L-Usnate-Urease Interactions: Binding Sites for the Ligand

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L-usnic acid inactivates urease by formation of high molecular weight aggregates which can reached by a maximum of 880 000. L-cysteine partially reverses the inactivation by stimulating the appearance of active high molecular weight polymers. The existence of two class of binding points for L-usnic acid on the urease molecule is proposed, the first showing high affinity for the ligand, related with the loss of activity, and the second, of low affinity, related to polymerization process.

Introduction

L-usnic acid is a powerful inactivator of native urease (molecular weight 480 000) through a process which implicates the formation of inactive aggregates with a molecular weight higher than the corresponding native enzyme. The highest molecular weight of these polymers are 880 000 [1] and 820 000 [2], respectively.

Enzyme monomers associate by the formation of L-alanyl-L-usnate-L-prolyl bridges between different polypeptide chains [3]. However, because 50 mM L-cysteine seems to reverse the inactivation of the highest molecular weight forms of urease [1], the existence of a third site of linkage in the protein can be supposed. It is suggested that the active site protected by L-cysteine is different from that which supports the interaction with L-usnate for the polymerization process, because of the retention of activity by polymers formed in the presence of both inactivator and amino acid does not involve any change in the association degree (or molecular weight).

This report is concerned with the aspects of the kinetic properties of L-usnate-urease interactions which may clarify the existence of at least two class of binding sites for the ligand.

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Experimental

Samples of 0.5 mg of crystalline urease (type III, Sigma Chemical Company) were incubated with 35 mM L-usnic acid in a final volume of 3.0 ml stabilized at pH 6.9 with 75 mM phosphate buffer at 37 °C for 5 minutes. Immediately afterwards, the samples were assayed for enzyme acitivity, using variable substrate concentration, according to the Conway microdiffusion method [4]. If indicated, 10, 30 or 60 mM L-cysteine was included in the incubation mixture. Protein was measured using the method of Warburg and Christian [5].

Molecular weights of the different aggregates were determined by passing the samples through a Sepharose 6 B column (21 cm in height × 3 cm in diameter), equilibrated with 75 mM phosphate buffer pH 6.9 using as markers tyroglobulin, glutamine synthetase, glutamate dehydrogenase, α-urease, catalase or myosin. Molecular weights were also determined by electrophoresis in 7.5% acrylamide gel using Fishbein's method [6] and staining with amido-black 10-B.

Binding experiments were conducted by mixing the samples with calcium phosphate gel (75.0 mg of dry gel per mg of protein), equilibrated with the same buffer to separate the proteins. The gel was collected by centrifugation. Aliquots of the solution were assayed for the content in free L-usnic acid by using the absorbance of the ligand at 295 nm before and after separation of the proteins. When L-cysteine was present, optical densities were corrected according to the extinction coefficient of the amino acid at 267 nm. Finally, μmol of the bound ligand per unit of protein were determined and Scatchard plots [7] were drawn.

Results and Discussion

The kinetic behaviour of urease for a range of substrate concentrations is shown in Fig. 1. Enzyme activity increases when urea concentration is varied from 5 to 40 mM, reaching its maximum for the latter concentration. Both 50 and 60 mM urea diminish the urease acitivity, which is a well-known fact of inhibition by excess of substrate [8]. In the same figure, the effects of L-usnic acid on urease activity are shown. Inactivation of the enzyme reaches its highest value at a substrate concentration of 40 mM. Preincubation of both enzyme and L-usnic acid

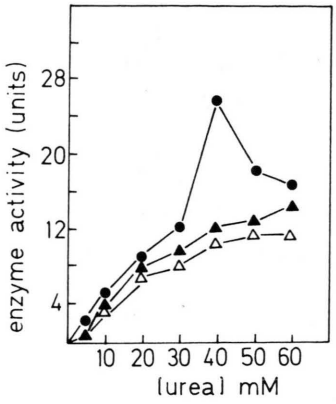

Fig. 1. Effect of L-usnic acid on urease activity. (●) control without the drug; (▲) both urea and L-usnic acid added at zero time; (△) both enzyme and inactivator preincubated for 5 minutes before addition of substrate.

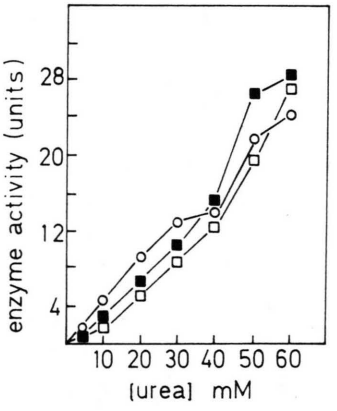

Fig. 2. Effect of L-cysteine, added at zero time, on the urease inactivation by L-usnic acid. (○) 10 mM L-cysteine; (■) 30 mM L-cysteine; (□) 60 mM L-cysteine.

prior to the addition of urea enhances the inactivation effect.

No protection of the enzyme activity for urea concentrations lower than 40 mM has been observed by including L-cysteine in the incubation mixture. However, there is an effective recovery of activity when the substrate concentration is higher than the expressed value (Fig. 2).

