4$ (313 g/l) was added slowly to the supernatant, which was stirred for a further 30 min and then centrifuged at 17000 ***g*** for 20 min. The resulting pellet was resuspended in a small volume of 10 mM-Tris/HCl buffer, pH 8, containing 0.5 mM-dithiothreitol, and desalted by passage through a Sephadex G-25 column (40 cm × 9 cm) previously equilibrated with 10 mM-Tris/HCl buffer, pH 8.

DEAE-cellulose chromatography. Dithiothreitol was added to the gel-filtered eluate to give a concentration of 0.5 mM, and the solution was loaded on to a DEAE-cellulose column (12 cm × 6.5 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 8, containing 0.5 mM-dithiothreitol. The column was washed with this buffer, and a linear 0–0.2 M-KCl gradient (2 litres) was applied. Fractions (14 ml) were screened for activity by using the accelerative assay for CoA production described above.

Procion Red–Sepharose 4B chromatography. Active DEAE-cellulose fractions were pooled and applied to a Procion Red–Sepharose 4B column (14 cm × 6.5 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 8, containing 0.5 mM-dithiothreitol and 0.1 mM-EDTA. The column was washed with this buffer, and the enzyme was eluted with a linear 0–0.6 M-KCl gradient (2 litres). Fractions (12 ml) were assayed for both activities (Fig. 1), and active fractions were pooled.

CoA elution from Blue Sepharose CL-6B. The Procion Red–Sepharose eluate was diluted with 1.5 vol. of 0.5 mM-dithiothreitol and loaded on to a Blue Sepharose column (12 cm × 3 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 8, containing 0.5 mM-dithiothreitol and 0.1 mM-EDTA. The column was washed with 0.12 M-KCl in this buffer, and the enzyme was eluted with 0.1 mM-CoA/0.1 M-KCl in 10 mM-Tris/HCl buffer, pH 8, containing 0.5 mM-dithiothreitol and 0.1 mM-EDTA.

Sephadex G-150 gel filtration. The active CoA-eluted material was concentrated by ultrafiltration under N_2 in an Amicon unit with a PM-10 membrane, and applied to a Sephadex G-150 column (80 cm × 3.2 cm) equilibrated with 50 mM-Tris-HCl buffer, pH 8, containing 0.5 mM-dithiothreitol and 0.1 mM-EDTA. The enzyme was eluted with this buffer, and fractions (3.8 ml) were assayed for both activities (Fig. 2). Both activities coincided with the protein peak, and active fractions were pooled.

The purified enzyme was concentrated to 0.5 mg/ml by ultrafiltration: when stored at −20°C it was found to be stable for at least 1 month.

The course of a typical purification is shown in Table 1.

Purity and M_r determination

The purity of the final enzyme preparation was examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and it was found

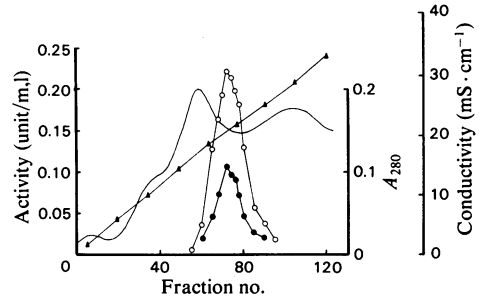

Fig. 1. *Elution of pantetheine phosphate adenylyltransferase* (○) *and dephospho-CoA kinase* (●) *from Procion Red–Sepharose 4B*

For full experimental details see the text. Conductivity (▲) and A_{280} (——) show KCl and protein concentrations.

Table 1. *Purification of pantetheine phosphate adenylyltransferase and dephospho-CoA kinase from pig liver (600 g)*

For full experimental details see the text.

