Arginase isoforms in frog and lizard tissues

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Isoforms of arginase in the liver and kidney tissues of the ureotelic frog (Rana tigerina) and uricotelic lizard (Calotes versicolor) were fractionated by DEAE-cellulose chromatography (pH 8.3). Four molecular forms, designated as A', A2, A3 and A4 based on the KCl concentration required for their elution from the ion-exchange column, were detected in lizard liver, while only two forms were found in lizard kidney (A3 and A4) and frog liver and kidney (A2 and A3). No major differences were found in the pH optimum, substrate affinity and molecular weight of the isoenzymes. The isoforms in lizard tissues were either totally unaffected or only partially immunoprecipitated by antibodies raised against rat liver and beef liver arginases, but those in frog tissues were significantly activated by the two antibodies. While the physiological importance of the presence of four isoforms in lizard liver remains enigmatic, different sets of isoenzymes were present in the liver of the two ureotelic vertebrates, rat and frog. Hence, it appeared that a given mode of nitrotelism was not associated with a specific set of isoenzymes. Also, the data were not consistent with the generally held view that a basic isoform of arginase served as a component of the urea cycle in liver and a neutral/slightly acidic form functions in the synthesis of proline, glutamate and polyamines in extra-hepatic tissues. The isoforms appeared to show considerable functional overlap.

Keywords: Arginase isoforms, Frog and lizard tissues, Nitrotelism, Chromatographic behavior, Electrophoretic mobility, Antigenic properties, Activation by antibodies

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1), which catalyzes the hydrolysis of L-arginine to urea and L-ornithine is believed to exist in two isozymic forms in mammalian tissues1-4. Arginase I (AI, hepatic form) is abundant in the liver, but present in low levels in extra-hepatic tissues, while arginase II (AII, extra-hepatic form) occurs at relatively high levels in tissues like intestine, brain, mammary gland and kidney, but only in low levels in liver. AI, a basic protein (pI = 9.3) of cytosolic location, functions as a component of the urea cycle, the pathway for ammonia detoxification in ureotelic animals. AII is neutral or slightly acidic (pI = 6.8), is present in the mitochondria and serves in the synthesis of proline, glutamate and polyamines as well as regulation of nitric oxide formation. The two isoforms share 58% sequence identity, but are immunologically distinct5.

Recent studies on relative distribution of the two isoforms in the liver of different mammals, patterns of their expression vis-à-vis the enzymes of proline, glutamate and polyamine synthesis in mouse tissues and the metabolic consequences in mouse knock-out models for AI and AII suggest that there might be a considerable functional overlap between the two isoenzymes5,6,7.

While a functional urea cycle appears to be absent in non-ureotelic animals, arginase is known to be ubiquitously distributed in ammonotelic, ureotelic, uricotelic and guanotelic animals8-12. Earlier, it has been suggested that arginasces from ureotelic and uricotelic species differed in their physical, kinetic and antigenic properties, but it was negated by later studies5. Multiple molecular forms of the enzyme are known in fish, frog and lizard tissues13-17 and CDNA clones of type I and II arginase genes and their expression have been reported in frog and trout tissues18-21. However, it is not clear, if animals practicing different modes of nitrotelism are equipped with different sets of arginase isoenzymes in their tissues. Hence, in the present study, we have fractionated the arginase isoforms in the liver and kidney of a ureotele (frog) and a uricotele (lizard) by

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DEAE-cellulose chromatography (pH 8.3) and studied their kinetic, physical and immunological properties.

Materials and Methods

Biologicals

Frogs (Rana tigerina) were obtained from commercial sources and maintained in aquaria in the laboratory. Lizards (Calotes versicolor) were supplied by local field collectors. Both animals were fed on insects. Lizards were provided with water ad libitum. The animals were dissected under ether anesthesia and tissues were excised for the fractionation of arginase isoenzymes.

Fractionation of arginase isoforms

Liver and kidney homogenates (10%, w/v) were prepared in 5 mM Tris-chloride buffer (pH 8.3) containing 5 mM MnCl₂ and centrifuged at 15,000 g for 15 min at 4°C. Arginase isoforms in the supernatants were fractionated on DEAE-cellulose columns, previously equilibrated with the same buffer and eluted with a linear KCl gradient (0 to 0.3 M). For CM-cellulose chromatography the same buffer of pH 7.5 was used. Details of the procedures have been described earlier.

