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REVERSAL BY DITHIOTHREITOL OF UREASE INACTIVATION BY L-USNIC ACID

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Key Word Index—Dithiothreitol; usnic acid; urease inactivation.

Abstract—L-Usnic acid inactivates urease through the formation of high M_r aggregates of the enzyme. In addition, L-usnic acid strongly binds to the protein, blocking the essential –SH groups of the urease molecule. Low concentrations of dithiothreitol (about 0.8 mM) reverse this blockade without any modification in the pattern of polymerization, inducing the appearance of active high M_r polymers.

INTRODUCTION

Usnic acids have been shown to be powerful inactivators of several enzymes. Inactivation of urease by sodium L-usnate is, in part, related to the formation of aggregates among different enzyme subunits [1] by using both L-alanine and L-proline residues in the urease molecule as binding sites for the inactivator. On the other hand, the presence of L-cysteine in the incubation mixtures reverses this inactivation because the highest M_r forms of the enzyme are now active [2]. On this basis, the existence of a third site of binding for L-usnic acid has been postulated [3].

Essential –SH groups of the enzyme, the integrity of which are necessary for enzyme activity [4], are defined as sites of high affinity for L-usnic acid and support the inactivation process [3]. The additional polymerization, which is not affected by chemicals protecting –SH groups, is carried out by the linkage of L-usnic acid to the low affinity sites, presumably both L-alanine and L-proline residues [5].

The present paper reports the action of dithiothreitol on the inactivation of urease by usnic acid.

RESULTS

Figure 1 shows the inactivation of urease by L-usnic acid with concentrations varying from 8.2 to 41 μM. However 0.8 mM dithiothreitol reverses this inactivation when the chemical is included in the incubation mixtures together with the inactivator, and when L-usnic acid is added after 5 min of incubation of the enzyme with 0.8 mM dithiothreitol. This concentration was chosen on the basis of the results shown in the insert in Fig. 1, which indicate that dithiothreitol concentrations higher than 1 mM inhibit urease activity.

Separation of larger fractions of urease polymers was achieved by filtration of the incubation mixtures through Sepharose 4B (data are not shown). Untreated enzyme elutes as a main peak at 160 ml of filtrate, retaining the highest enzyme activity. This peak is homogeneous on polyacrylamide gel electrophoresis (PAGE) and identified as native α-urease, with an M_r of 483 000. Inactive polymers of urease, produced by the action of 41 μM L-usnic acid, elute in the void volume, the main peak of active enzyme being formed at 110 ml of filtrate.

The protein treated with 0.8 mM dithiothreitol shows as a main peak of activity at 120 ml of filtrate. A different pattern of elution is observed when the enzyme is incubated with both 41 μM L-usnic acid and 0.8 mM dithiothreitol. Different polymers which retain some enzyme activity are eluted at 70 ml, although the main

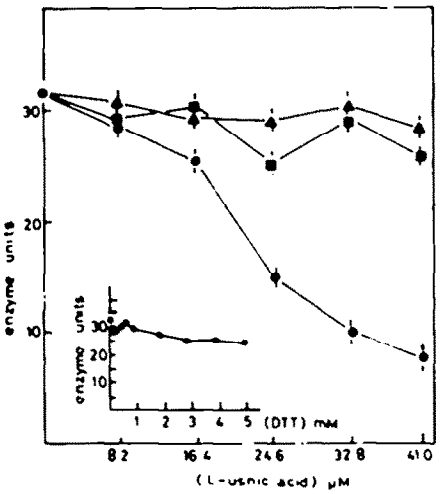

Fig. 1. Effect of both L-usnic acid and dithiothreitol on urease activity. (●) 0.5 mg urease and L-usnic acid were incubated for 5 min at 37° in 3 ml of 75 mM phosphate buffer, pH 6.9; (▲) 0.8 mM dithiothreitol added at zero time; (■) 0.5 mg urease and 0.8 mM dithiothreitol were preincubated for 5 min at 37° prior to the addition of L-usnic acid. Each point is the average of three replicates. Vertical bars give the standard error. The insert shows the effect of several concentrations of dithiothreitol on urease activity after 5 min of incubation at 37°.

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Table 1. Active and inactive forms of urease produced by L-usnic acid and dithiothreitol

Chemical*	Fraction from Sepharose 4B (ml)	M_r† × 10^3	Number of monomers	Protein (mg)	% of total protein	% of remaining activity	Homogeneity from electrophoretic mobility
L-Usnic	70	1040	65	0.24	2.80	—	0.050
acid	90	995	62	0.22	2.54	—	0.055
L-Usnic	70	1040	65	0.24	2.80	0.73	0.050
acid†	90	995	62	0.22	2.80	63.70	0.055
DTT	110	880	55	0.67	7.75	15.00	0.055
							0.150
							0.175
							0.210
							0.230

*41.0 μM L-Usnic acid and 0.8 mM dithiothreitol in the incubation mixtures.

