

Equilibrium between active and inactive forms of rat liver ornithine decarboxylase mediated by L-ornithine and salts

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The mechanisms controlling the activity of ornithine decarboxylase (ODC) are complex and only partly understood. This study shows that ODC can exist as two different aggregation states, that differ in catalytic activity, the dimeric form being active and the monomeric form inactive. While L-ornithine shifts the association-dissociation equilibrium to the dimeric form, salts produce an opposite effect leading to subunit dissociation. α -DFMO, an enzyme-activated irreversible inhibitor of ODC, does not react with the monomeric form and therefore the influence of substrate and salts on the aggregation equilibrium must be taken into account in titration experiments with α -DFMO of the total amount of ODC in tissue preparations.

Ornithine decarboxylase Polyamine Association equilibrium Rat liver Ionic strength

1. INTRODUCTION

Ornithine decarboxylase (L-ornithine carboxylase, EC 4.1.1.17) is a pyridoxal 5'-phosphate requiring-enzyme which catalyses the formation of putrescine from L-ornithine, the first and rate-limiting step in the biosynthesis of polyamines [1–3]. This enzyme, which is considered to play an important role in the control of cell growth, proliferation and differentiation [4], exhibits an extremely short half-life [5] and its activity is affected by a great variety of agents. To account for the rapid changes in its activity, several *in vivo* regulatory mechanisms have been reported, such as post-translational modifications [6,7] and regulatory proteins [8,9]. The existence of cryptic or latent forms of the enzyme has also been suggested [10]. In most cases, the changes in activity are correlated with the amount of enzymatic protein, as shown by radioimmunoassay titration [11] or by means of radiolabelled α -DFMO, a suicide substrate of ODC [11,12].

Abbreviations: ODC, ornithine decarboxylase; α -DFMO, α -difluoromethylornithine

Although the activity of ODC is probably not mainly regulated by any low molecular mass effectors at physiological level, *in vitro* experiments have shown that ODC activity is also affected by a variety of chemical agents such as salts [13,14], polyanions [15] or phospholipids [16]. Moreover, the ionic strength influences the aggregation state of the enzyme, since different forms have been observed in gel-filtration experiments to depend on the ionic conditions [13,17]. This report shows that the enzyme occurs basically as an equilibrium between a monomeric inactive form and a dimeric active form. The aggregation state of ODC can be shifted in opposite directions by salts and L-ornithine. The inactivity of the monomer, shown here, and the reversibility of the equilibrium, are very important points to be taken into account when irreversible inactivation or affinity labelling by suicide substrates, such as α -DFMO, are carried out.

2. EXPERIMENTAL

DL-[1-¹⁴C]Ornithine (57 mCi/mmol) was obtained from Radiochemical Centre (Amersham,

England). Sephadex G-200 and DEAE-Sephadex A-50 were purchased from Pharmacia (Uppsala). α -DFMO was a generous gift from Merrell International (Strasbourg, France). Unlabelled L-ornithine, pyridoxal 5'-phosphate, dithiothreitol, EDTA, thioacetamide, trypsinogen, bovine serum albumin and catalase were purchased from Sigma (London, England). All other chemicals and reagents were of analytical grade.

ODC was obtained from liver cytosol of adult Wistar rats treated with thioacetamide (150 mg/kg) 24 h before killing. The enzyme was partially purified by ammonium sulphate fractionation, ion-exchange chromatography on DEAE-Sephadex and gel permeation chromatography on Sephadex G-200 [13].

The activity of ODC was assayed by measuring the rate of $^{14}\text{CO}_2$ evolved from L-[1- ^{14}C]ornithine. The assay mixture contained 25 mM sodium phosphate buffer (pH 7.2), 2 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM pyridoxal phosphate (buffer A), variable amount of L-ornithine and 50 μl enzyme solution in a total volume of 200 μl . The reaction was carried out at 37°C in tubes sealed with rubber stoppers suspending paper filter discs containing hyamine hydroxide. After 1 h the reaction was stopped by the addition of 0.5 ml of 3 M citric acid. After 45 min, paper discs were removed and counted on toluene based scintillation mixture. Activity was expressed in nmol CO_2 released/h.

