Arginase in Chang's Liver Cells

GUNILLA LARSSON, GEORGE BÖLCSFÖLDI and EVA ELIASSON

The Wenner-Gren Institute, University of Stockholm, Norrtullsgatan 16, S-113 45 Stockholm, Sweden

Arginase activity has been studied in lysates of Chang's liver cells, an established cell line derived from human liver. It was found that the enzyme of Chang cells differed only slightly from human liver arginase with respect to activation by manganese, pH dependence, Michaelis constant, and inhibition by basic amino acids. On the other hand there was a marked difference in electrophoretic mobility — the Chang cell enzyme moved towards the anode while liver arginase moved towards the cathode at pH 8.6. Furthermore, cell fractionation studies revealed, that the localization of the enzyme was different, the bulk of the Chang cell arginase being firmly bound to cytoplasmic particles sedimenting with mitochondria.

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) is a wide spread enzyme present in a variety of animal and plant cells.¹ By far the highest enzyme activity is found in the liver of ureotelic animals, where it mediates the final step in the ornithine-citrulline-urea cycle. When liver cells are maintained in cell culture the urea cycle is lost.² Thus, Chang's liver cells, an established cell line derived from human liver have no ornithine transcarbamylase activity² and consequently citrulline can not be formed from ornithine by these cells. However, arginase is still present but with a specific activity in the range of 0.1 % of the activity reported for intact liver.^{3,4} The functional role of arginase in cells and tissues lacking the urea cycle has generally been ascribed to the catabolism of arginine and to the synthesis of proline by way of ornithine.⁵

Four isoenzymes with arginase activity present in varying amounts in different rat organs have been separated by means of chromatography.⁶ It has also been shown that arginases isolated from rat kidney and liver differ in many properties.⁷

In human liver Bascor *et al.*⁸ found two protein fractions with arginase activity, which could be separated by chromatography. Using immunological methods and electrophoresis Cabello *et al.*⁹ compared purified liver arginase with arginase isolated from red blood cells. They found two, possibly identical isoenzymes in both preparations.

The aim of the present investigation was to compare arginase from Chang's liver cells with arginase from human liver. It seemed of interest to know whether the cultured cells contained the liver enzyme in reduced amounts or if they contained an arginase with different properties.

MATERIAL AND METHODS

Chang's liver is an established cell line originally derived from human liver.¹⁰ Chang cells were grown as spinner cultures in Eagle's minimal medium for suspension cultures, containing 10 % horse serum.¹¹ The cultures were diluted with fresh medium three times a week and were routinely examined for bacterial contamination. (No contamination was found).

Arginase activity was determined according to the micromethod of Schimke,¹² using guanido labelled arginine as substrate followed by determination of ¹⁴CO₂ liberated by urease. Cell samples from the suspension cultures were centrifuged at 700 *g* for 3 min and resuspended in 50 mM Tris-HCl buffer, pH 7.8, at a density of approximately 10⁸ cells per ml. The cell suspension was frozen and stored at -17°. As pointed out by Schimke¹³ preparations made by freezing and thawing gave the same arginase activity as cell homogenates prepared in other ways. After thawing MnCl₂ was added to a concentration of 10 mM. The cell suspension was then heated to 55° for 5 min in order to activate arginase. 100 μ l samples of the activated suspension were transferred to test tubes with screw caps. The reaction was started by the

addition of 100 μ l 0.2 M guanido- 14 C-arginine pH 9.7 (sp. act. 0.01 mC/mmol), the pH of the reaction mixture was 9.0. After incubation at 37° for appropriate times (usually 30 or 60 min) the reaction was stopped by the addition of 50 μ l 0.4 M HCl. Strips of filterpaper were attached inside the screw caps with one end of the strip inserted under a plastic disc fitting into the bottom of the cap. 25 μ l of hyamine hydroxide (1 M in methanol) was placed on the paper strip. Urease was dissolved in 0.2 M potassium phosphate buffer pH 6.3 to a concentration of 5 mg per ml. One ml of urease solution was added to the reaction mixture and the test tubes were immediately closed with the screw caps. The tubes were then placed in a rotating test tube rack at 37° for 45 min. The part of the paper strips containing hyamine was cut off and placed in scintillation vials. Radioactivity was determined, using Omnifluor (NEN) in toluene, in an Intertechnique SL 36 liquid scintillation spectrometer at 4°. The amount of enzyme added and the incubation time during the arginase reaction was modified so that the amount of urea formed did not exceed 0.5 μ mol (2.5 % of the substrate). Standard solutions containing 0.02 to 0.6 μ mol of 14 C labelled urea with a specific activity of 0.01 mC/mmol were used for incubations with urease under identical conditions. 1 μ mol urea gave 16 000 cpm. One unit of arginase was defined as the amount of enzyme which produced 1 μ mol urea per hour under the above conditions. Arginase activity was determined in the same way in subcellular fractions of Chang cells. For kinetic studies guanido- 14 C-arginine with a specific activity of 0.02 mC/mmol was used.

