Studies on the Advent of Ureotelism

THE EFFECTS OF BIVALENT CATIONS ON THE CAPACITY OF THE HEPATIC ARGINASE OF THE MEXICAN AXOLOTL TO HYDROLYSE ENDOGENOUS ARGININE

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1. The effects of 19 bivalent cations on the activity, stability and capacity to hydrolyse endogenous arginine of axolotl liver arginase were studied. 2. It was found that Fe^{2+} , Co^{2+} , Ni^{2+} and Zn^{2+} , as well as Mn^{2+} , stabilize the enzyme and render it able to hydrolyse endogenous arginine. 3. By using different concentrations of Co^{2+} and Ni^{2+} it was possible to dissociate the effect of each of the metal ions on the activity and stability of arginase from that on its capacity to hydrolyse endogenous arginine. 4. The effects of Mn^{2+} , Co^{2+} and Ni^{2+} on the activity and stability of arginase in the homogenate were also observed with purer preparations of the enzyme. 5. It is suggested that the capacity to hydrolyse endogenous arginine is a consequence of the integration of arginase with the arginine-formation sites.

We have suggested the possibility that the urea-cycle enzymes are physically linked to form a functional particle (Soberón, Ortiz-Pineda & Tarrab, 1967). The existence of protein aggregates constituting multienzyme systems has been substantiated in several instances (Sanadi, 1961; Koike, Reed & Carroll, 1963; Lynen, 1967). The experimental model that has been studied in our laboratory deals with the biosynthesis and hydrolysis of arginine by liver homogenates. It has been previously reported that rat liver homogenate readily converts endogenous arginine (i.e. the arginine formed from citrulline, aspartic acid and ATP) into urea and ornithine (Mora, Martuscelli, Ortiz-Pineda & Soberón, 1965a). When axolotl liver homogenate is used, endogenous arginine accumulates, probably because, unlike the rat enzyme, axolotl arginase is highly unstable (Palacios, Tarrab & Soberón, 1968). The enzyme is stabilized by Mn^{2+} , which also renders it able to hydrolyse endogenous arginine (Palacios *et al.* 1968). The latter effect could be due either to preservation of the activity of arginase or to the metal ion associating with the enzyme to produce a conformation suitable to form a proper fit with the elements of the endogenous arginine-producing system.

It has been consistently demonstrated that Co^{2+} and Ni^{2+} , as well as Mn^{2+} , affect the activity and other properties of arginase from different sources (Mohamed & Greenberg, 1945; Greenberg, Bagot & Roholt, 1956; Robbins & Shields, 1956). Moreover,

purified horse liver arginase has different thermal stabilities in the presence of Mn^{2+} , Co^{2+} and Ni^{2+} (Greenberg *et al.* 1956). Accordingly it was decided to examine the effects of a wide variety of bivalent cations on the activity, stability and capacity of the enzyme to hydrolyse endogenous arginine in relation to the problem being investigated. It was considered possible that different enzyme-metal ion complexes could produce different conformations whose characteristics might help to decide between the two alternatives proposed above.

Because the main objective of this work was to elucidate the factors involved in the production and disposal of arginine, it was necessary to use liver homogenate, where the elements of biosynthesis and hydrolysis of this amino acid are present.

The results reported here suggest that the capacity to hydrolyse endogenous arginine is a consequence of the integration of arginase with the arginine-formation sites.

MATERIALS AND METHODS

The source of the axolotls used, the handling of them, the preparation of liver homogenates and the determination of urea and arginine were carried out as described by Palacios *et al.* (1968), except that liver was homogenized in water instead of cetyltrimethylammonium bromide. It was established that no differences in the formation and hydrolysis of arginine resulted as a consequence of this change.