†Determined from filtration pattern.

‡The numbers indicate the electrophoretic mobility of each band which is revealed by staining with Coomassie blue dye.

fractions of protein are heterogeneous on PAGE (Table 1). The polymeric form retaining the highest enzyme activity is eluted only at 90 ml without any similarity to that of α-urease described.

The increase of free thiol groups in the protein are shown in Fig. 2. Incubation with buffer (Fig. 2A), .8 mM dithiothreitol (Fig. 2B), 41 μM L-usnic acid (Fig. 2C) or both inactivator and protector (Fig. 2D) were carried out for 5 min at 37° and denaturation with 8 M urea and reaction with *N*-ethylmaleimide was carried out at least for 70 min using samples of enzyme containing 0.5 mg of protein. The -SH concentration in the untreated enzyme reaches a value of 84.7 mol of thiol/mol of protein (Fig. 2A). When urease is incubated with 0.8 mM dithiothreitol and then dialysed for 24 hr against 4 l. of phosphate buffer, the final concentration of free -SH groups, for 45 min of *N*-ethylmaleimide reaction, was 69.7 mol/mol of protein (Fig. 2B). Figure 2C shows the results obtained when urease is incubated for 5 min with 41 μM L-usnic acid and then dialysed for 24 hr against 4 l. of 2.5% sodium chloride in 75 mM phosphate buffer to remove L-usnic acid. In these conditions, free -SH groups are not detectable, whereas when urease is incubated with L-usnic acid in the same conditions but 0.8 mM DTT is included in the reaction mixtures, the concentration of free thiol groups reaches a value of 54.7 mol/mol of protein for 60 min reaction with *N*-ehtylmaleimide (Fig. 2D). L-Usnic acid strongly binds to the urease molecule since -SH groups are still buried after various treatments (Table 2).

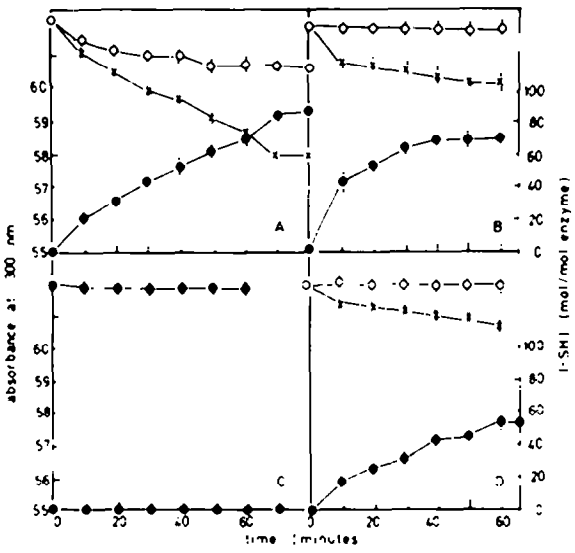

Fig. 2. Quantitative determination of free -SH groups of: (A) untreated urease; (B) enzyme incubated with 0.8 mM dithiothreitol; (C) urease incubated with 0.8 mM dithiothreitol and 41 μM L-usnic acid; (D) urease incubated with 41 μM L-usnic acid. (×) absorbance at 300 nm; (○) reference without protein; (●) concentration of free thiol groups. Each point is the average of three replicates. Vertical bars give the standard error.

DISCUSSION

Incubation of urease with dithiothreitol concentrations higher than 0.8 mM causes loss of enzymes activity. This is uncommon, but an analogous effect has been reported for carbamyl phosphate synthetase [6]. This has been explained as an oxidation of an amino acid residue in the active site of the enzyme due to the protection of hydrogen peroxide formed by an oxidation of dithiothreitol in the presence of molecular oxygen. Although this hypothesis could be supported by the low reactivity of denatured urease with *N*-ethylmaleimide (Fig. 2B), when dithiothreitol-treated enzyme is used in -SH titration [7], we have not found any oxygen uptake or hydrogen peroxide production when urease is incubated with dithiothreitol at concentrations greater than 1 mM. The increase in the M_r of the most active fraction of dithiothreitol-treated urease can be interpreted as a true interaction between dithiothreitol and one or more monomers of enzyme.

