Purification and Properties of Arginase from Human Liver and Erythrocytes

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Arginase was isolated from human liver and erythrocytes. The purification procedure used acetone precipitation, heat-treatment, $(\text{NH}_4)_2\text{SO}_4$ precipitation, DEAE-cellulose chromatography and gel filtration on Sephadex G-200 in the presence of 2-mercaptoethanol. Both enzymes migrated to the anode at pH 8.3 on polyacrylamide-gel electrophoresis. After incubation at pH 8.0 and 37°C the purified anionic liver arginase migrated to the cathode on polyacrylamide-gel electrophoresis. It is assumed that the multiple forms of the enzyme reported in the literature are partly artifacts of the purification procedure. The liver arginase showed a mol.wt. of 107000 determined by gel filtration and a sedimentation coefficient of 5.9S. Treatment of the liver enzyme with 0.25% sodium dodecyl sulphate at pH 10 demonstrated an oligomeric structure of the enzyme with a mol.wt. of the subunit of 35000. The kinetic properties determined for the purified liver arginase showed an optimum pH of 9.3 and an optimal MnCl_2 concentration of 2 mM. The K_m for L-arginine was 10.5 mM and for L-canavanine 50 mM, and L-lysine exhibited a competitive type of inhibition with a K_i of 4.4 mM. L-Homoarginine was not a substrate for liver arginase.

L-Arginase (L-arginine amidinohydrolase, EC 3.5.3.1), one of the urea-cycle enzymes, catalyses the cleavage of L-arginine to ornithine and urea. At present the best-characterized arginases are liver enzymes from rat (Schimke, 1964; Hirsch-Kolb & Greenberg, 1968; Tarrab *et al.*, 1974), rabbit (Vielle-Breitburd & Orth, 1972), horse (Greenberg, 1951) and calf (Grassmann *et al.*, 1958), which have been highly purified. Some of the physicochemical properties, e.g. molecular-weight and oligomeric structure of the native enzymes (Hirsch-Kolb & Greenberg, 1968; Vielle-Breitburd & Orth, 1972), are very similar. But debate continues over possible differences in electrophoretic mobility between and within species and the existence of arginase isoenzymes.

It was shown that the addition of dithiothreitol or 2-mercaptoethanol during purification produced a single enzyme, from rabbit and pig liver respectively, that migrated to the anode on polyacrylamide-gel electrophoresis (Vielle-Breitburd & Orth, 1972; Sakai & Murachi, 1969). This raises the question of whether the two cationic isoenzymes from both human liver and erythrocytes described in previous reports (Cabello *et al.*, 1965; Bascur *et al.*, 1966) are partly the result of procedural artifacts.

The present study was undertaken to purify and characterize arginase from human liver and erythrocytes. We decided to find out whether purification of human arginase in the presence of 2-mercaptoethanol would result in isoenzymes with characteristics similar

to those that were obtained in the absence of the reducing agent (Cabello *et al.*, 1965; Bascur *et al.*, 1966). In addition, it has been proposed that arginase isoenzymes play a role in argininaemia, a disease characterized by arginase deficiency (Shih *et al.*, 1972; Colombo *et al.*, 1976). In this context, information on the existence and properties of human arginase isoenzymes from liver and erythrocytes is important.

Experimental

Materials

Analytical-reagent-grade chemicals were obtained from the following commercial sources. Tris, 2-mercaptoethanol, L-arginine, glycylglycine, anti-pyrine and diacetylmonoxime, from Fluka, Buchs, Switzerland; glycine, urease, L-lysine, from Sigma Chemical Co., St. Louis, MO, U.S.A.; L-homo-arginine and L-canavanine, from Calbiochem, San Diego, CA, U.S.A.; Sephadex G-25, G-150 and G-200 from Pharmacia, Uppsala, Sweden; DEAE-cellulose, horse heart cytochrome c, ovalbumin, bovine serum albumin, pepsin, trypsin, γ -globulin and Coomassie Brilliant Blue, from Serva, Entwicklungslabor, Heidelberg, Germany.