Characterization of isoforms

Arginase assays in the fractions, polyacrylamide gel electrophoresis of the fractionated isoforms and determination of their molecular weights by gel filtration on Sephadex G-100 or G-200 were performed as described previously.

Immunological studies

Preparation of antibodies against purified rat and beef liver arginases and immunotitration of arginase isoforms in lizard and frog tissues with the antibodies were carried out as described earlier. The molecular forms were allowed to cross-react with different concentrations of each immunoglobulin. The antigen-antibody complexes resulting from the interaction were removed by centrifugation at 13,000 g and 4°C for 30 min and the residual enzyme activity in the supernatants and immunoprecipitates was assayed.

Results

The molecular forms of arginase in lizard and frog tissues, like those in rat and beef tissues were designated as A₁, A₂, A₃ and A₄ based on the concentration of KCl (0, 20-70, 100-140 and 190-240 mM, respectively) required for their elution from the DEAE-cellulose column (pH 8.3). Furthermore, A₁ was distinguished into A₁ and A'₁ depending upon whether it was excluded only by DEAE-cellulose (pH 8.3) or by both DEAE-cellulose (pH 8.3) and CM-cellulose (pH 7.5).

Arginase in lizard liver extracts resolved into four fractions by DEAE-cellulose chromatography at pH 8.3 (Fig. 1A). The first fraction, which eluted with the buffer front on DEAE-cellulose was also excluded by CM-cellulose (pH 7.5) (Fig. 1A). The other three forms were retained on DEAE-cellulose (pH 8.3) and eluted out with 54, 114 and 208 mM KCl (Table 1). The four isoforms present in more or less equal proportions were chromatographically similar to A'₁, A₂, A₃ and A₄ earlier reported by us in rat kidney. As with the rat kidney isoforms, the electrophoretic mobility of the four molecular forms from lizard liver to the anode on native polyacrylamide gels (A'₁<A₂<A₃<A₄) at pH 8.3 was consistent with their chromatographic behavior.

On the other hand, in lizard kidney and in the liver and kidney of frog, only two isoforms of arginase were detected (Fig. 1B, 2A, 2B). Both forms in these...
tissues were adsorbed on DEAE-cellulose (pH 8.3) and eluted by KCl (Table 1). The same two forms corresponding to $A_2$ and $A_3$ appeared to be present in both tissues of the frog as they were eluted at the same concentration of KCl viz., 47 and 115-117 mM, respectively. However, in lizard kidney, the isoforms were eluted with 105 and 190 mM KCl and corresponded to $A_3$ and $A_4$ of rat kidney. Consistent with this chromatographic behavior, the electrophoretic mobility of $A_3$ from all the three tissues was intermediate to those of $A_2$ from frog tissues and $A_4$ from lizard kidney (Fig. 3). The major isoenzyme in frog tissues was $A_2$, but in lizard kidney it was $A_4$ (Table 1).

The isoforms in liver and kidney tissues of the two species had their pH optimum between 9.6 and 10.2. No significant differences were noticed in the $K_m$ values of the isoforms, except that both isoforms in lizard kidney and $A_3$ in frog kidney exhibited a slightly higher affinity for L-arginine (Table 1). While lizard kidney $A_3$ showed mixed-type inhibition by

