EVIDENCE THAT YEAST ACETYLORNITHINASE IS A CARBOXYPEPTIDASE

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1. Introduction

Ornithine is an obligatory intermediate in de novo arginine biosynthesis. In *E. coli,* an acetylornithinase (EC 3.5.1.16) produces it by deacylating N-α-acetylornithine* [1,2]. In other bacteria [2] and all eucaryotes tested [3,4], an ornithine acetyltransferase (EC 2.3.1.-) transfers the acetyl group of *N*-α-acetylornithine to glutamate, hereby establishing an energetically favourable 'acetylornithine cycle'. Although the latter organisms require this enzyme for ornithine biosynthesis [3], most of them also possess a metal-activated 'acetylornithinase' activity [2–4]. De Deken suggested that, in yeast, the acetylornithinase involved was an enzyme the functional substrate of which was unknown or a relic left over after evolution from the type of ornithine biosynthesis observed in *E. coli* towards the acetylornithine cycle [3].

Because an 'acetylornithinase' activity is a possible threat to the efficiency of the acetylornithine cycle, its presence may be regarded as paradoxical unless the enzyme involved is shown to bear no relationship to its *E. coli* counterpart. We report evidence that the 'acetylornithinase' activity present in yeast is no artefactual side-reaction of ornithine acetyltransferase, that the enzyme involved is different from *E. coli* acetylornithinase and that it shows sufficient relatedness with baker's yeast peptidase α (5) and pancreatic carboxypeptidase B (EC 3.4.2.2) [6] to be regarded as a carboxypeptidase.

* *Abbreviations:* acetylornithine = *N*-α-acetyl-L-ornithine; acetylglutamate = *N*-α-acetyl-L-glutamate; cbzglycine leucine = *N*-α-carbobenzoxy-L-glycine-L-leucine; hippurylysine = *N*-α-benzoyl-L-glycine-L-lysine; benzoylarginine = *N*-α-benzoyl-L-arginine; hippurate = *N*-α-benzoyl-L-glycine; DFP = diisopropyl phosphfluoridate; EDTA = ethylenediaminotetra acetage; *p*MB = *para* chloro mercury benzoate.

2. Materials and methods

Saccharomyces cerevisiae strain 1278b or derivatives were grown and harvested as previously described [7]. The supernatant of centrifuged cell-free extract was dialyzed on Sephadex G-25 equilibrated with potassium phosphate buffer M/50 at pH 7.2. Acetylornithinase activity was assayed [1] in 0.1 M potassium phosphate buffer at pH 7.2. $CoCl_2$ concentration was 2×10^{-4} M and acetylornithine 2×10^{-2} M. Incubation were for one hour at 30°C. The ornithine produced was measured colorimetrically [1]. Specific activity in crude extracts varied between 0.03 and 0.08 μM/hr/mg prot. Higher activities (0.20 μM/hr/mg prot.) were obtained from cells harvested in the late exponential phase. Ornithine acetyltransferase was assayed by measuring the exchange reaction taking place between acetylornithine (2×10^{-3} M) and ornithine (8×10^{-3} M), in 0.1 M phosphate buffer pH 7.2 and a total volume of 0.3 mL. The reaction was terminated after 120 min at 30°C by addition of 1.2 mL ice-cold water. Columns containing 1 mL of Dowex 50 W × 8 equilibrated with M/50 potassium phosphate buffer pH 6.8 were quickly loaded with the samples. The radioactive acetylornithine formed during the reaction was eluted with 3 ml of the same buffer. After evaporation, the residue was dissolved in 0.5 ml water and its radioactivity

was counted in a mixture of 60% dioxane cocktail (100 g naphtalene and 5 g 2,5-diphenyloxydazole per liter) and 40% Triton X 100. Protein was determined according to Lowry et al. [8] with bovine serum albumin as a standard. Specific activity is expressed as μM/hr/mg protein.