In order to analyze the action of L-cysteine on the inactivation process the incubation mixtures (containing L-cysteine) are filtered though a Sepharose 6 B column. Table I demonstrates the analysis data of the highest molecular weight polymers formed in the presence of the amino acid. The number of monomers has been calculated assuming a monomer molecular weight of 16 000 [9].

α-Urease recovered in the fraction which elutes at 130 ml of filtration, inactive polymers eluted at 10 ml are the high molecular weight aggregates whose formation is not prevented by both 10 and 30 mM L-cysteine. However, when 60 mM L-cysteine is mixed with both enzyme and inactivator in the incubation mixture, polymers filtered at 10 ml from Sepharose 6 B retains a discreet percentage of activity.

It has been reported that L-usnic acid is bound to urease in an aggregation process involving, at least, both L-alanyl and L-prolyl rests [3]. If L-cysteine prevents the inactivation without any modification of polymeric behaviour [1], a third site of linkage may be predicted. The results are demonstrated by Scatchard plot [7], showing in Fig. 3A a plot of bound L-usnate per protein unit in relation with the free ligand *versus* bound ligand per unit of protein

Table I. The highest molecular weight polymers of urease formed by action of both L-cysteine and L-usnic acid. Incubation mixture contained 10 mg urease, 350 μmol L-usnic acid and variable concentrations of L-cysteine in a final volume of 10 ml. Mixtures were incubated for 5 min at 37 °C and then, filtered through Sepharose 6B, as it was specified in Experimental. Relations between electrophoretic mobility-molecular weight-number of monomers were 0.14-880 000-55 and 0.24-820 000-51 respectively.

L-cysteine [μmol/ml]	Fraction from Sepharose 6B [ml]	Electrophoretic mobility	Enzyme activity [units]	% of remaining activity	Protein [mg]	% of the total protein	
						observed	expected
–	10	0.14 0.24	–	–	0.35	3.80	3.5
10	10	0.14 0.24	–	–	0.70	8.29	7.0
30	10	0.14 0.24	–	–	0.45	5.33	4.5
60	10	0.14 0.24	0.20	2.2	0.8	8.90	8.0

as a function of enzyme concentration. Binding kinetics is not changed upon increase of the amount of protein in the incubation mixture. This behaviour differs from that reported for other enzymes, *e. g.* glutamate dehydrogenase [10].

Biphasic curves (Fig. 3 A) are generally interpreted to indicate a limited number of binding sites of high affinity for the ligand together with a larger number of lower affinity sites [11, 12]. In addition, the first phase of these curves, the zone of accelerated binding, provides evidence that L-usnate itself enhances its linkage rate on the urease molecule in a process of positive cooperativity [10]. The anomalous stabilization of the high binding values may be interpreted as a consequence of the low solubility of L-usnic acid in aquous solvent [13, 14]. As a result, ligand saturation is not reached in the described experiments.

Inclusion of L-cysteine in the incubation mixture deccelerates the cooperative binding of L-usnic acid (Fig. 3 B), without any modification of binding behaviour on the hypothetic low affinity sites.

As the integrity of free −SH groups for urease activity is necessary [15, 16], it can be postulated that L-cysteine protects these groups of the enzyme against L-usnic acid and that −SH groups constitute the linkage sites of high affinity for the ligand, exclusively related with enzyme activity. However, it cannot be disgarded an interaction between −SH groups of urease and L-cysteine with formation of disulfur bridges, because these relations, in some extension, have been reported for the native enzyme [17]. In addition, the observed activity at 60 mM L-cysteine (Fig. 2), which is lower than that at 10 and 30 mM L-cysteine concentrations, can be explained in this way.

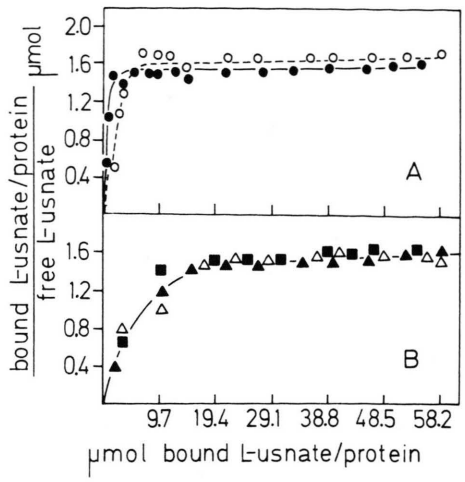

Fig. 3. Scatchard plots for the binding of L-usnic acid on urease: A) at two enzyme concentrations being (●) 0.166 and (○) 0.033 mg of protein per ml, and B) at only one enzyme concentration (0.166 mg/ml) with (▲) 10 mM L-cysteine, (△) 30 mM L-cysteine and (■) 60 mM L-cysteine.

The sites of low affinity for the ligand may be related with the polymerization process which is not affected by L-cysteine (see Table I and Fig. 3 B).

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