Arginase (L-arginine ureohydrolase, EC 3.5.3.1) activity

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was determined in a system containing 30 mg. of homogenized tissue, 1 μ mole of L-arginine and 50 μ moles of potassium phosphate buffer, pH 7.0, in a final volume of 1.0 ml.; the temperature of incubation was 37°. The activity was determined by measuring urea formation after 1 or 2 min. of incubation. The capacity to synthesize arginine and the further conversion of this into urea was followed by measuring arginine and urea formation as a function of time in a system containing 30 mg. of homogenized tissue, 5 μ moles of L-citrulline, 5 μ moles of L-aspartic acid, 5 μ moles of ATP, 5 μ moles of MgSO_4 and 50 μ moles of potassium phosphate buffer, pH 7.0, in a final volume of 1.0 ml.; the temperature of incubation was 37°. When liver homogenate was pre-incubated, this was done at 37° in a system containing 30 mg. of tissue and 50 μ moles of potassium phosphate buffer,

pH 7.0, in a volume of 0.8 ml. The reaction was started by adding all other ingredients in a volume of 0.2 ml.

It was found that 1 μ mole of Mn^{2+} , Rh^{2+} , Pd^{2+} and Pt^{2+} increases the colour intensity for the urea determination by 30, 25, 15 and 40% respectively. The same quantity of Cu^{2+} and Sn^{2+} decreases the colour intensity by 30 and 25% respectively. Standard curves of urea in the presence of various concentrations of the metal ions were obtained so that the appropriate corrections could be made.

Arginase activity was purified by the procedure described by Shimke, Brown & Smallman (1963), which includes acetone precipitation, heat denaturation, ethanol precipitation and chromatography on CM-cellulose. Unlike the rat liver arginase, the bulk of the axolotl liver arginase is precipitated with the addition of 1 vol. of cold ethanol and

Fig. 1. Effects of different bivalent cations on the activity, stability and capacity to hydrolyse endogenous arginine of axolotl liver arginase. The arginase activity, the preincubation of the homogenate and the formation and hydrolysis of endogenous arginine were carried out in the presence of a 1 mM concentration of the indicated metal ion. 'None' refers to the control. In the upper part the arginase activity is expressed as percentage of that obtained with the control. The results presented were obtained in two different experiments: for Mg^{2+} , Ca^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} , Cu^{2+} , Zn^{2+} , Sr^{2+} and Ba^{2+} the control activity was 11 μ moles of urea/min./g. of wet tissue; for Be^{2+} , Ti^{2+} , Rh^{2+} , Pd^{2+} , Sn^{2+} , Pt^{2+} and Pb^{2+} the control activity was 6 μ moles of urea/min./g. of wet tissue. Arginase was assayed at zero time ($\square$) and after 15 min. of preincubation of the homogenate at 37° ($\blacksquare$). In the lower part the distribution of arginine ($\boxtimes$) and urea ($\blacksquare$) formed from L-citrulline and L-aspartic acid is presented on a percentage basis. The sum of arginine plus urea at 30 min. (left half of the bar) and 60 min. (right half) is defined as 100%. In the cases where inhibition of arginine formation was noted, this is indicated below the corresponding bar by a number expressing the percentage of activity remaining in the presence of the metal ion (% AS). The sensitivity of the procedures used for the determination of urea and arginine was not precise enough to establish the distribution of urea and arginine when Pd^{2+} , Cd^{2+} and Hg^{2+} were employed. See the Materials and Methods section for conditions of the assay systems. See the text for the special case of Fe^{2+} .

also this fraction is eluted with the exclusion volume on CM-cellulose chromatography. The activity of the preparation obtained after the ethanol precipitation, selected for testing the effects of some bivalent cations, had a specific activity of 126 μ moles of urea/min./mg. of protein, which represented a 35-fold purification with a yield of 60%. The enzyme activity during the purification was assayed in a system containing 25 μ moles of glycine-NaOH buffer, pH 9.5, 100 μ moles of L-arginine adjusted to pH 9.5 with 1 M-NaOH and 0.25 μ mole of MnCl_2 in a final volume of 1.0 ml. The temperature of incubation was 37°. Protein was measured by the method described by Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

