BBA 65962

THE FERROUS ION AS THE COFACTOR OF ARGINASE *IN VIVO*

I. PROPERTIES OF YEAST ARGINASE METALLO-COMPLEXES OF KNOWN COMPOSITION AND OF NATIVE ARGINASE

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(Received May 19th, 1969)

SUMMARY

A comparative study has been made of metallo-complexes of yeast arginase (L-arginine ureohydrolase, EC 3.5.3.1) prepared *in vitro* and of yeast arginase demonstrable in fresh cell-free extracts without the addition of activating cations (native arginase). Since native yeast arginase is strongly inhibited by phosphate, yeast grown with α-glycerophosphate as sole source of phosphorus has been used for this purpose.

The activation of yeast arginase by Fe^{2+} *in vivo* is stated on account of the following observations:

1. The pH–activity curves of native arginase and of Fe^{2+}–arginase were similar, showing shapes and optima different from those of Mn^{2+}–, Co^{2+}–, Ni^{2+}– and Mg^{2+}–arginases.

2. The specific activity of native arginase at pH 8.5 was hardly increased by previous incubation of the cell-free extracts with Fe^{2+} salts.

3. Both native and Fe^{2+}–arginase were inhibited by phosphate in contrast with other metallo–arginases.

4. The Michaelis constants, as well as the inhibition constants for ornithine, were equal for native and for Fe^{2+}–arginase at 4 pH values; they were significantly different from those of other metallo–arginases.

INTRODUCTION

Arginase (L-arginine ureohydrolase, EC 3.5.3.1) catalyzes the hydrolysis of L-arginine to equimolar amounts of L-ornithine and urea. Since the first demonstration of arginase in liver by KOSSELL AND DAKIN[1] in 1904, many investigations on its action have been carried out, especially after the elucidation of the role of this enzyme in the urea synthesis in the liver of ureotelic organisms by KREBS AND HENSELEIT[2] in 1932. Liver arginase is a metallo-enzyme[3], the presence of either Mn^{2+}, Co^{2+}, Fe^{2+} or

Ni^{2+} being required for its action. The apoenzyme forms stable complexes with the activating cations[4]. For activation of arginase preparations, incubation with an excess of the cations prior to arginine addition is necessary[4]. Complete activation requires incubation at elevated temperatures or for long periods[4]. The enzyme is protected against heat denaturation by the presence of the activating cations[5]. The pH–activity curve of liver arginase varies with the activating cation. Mn^{2+}–arginase shows its optimum at pH 10, Co^{2+}– and Ni^{2+}– arginases at pH 7–9.5 (ref. 4).

EDLBACHER AND BAUR[6] were the first to demonstrate the presence of arginase in baker's yeast. Afterwards, MIDDELHOVEN[7] has shown the inducibility of the enzyme by arginine and its participation in the arginine breakdown in yeast. Yeast arginase shows a requirement for bivalent cations like liver arginase[6]. It differs from the latter in being more readily inactivated by dialysis; reactivation by addition of activating cations is possible[6].

In liver homogenates and in cell-free extracts of yeast, an arginase activity was found to be demonstrable, without the addition of activating cations. This activity (to be referred to as native arginase) was strongly inhibited by chelating agents such as 8-hydroxyquinoline. Hence it must be concluded that its activity depends on bivalent cations as well. Because of the slow reaction of apoarginase with its activating cations[4], it is improbable that the native arginase activity originates from the reaction of arginase with free cations during the preparation of cell-free extracts. Hence the native arginase may be assumed to be identical with the metallo–arginase that is active *in vivo*. The question arises as to which of the cations, capable of activating the enzyme *in vitro*, takes part in the enzyme reaction *in vivo*. The answer to this question may be given by a comparative study of native arginase and of metallo–arginases, prepared *in vitro*, of known composition. During the thirties and early forties, several studies on the nature of the cation, activating liver arginase *in vivo*, have been undertaken. The results of these studies will be briefly summarized.