Purification of arginase from human liver

Arginase was purified from a human liver obtained *post mortem* from an adult man. The liver was

removed 2h after death and chilled in chopped ice. For the purification of the enzyme the procedure of Vielle-Breithurdt & Orth (1972) was used. The whole liver was minced and homogenized with 3 vol. of 0.05M-Tris/HCl buffer, pH 7.5, containing 0.05M-MnCl₂. The procedure was followed by heat-treatment, acetone precipitation, (NH₄)₂SO₄ precipitation, DEAE-cellulose chromatography and gel filtration on Sephadex G-200 in the presence of 2-mercaptoethanol.

Purification of arginase from human erythrocytes

Human erythrocytes were obtained from citrated blood-bank blood. The erythrocytes were washed four times by centrifugation with 0.9% NaCl, stored at -20°C and thawed. For the purification of erythrocyte arginase the first three steps were adapted from Greenberg (1951). The erythrocytes (2000ml) were mixed by stirring with an equal volume of water. The pH was adjusted to 7.5 with 0.1M-NaOH, and 2M-MnCl₂ was added to bring the final Mn²⁺ concentration to 0.15M. More water was added so that the final extraction mixture contained 1 part (by vol.) of erythrocytes and 2 parts of water. The pH was again adjusted to 7.5 and toluene (corresponding to 2% of the total volume) was added. The extraction mixture was stirred for 30min and then left overnight at 4°C. After removal of the toluene phase the purification used acetone precipitation and heat-treatment. 2-Mercaptoethanol was then added to the active fraction and the final three steps were identical with those in the liver arginase purification.

Assay methods

Arginase activity during purification was assayed in a system containing 6μmol of glycylglycine buffer (pH 9.5), 31μmol of L-arginine adjusted to pH 9.5 with 10M-NaOH and 0.3μmol of MnCl₂ in a final volume of 0.12ml. The reaction mixture was incubated for 15min at 37°C. The incubation was stopped with 0.2ml of 0.66M-HClO₄. After adding 0.2ml of 1.2M-K₂HPO₄, the resulting urea was assayed spectrophotometrically as NH₃ after incubation with urease followed by the Berthelot reaction (Brown & Cohen, 1959).

For kinetic studies, arginase activity was assayed in a medium containing 100μmol of glycine/NaOH buffer, pH 9.5, 60μmol of L-arginine adjusted to pH 9.5 with 10M-NaOH, and 2μmol of MnCl₂ in a final volume of 1.0ml. The reaction mixture was incubated at 37°C for 10min and then stopped with 1.0ml of 10% (v/v) H₂SO₄. The urea was then assayed spectrophotometrically as described by Ceriotti & Spandrio (1963). It was found that the determination of urea at lower concentrations was

more sensitive with the latter method. With both assay methods, urea liberation was linear with time and arginase activity with enzyme concentration.

One unit of enzyme activity is defined as the amount of the enzyme that produces 1μmol of urea/min at 37°C. Specific activity is expressed in enzyme units per mg of protein.

Protein was measured by A₂₈₀ or by the procedure of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Polyacrylamide-gel electrophoresis

Gels containing 7% (w/v) acrylamide were prepared and used as described by Davis (1964). Electrophoresis at pH 8.3 was performed in Tris/glycine buffer (Davis, 1964). The application buffer was the same buffer diluted 10-fold and containing 0.01M-2-mercaptoethanol. Gels containing 10% acrylamide and 0.1% sodium dodecyl sulphate were prepared as described by Dunker & Rueckert (1969), and were run in 0.1M-sodium phosphate buffer, pH 7.2, and 0.1% sodium dodecyl sulphate.

The gels were stained with Coomassie Brilliant Blue, 0.25% in methanol/water/acetic acid (227:227:46, by vol.) and destained with several changes of methanol/acetic acid/water (10:3:27, by vol.).

For the location of bands of arginase activity on the gel, the enzyme was determined in latitudinal segments of the gel. After electrophoresis, the gel was cut into 5mm segments and arginase activity in each slice was measured by the arginase assay described above. Each segment was immersed in 200μl of 0.017M-Tris/HCl buffer, pH 8.0, homogenized with a glass rod, and extraction was allowed to proceed at 4°C for 12h. After centrifugation for 5min at 12000g a sample of the supernatant was used for arginase assay.

Molecular-weight determination

The molecular weight of human liver arginase was estimated by Sephadex G-150 chromatography at 4°C as described by Andrews (1964). Horse heart cytochrome *c* (mol.wt. 12400), ovalbumin (45000), bovine serum albumin (71000) and bovine γ-globulin (205000) were used as molecular-weight standards. The above-mentioned values are the apparent molecular weights determined by gel filtration (Andrews, 1965).