The amino acids utilized were purchased from Calbiochem (Los Angeles, Calif., U.S.A.). Isonitrosopropiophenone and ATP were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). CaCl_2 , MgCl_2 , ZnCl_2 , HgCl_2 , BaCl_2 , CoCl_2 , CdCl_2 , SrCl_2 , NiCl_2 , PtCl_2 , CuCl_2 , TiCl_2 and FeSO_4 were purchased from Mallinckrodt Chemical Works (New York, N.Y., U.S.A.); SnCl_2 was from J. T. Baker (Ecatepec, Mexico); PbCl_2 was from May and Baker Ltd. (Dagenham, Essex); BeCl_2 was from K & K Laboratories Inc. (Plainview, N.Y., U.S.A.); RhCl_2 was from Fisher Scientific Co. (New York, N.Y., U.S.A.); PdCl_2 was from American Platinum Works (Newark, N.J., U.S.A.). Other inorganic salts were from Merck, Sharp and Dohme Inc. (Rahway, N.J., U.S.A.).

RESULTS

The effects of 18 different bivalent cations on the activity, stability and capacity to hydrolyse endo-

genous arginine of the liver arginase of the Mexican axolotl are presented in Fig. 1. The addition of Mn^{2+} , Co^{2+} or Ni^{2+} increases the activity of arginase compared with that observed in the absence of metal ion. These ions are also efficient in stabilizing the enzyme, as judged by the activity remaining after 15 min. of preincubation of the homogenate in the presence of the metal ion. Co^{2+} and Ni^{2+} have the most striking effect, since the activity after preincubation is much higher than that measured without preincubation; Mn^{2+} preserves only about 60–70% of the original activity, and Zn^{2+} is less effective. Several metal ions, e.g. Cu^{2+} and Hg^{2+} , slightly inhibit the enzyme activity. As already reported, Mn^{2+} renders the enzyme able to hydrolyse endogenous arginine (Palacios *et al.* 1968). Zn^{2+} also produces an increase in the urea formation from the arginine being synthesized. Again the effects of Co^{2+} and Ni^{2+} are remarkable since at the concentration tested all the arginine produced is readily transformed into urea. No urea is formed when the system contains Be^{2+} , Ti^{2+} , Rh^{2+} , Sn^{2+} or Pt^{2+} . The activity of the arginine synthetase system (argininosuccinate synthetase and argininosuccinase) is inhibited by about 50% by Ca^{2+} , Mn^{2+} or Pt^{2+} at a concentration of 1 mM. Inhibition is almost complete with Pd^{2+} , Cd^{2+} or Hg^{2+} at the same concentration.

Fig. 2. Effects of different concentrations of Co^{2+} and Ni^{2+} on the activity, stability and capacity to hydrolyse endogenous arginine of axolotl liver arginase. The arginase activity is expressed as μ moles of urea/min./g. of wet tissue. Other conditions and symbols are the same as those given for Fig. 1.

The tests with Fe^{2+} deserve special comment. It was not possible to use ferrous chloride because this salt is highly unstable. The addition of cysteine to maintain the metal ion in the ferrous state proved to be impossible because this amino acid strongly inhibited arginase activity. [This inhibition was rather surprising since it was previously reported that Fe^{2+} , in the presence of cysteine, activates arginase (Hellerman & Perkins, 1935).] When ferrous sulphate was added at the concentration tested, ferrous phosphate was precipitated in the system. With tris-hydrochloric acid buffer, pH 7.0 (50 mM final concentration), instead of phosphate buffer the arginase is activated threefold and after 15 min. of preincubation in the presence of ferrous sulphate the activity reaches 4.5 times that of the control. However, it was also observed that tris-hydrochloride buffer alone strongly inhibited the formation of arginine. Incubation of the system without buffer, the pH being adjusted with sodium hydroxide as required during the progress of the reaction, showed that Fe^{2+} caused the conversion into urea of 60% of the arginine produced. Under these conditions arginine synthetase was thus inhibited 40% by Fe^{2+} .