Protein content was determined using Biuret reagent¹⁴ or in clear supernatant fractions by the method of Warburg and Christian.¹⁵

Electrophoresis. Chang cell arginase activity could be removed from particulate cell fractions by a non-ionic detergent. Cells were washed in Hank's balanced salt solution¹⁶ and suspended in 50 mM Tris buffer, pH 7.8. MnCl_2 and Lubrol (I.C.I.) were added to final concentrations of 10 mM and 0.5 %, respectively, and the cell suspension was shaken on a Vortex mixer. After adjusting the pH to 7.4 with Tris base the suspension was heated to 55° for 20 min and then centrifuged at 15 000 *g* for 20 min. Before electrophoresis the supernatant was concentrated 6 times by dialysis for 4 h against a 10 mM Tris-HCl buffer, pH 7.8, containing 10 mM MnCl_2 , 10 mM NaCl, 1.5 mM MgCl_2 , and 15 % polyethyleneglycol (MW 6000). After dialysis the preparation contained 5 to 10 units of arginase activity per ml.

Human foetal liver from a 20–25 week old foetus was washed in Hank's salt solution and homogenized in a Potter-Elvehjem homogenizer in 3 volumes cold 10 mM Tris-HCl buffer, containing 150 mM KCl, 20 mM MnCl_2 , 10 mM NaCl, 1.5 mM MgCl_2 , pH 7.8. The homogenate

was then centrifuged at 15 000 *g*. The supernatant containing the entire arginase activity was finally heat activated for 20 min at 55° and centrifuged. Immediately before electrophoresis the liver enzyme was diluted 200 times with 10 mM Tris buffer, containing 10 mM MnCl_2 , in order to obtain an arginase activity in the same order of magnitude as that of the Chang cell preparation.

Three identical 30 μ l samples were applied to each glass slide covered with 1 % buffered agarose gel. Sodium barbital buffer, pH 8.6, ionic strength 0.1, was used. Potential difference at the electrodes was 100 V and the current intensity 3 mA. Electrophoresis was performed during 4 h. The agarose gel was sectioned in 3 stripes longitudinally, each containing one sample well, and in 13 cross sections giving pieces of gel approx. 100 μ l in volume. These were transferred to test tubes, minced with a glass-rod, and 10 μ l 0.1 M MnCl_2 was added. After heating for 5 min at 55° arginase activity was determined as described above.

Cell fractionation. Lysates of Chang cells were prepared according to Holtzman *et al.*¹⁷ After washing the cells with cold Hank's salt solution, the cells were suspended in ice cold hypotonic buffer, 10 mM Tris pH 7.8, containing 10 mM NaCl and 1.5 mM MgCl_2 , and allowed to swell at 0–4° for 20 min. The cells were then homogenized in a tight fitting Dounce homogenizer. Microscopic inspection showed very few intact cells. MnCl_2 and KCl were then added to the homogenate to final concentrations of 10 mM and 150 mM, respectively. Homogenate of foetal human liver was prepared as described above. Centrifugation at 15 000 *g* for 15 min gave pellets containing *nuclei and larger cytoplasmic particles*. For arginase activity determination pellets were suspended in 50 mM Tris, pH 7.8.

Separation of nuclei from cytoplasm was performed using the above mentioned hypotonic buffer but containing 0.05 mM spermine. Cells were washed, lysed, and homogenized as above. Nuclei were sedimented at 1600 *g* for 2 min. The nuclear pellet was resuspended in buffer and adhering cytoplasm was removed from the nuclei by pipetting several times with a narrow Pasteur pipett. After centrifugation at 1600 *g* the supernatant was added to the first supernatant. The combined supernatants, which did not contain any visible nuclei on microscopic inspection, were centrifuged at 15 000 *g* for 15 min. The resulting pellet, which contained *cytoplasmic particles, mainly mitochondria*, was suspended in 50 mM Tris, pH 7.8 and used for arginase activity and protein determinations.