3. Results and discussion

The acetylornithinase activity does not result from metal-activated hydrolysis of an acetyl-enzyme intermediate formed on ornithine acetyltransferase. First, such an acetyl-enzyme intermediate occurs since we can observe an exchange reaction between acetylornithine and ornithine, in the absence of glutamate (table 1). Second, this exchange reaction, which is indicative of a ping-pong mechanism, is absent from the two ornithine acetyltransferase-less mutants available while the acetylornithinase activity is still present (table 1); therefore these activities are due to different enzymes. This evidence suggests that the acetylornithine hydrolytic activity found to accompany *Chlamydomonas* ornithine acetyltransferase during partial purification is not due to the latter enzyme [9].

E. coli acetylornithinase is activated by Co^{2+} [1]; yeast 'acetylornithinase' activity is activated by Zn acetate and $CoCl_2$ (optimum concentration for both between 10^{-4} and 2×10^{-4} M in crude extracts) but is inhibited at higher concentrations. EDTA [3] and 8-hydroxyquinoline inhibit yeast 'acetylornithinase' almost completely; addition of Zn^{2+} or Co^{2+} to an EDTA inhibited extract restores the activity. Peptidase α exhibits a closely similar behaviour [5].

E. coli acetylornithinase is activated by glutathione [1]; the yeast activity, as well as peptidase α [5] is slightly inhibited by glutathione (27.5% inhibition in 0.1 M phosphate buffer and 5×10^{-4} M glutathione without added metal). Glutathione acts by virtue of its metal complexing properties since the enzyme may be reactivated by increasing the concentration of Co^{2+} ions.

Yeast 'acetylornithinase' is sensitive to PMB; as much as 78% inactivation is observed after preincubating 0.8 mg protein at 30°C in the presence of 10^{-3} M PMB. No effect of DFP, known for alkylating active serines, could be demonstrated. This behaviour of yeast 'acetylornithinase' towards PMB and DPF is similar to that of peptidase α [5].

The saturation curve of yeast 'acetylornithinase' reveals a reproducible shoulder (fig. 1) at 8 mM acetylornithine, whereafter the activity increases linearly with substrate concentration. This pattern does not result from an increase of ionic strength (as tested by addition of NaCl) nor does it seem to reflect the presence of two enzymes acting on acetylornithine. Indeed, the determination of the pH optimum reveals only one peak at 6.8 (the pH optimum of peptidase α acting on CBZ–gly–leu is 6.2 [5]).

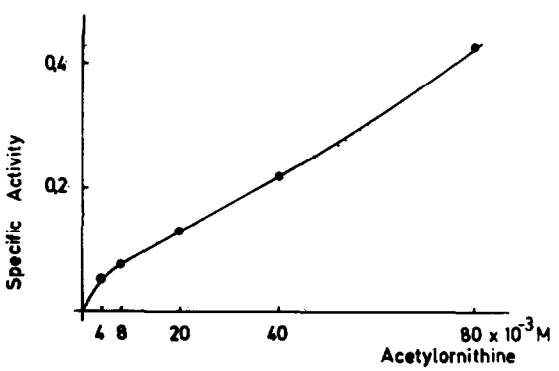

Fig. 1. Saturation curve for yeast acetylornithinase.

Table 1
Ornithine–acetylornithine exchange and acetylornithinase activity in extracts of wild-type (Σ1278b) and ornithine acetyltransferase-less mutants (MG649 and MG810)

	Σ1278b	MG649	MG810
Ornithine acetyltransferase* Δ cpm	10.251	85	250
Specific activity	0.176	–	–
Acetylornithinase activity. Specific activity*	0.088	0.266	0.142

* Acetylornithine was 8×10^{-2} M.

Table 2
Activation and inhibition of yeast acetylornithinase activity

		Relative activities (%)
Activation:		
Control:	acetylornithine 10^{-2} M	100
	+ ornithine 5×10^{-5} M	180
	+ ornithine 10^{-4} M	210
	acetylornithine 2×10^{-2} M	205

Activity calculated from non-incubated controls.