EDLBACHER AND ZELLER[8] suggested that either Fe^{2+} or Mn^{2+} activate the native liver arginase. They considered the inhibition of native liver arginase by KCN at pH 6–7 (ref. 9) as an argument in favour of Fe^{2+}. Nevertheless, they stated that Mn^{2+} is the activating cation in native liver arginase, on account of the excellent activation of the enzyme by Mn^{2+} *in vitro* and on account of the ability of Mn^{2+} to protect the liver arginase against inactivation by trypsine; Fe^{2+} failed to protect the enzyme under such conditions. RICHARDS AND HELLERMAN[10] purified native liver arginase 100-fold without addition of activating cations during their fractionating procedure. Analyses of their preparations by flame spectroscopy demonstrated that, of all metals capable of activating arginase *in vitro*, only Mn^{2+} and Fe^{2+} were present. They suggested that Mn^{2+} is the activating cation *in vivo*, without completely excluding the possibility of participation of Fe^{2+}, on account of the superior reactivation of an inactivated preparation by minor concentrations of Mn^{2+}.

Assays of liver arginase in Mn-deficient rats and mice[11,12] have learned that the arginase activities in Mn-deficient animals were significantly smaller than those in the control group. The Mn-deficient animals showed growth retardation and failed to reproduce. No effect on urea excretion was observed[11,12], however, even after the administration of large doses of ammonium citrate[12]. The observed decrease in liver arginase activity, caused by Mn deficiency, is generally accepted as the definite proof of Mn^{2+} being involved in the liver arginase reaction *in vivo*. This conclusion has

never been substantiated by a comparative study of native liver arginase and of metallo–arginases of known composition, however.

The aim of the present research was to study the native arginase in cell-free extracts of baker's yeast and to compare its properties with those of metallo-complexes of yeast arginase prepared *in vitro*. In this paper evidence is presented for the involvement of Fe^{2+} in the action of yeast arginase *in vivo*. The results of the present research have already been published as a preliminary report[13].

MATERIALS AND METHODS

Cultivation of yeast

A strain of *Saccharomyces cerevisiae* Hansen, isolated from "Koningsgist" of the Koninklijke Nederlandsche Gist- en Spiritusfabriek (Delft, The Netherlands) was used throughout this investigation. The growth medium contained per l: 40 g of glucose; 10 g of peptone (acid hydrolysate of casein); 1.36 g (10 mmoles) of KH_2PO_4; 400 mg of $MgCl_2 \cdot 6\ H_2O$; 100 mg of $CaCl_2 \cdot H_2O$; 100 mg of NaCl; 2 mg of $FeCl_3 \cdot 6\ H_2O$; 0.5 mg of H_3BO_3; 0.1 mg of $CuSO_4 \cdot 5\ H_2O$; 0.1 mg of KI; 0.4 mg of $MnSO_4 \cdot H_2O$; 0.2 mg of Na_2MoO_4; 0.4 mg of $ZnSO_4 \cdot 5\ H_2O$; 0.2 mg of thiamine; 0.1 mg of riboflavine; 5 mg of nicotinic amide; 0.3 mg of *p*-aminobenzoic acid; 1 mg of pyridoxine; 2 mg of calcium pantothenate; 10 mg of *myo*-inositol; 0.02 mg of biotin; pH 4.5. The yeast was grown aerobically at 30° in a Kluyver flask, was harvested in the stationary phase of growth and was washed 3 times with water at 0°. Yeast poor in phosphate was grown in the same way, using 0.63 g (2 mmoles) of disodium α-glycerophosphate per l of medium as a source of phosphorus, instead of KH_2PO_4.

Preparation of crude cell-free extracts

2-g yeast samples were sonically disintegrated for 5 min at 0° in a M.S.E. Ultrasonic Power Unit, after addition of 1 ml of a solution of 1% $NaHCO_3$ in 10 mM β-mercaptoethanol. After dilution with 3 ml of H_2O, nuclei and cell debris were removed by centrifugation (10 min, $4000 \times g$).

Preparation of metallo–arginases

Bivalent cations were removed from the crude cell-free extract by reaction with 10 mM EDTA (pH 7.5) for 10 min at 30° and by subsequent gel filtration on a column of Sephadex G-25 (10 mM Tris–HCl, pH 7.5). In this way, purified cell-free extracts were obtained which were devoid of EDTA and other low-molecular substances (*e.g.* inhibiting amino acids). In these extracts no arginase activity was observed, unless activating cations were added. Metallo–arginases of known composition were prepared from these purified cell-free extracts by incubation with 40 mM bivalent cations (as hydrochlorides of A.R. quality) and 40 mM Tris–HCl (pH 7.5). The activations with Mn^{2+} and Mg^{2+} salts were performed at 50° for 15 min, those with other cations at 45° for 15 min.

