ISOLATION OF AN IMMUNOLOGICALLY PURE PREPARATION OF CARBAMYLPHOSPHATE SYNTHETASE (AMMONIA) USING CHROMATOFOCUSING

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Received 24 August 1981

1. Introduction

Carbamylphosphate synthetase (ammonia, EC 2.7.2.5) catalyzes the first reaction in urea biosynthesis in mammalian liver. It is the major protein found in the mitochondrial matrix in liver cells [1–6], and appears to be critically important in the regulation of urea biosynthesis [5,7]. A number of purification procedures have been reported for isolation of the enzyme from rat liver [2–4,6]. The enzyme is very susceptible to proteolytic modification [8–10], and existing methods either produce yields of <15% or take a considerable time to perform. A method employing high-performance liquid chromatography has been described [11], but only small quantities of enzyme can be isolated using this approach. Here, a simple purification procedure is described involving ion-exchange chromatography, gel filtration and chromatofocusing which results in the isolation of an enzyme preparation which is >99% pure, has a specific activity equal to the highest reported and can be obtained with a yield of 30%. A method is also described for testing whether antibodies produced against this preparation of carbamylphosphate synthetase are monospecific.

This procedure is an improvement on existing methods in that it is rapid and therefore minimizes problems arizing from the susceptibility of carbamylphosphate synthetase to proteolysis. The enzyme obtained chromatographs as a single isoelectric species using the chromatofocusing technique and produces monospecific antibodies in rabbits which bind only to carbamylphosphate synthetase in extracts of rat liver.

2. Materials and methods

2.1. *Materials*

Male Wistar rats (250–300 g) were fed on a standard laboratory chow and starved overnight before use. Diethylaminoethyl (DEAE) Sephacel, Sephacryl S-300, polybuffer exchanger PBE 94 and polybuffer 74 were purchased from Pharmacia. Dithiothreitol, crystalline bovine serum albumin, leupeptin, Triton X-100, SDS, Coomassie brilliant blue R250, and G250, *N*-acetylglutamate and *N*-2-hydroxyethylpiperazine *N'*-2-ethanesulfonic acid (Hepes) were from Sigma. Sodium [^{14}C]carbonate (58.9 mCi/mmol, 2.18 GBq/mmol) was from New England Nuclear Corp. Acrylamide, methylene-bisacrylamide and *N,N,N',N'*-tetra-methylene diamine were from Merck. 1,4-Butane diol diglycidyl ether and 2-aminothiophenol were obtained from Aldrich Chemical Co. Ltd. ^{125}I-Labelled protein was prepared by the chloramine-T method to spec. act. 6.6 mCi/mg.

2.2. *Carbamylphosphate synthetase assay*

The enzyme was assayed by a radiochemical procedure based on the conversion of $^{14}CO_2$ to [^{14}C]carbamylphosphate, which was then converted to hydroxy [^{14}C]urea on the addition of hydroxylamine [12]. Appropriate samples of enzyme (1–10 μl) were added to 0.2 ml stock solution containing 50 mM Na-Hepes (pH 7.4), 100 mM KCl, 10 mM ATP, 15 mM $MgSO_4$, 10 mM NH_4Cl and 1 mM *N*-acetylglutamate in 0.4 ml total vol. Reactions were initiated by the addition of 50 μl Na-[^{14}C]bicarbonate (400 mM; 0.2 mCi/ml) to 50 mM final conc. One unit is the amount of enzyme that synthesizes 1 μmol carbamylphosphate/min at 37°C and pH 7.4. The column frac-

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.75

tions were assayed by a modified procedure using [^{14}C]bicarbonate to 5 mM final conc.

2.3. *Determination of protein*

Protein was measured as in [13] using Coomassie brilliant blue G250, and by measuring the absorbance at 280 nm (extinction coefficient 1.16, [1,4]).