3. RESULTS

The activity of ODC decreases considerably by increasing salt concentration in the assay mixture. This effect was not dependent on the nature of the salt, although slight variations were observed (fig.1) and sodium chloride was used as a standard for increasing the ionic strength in the enzyme media. The double-reciprocal plot of fig.2 shows that the apparent K_m of the enzyme for L-ornithine increases with NaCl concentration, but V_{max} remains constant. This indicates that NaCl acts as a competitive inhibitor of ODC. However, NaCl cannot be considered as a classical competitive inhibitor, but rather that NaCl and L-ornithine exert opposite effects on ODC activity. The fact reported by Kitani and Fujisawa [13], that salts produce subunit dissociation of the enzyme, and

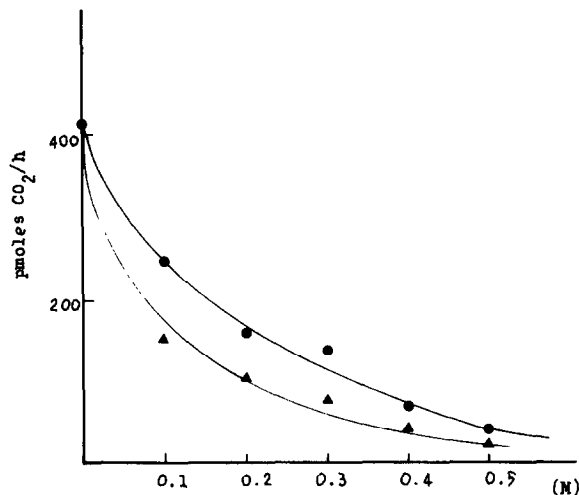


Fig.1. Inhibition of ODC activity by salt concentration. The assay medium was 15.6 μM [1- ^{14}C]ornithine and variable concentration (M) of salt in buffer A. (●) NaCl, (▲) KNO_3 .

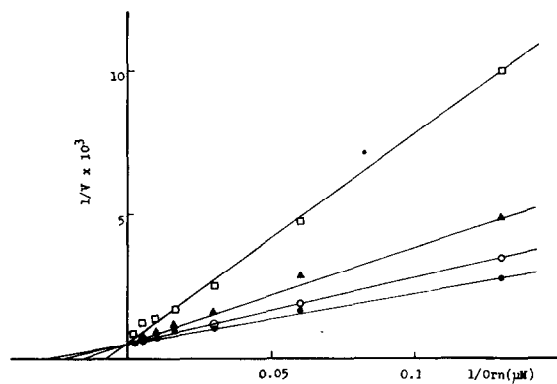
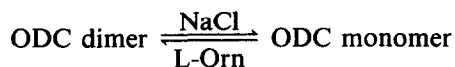


Fig.2. Lineweaver-Burk plot of the inhibition of ODC activity by NaCl. The assay medium was 15.6 μM [1- ^{14}C]ornithine and variable concentrations of unlabelled L-ornithine and NaCl in buffer A. (●) Control. NaCl: 0.05 M (○), 0.1 M (▲) and 0.2 M (□).

the above results similar to those reported by these authors, can be explained assuming that ODC exists as an equilibrium of dimeric and monomeric forms, and that NaCl enhances the dissociation while L-ornithine decreases the effect produced by the salt.

