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

II. EXPERIMENTS ON THE REPLACEMENT OF FERROUS IONS IN NATIVE YEAST ARGINASE BY OTHER CATIONS *IN VIVO*

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SUMMARY

The possibility of the replacement of Fe^{2+} in yeast arginase (L-arginine ureohydrolase, EC 3.5.3.1) by Mn^{2+} or Co^{2+} *in vivo* has been studied. Neither the conditions of moderate Fe deficiency nor the addition of Mn^{2+} salts to the Fe-deficient culture medium exerted any effect on the properties and on the activity of native yeast arginase. The same applies to the addition of Co^{2+} salts; only when the selected concentration ratio of Co^{2+}: Fe^{2+} was extreme, were the two effects observed: primarily the appearance of unactivated apoenzyme and secondly that of some Co^{2+}–arginase. It is dubious, however, whether the latter had arisen *in vivo* or during the preparation of the cell-free extract.

INTRODUCTION

In many organisms, yeast included[1], the pathway of arginine breakdown is initiated by the action of arginase (L-arginine ureohydrolase, EC 3.5.3.1). Especially the liver arginase of ureotelic animals has been studied by numerous investigators. Liver arginase requires the presence of bivalent cations (Mn^{2+}, Co^{2+}, Fe^{2+} or Ni^{2+}) for its action[2]. The enzyme forms stable complexes with its activating cations[3]. These complexes can be prepared *in vitro* by reaction of apoarginase and metal ions. Liver arginase has been considered to be activated by Mn^{2+} *in vivo*[4,5].

The arginase of baker's yeast is similar to that of liver[6–8]. Its apoenzyme *in vitro* forms enzymically active complexes with the same cations as does liver arginase; in addition to these cations, Mg^{2+} is activating yeast arginase, though less effectively[8]. The metallo-complexes of yeast arginase are more readily inactivated by dialysis than those of liver arginase[6]. The various metallo-complexes of yeast arginase, which are readily prepared *in vitro*, are distinguished from each other by their pH–activity curves, Michaelis constants, specific activities and sensitivities to inhibiting reagents[8].

The metallo-complex active *in vivo* (native yeast arginase) can be isolated by careful extraction of the yeast[7,8]. A comparative study of native arginase and of me-

tallo–arginases of known composition has shown that the yeast arginase is activated *in vivo* by Fe^{2+} (refs. 7, 8).

The aim of the present study was to investigate the possibility of the replacement of Fe^{2+} in native yeast arginase by other cations *in vivo*. For this purpose the yeast was grown in Fe-deficient media supplied with Co^{2+} or Mn^{2+} salts. The nature of the activating cation in the native arginase was deduced from the shape of the pH–activity curve and from the sensitivity to inhibition by phosphate.

MATERIALS AND METHODS

The yeast strain was the same as previously used[1,7,8]. The Fe-deficient medium contained per l: 20 ml of DL-lactic acid, 10 mmoles L-arginine · HCl (N.B.C.), 40 mmoles L-glutamic acid, 2 mmoles disodium α-glycerophosphate, 400 mg of $MgCl_2 \cdot 6H_2O$, 100 mg of $CaCl_2 \cdot 2H_2O$ and 100 mg of NaCl. Vitamins were added as described previously[8]. All ingredients were of analytical standard (A.R.) quality and were dissolved in glass-distilled water. Fe was removed from the medium by adding 100 mg of 8-hydroxyquinoline. After adjusting the pH to 7.0 with KOH, the medium was boiled for 10 min, left overnight at room temperature and afterwards shaken with 100 ml of chloroform. After discarding the chloroform layer, the pH was brought to 3.5 with H_2SO_4 (A.R.). The residual 8-hydroxyquinoline was removed by repeated extraction with chloroform, the last traces of which were removed by boiling the medium for 10 min. Trace elements (B, Cu, I, Mn, Mo and Zn) were added as described previously[8].

The growth of the yeast in Fe-deficient media, prepared in the described way, was found to be 30–60% of that in identical media supplied with excess of $FeSO_4$ (2 mg Fe^{2+} per l). Cell yield of the Fe-deficient medium was determined as the dry weight of the culture (10 ml of medium in a 100-ml conical flask), after shaking for 48 h at 30°. This culture was inoculated with 0.1 ml of an end-log-phase culture in the same medium, supplied with 0.5 mg Fe^{2+} per l.

