PURIFICATION AND CHARACTERISATION OF CARBAMOYL PHOSPHATE SYNTHETASE FROM BEEF LIVER

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1. Introduction

The *N*-acetyl-L-glutamate dependent carbamoyl phosphate synthetase (CPS) (EC 2.7.2.5) has been purified from frog liver [1] and rat liver [2]. This enzyme is thought to play a key role in the regulation of the urea cycle in vivo [3]. In preparation for kinetic studies we have purified and characterised CPS from beef liver.

2. Materials and Methods

2.1. *Materials*

Pyruvate kinase and lactate dehydrogenase (pig muscle) in 50% glycerol, β-galactosidase, malate dehydrogenase, ATP, NADH (grade II), oxaloacetate and phosphoenolpyruvate were obtained from Boehringer (Mannheim). Acetyl glutamic acid was obtained from Koch-Light Laboratories Ltd. All other reagents were obtained from BDH (Chemicals) Ltd.

2.2. *Methods*

Polyacrylamide gel electrophoresis (PAGE) was performed by the method of Davis [4] using 6.5% gels with 5% cross linking. The molecular weight (mol. wt) was determined by gel filtration on Sephadex G-200 [5] using β-galactosidase (mol. wt 520 000 [6]), pyruvate kinase (mol. wt 237 000 [7]), lactate dehydrogenase (mol. wt 140 000 [8]) and malate dehydrogenase (mol. wt 65 000 [5]) as markers. The subunit mol. wt was determined by PAGE in the presence of sodium dodecyl suphate (SDS) [9] using 10% gels with 1.33% cross linking and β-galactosidase (mol. wt 130 000 [6]), bovine serum albumin (mol. wt 68 000 [10]), pyruvate kinase (mol. wt 57 000 [7]), aldolase (mol. wt 40 000 [11]), pepsin (mol. wt 35 000 [12]) and lysozyme (mol. wt 14 000 [9]) as markers.

Amino acid analysis was performed after a 24 hr hydrolysis using a Beckman 120C automatic analyser. Tryptophan was estimated spectrophotometrically [13]. Biotin content was estimated by a bioassay using *Lactobacillus arabinosus* [14]. The N-terminal amino acid was determined using dansyl chloride [15, 16].

The isoelectric point was measured using an isoelectric focusing column by the method described by Illingworth [17].

2.3. *Assay of CPS activity*

CPS activity was assayed at 30°C by coupling ADP formation to NAD^+ formation with pyruvate kinase and lactate dehydrogenase [18] and measuring the decrease in absorbance at 340 nm. The assay mixture contained CPS, 2.5 mM phosphoenolpyruvate, 0.2 mM NADH, 10 mM NH_4Cl, 100 mM $KHCO_3$, 5 mM ATP, 10 mM $MgSO_4$, 10 mM acetyl glutamate, 20 μg pyruvate kinase, 20 μg lactate dehydrogenase and 50 mM glycylglycine at pH 7.6, which was found to be the optimum pH for activity. The reaction could be started by addition of either acetyl glutamate or CPS. The initial velocity was directly proportional to CPS concentration and one unit of CPS activity corresponds to the formation of 1 μmole ADP/min at 30°C.

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Table 1
Purification of carbamoyl phosphate synthetase.

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification
CTAB extract	500	6650	0.075	(100)	(1)
50–55% $(NH_4)_2SO_4$	300	2060	0.15	60	2
DEAE-cellulose column	150	131	1.14	30	15
Sephadex G 200 gel filtration	111	73.5	1.5	22.2	20