To assess the above mentioned hypothesis, represented by the scheme,



the molecular mass of the enzyme was determined by gel permeation chromatography on a calibrated column of Sephadex G-200, under 3 different sets of conditions: 1, in the absence of NaCl and L-ornithine; 2, in the presence of 0.25 M NaCl and the absence of L-ornithine, and 3, in the presence of 0.25 M NaCl and 0.1 mM L-ornithine. Fig.3 shows the molecular mass determined for ODC under the above mentioned conditions. In the absence of NaCl and L-ornithine the apparent M_r was approx. 100000, but in the presence of 0.25 M NaCl the M_r was 55000. When the column was equilibrated and eluted with buffer containing both 0.25 M NaCl and 0.1 mM L-ornithine the elution profile of enzyme activity exhibited a broad peak, but the fraction with highest activity again indicated an M_r of approx. 100000.

The reversibility of the equilibrium was verified since samples of enzyme incubated with high NaCl concentrations showed the same activity after dilution of salt concentration in the assay mixture as samples not preincubated.

The parallel inhibitory and disaggregating effects of NaCl clearly show that the monomer is less active than the dimer. In turn, chromatography shows that at NaCl concentration about 0.25 M or higher the main form of ODC is the monomer. The

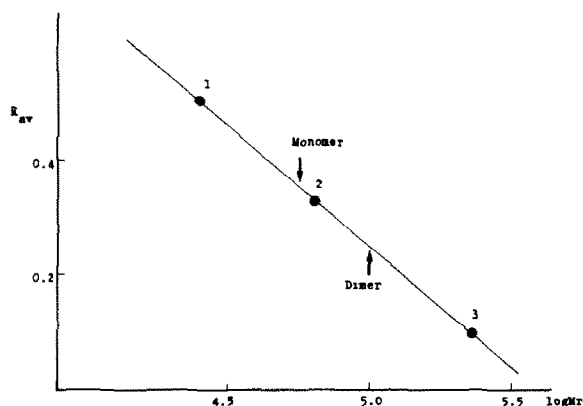


Fig.3. Determination of M_r of ODC by gel-permeation in Sephadex G-200. The column (1.2×28 cm) was equilibrated with 3 different conditions as detailed in the text. Standard calibration proteins: 1, trypsinogen; 2, bovine serum albumin; 3, catalase. Monomer: buffer A containing 0.25 M NaCl (condition 2). Dimer: buffer A (condition 1). Buffer A containing 0.25 M NaCl and 0.1 mM L-ornithine (condition 3).

Table 1

Pre-treatment	Activity (nmol $\text{CO}_2 \cdot \text{h}^{-1}$)	% inactivation
(a) None	1.49	—
(b) 1 M NaCl	1.48	—
(c) 1 μM α -DFMO	0.36	76
(d) 1 M NaCl plus 1 μM α -DFMO	1.50	—

Samples of 100 μl of enzyme were incubated for 5 h in buffer A (control, a), 1 M NaCl (b), 1 μM α -DFMO (c) or 1 M NaCl and 1 μM α -DFMO (d). Activity was determined by adding 25 μl preincubated enzyme to the assay media containing 31 μM L-[1- ^{14}C]ornithine and 69 μM L-ornithine. All assays were normalized to reach final concentration of α -DFMO and NaCl of 0.2 μM and 0.2 M, respectively

activity of this form cannot be clearly determined since the presence of L-ornithine enhances the formation of the more active dimeric form. To test the activity of the monomer we studied the inactivation of ODC by means of the suicide substrate analogue α -DFMO. It follows that conditions decreasing the enzyme activity will also prevent the inactivation of this enzyme. Samples of enzyme were preincubated under different conditions: 1 μM α -DFMO; 1 μM α -DFMO plus 1 M NaCl, and in the absence of both. After 5 h of preincubation, the activity was determined from aliquots in the presence of L-ornithine, being all activity measurements normalized to the activity of control reaction containing identical concentration of α -DFMO and NaCl. The concentration of these compounds in the assay mixture was 0.2 μM and 0.2 M, respectively. Table 1 shows that preincubation with 1 M NaCl does not affect, in a time dependent way, the activity of ODC. The preincubation of ODC with 1 μM α -DFMO considerably decreased the enzyme activity. However, when the enzyme was preincubated with 1 μM α -DFMO in the presence of 1 M NaCl, no inactivation, at all, could be detected. Therefore, ODC in the presence of 1 M NaCl and the absence of the substrate, L-ornithine, is completely inactive and consequently it cannot be inactivated by the suicide substrate, since this inactivation depends on the presence of active enzyme.