The molecular weight of the arginase subunit was estimated by the procedure of Shapiro *et al.* (1967) by electrophoresis in 10% polyacrylamide gels containing 0.1% sodium dodecyl sulphate, and with cytochrome *c*, trypsin [mol.wt. 23800 (Dunker & Rueckert, 1969)], pepsin [mol.wt. 35500 (Dunker & Rueckert, 1969)], ovalbumin and bovine serum albumin, as molecular-weight standards. The standards were previously incubated with sodium dodecyl

sulphate as described by Dunker & Rueckert (1969). For arginase, 9 vol. of enzyme (2.5 mg/ml) in 0.017 M-Tris/HCl buffer, pH 8.0, was added to 1 vol. of concentrated dissociating mixture containing 0.5 M-glycine/NaOH, pH 10.8, 2.5% sodium dodecyl sulphate, and 1 M-2-mercaptoethanol adjusted to pH 9. The final mixture has a pH of 10 and was incubated at 37°C for 10 h. The gels were stained with Coomassie Brilliant Blue as described above. The rate of migration of the proteins was evaluated by measuring on photographs the distance from the top of the gel to the middle of the protein band.

Professor P. von Tavel (Theodor Kocher Institut, University of Berne) kindly measured the sedimentation coefficient of the purified liver arginase by ultracentrifugation.

Results and Discussion

Purification of human liver and erythrocyte arginase

The results from the purification of arginase from liver and erythrocyte are summarized in Table 1.

The specific activity of the purified liver enzyme was 410-fold that of the crude liver extract, having a specific activity of 2090 units/mg of protein (Table 1). The overall recovery was 21%.

The total activity of the liver enzyme was increased after heat-treatment and Sephadex G-200 chromatography (Table 1). Other workers (Sakai & Murachi, 1969; Nishibe, 1973; Tarrab *et al.*, 1974) have also reported an increase in total arginase activity after heat-treatment, which is due to a heat activation of the enzyme. The increase after Sephadex G-200 chromatography (step 6) is caused by a lowered total activity after DEAE-cellulose chromatography (step 5). The KCl present in the assay mixture of the step-5

enzyme interferes in arginase-activity determination, producing low-activity results.

The erythrocyte arginase was purified 520-fold after $(\text{NH}_4)_2\text{SO}_4$ precipitation, corresponding to a specific activity of 27.5 units/mg of protein. The specific activity, however, decreased again to 12.2 units/mg of protein after DEAE-cellulose and Sephadex G-200 chromatography. Decrease of activity during the last steps of purification is due to inactivation of the enzyme. The highly purified step-6 erythrocyte arginase was totally inactive after storage at 4°C for 3 days. A similar inactivation during purification of calf liver arginase was observed by Grassmann *et al.* (1958). In their work addition of Mn^{2+} to all purification steps decreased the inactivation effect. However, the presence of Mn^{2+} is unfavourable, since the oxidation of Mn^{2+} to Mn^{4+} which takes place at alkaline pH also affects arginase activity. Other authors showed that the addition of 2-mercaptoethanol has a protective effect on rabbit liver arginase (Vielle-Breitburd & Orth, 1972). However, in our work, the presence of 2-mercaptoethanol did not satisfactorily prevent loss of activity.

The purification of arginase from erythrocytes is generally considered difficult. Cabello *et al.* (1961) report only a 6% yield from erythrocytes. Nishibe (1973) could increase the yield to 17%, but his method of purification included a rather drastic step that consisted of extraction of an air-dried acetone precipitate of the haemolysate.

We favoured using as far as possible the same purification procedure for both liver and erythrocyte enzymes, since the purification procedure seems to affect the electrophoretic behaviour of the purified arginase.

In contrast with reports of multiple forms of arginase demonstrated by CM-cellulose chromato-

Table 1. Purification of arginase from human liver and human erythrocytes
Comparison of arginase activity from human liver and erythrocytes during the course of purification.