For arginase experiments the yeast was grown in Fe-deficient medium supplied with small amounts of $FeSO_4$ to increase the maximal cell yield to 80% of that in the same medium, supplied with 2 mg Fe^{2+} per l. Amounts of 100 ml of Fe-deficient medium were inoculated with 1 ml of an end-log-phase culture in the same medium supplied with 0.5 mg Fe^{2+} per l. The cultures were shaken in 1000-ml conical flasks at 30°. The yeast was harvested in the log phase, before the pH of the culture rose above 6.5.

The methods concerning the determinations of the specific activity of total arginase (as Mn^{2+}-activated enzyme, pH 9.5), of the pH–activity curve of native arginase and of the protein content of the cell-free extracts were described previously[8]. All specific activities were expressed as μmoles urea produced per h per mg protein. The inhibition of the native arginase by phosphate was studied by incubating the cell-free extract for 10 min at 20° in 100 mM potassium phosphate (pH 6.0), followed by determination of the specific activity of native arginase at pH 8.75.

RESULTS

The native arginase of Fe-deficient yeast

The possibility of replacing Fe^{2+} in native arginase by other cations was in-

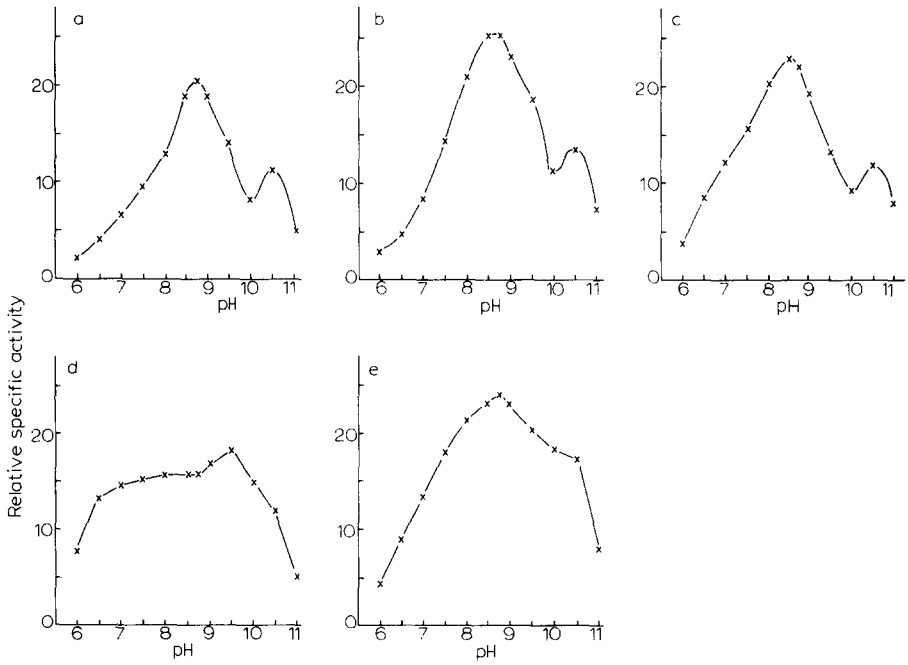

Fig. 1. pH–activity curves of native arginase. Ordinate: relative specific activity (percent of the specific activity of total arginase, determined as Mn^{2+}–arginase at pH 9.5). a, Fe-deficient; b, Fe-deficient, 600 mg Mn^{2+}/l; c, Fe-deficient, 10 mg Co^{2+}/l; d, Fe-deficient, 40 mg Co^{2+}/l; e, 2 mg Fe^{2+}/l and 40 mg Co^{2+}/l.

vestigated by studying the native arginase of yeast grown in a Fe-deficient medium supplied with a growth-retarding amount of a Mn^{2+} or a Co^{2+} salt. When replacement of Fe^{2+} in native arginase by Mn^{2+} or Co^{2+} would be possible *in vivo*, this phenomenon might be expected to occur in yeast grown in the above-mentioned medium because of the selected extreme concentration ratio of Mn^{2+} or Co^{2+} and Fe^{2+}. The other growth conditions in the lactate–arginine–glutamate medium were expected to favour the replacement of Fe^{2+} as well because of the following reasons:

(1) Lactate is metabolized by the yeast exclusively aerobically. Hence the available Fe^{2+} might be expected to be preferably incorporated in cytochromes.