4. DISCUSSION

The mechanisms controlling ornithine decarboxylase activity seem to be complex and partially understood. Several modified forms of the enzyme have been reported, including phosphorylated [7] and transamidated forms [6], and the existence of an equilibrium between active and cryptic forms of ODC has also been suggested [10]. Moreover, different investigators have observed multiple forms of ODC after fractionation by gel permeation chromatography [13,17], but the physiological significance of these forms remains unknown.

Salts are reported to be strong effectors of ODC activity both in vitro [13,14] and in vivo conditions [20,21]. Our results show that ODC activity is greatly dependent on ionic strength, and that salts affect the aggregation state of the enzyme. Kitani and Fujisawa [13] showed that ionic strength increases the K_m for L-ornithine and causes subunit dissociation of the rat liver enzyme. ODC from *Phycomyces* is also inhibited by salts [14] and an equilibrium between dimeric and monomeric forms affected by salts and polyamines has been described for ODC of *Physarum* [17]. However, no correlation between the changes in activity and aggregation state has been clearly stated. The results presented in this paper indicate that the enzyme can exist as two different states, dimeric or monomeric. Two important factors, L-ornithine and ionic strength, have an opposite effect, the latter favouring the dissociation to the monomeric form and the substrate preventing the dissociation. So, NaCl acts as a competitive inhibitor.

This mechanism explains the experimental data only if the monomeric form is inactive. This hypothesis cannot be simply tested by measuring ODC activity since the presence of substrate shifts the equilibrium to the dimeric active form. To elude this difficulty, the activity was tested in the absence of substrate by using a potent suicide inhibitor, α -DFMO, that acts only on the active enzymic form. The absence of inactivation of ODC at high NaCl concentration in the presence of α -DFMO demonstrates that ODC monomer is devoided of catalytic activity.

The relationship between activity and quaternary structure of enzymes as well as the participation of substrates and products in the association-dissociation equilibria has been reported [18,19].

Although this relation could be significant for the regulation of intracellular activity in some enzymes, the implication of this mechanism in the control of intracellular ODC activity cannot be evaluated prior. Experiments using different types of cell culture have shown that ODC activity is greatly affected by ionic content of the media [20,21]. If such equilibrium occurs under physiological conditions, its relative importance in the regulation of ODC activity could be related to the existence of two forms of different metabolic stability rather than to forms of different catalytic activity, since it is known that the structure can play an important role in the process of enzyme degradation, which is an important but unknown event in the regulation of the turnover of this enzyme.

On the other hand, the existence of inactive forms of ODC readily convertible in active forms by either L-ornithine or decreasing ionic strength, can be of interest in experiments based on the treatment of ODC with α -DFMO. L-Ornithine must not be present in the labelling mixture since it can compete with α -DFMO for the enzyme. The relative amount of monomeric forms to the total enzyme protein can be increased dramatically by the ionic strength under conditions in which L-ornithine is not present. α -DFMO produces inhibition of growth and replication and has been used in cancer chemotherapy [22]. Labelled α -DFMO has proved to be particularly useful in enzymatic studies because of its specific covalent binding to ODC. The low percentage of ODC resistant to α -DFMO reported in some preparations [23] could be related to this 'cryptic' monomeric form of ODC. Thus, the wide variation in sensitivity of ODC to α -DFMO depending on the state of the enzyme would be taken into account to maximize the usefulness of this drug in medicine and biochemical research purposes.

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