Step	Procedure	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)
(a) Liver						
1	Homogenate supernatant	3000	225 000	55 000	5.0	
2	Heat at 60°C	2830	413 000	11 900	34.9	7
3	Acetone	180	206 000	5150	40.0	8
4	40–55% satd. $(\text{NH}_4)_2\text{SO}_4$	37.5	146 000	1170	125	25
5	DEAE-cellulose	50	20 300	85	238	48
6	Sephadex G-200	84	47 000	22.5	2090	418
(b) Erythrocytes						
1	Extract from erythrocytes	5400	33 480	621 300	0.053	
2	Acetone	1800	24 300	192 900	0.125	2.4
3	Heat at 60°C	1180	16 638	10 980	1.51	28.5
4	40–55% satd. $(\text{NH}_4)_2\text{SO}_4$	23	3027	110	27.50	520
5	DEAE-cellulose	56	56	3.3	16.90	320
6	Sephadex G-200	40	10	0.8	12.2	230

Fig. 1. DEAE-cellulose chromatography of human liver and erythrocyte arginase after $(\text{NH}_4)_2\text{SO}_4$ fractionation (a) Human liver arginase. The step-4 enzyme (1170 mg, 37 ml) was applied to a DEAE-cellulose column (2.5 cm $\times$ 10 cm). Elution was carried out with a linear gradient (600 ml volume) of KCl from 0 to 50 mM in 0.017 M-Tris/HCl buffer/5 mM-2-mercaptoethanol, pH 8.0; 10 ml fractions were collected. (b) Human erythrocyte arginase. The step-4 enzyme (110 mg, 23 ml) was applied to a DEAE-cellulose column (2.5 cm $\times$ 10 cm) and eluted as in (a). ———, A_{280} ; ●, arginase activity; —.—, KCl concentration.

graphy of human liver arginase (Bascur *et al.*, 1966), we found both the liver and the erythrocyte enzyme to be eluted as single symmetrical peaks on DEAE-cellulose (Figs. 1 and 2).

Multiple forms have also been described for rabbit liver arginase (Vielle-Breitburd & Orth, 1972). The presence of 2-mercaptoethanol during the purification procedure was shown to decrease the heterogeneity of rabbit liver arginase. Vielle-Breitburd & Orth (1972) suggested that the five closely related forms result from the slight degradation of a single species.

Also, in our experiments the use of 2-mercaptoethanol during the purification of human liver and erythrocyte arginase seemed to inhibit the formation of isoenzymes.

Electrophoretic properties of liver and erythrocyte arginase

The arginase preparations for liver and erythrocyte obtained after step 6 gave a single diffuse protein band that migrated towards the anode after polyacrylamide-gel electrophoresis at pH 8.3. This band corresponded to arginase activity after extraction of the gel. The anionic behaviour in electrophoresis relates the human liver and erythrocyte enzyme to rabbit, horse, calf and pig liver arginases (Vielle-

Breitburd & Orth, 1972; Greenberg, 1951; Grassmann *et al.*, 1958; Sakai & Murachi, 1969). This finding, however, is in contrast with previous reports (Cabello *et al.*, 1965; Bascur *et al.*, 1966; Nishibe, 1973) on cationic enzymes purified from human liver and erythrocytes. It is assumed that the presence of 2-mercaptoethanol has an influence on the electrochemical properties of the isolated enzymes. The reducing agent may prevent oxidation of a native enzyme during purification and thus decrease the formation of multiple active forms. The same effect was also observed for the rabbit arginase (Vielle-Breitburd & Orth, 1972).

With rat liver arginase, findings on a number of molecular species and electrochemical behaviour of the enzyme vary considerably. Although the enzyme is reported to be homogeneous (Schimke, 1964; Hirsch-Kolb & Greenberg, 1968), three different isoenzymes were separated on CM-cellulose (Tarrab *et al.*, 1974) and two on DEAE-cellulose (Gasirowska *et al.*, 1970). The purified enzymes (Schimke, 1964; Hirsch-Kolb & Greenberg, 1968; Tarrab *et al.*, 1974) were cationic in the physiological pH range. In crude homogenates of rat liver only two bands of activity were observed; however, one migrates towards the anode and the other to the cathode (Porembska *et al.*, 1971).