It was thought that investigation of the effects of various concentrations of metal ions on the arginase activity, stability and capacity to hydrolyse endogenous arginine would throw light on the problem being studied. Indeed, if the last property is independent of the other two, the concentrations of the metal ions that induce the expression of these different properties might well not be the same. Because Mn^{2+} and Fe^{2+} inhibited the arginine synthetase system mentioned above and because Co^{2+} and Ni^{2+} proved to be more efficient with regard to the effects being explored, the study was continued with only these two metal ions.

Fig. 2 shows the effects of different concentrations of Co^{2+} and Ni^{2+} ions on the activity, stability and capacity to hydrolyse endogenous arginine of axolotl liver arginase. Concentrations of Co^{2+} lower than $5 \mu\text{M}$ do not affect the stability and activity of the enzyme; higher concentrations up to $10 \mu\text{M}$ increase the stability so that at this concentration the activity remaining after 15 min. of preincubation is the same as the original. At $25 \mu\text{M}$ there is a definite effect on the activity, which increases to almost double that of the control, this increment being more noticeable when the enzyme is preincubated in the presence of the metal ion. Activity and stability reach a maximum at a concentration of $50 \mu\text{M}$. The hydrolysis of endogenous arginine increases progressively from $5 \mu\text{M}$ and also reaches a maximum at $50 \mu\text{M}$.

With $50 \mu\text{M-Ni}^{2+}$ the immediate increase in the activity of the enzyme becomes more apparent after incubation. Both activity and stability reach a max-

Fig. 3. Effects of Co^{2+} ($50 \mu\text{M}$) and Ni^{2+} ($100 \mu\text{M}$) on the activity, stability and capacity to hydrolyse endogenous arginine of axolotl liver arginase. Arginase activity is expressed as $\mu\text{moles of urea/min./g. of wet tissue}$. Symbols are the same as those given for Fig. 1. See the Materials and Methods section for conditions of the assay systems.

imum at a concentration 0.10 mM or higher. Nevertheless the concentration necessary to produce the maximal hydrolysis of endogenous arginine is 0.75 mM . Over the range $0.10\text{--}0.75 \text{ mM}$ activity and stability remain constant whereas the hydrolysis of endogenous arginine keeps increasing.

It seemed feasible from the above results to separate the effect of the metal ions on the activity and stability of the arginase from the effect on the capacity to hydrolyse endogenous arginine. We have acquired enough experience to be able to predict the concentrations of Co^{2+} and Ni^{2+} required to cause similar activity and stability but that, however, would produce different amounts of urea from the arginine synthesized. Such an experiment is illustrated in Fig. 3. Concentrations of $50 \mu\text{M-Co}^{2+}$ and 0.10 mM-Ni^{2+} give the same activity and stability; with Co^{2+} all the arginine is converted into urea but with Ni^{2+} 60% of the arginine formed remains unchanged.

It was considered that the effects of bivalent cations on arginase activity and stability, described above, needed to be substantiated with

Fig. 4. Effects of Mn^{2+} (a and d), Co^{2+} (b and e) and Ni^{2+} (c and f) on the activity, stability and restitution of the activity of denatured axolotl liver arginase. The experiment was carried out with homogenate (a, b and c) and with a purified preparation of arginase (d, e and f). The preincubation of the homogenate was carried out in the presence (—) and in the absence (---) of the corresponding metal ion. The enzyme activity was determined in the presence (●) and in the absence (○) of each metal ion. The concentration used was 1 mM in each case. Arginase activity is expressed as $\mu\text{moles of urea/min./ml. of homogenate or enzyme solution}$. See the Materials and Methods section for characteristics of the purified arginase and conditions of the assay systems.

purier preparations of the enzyme, and the experiment described in Fig. 4 was therefore carried out. The livers of 25 axolotls were removed, pooled and minced in the cold. A 2g. portion of the tissue was homogenized as indicated in the Materials and Methods section. To purify arginase activity the remaining 18g. of liver tissue was treated as indicated by Schimke *et al.* (1963). The fraction obtained after the first ethanol addition was extensively dialysed against water at 4° (five changes, 2l. each, were performed during a period of 18hr.). The effects of 1mM solutions of Mn^{2+} , Co^{2+} and Ni^{2+} were tested on the original homogenate and on the purified enzyme.