### Table 1—Properties of arginase isoforms in lizard and frog tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Isoform</th>
<th>KCl (mM)</th>
<th>Activity (%)</th>
<th>$K_m$ (mM)</th>
<th>pH Optimum</th>
<th>Mol. wt ($\times 10^{-3}$)</th>
<th>Inhibition* by</th>
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<tbody>
<tr>
<td>Lizard liver</td>
<td>$A_1^*$</td>
<td>0</td>
<td>21 $\pm$ 6</td>
<td>38 $\pm$ 7</td>
<td>9.6</td>
<td>122</td>
<td>Ornithine Lysine</td>
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<td></td>
<td>$A_2$</td>
<td>54 $\pm$ 3</td>
<td>24 $\pm$ 6</td>
<td>47 $\pm$ 9</td>
<td>9.8</td>
<td>122</td>
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<tr>
<td></td>
<td>$A_3$</td>
<td>114 $\pm$ 7</td>
<td>31 $\pm$ 10</td>
<td>44 $\pm$ 1</td>
<td>10.0</td>
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<td>C C</td>
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<tr>
<td></td>
<td>$A_4$</td>
<td>208 $\pm$ 5</td>
<td>18 $\pm$ 4</td>
<td>31 $\pm$ 8</td>
<td>10.0</td>
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<td>C C</td>
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<tr>
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<td>$A_3$</td>
<td>105 $\pm$ 2</td>
<td>22 $\pm$ 3</td>
<td>20, 25</td>
<td>9.6–9.7</td>
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<td>78 $\pm$ 3</td>
<td>25, 28</td>
<td>9.6–9.7</td>
<td>122</td>
<td>C C</td>
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<td>Frog liver</td>
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<td>47 $\pm$ 2</td>
<td>86 $\pm$ 3</td>
<td>46 $\pm$ 11</td>
<td>10.2</td>
<td>28</td>
<td>C C</td>
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<td>14 $\pm$ 3</td>
<td>41 $\pm$ 7</td>
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<td>C C</td>
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<td>71 $\pm$ 7</td>
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<td>M M</td>
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<td>117 $\pm$ 3</td>
<td>29 $\pm$ 7</td>
<td>18 $\pm$ 6</td>
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<td>10.2</td>
<td>28</td>
<td>C C</td>
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\*Concentration required for elution from DEAE-cellulose (pH 8.3)

#C = Competitive inhibition; M = Mixed-type inhibition

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Fig. 2—Fractionation of arginase isoforms in frog liver (A) and kidney (B) [Tissue extracts were subjected to DEAE-cellulose chromatography at pH 8.3 ( ). The adsorbed isoforms were eluted by a 0–0.3 M KCl gradient ( )].

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Fig. 3—Anodal electrophoresis (pH 8.3) of arginase isoforms on polyacrylamide gels [Frog liver $A_2$ ( ) and $A_3$ ( ); frog kidney $A_2$ ( ) and $A_3$ ( ); lizard kidney $A_3$ ( ) and $A_4$ ( )]. Isoforms fractionated by DEAE-cellulose chromatography were loaded on separate tube gels and electrophoresis was carried out simultaneously. The gels were sliced and arginase assayed in the slices].
L-ornithine and competitive inhibition by L-lysine, A2 from frog kidney exhibited mixed-type inhibition in the presence of both amino acids. All the isoforms in lizard and frog livers, A4 from lizard kidney and A3 from frog kidney were inhibited competitively by the two basic amino acids.

All the isoforms in lizard tissues gave a molecular weight of 122,000 by gel filtration. Arginase isoforms in both tissues of frog, however, were found to be substantially smaller in size (mol. wt. 27,000 to 28,000).

Despite prolonged incubation (48-56 h), none of the isoforms in lizard tissues gave precipitin bands with antibodies raised against purified rat and beef liver arginases by Ouchterlony double immunodiffusion technique. In immunotitration experiments, A1' and A2 from lizard liver were precipitated to the extent of about 40% and 15%, respectively by anti-rat liver arginase at an antibody concentration of 30 mg per unit enzyme, which was 1,500-fold higher than that required for the complete precipitation of rat liver arginase. The A3 and A4 from lizard liver were totally unaffected by anti-rat liver arginase (Fig. 4A). Beef liver arginase antibody caused immunoprecipitation of only A1', but not of the other three isoforms, to the extent of 60% at a concentration of about 58 mg per unit enzyme which was 20-times higher than that required for the complete precipitation of the homologous enzyme (Fig. 4B). The lizard kidney isoforms were not precipitated by the two antibodies (Fig. 4A, B).