Isolation of native arginase

Cell-free extracts were prepared from yeast poor in phosphate in the way described above. Low-molecular substances were removed from the crude cell-free extracts by gel filtration on a column of Sephadex G-25 (10 mM Tris–HCl, pH 8.5,

bed dimensions 18 cm × 1.6 cm), within 10 min at 0°. Maintenance of the pH at 8–9 was found to be a requirement for obtaining active preparations. For this purpose $NaHCO_3$ was added prior to the disintegration of the yeast.

pH–activity curves

The activities of native arginase and of the metallo–arginases were determined at various pH values by incubating enzyme, 100 μmoles L-arginine · HCl (pH adjusted) and 200 μmoles of buffer in a total volume of 2 ml. The tubes were incubated at 30° for 1 h, unless otherwise indicated. No more than 5 μmoles urea were allowed to be formed. Buffers used were sodium succinate for pH 5.0, 5.5 and 6.0; sodium cacodylate for pH 6.5 and 7.0 (potassium phosphate for Ni^{2+}–arginase pH 6.5, 7.0 and 7.5); Tris–HCl for pH 7.5, 8.0, 8.5 and 8.75; and sodium glycinate for pH 9.0, 9.5, 10.0 and 11.0. The final concentrations of the activating cations were 1 mM. The reactions were stopped with $HClO_4$. The protein precipitates were removed by centrifugation, if necessary. Urea was determined spectrophotometrically; the isonitrosopropiophenone method of ARCHIBALD[14] was used. Since the presence of arginine in the aliquots was shown to decrease the absorbance, corrections were made by addition of arginine to the urea standard series. Similar corrections were made for the presence of bivalent cations, especially Mn^{2+}, which tended to increase the absorbance. The activities of arginase at various pH values were expressed as specific activities, *i.e.* units per mg protein. One unit was defined as the quantity of enzyme, catalyzing the production of 1 μmole urea per h at 30°. Protein was determined in the cell-free extracts[15], using crystalline bovine serum albumin as the standard.

Determinations of the Michaelis constant and of the inhibition constant for ornithine

Equal amounts of enzyme were added to a series of test tubes containing the same buffer as used for the pH–activity curves (final concentration 100 mM) and arginine (variable concentration). After incubation at 30° for 1 h, the amount of urea was determined[14], corrections being made for arginine and for bivalent cations. The series was run in triplicate. The substrate concentrations (S) and the calculated rate of reaction (v) were plotted reciprocally according to LINEWEAVER AND BURK[16]. From this plot the Michaelis constant (K_m) was calculated graphically (see Fig. 1).

TABLE I

SPECIFIC ACTIVITIES OF DIFFERENT METALLO-COMPLEXES OF YEAST ARGINASE

Native yeast arginase and different metallo-complexes of yeast arginase were prepared and their specific activities determined as described in MATERIALS AND METHODS. The specific activities (μmoles urea produced per h per mg protein), observed in various cell-free extracts, were expressed as per cent of the specific activity of Mn^{2+}-activated arginase at pH 9.5.

Activating cation	*pH*	*Buffer*	*% Specific activity*
Mn^{2+}	9.5	Sodium glycinate	100
Mg^{2+}	9.5	Sodium glycinate	5–15
Native	8.5	Tris–HCl	20–25
Fe^{2+}	8.5	Tris–HCl	15–25
Co^{2+}	8.5	Tris–HCl	20
Ni^{2+}	7.0	Potassium phosphate	10–20

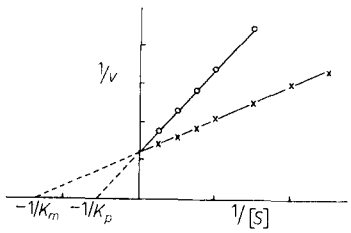

Fig. 1. Determination of the Michaelis constant and of the inhibition constant for ornithine. ×—×, no ornithine; ○—○, 5 mM L-ornithine.