2.4. *Gel electrophoresis and electrophoretic transfer*

Polyacrylamide gel electrophoresis was performed as in [15] using the buffer system in [16] and 5% SDS–polyacrylamide gels. Two replica sets of samples (25–50 μg protein) from each stage of the purification procedure were electrophoresed. The gel was cut in half after electrophoresis and one half stained and the other half transferred to a diazotized paper. Staining was for 3 h in 0.25% Coomassie blue in methanol:acetic acid:H_2O (5:1:5), and gels were destained at 37°C in an aqueous solution 20% in methanol and 10% in acetic acid. Electrophoretic transfer was performed on the apparatus in [17]. 2-Aminothiophenol paper was prepared by the method of Brian Seed (Caltech, personal communication): Whatman 540 paper (14 × 10 cm; 20 g) was mixed by end-over-end shaking with 70 ml 0.5 M NaOH containing 2 mg/ml $NaBH_4$ and 30 ml 1,4-butanediol diglycidyl ether for 16 h in a sealed bag. The paper was washed for 1 h in 0.5 M NaOH:ethanol (1:1, v/v) then mixed with 100 ml 0.5 M NaOH:2-aminothiophenol (1:1, v/v, aminothiophenol in a 5% solution in ethanol) for 2 h. Paper was extensively washed with ethanol, followed by 0.1 M HCl and water. The dried papers were stored in the dark at room temperature before transfer. Gels were washed 3 times for 10 min in 25 mM phosphate buffer (pH 6.5). The amino paper was diazotized [18] and transfer of proteins was carried out by gel electrophoresis at 25 V (0.8 A) for 1 h at room temperature in 25 mM phosphate buffer (pH 6.5). Excess diazo groups were quenched by incubating paper at 37°C for 2 h in 0.1 M Tris–HCl (pH 9.0)/10% ethanolamine (v/v)/0.25% gelatin (w/v). The paper was probed with 1 ml antisera obtained from rabbits innoculated with carbamylphosphate synthetase as in [18]. The paper was also probed with 1 ml pre-immune serum after regeneration of the paper with 8 M urea and 0.1 M β-mercaptoethanol [18].

Antisera to carbamylphosphate synthetase was also tested by Ouchterlony double-diffusion analysis under non-dissociating [19], and dissociating conditions [1] and by immunoelectrophoresis [20].

2.5. *Production of antibodies*

Antibodies against carbamylphosphate synthetase were produced in rabbits using the following innoculation schedule: 250 μg was injected with complete Freund's adjuvant, followed by 2 further injections of 100 μg emulsified with incomplete Freund's adjuvant at monthly intervals. Blood was collected 7 days later.

3. Results and Discussion

3.1. *Purification of carbamylphosphate synthetase*

A summary of the purification procedure is given in table 1. The livers of 2 rats were excised (24.8 g)

Table 1
Purification of carbamylphosphate synthetase from rat liver

Fraction	Activity[a] (total, units)	Protein (total, mg)	Spec. act. (units/mg)	Purification (fold)	Recovery (%)
Crude homogenate	309.86	3456	0.090	–	–
Triton X-100 extract	330.20	480	0.688	1.0	100
DEAE-Sephacel fraction	156.6	70	2.24	3.25	47.4
Sephacryl S-300 fraction	132.86	34.6	3.84	5.15	42.9
Chromatofocusing fraction	94.62	24.9	3.80	5.1	30.5

[a] Enzyme activity was determined as in section 2; 1 unit activity representing the formation of 1 μmol carbamylphosphate/min at 37°C