(2) Arginine induces the synthesis of arginase in yeast[1]. This induction is hardly counteracted by the presence of glutamate in the medium[12]. Since glutamate is an excellent source of nitrogen, the activity of arginase in the yeast is no requirement for the growth in this medium. Even if Mn^{2+}– and Co^{2+}–arginase would be unable to degrade arginine *in vivo*, as efficiently as does Fe^{2+}–arginase, the growth of the yeast would not be retarded.

The pH–activity curve of the native arginase of yeast grown in the Fe-deficient medium (Fig. 1a) had the same shape as the curve observed earlier[8], in a study on the native arginase of yeast grown in media containing an excess of Fe (2 mg of $FeCl_3 \cdot 6H_2O$ per l). The inhibition of native yeast arginase by phosphate (Table I) demonstrated its identity with Fe^{2+}–arginase as well. The specific activity of native arginase at the

TABLE I

THE ARGINASE OF YEAST, GROWN IN MEDIA OF DIFFERENT MINERAL COMPOSITION

	Medium				
	Fe-deficient	*Fe-deficient 600 mg Mn^{2+}/l*	*Fe-deficient 10 mg Co^{2+}/l*	*Fe-deficient 40 mg Co^{2+}/l*	*Fe^{2+} 2 mg/l 40 mg Co^{2+}/l*
Growth period (h)	24	48	40	48	36
Yield (g yeast per 100 ml)	1.0	1.3	1.4	1.1	1.2
Specific activity of total arginase (units/mg protein, as Mn^{2+}–enzyme at pH 9.5)	31	30	25.5	32	28
Specific activity of native arginase (units/mg protein, at pH 8.75)	6.3	7.5	5.1	4.3	6.9
Inhibition (%) of native arginase by phosphate	85	83	80	59	70
Relative specific activity of native arginase (% of the specific activity of total arginase)	21	25	20	13.5	24.5

optimal pH 8.75 (Table I) was 25% of that of total arginase (determined at pH 9.5 after heating the cell-free extract with $MnCl_2$). Both specific activities were the same as those usually observed in yeast grown in similar media with an excess of Fe (ref. 8). These results demonstrate that the arginase of Fe-deficient yeast *in vivo* was activated completely and exclusively by Fe^{2+}. The conditions of moderate Fe deficiency obviously did not affect the total amount of arginase in the yeast. Under these conditions all native arginase of the yeast was present as Fe^{2+}–arginase; there was no evidence for the presence of unactivated apoarginase.

The effect of Mn^{2+} on yeast arginase in vivo

The yeast was grown in the Fe-deficient medium to which 600 mg of Mn^{2+} per l (as $MnSO_4 \cdot H_2O$, A.R.) had been added. As shown in Table I, the growth of the yeast was much retarded by this Mn^{2+} concentration. The native arginase of yeast, grown in the Mn^{2+} medium, was inhibited by phosphate (Table I). Its pH–activity curve (Fig. 1b) was found to be identical with that of native arginase of yeast, grown in the Fe-deficient medium (Fig. 1a). Furthermore, its specific activity at pH 8.75 was 25% of that of total arginase. These results clearly demonstrate that neither the total amount of arginase in the cell nor the specific activity of the native arginase nor the properties of the latter were influenced by the addition of Mn^{2+} to the culture medium. No evidence was presented for the incorporation of Mn^{2+} in yeast arginase *in vivo*.

It must be emphasized that the results as described above were only obtained when the yeast was harvested before the pH of the culture had risen to 7.0. The native arginase of yeast harvested from old cultures often showed the properties of Mn^{2+}–arginase. In such cultures, in which the pH may have been as high as 8.5 for several hours, the yeast was dying and its cell membranes became more permeable, as was readily demonstrated by adding methylene blue. It is evident that the observed Mn^{2+}–arginase originated from a secondary reaction, occurring in old cultures. Hence it must be considered as an artifact. The presence of Mn^{2+}–arginase as native yeast arginase

has never been demonstrated in cultures with a pH below 7.0, even when the Mn^{2+} concentration was as high as 1200 mg per l.

The effect of Co^{2+} on yeast arginase in vivo

Fig. 1c shows the pH–activity curve of native arginase, extracted from Fe-deficient yeast grown in the presence of 10 mg of Co^{2+} per l (as $CoCl_2 \cdot 6H_2O$, A.R.). The shape of the curve and the other properties of the native arginase (Table I) show that this Co^{2+} concentration is without effect on the nature and the specific activity of yeast arginase. Hence it is concluded that the arginase of Fe-deficient yeast, grown in the presence of a slightly growth-retarding Co^{2+} concentration, is activated *in vivo* completely and exclusively by Fe^{2+}.