The *nuclei* were further purified by suspension in 3.75 ml of 0.34 M sucrose in the above buffer containing spermine and layered over 1.25 ml of 1.35 M sucrose in the same buffer. After centrifugation at 15 000 *g* for 15 min the pellet was suspended in 2.5 ml of 2.1 M sucrose

and layered over 2.5 ml of the same sucrose solution. After centrifugation for 45 min at 75 000 *g* the nuclear pellet was washed with 5 ml of 0.34 M sucrose and sedimented at 15 000 *g* for 15 min. The pellet was suspended in 50 mM Tris buffer, pH 7.8. The majority of the nuclei looked intact, but some were severely fragmented. The suspension was used for arginase activity and protein determinations.

L-Arginine-(guanido)- ^{14}C was obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England. Hyamine hydroxide from the Packard Instrument Company Inc., Ill. USA. Omnifluor and urea- ^{14}C from NEN Chemicals GmbH, Frankfurt, West Germany, urease (Jack bean type III) from Sigma Chemical Company, St. Louis, USA and Eagle's medium and horse serum from Statens Bakteriologiska Laboratorium, Solna, Sweden.

RESULTS AND DISCUSSION

Kinetic properties. Arginase is activated by bivalent cations.¹ Preincubation of Chang cell lysates at 55° for 5 min in the presence of 10 mM MnCl_2 increased the arginase activity by 70 %. Longer preincubation times did not increase the activity further. Fig. 1 shows the pH dependence of the reaction. Maximum activity was above pH 9, which is in agreement with results using arginase from ureotelic liver. On

Fig. 1. pH dependence. Cell lysate in 5 mM Tris-HCl, pH 7.8, was activated by heating in the presence of 10 mM MnCl_2 prior to determination of arginase activities. Incubation mixtures contained 5 mM MnCl_2 , 0.1 M ^{14}C -(guanido)-arginine (0.01 mC/mmol) and 50 mM Tris-HCl (pH 7.0–8.8 ★) or 50 mM glycine-NaOH (pH 8.5–9.6 □). Ordinate: pH, abscissa: arginase activity (cpm in $^{14}\text{CO}_2$, liberated by urease).

Fig. 2. Michaelis constant and inhibition by lysine and ornithine. Cell lysates were prepared as in Material and Methods, and arginase activated by heating in the presence of 10 mM MnCl_2 . Incubation mixtures contained 2×10^7 lysed cells, 50 mM glycine-NaOH (pH 9.7), 25 mM Tris-HCl, 5 mM MnCl_2 , 1.5–10 mM ^{14}C -(guanido)-arginine (0.02 mC/mmol). No addition or 10 mM lysine or 10 mM ornithine, respectively. Final pH in the reaction mixtures was 9.0.

K_m and $1/V$ were determined graphically. K_i 's for lysine and ornithine were determined using the formula: $\text{slope} = (K_m/V)(1 + I/K_i)$ (Ref. 20).

the other hand, Gasiorowska *et al.* obtained two pH optima at 7.5 and 9.5 for arginase from kidney, submaxillary gland and brain of the rat.⁶

The K_m for arginase as measured in crude Chang cell preparations varied in different experiments between 4 and 7 mM. Lysine and ornithine were both competitive inhibitors. A typical experiment is shown in Fig. 2 where the K_m was 4.5 mM, and the K_i values for lysine and ornithine were 1.6 mM and 1.5 mM, as calculated from the experimental results. With respect to substrate affinity and competitive inhibition by lysine, Chang cell arginase does not differ significantly from human liver arginase, as determined by Baseur *et al.*⁸ These authors separated arginase, from human liver, by chromatography, into two components which differed with respect to their inhibition by ornithine. The larger component, corresponding to more than 90 % of the total activity, showed a mixed type of inhibition, while the

Fig. 3. Agar gel electrophoresis of arginase. A, 30 μ l of Chang cell lysate prepared as in Material and Methods (1 mg protein/ml). B, 30 μ l liver enzyme (0.03 mg protein/ml). C, Mixture of approx. 15 μ l Chang cell arginase and 15 μ l liver arginase. Ordinate: agar section, abscissa: arginase activity, adjusted to the total of 15 μ l Chang cell enzyme (A), 15 μ l liver enzyme (B), and 15 μ l Chang cell enzyme + 15 μ l liver enzyme (C).

smaller component was competitively inhibited, with a K_i of 3.1. Evidently the Chang cell arginase resembles the minor component of the liver enzyme with respect to inhibition by ornithine.