The inhibition constant for ornithine (K_{Orn}) was determined by measuring the reaction rate in a series of incubation mixtures, identical with the series used for the K_m determination, except for the addition of 5 mM L-ornithine·HCl (Fluka) to each tube. These determinations were also run in triplicate. Ornithine was shown to be a competitive inhibitor of liver arginase by HUNTER AND DOWNS[17]. The Lineweaver-Burk plot gave a straight line, intersecting that of the series without ornithine at the $1/v$ axis thus demonstrating that the inhibition was of the purely competitive type[18] (see Fig. 1). The $1/[S]$ axis was intersected by this line at $-1/K_p$. K_{Orn} was calculated from K_p by the following equation (see ref. 18 for the derivation):

$$K_p = K_{Orn}(1 + [\text{Ornithine}]/K_{Orn}),$$

in which the ornithine concentration is 5 mM.

RESULTS

pH–activity curves of metallo–arginases prepared in vitro

Cell-free extracts of baker's yeast freed from bivalent cations by reaction with EDTA and subsequent gel filtration, showed no arginase activity unless activating cations had been added. Metallo–arginase preparations of known composition were obtained by reaction of cell-free extracts with 40 mM cations (as hydrochlorides) at pH 7.5. Only the addition of Mn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+} or Mg^{2+} resulted in arginase activity. The bivalent cations: Ba^{2+}, Be^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, Pd^{2+}, Sn^{2+}, Sr^{2+}, UO_2^{2+}, ZrO^{2+} and Zn^{2+}; and the trivalent cations: Al^{3+}, Ce^{3+}, Fe^{3+} and La^{3+} did not activate yeast arginase.

The various metallo–arginases showed different pH–activity curves (see Figs. 2a–2e). The Mn^{2+}– and Mg^{2+}–arginases had optima at pH 9.5 and 10.0, respectively; the optimum of Fe^{2+}–arginase was at pH 8.5–9.0. The curve of Co^{2+}–arginase was more complicated, showing a shoulder at pH 7–9 and an optimum at pH 9.5. Ni^{2+}–arginase was found to be inhibited by cacodylate and Tris buffers; it showed its optimum at pH 7.0 in phosphate buffer, and its activity decreased gradually with increasing pH.

The observed specific activities of arginase varied with the activating cation. Table I shows the specific activities of various metallo-complexes of yeast arginase. Mn^{2+} was the most potent activator, similar to the case with liver arginase[4]. Because of the great heat resistance of Mn^{2+}–arginase, the activation by this cation was found to be excellently reproducible. The activation of yeast arginase by the other cations