When the yeast was grown in the Fe-deficient medium in the presence of 40 mg of Co^{2+} per l, a native arginase of other properties was observed. The pH–activity curve of this native arginase (Fig. 1d) resembles the curve of Co^{2+}–arginase[8] in showing an optimum at pH 9.5 and a broad shoulder at pH 7.0–9.0. The specific activity of Co^{2+}–arginase at pH 7.0–9.0 is about $^2/_3$ of that at the optimal pH (ref. 8); in the curve of Fig. 1d the shoulder is relatively more active, however. Hence the native arginase of Fe-deficient yeast, grown in the presence of 40 mg of Co^{2+} per l, is considered as a mixture of Co^{2+}– and Fe^{2+}–arginases, as is shown by its susceptibility to phosphate inhibition as well (Table I). This native arginases was inhibited by phosphate much less strongly than normal native yeast arginase. This is an indication for the involvement of a cation, distinct from Fe^{2+}, because phosphate is inhibiting only Fe^{2+}–arginase and not the other metallo–arginases[8].

The total amount of arginase in Fe-deficient yeast grown in the presence of 40 mg of Co^{2+} per l was the same as that in yeast grown in normal Fe-deficient medium (Table I). The specific activity of native arginase (pH 8.75) was only 13.5% of that of the total arginase activity, instead of 20–25% as usual (Table I). Since the specific activities of Fe^{2+}– and Co^{2+}–arginases at pH 8.75 are about the same[8], it is concluded that the native arginase of Fe-deficient yeast grown in the presence of 40 mg of Co^{2+} per l comprised, next to Co^{2+}– and Fe^{2+}–arginase a large amount (35–50% of the total) of unactivated apoenzyme. This suggests that the effect of Co^{2+} on yeast arginase *in vivo* is primarily a decreased incorporation of Fe in the enzyme. This conclusion is in accordance with that of HEALY *et al.*[9], who state that the main phenomenon caused by Co^{2+} intoxication in Neurospora is a severe Fe deficiency, resulting in decreased activities of many Fe-enzymes. This is probably not caused by an inhibition of the Fe^{2+} assimilation from the medium but by a competition of Co^{2+} and Fe^{2+} in the system that accomplishes the incorporation of Fe^{2+} in enzymes[10].

The addition of 40 mg of Co^{2+} per l culture medium, containing an excess of Fe^{2+} (2 mg/l) did not influence the specific activity and the susceptibility to phosphate inhibition of native yeast arginase (Table I). The pH–activity curve of this native yeast arginase (Fig. 1e) has its optimum at pH 8.75. It differs from the normal native yeast arginase in showing a greater activity at unfavorable pH values. It closely resembles the curve of Fe^{2+}–arginase, determined in the presence of excess Fe^{2+} (ref. 8). Possibly, the addition of 40 mg of Co^{2+} per l of Fe^{2+}-sufficient medium results in the formation of a more stable native Fe^{2+}–arginase complex.

Since the addition of large amounts of Co^{2+} to a Fe-deficient medium caused a reduced native arginase activity, the toxicity of Co^{2+} for yeast might be more severe

TABLE II

THE TOXICITY OF Co^{2+} FOR YEAST IN Fe-DEFICIENT LACTATE–ARGININE MEDIA, WITH AND WITHOUT GLUTAMATE

The growth was determined as the dry weight of the culture (expressed as mg/10 ml).

Additions	*Concn. mg/l*	*Glutamate concentration*	
		0	*40 mM*
Fe^{2+}	2	25.8	36.0
	0	21.6	30.0
Co^{2+}	10	15.7	25.2
	20	11.1	25.9
	30	9.5	18.9
	40	3.2	15.2

when the organism is grown with arginine as the sole nitrogen source than in the presence of an additional nitrogen source. In order to investigate this hypothesis, the yeast was grown with different Co^{2+} concentrations in Fe-deficient lactate–arginine media, with and without L-glutamate. The growth was measured by determining the dry weight of 10 ml of the culture after growing the yeast at 30° for $2\frac{1}{2}$ days in the usual way. As shown in Table II, Co^{2+} was significantly more toxic in the lactate–arginine medium than it was in the medium containing an additional amount of glutamate.