Electrophoretic mobility. Electrophoresis of Chang cell and foetal human liver preparations were carried out according to Material and Methods. The liver enzyme moved towards the cathode (Fig. 3B), which is in accordance with the results of Cabello *et al.*⁹ using arginase from human liver and erythrocytes, where arginase from both sources moved towards the cathode at pH 8.6. Chang cell arginase on the other hand moved towards the anode (Fig. 3A). The activity from Chang cells recovered in the peak (Fig. 3A) corresponded to 100 % of the activity added. (Since Chang cell enzyme was prepared

in the presence of 0.5 % Lubrol, a control experiment with human liver in the presence of Lubrol was performed which indicated that the electrophoretic mobility was unaffected). A mixture of the enzyme from human liver and Chang cells was separated into two peaks with maximum activities on the anodal side, corresponding to Chang cell arginase, and on the cathodal, corresponding to human liver arginase (Fig. 3C).

Therefore, the electrophoretic mobility of Chang cell arginase differs from that of human liver. Further studies using purified enzyme preparations may indicate whether this difference is due to the association of Chang cell arginase with some other molecule, thus altering the electrophoretic mobility, or if there is a true difference in the enzyme proteins.

Localization of the enzyme. Using standard procedures for extraction of arginase from liver, in the presence of 10 mM $MnCl_2$ and 150 mM KCl, the activity was found exclusively in the supernatant after centrifugation at 15 000 g for 15 min (Table 1). In order to homogenise Chang cells it was first necessary to swell the cells in hypotonic buffer. Immediately after homo-

Table 1. Arginase activity in subcellular fractions (cpm in $^{14}CO_2$).

Fraction	Chang cells	Liver
Whole homogenate	2800 ^a	45 000 ^b
15 000 g pellet	2500	—
15 000 g supernatant	300	46 000

^a 100 μ l, incubation time 60 min. ^b 10 μ l, incubation time 10 min.

Table 2. Release of arginase from the particulate fraction by Lubrol treatment for 3 min at 37°.

Fraction	Arginase activity (cpm in $^{14}CO_2$)
15 000 g pellet before Lubrol treatment	3150
15 000 g supernatant after Lubrol treatment	
0.1 % Lubrol	3280
0.2 % Lubrol	3200
0.5 % Lubrol	3010

Table 3. Arginase activity in cytoplasmic particles and purified nuclei.

Fraction	Protein ^a (mg)	Arginase ^a (cpm in CO ₂)	Arginase ^a (μmol urea per hour)	Arginase specific activity
A. 15 000 g pellet	0.68	1690	0.106	0.156
B ₁ . "mitochondrial fraction"	0.24	1180	0.073	0.310
B ₂ . purified nuclei	0.25	80	0.005	0.020

^a per 100 μl sample.

genisation KCl was added to 150 mM and MnCl₂ to 10 mM, i.e. ion concentrations that completely remove arginase from the particulate fractions of liver.¹⁸ After centrifugation 90 % of the arginase activity was consistently found in the pellet (Table 1). This was also true when the MnCl₂ concentration was increased to 50 mM. The enzyme could be released from the particulate fraction by treatment with a non-ionic detergent, e.g. Lubrol (Table 2).

In order to further clarify, whether arginase was bound to nuclei or to cytoplasmic particles, cells were lysed in a hypotonic buffer containing spermine, which maintains the integrity of the nuclear membrane.¹⁹ One part of the lysate was centrifuged at 15 000 g yielding nuclei together with cytoplasmic particles. The other part was fractionated as described in Material and Methods, separating the cytoplasmic particles from the nuclei. 70 % of the arginase activity of the combined nuclear and mitochondrial pellet was found in the cytoplasmic particle fraction (Table 3). Attempts to purify the nuclei by centrifugation in hypotonic sucrose, yielded only 3 % activity in the pure nuclear fraction, while the remaining 25 % was lost during the purification procedure.

In summary, unlike liver, most of the arginase activity in Chang cells was firmly bound to cytoplasmic particles sedimenting with mitochondria. The activity cannot be removed from these particles by salt, but it can be released by non-ionic detergents. A similar localization of arginase activity in the mitochondrial fraction has been reported by Kaysen⁷ for rat kidney.

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