and homogenised in 250 ml buffer containing 280 mM sucrose, 4 mM Tris–H_2SO_4 (pH 7.4), 0.12 mM EDTA and 0.1% (w/v) bovine serum albumin. Mitochondria were isolated by differential centrifugation [2] and lysed in 50 ml buffer containing 0.5% (v/v) Triton X-100, 10 mM Tris–acetate (pH 7.6), 0.2 mM dithiothreitol and 0.5 mM leupeptin at 4°C for 20 min. The supernatant fraction was passed through a DEAE-Sephacel column (3.3 × 12 cm) pre-equilibrated with 10 mM Tris–acetate (pH 7.6), 0.2 mM dithiothreitol and 10% (v/v) glycerol. The column was washed with buffer at a flow rate of 1.8 ml/min until the absorbance at 280 nm returned to zero. The enzyme was then eluted with a 400 ml linear gradient of sodium acetate from 0–150 mM in the equilibration buffer, collecting 8.3 ml fractions. Fractions containing the highest specific activity carbamylphosphate synthetase (32–43) were combined and concentrated by adding ammonium sulphate (60 g/100 ml) and stirring for 30 min at 4°C. After centrifugation at 10 000 × *g*, 10 min at 4°C, the precipitate was resuspended in 3 ml Tris–acetate buffer and chromatographed on a 2.2 × 50 cm Sephacryl S-300 column equilibrated and run in the same Tris–acetate buffer at 0.68 ml/min, collecting 6.8 ml fractions. The enzyme activity eluted as a sharp peak with the bulk of the protein, and was separated from 2 major impurities of lower molecular size. Fractions with the highest specific activity (12–16) were pooled (35 ml) and further chromatographed on a 1.6 × 24 cm chromatofocusing column equilibrated in 25 mM imidazole–HCl (pH 7.4) containing 1.0 mM dithiothreitol. The enzyme was run onto the column which was washed with 5 ml imidazole buffer followed by a 1/8 dilution of polybuffer 74–HCl (pH 4.0) containing 1 mM dithiothreitol at 1.1 ml/min. All buffers were degassed. Enzyme activity eluted as a sharp peak at pH 5.8 and was separated from a number of minor impurities (fig.1). The isoelectric point of the rat enzyme is therefore very close to that of the human enzyme which is pH 6.05 [11]. As shown in fig.1, the enzyme obtained after chromatofocusing, eluted as a homogeneous peak, suggesting that it is a single isoelectric species. The fractions with enzyme protein were adjusted to pH 7.4 with 1 M Tris base as they were eluted. Enzyme was concentrated by adding solid ammonium sulphate (60 g/100 ml). Overall a purification of some 5-fold was obtained over enzyme present in the mitochondria. The specific activity was 1.96 units/mg at pH 7.4, 30°C and 3.80 units/mg at pH 7.4 and 37°C. The method in [3] for the isolation of the enzyme from rat liver gives the highest specific activity and the highest yield out of all existing meth-

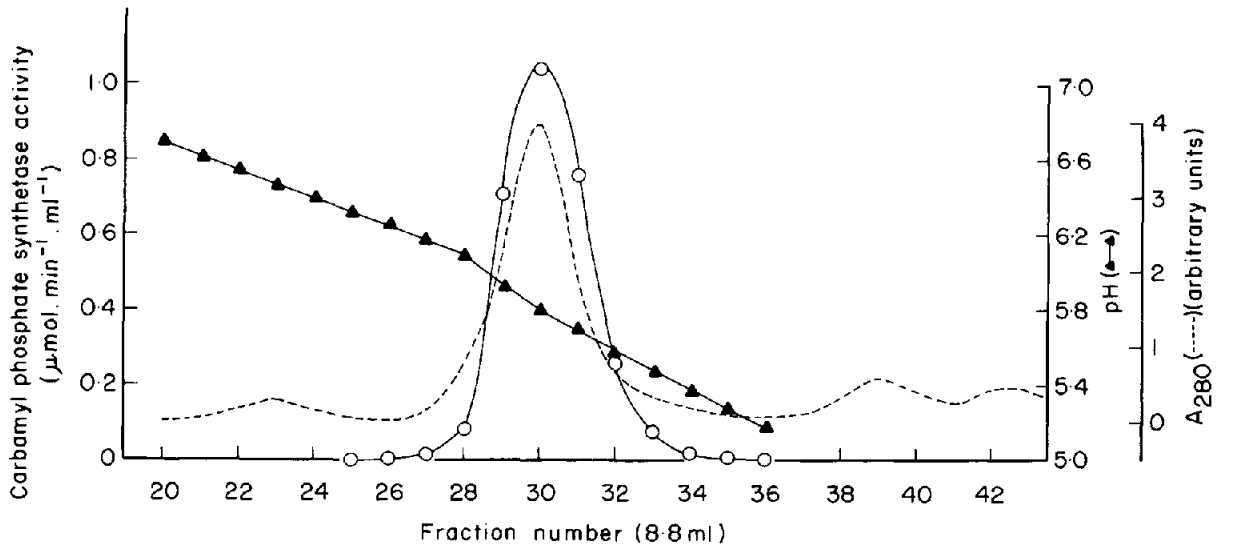

Fig.1. Chromatography of carbamylphosphate synthetase on a chromatofocusing resin. Sample was applied to the column and separated using a pH gradient generated by Polybuffer 74 as in section 2: (○) carbamylphosphate synthetase activity; (– – –) A_{280} profile; (▲) pH gradient.

ods. However, the enzyme migrated as 3 isoelectric species on isoelectric focusing gels. This method results in the isolation of carbamylphosphate synthetase with a higher specific activity and with a clear improvement in yield. Furthermore, a single isoelectric species was obtained on chromatofocusing suggesting that the enzyme is not appreciably modified during the isolation procedure.