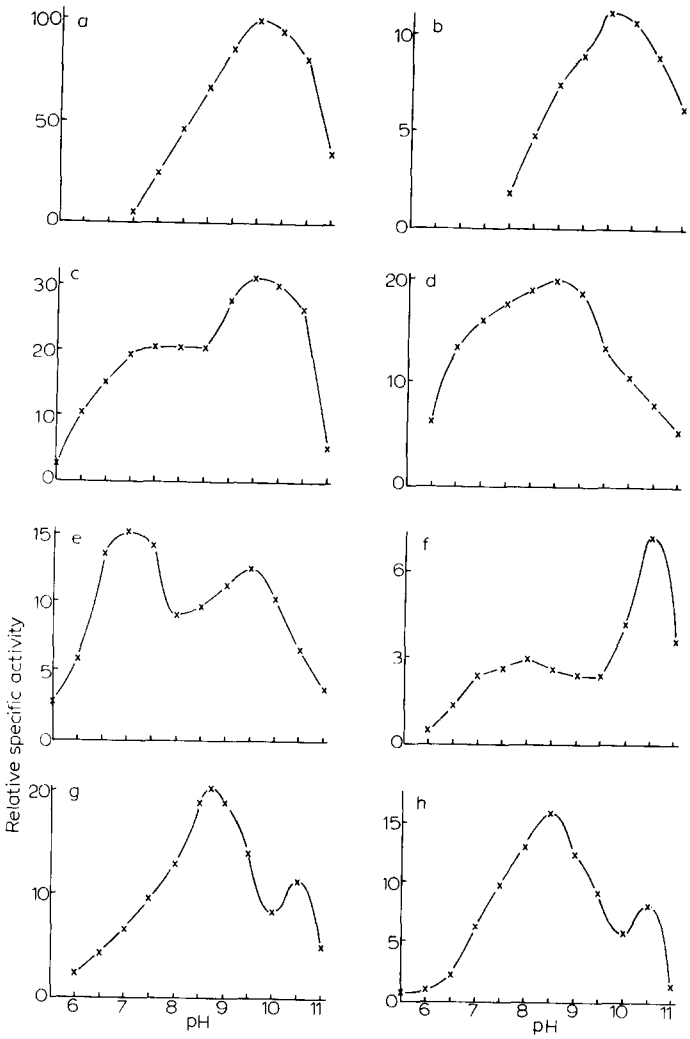

Fig. 2. pH–activity curves of various metallo-complexes of yeast arginase. Ordinate: relative specific activity, given as percent of the specific activity of Mn^{2+}–arginase (pH 9.5) (see Table I). a, Mn^{2+}; b, Mg^{2+}; c, Co^{2+}; d, Fe^{2+}; e, Ni^{2+}: f, native with phosphate; g, native phosphate-poor; h, Fe^{2+}, excess Fe^{2+} removed.

was variable. For the sake of clearness, all specific activities in Fig. 2 and in Table I have been expressed as percent of the specific activity of Mn^{2+}–arginase at pH 9.5.

Native arginase

It is possible to demonstrate an arginase activity in cell-free extracts of commercial baker's yeast, without the addition of bivalent cations prior to or during the incubation with arginine (native arginase). The specific activity of this arginase is small in comparison with that of arginase activated by bivalent cations. Its pH–activity curve shows a sharp peak at pH 10.5 and another maximum at about pH 8 (see Fig. 2f).

In early experiments it has been demonstrated that especially the native arginase activity (pH 8) is inhibited by phosphate. Since the phosphate concentration in commercial baker's yeast is sufficient for inhibition of the native arginase during the preparation of the cell-free extracts, the native arginase in cell-free extracts of yeast poor in phosphate has been studied as well. For that purpose the yeast was grown with α-glycerophosphate, instead of KH_2PO_4, as the phosphorus source. In the pH–activity curve of the native arginase of yeast poor in phosphate (Fig. 2g), the peak at pH 8.5–9.0 is predominant, in contrast with that of commercial baker's yeast; besides this optimum there is a smaller peak at pH 10.5. The specific activity at pH 8.5 approached that of Fe^{2+}–arginase (Table I). The period of incubation in the determination of this curve was 12 min, instead of 1 h as usual for the other pH–activity curves. The instability of native arginase did not allow longer periods of incubation.

Native arginase was found to be rather stable at pH 8–10. At other pH values, preparations of native arginase readily lost their activities (Table II), most probably due to dissociation of the metallo–arginase complex. For this reason it must be emphasized that the preparation of a cell-free yeast extract with strong native arginase activity is successful only if the pH during the whole procedure is above 8 and when the yeast has been grown in a medium rich in arginine, containing α-glycerophosphate as the phosphorus source.

TABLE II

STABILITY OF NATIVE ARGINASE

A cell-free extract with a strong native arginase activity was incubated for 30 min at 30° in 20 mM buffers of various pH's (see MATERIALS AND METHODS). Afterwards the specific activity of native arginase was determined at pH 8.5, as described in MATERIALS AND METHODS. The results are given as μmoles urea produced per ml of cell-free extract per h and as percent of the control (untreated cell-free extract).

pH	*Specific activity*	*% Specific activity*
6.0	400	21
7.0	1360	71.5
8.0	1730	91
8.5	1900	100
9.0	1770	93
10.0	1820	96
11.0	1520	80
Control	1900	100

The native arginase activity in the cell-free extracts was lost as soon as these extracts were submitted to protein fractionation procedures, such as precipitation with $(NH_4)_2SO_4$ or with cold acetone, unless the enzyme was protected by activating cations. In this respect it differs from native liver arginase[10]. Therefore, all the work for the elucidation of the nature of the cofactor of native yeast arginase had to be done with crude cell-free extracts; in purified enzyme preparations the native cofactor most probably would have been exchanged for added cations.