Fig. 2. Sephadex G-200 gel filtration of human liver and erythrocyte arginase after DEAE-cellulose chromatography (a) Human liver arginase. The step-5 enzyme (85 mg, 5 ml) was applied to a Sephadex G-200 column (2.5 cm $\times$ 190 cm) and eluted with 0.017 M-Tris/HCl buffer/5 mM-2-mercaptoethanol, pH 8.0; 10 ml fractions were collected. Active fractions were pooled as indicated by the arrows and freeze-dried. (b) Human erythrocyte arginase. The step-5 enzyme (3.3 mg, 5 ml) was applied to a Sephadex G-200 column (2.5 cm $\times$ 100 cm) and eluted as in (a). ----, A_{280} ; ●, arginase activity.

It could further be demonstrated that incubation of the purified human liver arginase at pH 8.0 in the presence of Mn^{2+} ions at 37°C produced a new form of arginase. On electrophoresis at pH 8.3 the new form migrated towards the cathode. After 24 h, about 30% of the initial arginase activity of the anionic enzyme was present as a cationic band on the gel. This result demonstrates the possibility that arginase isoenzymes may be formed during the isolation procedure.

Molecular weight: sedimentation coefficient of liver arginase

The molecular weight for human liver arginase, estimated by gel filtration as described by Andrews (1964) on a Sephadex G-150 column, was 107 000.

The sedimentation coefficient of the liver enzyme was evaluated as 5.9 S in 0.017 M-Tris/HCl buffer, pH 8.0.

These results agree with reported values of other liver arginases, such as rat arginase (mol.wt. 118 000; $s = 6.1$ S) (Hirsch-Kolb & Greenberg, 1968) and rabbit arginase (mol.wt. 110 000; $s = 5.9$ S) (Vielle-Breitburd & Orth, 1972).

Dissociation into subunits of liver arginase

The purified liver enzyme (step 6) was incubated at 37°C, at pH 10 in the presence of 0.25% sodium dodecyl sulphate and 0.1 M-2-mercaptoethanol. After

incubation the arginase gave rise to a main protein band after gel electrophoresis in sodium dodecyl sulphate. The rate of migration of this polypeptide allowed the estimation of a mol.wt. of 35 000. This result indicates the oligomeric structure of liver arginase. The values do not show a tetrameric structure like that inferred from the molecular weights of the native rat liver arginase (118 000) and its subunit (30 800) determined by the method of sedimentation equilibrium (Hirsch-Kolb & Greenberg, 1968). The value is closer to the subunit (36 500) of purified rabbit liver arginase (110 000) (Vielle-Breitburd & Orth, 1972) determined with the method used in the present study.

Kinetic properties of liver arginase

The step-6 liver enzyme reached optimum activity at pH 9.1–9.5 in 0.1 M-glycylglycine buffer. Maximum activity was also obtained when the enzyme was incubated in the presence of 2 mM- $MnCl_2$ at pH 9.5. The Michaelis constant of the purified liver enzyme for L-arginine was graphically evaluated to be 10.5 mM in glycine/NaOH buffer, pH 9.5, in the presence of 2.0 mM- $MnCl_2$ (Fig. 3).

On substituting L-canavanine for L-arginine as substrate, a K_m value of 50 mM was obtained (Fig. 4). L-Lysine behaved as a competitive inhibitor against L-arginine of the liver enzyme, with a K_i value of 4.4 mM.

Fig. 3. Double-reciprocal plot of human liver arginase activity versus L-arginine concentration. Arginase activity was assayed as described in the text. Velocities are expressed as μmol of urea produced/min per ml.

Fig. 4. Double-reciprocal plot of human liver arginase activity versus canavanine concentration. Arginase activity was assayed as described in the text. Velocities are expressed as μmol of urea produced/min per ml.

It was further demonstrated that L-homoarginine, the next highest homologue of arginine, had no substrate affinity for the purified liver arginase.

For L-arginine with arginase in lysed erythrocytes a K_m of 22 mM was obtained. The similar kinetic properties of liver and erythrocyte arginase are accompanied by similar electrochemical properties in the

purified enzymes. After the purification in the presence of 2-mercaptoethanol, both enzymes were anionic on gel electrophoresis. This behaviour of the purified arginase differs from the findings of Cabello *et al.* (1965), who report two cationic enzymes for both the human liver and the erythrocyte. The fact that the purified anionic liver enzyme can be converted into a cationic form strongly suggests, however, that isoenzymes of arginase described in the literature may be formed during the purification procedure from a native form.

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