3.2. *Gel electrophoresis*

Samples from each stage of the purification electrophoresed under dissociating conditions (fig.2a) show that the preparation after DEAE-Sephacel chromatography contained a number of major impurities of lower M_r than carbamylphosphate synthetase. These were substantially removed by gel filtration chromatography although a number of lower molecular size impurities remained. Further purification on the chromatofocusing column allowed the remaining impurities to be almost completely removed. Minor amounts of 2 low molecular size impurities were faintly visible on dissociating gels when sample was loaded at 50 μg and above (these cannot be seen on the photographs of the gel shown in fig.2a). The minor contaminants, which represent <1% of the protein appeared to be proteolytic degradation products of carbamylphosphate synthetase, since antibodies directed against the purified enzyme did not bind to proteins of this size in crude liver extracts (fig.2b).

3.3. *Immunological purity*

The immunological purity of the preparation of carbamylphosphate synthetase was assessed by Ouchterlony double-diffusion analysis and by immuno-electrophoresis. By both methods, a single precipitation band was obtained with both the pure enzyme and with the crude-liver extract. Nevertheless, the sensitivity of these analyses is such that a finding of a single precipitin band with liver extract cannot be taken as proof that the antisera is monospecific. Consequently, the antiserum was further characterized by transferring proteins from SDS–polyacrylamide gels, obtained at the various stages of the purification procedure, to diazotized paper and probing the paper with antibodies raised against the purified enzyme. The antibodies raised against the purified enzyme interacted strongly with a single band with a mobility identical to that of carbamylphosphate synthetase (fig.2b). In contrast to the result obtained with immune serum, no bands appeared when the paper

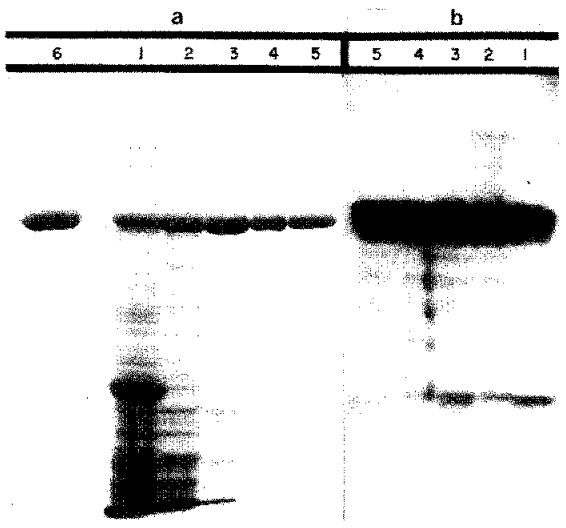

Fig.2. Electrophoretic transfer of proteins from an SDS–polyacrylamide gel to diazotized paper: (a) Whole liver extract (track 1), Triton X-100 extract of mitochondria (2), combined fractions after DEAE–Sephacel chromatography (3), combined fractions after Sephacryl S-300 chromatography (4) and combined fractions after chromatofocusing (20 μg, track 5; 50 μg, track 6) were electrophoresed as in section 2. (b) Replica lanes 1–5 were transferred to a sheet of diazotized paper as in section 2 and probed with antiserum and with ^{125}I-labelled protein A (1 μCi) for 2 h at room temperature [18]. The paper was autoradiographed for 2.5 h.

was probed with pre-immune serum. In addition to the interaction of antibodies with carbamylphosphate synthetase, the antibody also interacted with 2 minor components present in the extracts. These were (1) minor bands of lower molecular size than the enzyme in all samples other than liver extract and (2) with a band present in liver extracts but not present in the purified enzyme. These results suggest that these minor components could represent proteolytic cleavage products derived from carbamylphosphate synthetase.

The method described for the isolation of carbamylphosphate synthetase enables an essentially homogeneous preparation of enzyme to be obtained in substantial quantities, and the enzyme preparation is suitable for the preparation of antibodies which bind specifically to carbamylphosphate synthetase. In addition, electrophoretic transfer of proteins from gels to diazotized paper and subsequent probing of the paper with immune serum has been found to be a highly sensitive technique for assessing the specificity of antibodies produced against purified proteins.

Acknowledgements

This project was supported by a grant from the National Health and Medical Research Council of Australia.

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