Purification step	Volume (ml)	Protein (mg/ml)	Pantetheine phosphate adenylyltransferase (units/ml)	Pantetheine phosphate adenylyltransferase (units/mg)	Dephospho-CoA kinase (units/ml)	Dephospho-CoA kinase (units/mg)	Ratio transferase/kinase	Yield (%)	Purification
17000 *g* supernatant	1650	83	—	—	0.0165*	1.98×10^{-4}	—	100	1
Protamine sulphate supernatant	1680	32	—	—	0.0156*	4.87×10^{-4}	—	96	2.5
$(NH_4)_2SO_4$ fraction + Sephadex G-25	605	30	—	—	0.0395*	1.32×10^{-3}	—	87	6.7
DEAE-cellulose chromatography	485	6.7	0.095	0.014	0.045	6.7×10^{-3}	2.1	80	33.8
Procion Red–Sepharose eluate	380	0.208	0.113	0.543	0.055	0.264	2.05	76	1330
Blue Sepharose CoA eluate	104	0.041	0.305	7.4	0.15	3.64	2.03	57	18380
Sephadex G-150	75	0.028	0.213	7.61	0.103	3.68	2.07	29	18570

* Kinase activities from accelerative CoA production assay.

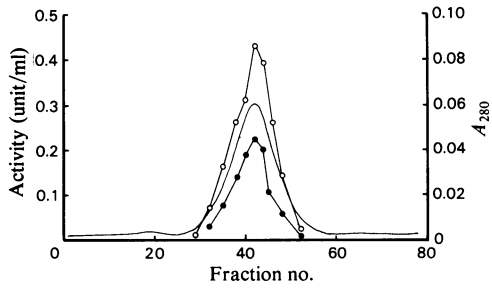

Fig. 2. *Pantetheine phosphate adenylyltransferase* (○) *and dephospho-CoA kinase* (●) *activities in Sephadex G-150 gel-filtration fractions (3.8 ml)*
For full experimental details see the text. ——, A_{280}.

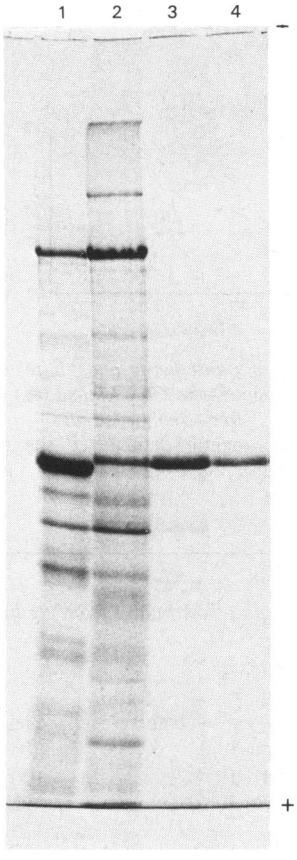

Fig. 3. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of preparations obtained at different stages during the purification of pantetheine phosphate adenylyltransferase and dephospho-CoA kinase*
For full experimental details see the text. Track 1, DEAE-cellulose eluate (50 μg of protein); track 2, Procion Red–Sepharose eluate (50 μg); track 3, CoA-eluted protein (20 μg); track 4, Sephadex G-150 eluate (10 μg).

to give essentially one band, with a subunit of M_r about 57000 (Fig. 3). Calibration of the Sephadex G-150 column yielded an M_r of approx. 115000 for the native enzyme, indicating that it is a dimer.

The isoelectric point of the enzyme was found to be 5.75.

Discussion

A procedure for the purification of both pantetheine phosphate adenylyltransferase and dephospho-CoA kinase has been developed, yielding a virtually homogeneous preparation, which is over 50 times more active than previously achieved. The ratio of specific activities remained constant throughout the purification, thus confirming the association suggested by Suzuki *et al.* (1967). A purification of 18000-fold was achieved, in which the use of immobilized triazine dyes and CoA affinity elution proved particularly successful. In unreported experiments PP_i and ATP have also been found specifically to elute both activities.

An M_r value of 115000 was obtained on Sephadex G-150 gel filtration, and the final preparation gave essentially one band on sodium dodecyl sulphate / polyacrylamide - gel electrophoresis, with a subunit M_r value of 57000. It therefore seems that the transferase and the kinase exist in pig liver cytosol as a bifunctional dimeric protein, which could conveniently be called CoA synthetase. The structural and catalytic relationships between the activities have yet to be investigated.

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