Fig. 4—Immunotitration of arginase isoforms from lizard tissues with (A) anti-rat liver arginase and (B) anti-beef liver arginase antibodies [Liver A1' (●), A2 (○), A3 (▲) and A4 (△); kidney A1 (●) and A4 (○)]. The percent residual activities in the supernatants obtained by centrifugation of antigen-antibody mixtures were plotted against antibody concentration. The upper scales on the X-axis in Fig. A (0 to 0.12 mg Ab per unit enzyme) and Fig. B (0 to 12 mg Ab per unit enzyme) were respectively for purified rat liver (A1, O) and beef liver (A2, O) arginases against which the antibodies were raised in rabbits.

Fig. 5—Immunooactivation of arginase isoforms from frog liver by anti-rat liver arginase [(A2, ●); A3, ○] and anti-beef liver arginase (A2, ▲; A3, △) antibodies. Enzyme activities in the supernatants obtained by the centrifugation of antigen-antibody mixtures expressed as percentages of enzyme activity in the absence of antibody were plotted against antibody concentration. Titration curves at very low antibody concentrations are shown in the inset.

On the other hand, the A2 and A3 from frog liver and kidney were activated by both antibodies at concentrations ranging from a fraction of a milligram to 25-35 mg per unit enzyme (Figs. 5, 6). The degree of activation was 6 to 7-fold for liver A2, 4 to 5-fold for liver A3 and kidney A2 and 2 to 2.5-fold for kidney A3 with either antibody. Thus, enzyme activation by the two antibodies was more pronounced with A2 than
with A3 from both tissues and both molecular forms in liver were activated to a greater degree than those in kidney.

Activation of frog liver A2 by anti-rat liver arginase was noticed in whole antigen-antibody mixtures as well as in the supernatants and immunoprecipitates obtained by the centrifugation of antigen-antibody mixtures (Fig. 7). In whole mixtures and in the supernatants, the enzyme activity increased with increase in antibody concentration to a greater degree than in immunoprecipitates, where the activity was very low. At any given antibody concentration, the sum of activities in the supernatant and immunoprecipitate was lower than the activity in the uncentrifuged antigen-antibody mixtures.

Discussion

We have earlier reported four molecular forms of arginase viz. A1, A2, A3 and A4 in mammalian tissue extracts using DEAE-cellulose chromatography (pH 8.3). The presence of four isoforms is consistent with the widely accepted concept of two distinct genes and trimeric structure for mammalian arginase. A1 and A2 are both present in rat liver, submaxillary gland and kidney, with two additional forms (A3 and A4) in the kidney. The predominant molecular form is

with A3 from both tissues and both molecular forms in liver were activated to a greater degree than those in kidney.

Activation of frog liver A2 by anti-rat liver arginase was noticed in whole antigen-antibody mixtures as well as in the supernatants and immunoprecipitates obtained by the centrifugation of antigen-antibody mixtures (Fig. 7). In whole mixtures and in the supernatants, the enzyme activity increased with increase in antibody concentration to a greater degree than in immunoprecipitates, where the activity was very low. At any given antibody concentration, the sum of activities in the supernatant and immunoprecipitate was lower than the activity in the uncentrifuged antigen-antibody mixtures.

Fig. 6—Immunoa ctivation of arginase isoforms in frog kidney by anti-rat liver arginase (A2, □□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□...
Renal arginase in *Rana catesbiana* (MW 143,000) and *Rana pipiens* (MW 152,000) appears to be a much larger protein. Molecular weights of 37,000 and 40,000 predicted for the hepatic and extra-hepatic forms of the enzyme from the cDNA clones of arginase genes in *Xenopus laevis* are apparently those of arginase monomers.

The lizard (*Calotes versicolor*) is a uricotelic species that excretes 89% of its urinary nitrogen in the form of uric acid and lacks a functional urea cycle. The presence of four molecular forms of arginase in its liver, as opposed to only two in frog liver is puzzling and finds a parallel in our earlier observation on the occurrence of four and two isoforms of the enzyme respectively in the kidney and liver of rat. Liver tissue in frog and rat, both of which are ureotelic species, is associated with a highly active urea cycle which is either absent or operates only at feeble levels in rat kidney. While the catabolic role of arginase in converting arginine to ornithine as a precursor for the synthesis of proline, glutamate and polyamines is probably common to all tissues, its presence of supernumerary forms of arginase in lizard liver and rat kidney than in the liver of ureotelic animals. The presence of supernumerary forms of arginase in lizard liver like those in rat kidney, remains enigmatic for now.