The pH–activity curve of native arginase resembles that of Fe^{2+}–arginase. Both enzyme activities are optimal at pH 8.5–9.0, quite distinct from those of Mn^{2+}–, Mg^{2+}–, Co^{2+}– and Ni^{2+}–arginases. Hence the cofactor of native yeast arginase is more

likely to be Fe^{2+} than any of the other activating cations. The activities of Fe^{2+}– arginase at unfavourable pH values were relatively greater than those of native arginase. This phenomenon might be explained by the instability of native arginase at unfavourable pH values. The excess of Fe^{2+}, present during the determination of the Fe^{2+} curve, might protect the enzyme at these pH values, thus causing apparently greater specific activities. This possibility has been investigated by the determination of the pH–activity curve of Fe^{2+}–arginase after removal of the excess of Fe^{2+} by gel filtration on a column of Sephadex G-25 (10 mM Tris–HCl, pH 8.5). Immediately before the gel filtration, 80 mM trisodium citrate was added to the Fe^{2+}–arginase solution (which contained 40 mM $FeCl_2$ and 40 mM Tris–HCl, pH 7.5). When the addition of citrate was omitted, all arginase activity was lost during the gel filtration, owing to adsorption of the enzyme by the $Fe(OH)_2$ precipitate. The pH–activity curve of Fe^{2+}–arginase, determined after removal of the excess of Fe^{2+}, is shown in Fig. 2h. It resembles that of native arginase both in shape and in optima. This resemblance points to the identity of native and Fe^{2+}–arginases. Further support for this assumption was found in studies on the behaviour of yeast metallo–arginases to inhibitors and in the determination of some kinetic constants.

Inhibition experiments with yeast arginase

Both liver and yeast arginase are strongly inhibited by reagents capable of binding bivalent cations, such as 8-hydroxyquinoline and EDTA. Native yeast arginase was also inhibited by these reagents. Many of the inhibitors behave unspecifically, reacting with all bivalent and trivalent cations. Some reagents, however, exhibit more specificity, *e.g.* Na_2S and dimethylglyoxim which react with heavy metals but not or less readily with Mn^{2+} and Mg^{2+}. A study of the sensitivity of native yeast arginase to these reagents was expected to give information about the nature of its activating cation.

TABLE III

INHIBITION OF NATIVE YEAST ARGINASE AND OF DIFFERENT METALLO–ARGINASES

The different arginase preparations, all derived from the same cell-free extract, were exposed to the action of inhibiting reagents for 30 min at 30° in 20 mM Tris–HCl (pH 7.5), the concentration of bivalent cations being 0.4 mM. Afterwards the specific activities of the arginases were determined as usual (final concentration of bivalent cations 0.1 mM; Fe^{2+}–, Co^{2+}–, Ni^{2+}–, native arginases in Tris–HCl (pH 8.5); Mn^{2+}– and Mg^{2+}–arginases in sodium glycinate (pH 9.5)). All data are specific activities (μmoles urea produced per h per mg protein).

Inhibitor	*Native*	Fe^{2+}	Co^{2+}	Ni^{2+}	Mn^{2+}	Mg^{2+}
Control	14	18.5	22	16	110	9
1 mM 8-hydroxyquinoline	0	0	0	0	0	0
0.4 mM Na_2S	0	0	0	0	75	8
4 mM dimethylglyoxim	3	0	0	0	60	6
20 mM potassium phosphate (pH 7.5)	3	1	22	16	110	9

Native yeast arginase and metallo–arginases of known composition, prepared *in vitro*, were exposed to the action of some inhibitors, and the activities of the various arginases were determined. As shown in Table III, native arginase resembles Fe^{2+}–, Co^{2+}– and Ni^{2+}–arginases in being inhibited by dimethylglyoxim and Na_2S. Mn^{2+}–