The responses of arginase to the presence of Mn^{2+} , Co^{2+} or Ni^{2+} are strikingly similar with either the homogenate or the purier preparation. The three metal ions increase the enzyme activity as compared with that observed in their absence, as also shown in the experiment reported in Fig. 1. If the enzyme is preincubated in the presence of the metal ions, the activity is partially preserved with Mn^{2+} , whereas noticeable increases are obtained with Co^{2+} or Ni^{2+} .

If the same metal ions are added when arginase has been already denatured by preincubation, Mn^{2+} effects a partial restitution of the enzyme activity, and there is total recovery with Co^{2+} or Ni^{2+} . This recovery is faster with Ni^{2+} .

DISCUSSION

It has been possible to separate the effects of the metal ions on the activity and stability of axolotl liver arginase from that on its capacity to hydrolyse endogenous arginine. This suggests that the metal ion in becoming associated with the enzyme moiety initiates the formation of different types of enzyme-metal ion complexes, depending on the ion species and its concentration. It is possible that one type of enzyme-metal ion complex stabilizes the enzyme activity, but is not enough to allow substantial hydrolysis of the arginine formed from citrulline and aspartic acid. A second type of enzyme-metal ion complex, in addition to being stable, has an adequate conformation to form a proper fit with the arginine-

formation sites. Another experimental situation distinguishing enzyme activity and stability from the capacity to hydrolyse endogenous arginine was presented by Palacios *et al.* (1968), who found that urea was continuously being formed from endogenous arginine in spite of the fact that arginase activity had disappeared. The distinction mentioned above is in further support of the idea that the urea-cycle enzymes, to function efficiently, must be physically integrated (Soberón *et al.* 1967). The finding that radioactive citrulline administered to the intact rat generates labelled arginine that is found in kidney proteins but is not significantly incorporated into liver proteins (Rogers & Freedland, 1968) is in accordance with this assumption. Even though a high activity of arginase is found in rat liver, it should be recalled that the K_m for arginine with this enzyme is rather high, of the order of $10^{-2}M$ (Mora *et al.* 1965a), compared with that with the arginine-activating enzymes, $10^{-6}M$ (Allende & Allende, 1964).

The situation found for the axolotl contrasts with that existing in *Neurospora crassa*, in which the enzyme activities corresponding to the urea cycle are not enough for the operation of the cycle. The fungal arginase, which is inducible, is involved in a metabolic path that converts arginine into proline; it also permits the utilization of exogenous arginine as a source of nitrogen, arginine being synthesized primarily for the biosynthesis of proteins (Cañedo, Martuscelli & Mora, 1967; Castañeda, Martuscelli & Mora, 1967; Davis & Mora, 1968). Some molluscs are similar, since arginine formed from citrulline is readily incorporated into protein in the presence of arginase (Campbell & Speeg, 1968).

We are therefore inclined to believe that, for the biosynthesis of urea, arginase must be coupled to the sites of production of arginine. To achieve this a specific protein with a proper conformation is required. It is very likely that such a conformation could be obtained by interaction with certain metal ions. Relevant to this is the description of the so-called 'ureotelic arginase' found in the liver of ureotelic animals (Mora, Tarrab, Martuscelli & Soberón, 1965b).

The present results have some other implications. The metals whose ionic species proved to be efficient have atomic numbers in the range 25–30 and therefore have rather similar properties. Copper (atomic number 29) did not have the same behaviour, but it has several characteristics distinctly different from

the others, e.g. $4s^1$ electronic configuration and bigger ionic radius.

Mn^{2+} , Co^{2+} and Ni^{2+} behave differently among themselves with respect to the recovery of the activity of the enzyme denatured by preincubation. These differences in behaviour should be the result of the different velocity of interaction of each metal ion with the denatured enzyme as well as a consequence of different characteristics of the enzyme-metal ion complexes formed.

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