Inhibition:		
Control:	acetylornithine 2×10^{-2} M	100
	+ glutamate 5×10^{-3} M	119
	+ *N*-α-acetyl-L-glutamate 10^{-2} M	107
	+ benzoyl-L-glycine 10^{-2} M	70
	+ bippuryllysine 10^{-2} M	0
	+ *N*-α-benzoyl-L-arginine 10^{-2} M	0
	+ benzoyl arginine 10^{-3} M	0
	+ benzoyl arginine 6×10^{-4} M	17
	+ benzoyl arginine 2×10^{-4} M	30

Activity calculated from controls in which the substrate was added after termination of the reaction.

Moreover, we were unable to change the relative contributions of the activities reflected by the two parts of the curve by: (a) addition or not of arginine to the ammonium basal medium (which means that the activity is not influenced by exogenous arginine); (b) assays with or without Co^{2+}; (c) assays in either of the following argininеless mutants: arg2 and 11 (which affects neither of the known arginine enzymes), arg7 (ornithine acetyltransferase-less), arg8 (acetylornithine aminotransferase-less, EC 2.6.1.11). The shape of the curve is at least partly accounted for by product activation: 5×10^{-5} M ornithine activates acetylornithine hydrolysis appreciably (table 2). Similar properties are known for pancreatic carboxypeptidase A (EC 3.4.2.1) [10].

The several similarities noted between yeast 'acetylornithinase', peptidase α and carboxypeptidase B (inhibition by excess of metal, inhibition by EDTA and subsequent reactivation by Zn^{2+} or Co^{2+}, slight inhibition by glutathione, inactivation by *p*MB, no effect of DFP, neutral pH optimum) as well as the analogy between carboxypeptidase activity and the hydrolysis of the peptide bond located next to the free carboxyl group of the *N*-α-acetylornithine, suggest that yeast 'acetylornithinase' is a carboxypeptidase. More decisive information about the stereospecificity requirements of the active site was obtained by studying the effect on acetylornithine hydrolysis of substrates and competitive inhibitors of known carboxypeptidases bearing structural resemblance to acetylornithine. As potential inhibitors of the acetylornithinase activity we took hippuryllysine and benzoylarginine, respectively substrate (K_M 2×10^{-4} M) and competitive inhibitor (K_i 4×10^{-5} M) of pancreatic carboxypeptidase B, which specifically splits off basic amino acids [6]. Besides, we tested acetylglutamate, where the *N*-α-acetyl group is present but charged negatively on its γ-carboxyl group and glutamate because of its metabolical relationship with acetylornithine. The results are given in table 2. Neither glutamate nor acetylglutamate have any influence on acetylornithinase activity over the whole range of the saturation curve (not shown). Hippurate which does not inhibit carboxypeptidase B [6] gives only a slight inhibition. We found significant inhibition by both hippuryllysine [also substrate for peptidase α [5]] and benzoylarginine: the latter's effect is comparable to that observed on carboxypeptides B.

The unrelatedness between yeast 'acetylornithinase' and the *E. coli* enzyme is further attested by the fact that 10^{-2} M hippuryllysine or hippurate inhibit the *E. coli* enzyme only 10%.

Our results therefore point to yeast 'acetylornithinase' as being a carboxypeptidase, as yet unidentified but very similar to yeast peptidase α and in some respect to pancreatic carboxypeptidase B. For the latter enzyme, however, we found acetylornithine to be a poor substrate. This difference reflects the structural requirements imposed on substrates in order to become hydrolyzed. A second peptide bond is needed for rapid hydrolysis by carboxypeptidase B; this requirement may be less pronounced in the case of the acetylornithinase activity of yeast.

It is possible that other 'acetylornithinases' detected in organisms endowed with an acetylornithine cycle are also the expression of a carboxypeptidase activity on acetyl derivatives of the susceptible amino acid. This hypothesis could account for the enzymic deacetylation of acetylglutamate in *Neurospora*

crassa, for which no metabolic explanation could be found [11].

The fact that the amount of carboxypeptidase acting on acetylornithine increases 3–6 times in the late exponential phase suggests that it plays a role, in concert with endopeptidases, in the provision of amino acids during unbalanced growth.

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