TABLE IV

K_m AND K_{Orn} OF NATIVE YEAST ARGINASE AND OF METALLO-ARGINASES OF KNOWN COMPOSITION

Activating cation	*pH*	*Buffer*	K_m *(mM)*	K_{Orn} *(mM)*
Co^{2+}	6.7	Potassium phosphate	6.0–7	2.7
Native	7.0	Sodium	4.3	1.5
Fe^{2+}	7.0	cacodylate	4.1–5.0	1.5–2.1
Co^{2+}	7.0		6.2	1.9
Native	7.5	Tris–HCl	4.0	1.4
Fe^{2+}	7.5		4.0	1.2
Native	8.5	Tris–HCl	3.3	0.8
Fe^{2+}	8.5		3.3	0.75
Native	9.5	Sodium	4.0	1.6
Fe^{2+}	9.5	glycinate	3.3	1.5
Co^{2+}	9.5		2.15	1.0
Ni^{2+}	9.5		1.3–1.6	1.3–2.1
Mn^{2+}	9.5		5.0–7.7	2.0
Mg^{2+}	9.5		7.0–9.0	4.5

and Mg^{2+}–arginases are rather unsensitive to these reagents. Since only native arginase and Fe^{2+}–arginase were inhibited by phosphate, it was concluded that Fe^{2+} is the activator of native yeast arginase.

Kinetic properties of yeast arginase

More evidence for the stated identity of native and of Fe^{2+}–arginase was provided by determining the Michaelis constant (K_m) and the inhibition constant for ornithine (K_{Orn}), both for native and for Fe–arginase at various pH values, and in comparison with those of other metallo-arginases. Table IV shows that the kinetic constants of native and Fe^{2+}–arginase are equal, within the error of the determination, and that they are significantly different from those of Co^{2+}–, Ni^{2+}–, Mn^{2+}– and Mg^{2+}–arginases. Ornithine was found to be a competitive inhibitor of yeast arginase under all circumstances investigated.

DISCUSSION

In the present investigation, the properties of various metallo-complexes of yeast arginase prepared *in vitro* have been studied. Only the bivalent cations: Mn^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+} were shown to activate the enzyme *in vitro*. The activation of yeast arginase[6] by Cd^{2+} was not observed. Mn^{2+}, Co^{2+}, Fe^{2+} and Ni^{2+} have been reported as activators of liver arginase *in vitro*[3,4]; the activation of an arginase by Mg^{2+} has never been described before. The various metallo-complexes of yeast arginase were readily distinguished from each other by the shapes and the optima of their pH–activity curves and by their sensitivities to various reagents that bind bivalent cations.

Carefully prepared cell-free extracts of yeast, grown in an α-glycerophosphate medium, also showed an arginase activity if no bivalent cations had been added prior to or during the incubation with arginine. This arginase activity (native arginase), which showed the characteristics of Fe^{2+}–arginase, was considered to be identical with

the metallo–arginase that is active *in vivo*. This identity is highly probable because the activation of arginase by bivalent cations is a slow process[4]. If native arginase would be an artifact, caused *e.g.* by an exchange reaction between genuine natural arginase and traces of Fe^{2+} present during the preparation of the cell-free extract, one might expect that native arginase would exhibit the properties of a mixture of different metallo–arginases. The observed pH–activity curve and especially the almost complete inhibition by phosphate demonstrated that this is not true; native arginase showed the properties of homogeneous Fe^{2+}–arginase. Another argument in favour of the identity of native arginase and genuine natural arginase is the great specific activity of native arginase. At the optimal pH this specific activity hardly increased after activation of the cell-free extract with Fe^{2+} salts (Table I). Hence most of the arginase *in vivo* must have been present as Fe^{2+}–arginase.

The assumption that the native arginase had arisen by an exchange reaction with Fe^{2+} during the preparation of the cell-free extract, is contradicted by the results of experiments reported in a subsequent paper[19]. In that investigation, a study was made of the native arginase in cell-free extracts of yeast grown in Fe^{2+}-deficient media supplied with toxic concentrations of Mn^{2+} or Co^{2+} salts. The native arginase of such yeast samples showed the properties of Fe^{2+}–arginase, as usual. Since both Mn^{2+} and Co^{2+} were accumulated from the medium and hence appeared in the cell-free extracts in concentrations largely exceeding that of Fe^{2+}, it was concluded that those ions were not able to replace Fe^{2+} in native arginase, either *in vivo* or during the preparation of the cell-free extracts. The supposed origin of Fe^{2+}–arginase from an exchange reaction proceeding during the extraction of the yeast is highly improbable.