Two renal forms of arginase, a major cytosolic component which functions in arginine catabolism and a minor one in the microsomes that is probably involved in urea transport have been reported in *Rana catesbiana*. Three arginases of different molecular weights, an electropositive (MW 80,000) and two electronegative (MW 160,000 and 80,000), have been found in the liver of *Rana esculenta*. We could detect neither the electropositive form nor the high molecular weight form in *Rana tigerina*. *Xenopus* liver contains two molecular forms with similar pH optima, molecular weights and Michaelis constants, but different isoelectric points. The presence of two hepatic forms of arginase, that are retained on DEAE-cellulose (pH 8.3) and hence electronegative, with similar pH optima, molecular weights and $K_m$ in *Rana tigerina* is consistent with the observations of Peiser and Balinsky. However, while the less electronegative form is predominant in the liver of *Rana tigerina* (present study) and *Rana esculenta*, the more electronegative form is the major hepatic form of arginase in *Xenopus*. It is not clear, if this difference is related to the different patterns of nitrogen excretion exhibited by the two species, viz., ammonotelism by *Xenopus laevis* and ureotelism by species of *Rana*.

While A$^1$ and A$^2$ from lizard liver are partially precipitated by anti-rat liver arginase, only A$^3$ is precipitated by anti-beef liver arginase. A$^3$ and A$^4$ from both tissues of the lizard are totally unaffected by either antibody. Activation of both molecular forms of arginase from frog tissues by the two antibodies suggests that the antigen-antibody interaction probably results in conformational changes, leading to an increase in enzyme-substrate affinity without forming large enough aggregates to immunoprecipitate. Stimulating antibodies have been reported for bacterial $\beta$-galactosidase, bovine pancreatic ribonuclease and monkey liver phenylalanine hydroxylase. Activation of amphibian arginases by antibodies is consistent with our earlier observation on rat submaxillary gland A2 which is activated by anti-beef liver arginase, though not by anti-rat liver arginase. Submaxillary gland A2 gives precipitin lines/arcs with anti-rat liver arginase, but not with anti-beef liver arginase in Ouchterlony and immunoelectrophoresis. Rat liver A$^1$ and A$^2$, submaxillary gland A$^1$, beef liver A$^2$ and beef kidney A$^1$ and A$^2$ are all either completely or partially precipitated and those in rat kidney are neither precipitated nor activated by the two antibodies. It is, therefore, tempting to speculate that lower vertebrates probably have only isoforms that are activated by antibodies to mammalian arginases. Isoforms of the enzyme that are either refractory to or immunoprecipitated by the antibodies probably appeared after the divergence of the amphibian and reptilian lines during vertebrate evolution. The ancestral type of isoform appears to have persisted sporadically in mammals as is probably the case with A$^2$ in rat submaxillary gland.

The tissue distribution of arginase isoforms in vertebrates is not consistent with the view that a basic isoform functions in the urea cycle in liver and an acidic or neutral form serves in generating ornithine for the synthesis of proline, glutamate and polyamines in extrahepatic tissues. Rat, ox and frog are all ureotelic vertebrates with a functional urea cycle in their liver, but have different sets of hepatic isoenzymes, A$^1$ and A$^2$ in rat, only A$^2$ in ox and A$^2$...
and A3 in frog. While the major isoform, A1 probably functions as a component of the urea cycle in rat liver, this function in frog liver is served by either A2 or A3 both of which are acidic. The solitary acidic form A2 in beef liver (pI 5.94) should function not only in the urea cycle but also in proline, glutamate and polyamine synthesis. The basic form A1, which is the major isoenzyme in rat submaxillary gland must function outside the urea cycle, since there is no evidence for the operation of the cycle in this tissue.

Thus, the evidence suggests that a given metabolic function is probably served by different isoforms in the tissues of different animals. This conclusion is consistent with the view that there might be a considerable functional overlap between the different isoforms of arginase. It also appears that arginase isoenzyme patterns do not show any correlation to the mode of nitrogen excretion in vertebrates.

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