2.4. *Purification of CPS*

The purification procedure was based on that of Guthöhrlein and Knappe [2] for rat liver CPS. Beef liver was obtained from freshly slaughtered animals and kept on ice during transport to the laboratory with all other steps then performed at 4°C. 400 g of liver were homogenised in 2 litres or 250 mM sucrose buffered to pH 7.3 with 10 mM Tris–Cl in a 'Kenwood Chef' liquidiser. The homogenate was centrifuged at 6 000 *g* and the supernatant was filtered through muslin before being subjected to further centrifugation at 200 000 *g* to yield a crude mitochondrial pellet. The pellet was homogenised in 1 litre 0.1% (w/v) cetyltrimethyl ammonium bromide (CTAB) in 20 mM Tris–Cl, pH 7.2, containing 5 mM β-mercaptoethanol. The insoluble material was separated by centrifugation and the supernatant was subjected to $(NH_4)_2SO_4$ fractionation. Fractions precipitating between 45%–50%, 50%–55% and 55%–60% saturation were collected by centrifugation and each was redissolved in a small volume of 50 mM Tris–Cl, pH 7.2, containing 5 mM β-mercaptoethanol, 5 mM KCl and 20% (v/v) glycerol.

The $(NH_4)_2SO_4$ fraction with the highest specific activity was desalted on Sephadex G 25 into the above buffer and applied to a DEAE-cellulose column (1.5 × 10 cm) equilibrated in the same buffer. After washing the column with about 5 column vol of the above buffer the CPS was eluted with a linear gradient of 5 mM–75 mM KCl in 50 mM Tris–Cl, pH 7.2, containing 5 mM β-mercaptoethanol and 20% (v/v) glycerol, over 10 column vol with the activity being eluted at about 40 mM KCl. The CPS was concentrated by ultrafiltration and applied to a Sephadex G 200 column (3 × 40 cm). A symmetrical peak of activity was eluted with a constant specific activity except for the last 30% which had a lower specific activity and was discarded. Table 1 shows the results of a typical purification.

Table 2
Amino acid composition of beef liver CPS.

Amino acid	% by weight	mole/125 000 mol.wt
Ala	5.5	78
Arg	7.25	52
Asx	10.0	94
Cys (half)	2.1	16
Glx	11.9	101
Gly	4.4	73
His	3.1	25
Ile	6.0	57
Leu	9.1	86
Lys	9.6	82
Met	1.6	14
Phe	5.2	39
Pro	4.3	46
Ser	4.6	55
Thr	4.75	50
Trp	–	13
Tyr	3.7	26
Val	6.78	72

3. Results

The preparation of CPS obtained was homogeneous as judged by PAGE, gel filtration and isoelectric focusing. On Sephadex G 200 a single peak of protein and activity was eluted at a position corresponding to a mol. wt of 250 000 (± 25 000) on the linear calibration curve. SDS-PAGE was performed in an attempt to determine the subunit molecular weight but when the native enzyme was subjected to this procedure it migrated at a rate corresponding to a mol. wt of about 215 000 (by extrapolation of the calibration curve). However if the CPS was first carboxymethylated a band of apparent mol. wt 165 000 was seen with a minor band corresponding to an approximate mol. wt 280 000 (probably corresponding to undissociated enzyme). This suggests that the native enzyme exists as a dimer. On isoelectric focusing the enzyme migrated in a sharp peak, corresponding to the

only protein peak, and was shown to have an isoelectric point of 5.3.

The amino acid composition based on a subunit mol. wt of 125 000 is shown in table 2. The N-terminal amino acid was found to be histidine. No biotin was found in the enzyme preparation.

4. Discussion

The modifications of the purification procedure which has previously been described for CPS from rat liver [2] were made in order to enable more convenient handling of larger volumes. The enzyme prepared in this way was stable for over one year when stored at $-10^{\circ}C$ in buffer containing 20% glycerol. When stored at $4^{\circ}C$ the enzyme activity slowly declined; some 50% of the starting activity being lost after 6 months. After this time 90% of the activity could be restored by incubation with 1 mM dithiothreitol for 12 hr at $4^{\circ}C$. The presence of KCN during the purification [2] was not found to have any appreciable effect on the stability of the enzyme. It was found that the preparation of mitochondria in 250 mM sucrose gave a greater yield of CPS than did the preparation in 20 mM Tris–Cl as previously recommended [2].

There is very little information available on the properties of CPS from other sources but the mol. wt of 250 000 and absence of biotin reported here are similar to results previously reported for the rat liver enzyme [2], although the enzyme from *E. coli* has been reported to contain biotin [19].

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