The pH–activity curves of native and of Fe^{2+}–arginase show their optima at pH 8.5–9.0. In addition to this optimum, the curve of native arginase shows a peak at pH 10.5 (Fig. 2g), which is also present in the curve of Fe^{2+}–arginase, provided that the excess of Fe^{2+} has been removed prior to the incubation with arginine (Fig. 2h); if not, the peak at pH 10.5 is masked (Fig. 2d). The cause and significance of the latter type of arginase activity are not clear. That it is due to the presence of a different arginase protein has not to be excluded. Its presence in preparations of Fe^{2+}–arginase suggests that it depends on Fe^{2+} as well. The predominance of the peak at pH 10.5 in the pH–activity curve of native arginase extracted from commercial baker's yeast (Fig. 2f) suggests that this arginase activity is more resistant to the action of phosphate. When the peak at pH 10.5 is really caused by a distinct arginase protein, the physiological role of the latter would be of minor importance because of its small specific activity and its extreme optimal pH. No more attention has been paid to this matter.

The identity of native and of Fe^{2+}–arginase is stated on account of their pH–activity curves, both showing optima at pH 8.5–9.0. The pH–activity curves of other metallo-arginases show optima at different pH values. Another argument in favour of the identity of native and of Fe^{2+}–arginase is their susceptibility to inhibition by phosphate. This phenomenon is not shown by any of the other metallo–arginases.

Determinations of some kinetic constants demonstrate the identity of Fe^{2+}–arginase and native arginase as well. The K_m and K_{Orn} values of native arginase at various pH values were the same as those of Fe^{2+}–arginase (Table IV). At pH 9.5 the K_m values of different metallo–arginases varied considerably. A decrease in K_m was observed in order of Mg^{2+}–, Mn^{2+}–, native and Fe^{2+}–, Co^{2+}– and Ni^{2+}–arginase. The inhibition by ornithine was always found to be of the purely competitive type. The

K_{Orn} was proportional to the K_m, being approx. 30–50% of the latter. The K_m of native and of Fe^{2+}–arginase varied with the pH. Close to the optimal pH, the K_m was minimal. The K_m–pH relationship of Fe^{2+}–arginase is related to the bell-shaped K_m–pH curve observed by ROHOLT AND GREENBERG[20] in their study on Mn^{2+}-activated liver arginase. These authors also report that near the optimal pH the K_m is minimal. A comparative study[21] has shown that the K_m at pH 9.5 of Mn^{2+}-activated liver arginase of various ureotelic animals is 10–20 mM, that of uricotelic animals 100–200 mM. The inhibition by ornithine of the latter is of the purely competitive type, that of the former is both competitive and noncompetitive[22]. MORA *et al.*[22] report that the arginase of *Neurospora crassa* is of the uricotelic type, with respect to the K_m, the inhibition by ornithine and a number of other characteristics. Although in the present research only the K_m and the K_{Orn} of yeast arginase have been determined, it is concluded that the yeast arginase is distinct from that of Neurospora. Because of its low K_m it shows more resemblance to that of ureotelic animals. It differs from the latter in its behaviour to ornithine, the inhibition being of the purely competitive type.

The activation of yeast arginase by Fe^{2+} *in vivo* is rather surprising because arginase is generally believed to be a Mn^{2+}-enzyme. Fe^{2+} has been considered hitherto as only a weak activator of liver arginase[10]; activation of yeast arginase by Fe^{2+} has been described to be impossible[6]. In those studies the arginase preparations had been activated in the presence of phosphate buffers, however. Furthermore the decrease of the specific activity of liver arginase, under conditions of Mn deficiency[22,12] is generally considered as an indication of the involvement of Mn^{2+} in the liver arginase reaction *in vivo*. Since native liver arginase is inhibited by KCN[9] and since its pH optimum is 7.7–9.0 (ref. 23), a role of Fe^{2+}, Co^{2+} or Ni^{2+} in liver arginase *in vivo* cannot be ruled out, however. It must be concluded that hitherto no definite answer has been given to the question as to which cation activates the liver arginase *in vivo*. A comparative study of native liver arginase and its metallo-complexes of known composition may give the solution to this problem.

ACKNOWLEDGMENTS

The author is greatly indebted to Professor E. G. Mulder for encouraging this work, to the Heineken's Brouwerij N.V., Rotterdam, and to the Landbouwhogeschoolfonds for research grants, and to Miss T. A. Wisserhof, Mr. G. K. Pesch and Mr. M. A. de Waard for their skillful assistance.

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