DISCUSSION

The influence of some variations in the mineral composition of the culture medium on the properties of native yeast arginase has been investigated. A similar study has recently been undertaken by CURDEL[11], who studied the D-lactate dehydrogenase (D-lactate:NAD^+ oxidoreductase, EC 1.1.1.28) of baker's yeast. This is a Zn^{2+}-enzyme in which the Zn^{2+} is replaceable by Co^{2+} or Mn^{2+} *in vitro* without loss of enzymic activity. When Zn^{2+} is omitted from the medium, the yeast produces unactivated apoenzyme. If $CoCl_2$ is added to the Zn-deficient medium, a Co^{2+}-enzyme is produced which, however, differs from the Co^{2+}-enzyme prepared *in vitro*.

By growing *Saccharomyces cerevisiae* under conditions of Fe deficiency and of intoxication by Mn^{2+} or Cc^{2+} salts, the possibility of incorporating these cations in the yeast arginase *in vivo* was studied. The nature of the activating cation of yeast arginase *in vivo* was deduced from the properties of the native arginase, especially the shape of the pH–activity curve and the susceptibility to phosphate inhibition were shown to be of great use for this purpose[8].

Besides a replacement of the Fe^{2+} in native yeast arginase, the conditions of Fe deficiency might change the amount of arginase in the cell. The appearance of unactivated apoarginase belonged to the possibilities as well. These phenomena were studied by the determination of the specific activities of native arginase and of total arginase (as Mn^{2+}–arginase, after heating the cell-free extracts with $MnCl_2$).

From the results obtained, it was concluded that the conditions of moderate Fe deficiency exerted no effect on the properties and the activity of the native arginase. Neither did the addition of growth-retarding amounts of $MnSO_4$ to the Fe-

deficient culture medium, provided that the yeast culture was harvested before the end of the log phase. In old cultures secondary exchange reactions may occur.

The effect to Co^{2+} on yeast arginase *in vivo* was more complex than that of Mn^{2+}. The native arginase retained the properties of Fe–arginase when 40 mg of Co^{2+} had been added per l of Fe^{2+}-sufficient medium or when a slightly growth-retarding amount of Co^{2+} (up to 10 mg per l) is added to the Fe-deficient medium. The specific activity of the arginase was not affected under these conditions. However, when more Co^{2+} (40 mg per l) had been added to the Fe-deficient medium, the native arginase had changed, both in properties and in specific activity; the total amount of arginase had not changed. Under these conditions, 35–50% of the total arginase was present as the apoenzyme; the rest showed the properties of a mixture of Co^{2+}– and Fe^{2+}–arginases, with respect to both the pH–activity curve and the inhibition by phosphate. Consistent with this finding of decreased native arginase activity was the observation that Co^{2+} is more toxic for baker's yeast when the organism is growing in a Fe-deficient lactate medium with arginine as the sole source of nitrogen than when glutamate is added to this medium at the same time.

In Neurospora, Co^{2+} intoxication brings about a severe Fe deficiency[9], probably caused by competition of Co^{2+} and Fe^{2+} in the system that achieves the incorporation of Fe^{2+} in enzymes[10]. This results in a considerable decrease in the activity of many Fe-enzymes[9]. The reduced native yeast arginase activity, caused by Co^{2+} intoxication of Fe-deficient yeast, is in agreement with the results of the Neurospora studies. It strongly suggests that the effect of Co^{2+} on yeast arginase *in vivo* is primarily an inhibition of the incorporation of Fe^{2+} in the apoarginase. Secondly, the apoarginase partially reacts with Co^{2+}. Since Co^{2+}–arginase is more resistant to heat inactivation and to storage at 0° than is Fe^{2+}–arginase (unpublished results), this activation by Co^{2+} apparently is only partial. Would it be complete, a native arginase of greater specific activity would be expected (the specific activities of Co^{2+}– and of Fe^{2+}–arginases are about the same at the pH concerned). The reaction of apoarginase and Co^{2+} may proceed directly or may be catalyzed by the same system that accomplishes the incorporation of Fe^{2+} in the arginase. This system has apparently a strong preference for Fe^{2+}, because the occurrence of Co^{2+}–arginase *in vivo* was observed only under extreme conditions. A direct reaction of apoarginase and Co^{2+}, either *in vivo* or during the preparation of the cell-free extracts, is more probable, however, because Co^{2+} was accumulated by the yeast (the cell-free extracts were pinkish).

The results of the present investigation confirm the conclusions of the previous report[8], in which it is stated that Fe^{2+} is the cofactor of yeast arginase *in vivo*.

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