Aggregation of enzyme monomers produced by L-usnic acid is achieved in the same way when 0.8 mM dithiothreitol is added to the incubation mixtures but, in this

Table 2. Effect of several denaturing agents on -SH group titration

Denaturing agent	Denaturation conditions (1 hr; temp)	Free thiol groups (mol -SH/mol enzyme)* Enzyme none treatment	41 μM L-usnic acid	0.8 mM DTT	Both DTT and L-usnic acid
6 M urea	20°	84.5 ± 3.1	0.0	69.7 ± 2.7	54.7 ± 2.9
6 M urea 1% SDS	26°	82.2 ± 3.0	0.0	74.0 ± 3.3	37.5 ± 2.7
0.24% SDS	26°	37.4 ± 1.9	0.0	74.0 ± 3.3	12.5 ± 2.2
5.8 M guanidine hydrochlorite	100°	26.0 ± 2.1	0.0	58.6 ± 2.9	16.9 ± 1.8
4 M sodium hypochlorite	100°	68.2 ± 3.1	0.0	69.8 ± 3.0	36.4 ± 2.3
2% SDS	100°	46.5 ± 2.6	0.0	69.0 ± 3.0	27.4 ± 2.3
2% 2-mercaptoethanol	100°	66.3 ± 0.0	0.0	71.4 ± 3.2	41.0 ± 2.5

*Values are the mean of three replicates.
†Incubations are carried out for 15 min at 37°.

case, the aggregates formed by 64–65 monomers (fractions collected between 70 and 90 ml filtrate, which are homogeneous on PAGE) are active. The number of monomers has been calculated [8], assuming an M_r of 16000 instead of that defined of 83000 [9]. M_r has been estimated from PAGE assuming that electrophoretic mobility and M_r do not follow a linear relationship for M_rs greater than 90000 [10].

From the estimation of the free -SH concentration in the protein, it is clear that L-usnic acid strongly binds to thiol groups in the protein producing the inactivation of the enzyme. The nature of this binding is not clear. Its resistance to chemical cleavage (Table 2) could indicate that L-usnic acid binds covalently to the protein, but the chemical structure of this compound (2,6-diacetyl-7,9-dihydroxy-8,9-dimethyl-[1,3(2H,9bH)-dibenzofurandione]) offers several possibilities of linkage to a thiol group. From the point of view of organic chemistry, the dione may oxidize -SH groups into intra or intermolecular disulphide bridgs (case 1 in Fig. 3). In a similar way, L-dihydro usnic is easily prepared by catalytic reduction of L-usnic acid [11]. But this hypothesis cannot explain the fact that L-usnic acid truly binds to urease molecule [3], and in addition, any change in absorbance due to a reduction of L-usnic acid has not been observed. It is probable that the blockade of thiol groups was achieved through a nucleophilic addition into a carbonyl bond according to that shown in case 2 in Fig. 3. The form II is the most probable, since form I is much more unstable. This bond could be reduced by dithiothreitol and, then the enzyme may partially recover its activity since, in the experimental conditions described here, 0.8 mM dithiothreitol only reduces about 65% of the oxidized thiol groups.

A similar case has been reported for glyceraldehyde 3-P dehydrogenase [12] that reacts as sulphenic acid with dimedone. This can also be related to the fact that when the pH of the enzyme reaction is high, the urease inactivation will be greater [13].

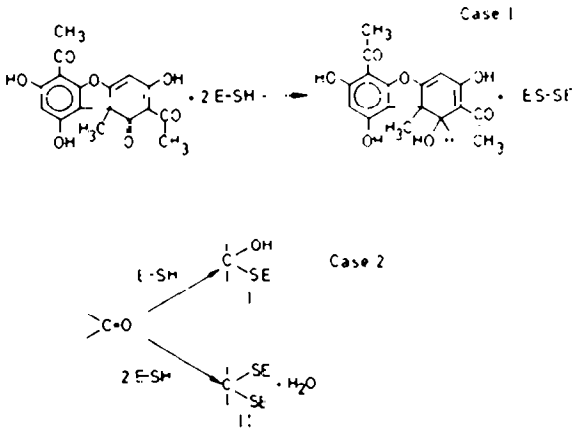

Fig. 3. Possible modes of linkage of L-usnic acid to urease molecule. Case 1 supposes that the protein forms disulphide bridges by reducing L-usnic acid. Case 2 supposes that urease blocks their free -SH groups by a nucleophilic addition into a carboxyl bond of L-usnic acid. The hydroxyl groups in the phenolic have not been used to bind urease, assuming that they are as a chelate ring.

EXPERIMENTAL

Samples of 0.5 mg of crystalline urease (Type III, Sigma), were incubated with variable concns of L-usnic acid in a final vol. of 3 ml, stabilized at pH 6.9 with 75 mM NaPi buffer, at 37° for 5 min. The samples were assayed for enzyme activity by the Conway microdiffusion method [14]. If indicated dithiothreitol was included in the incubation mixtures at variable concns alternatively, 0.8 mM was chosen on the satandard assay concn for this chemical. Protein was measured by using the method of ref. [15].

A first separation of the different aggregates was carried out by passing the samples through a Sepharose 4B column (20 cm × 3.2 cm) equilibrated with 75 mM NaPi buffer, pH 6.9. The void vol. was determined with dextran blue 2000. Homogeneity of the different fractions, as well as the M_r of the different aggregates, was achieved in 7.5% PAGE by using the method of ref. [16] and staining the protein with Coomassie blue dye. Myosin (M_r 220000), catalase (M_r 232000), phosphatase a (M_r 370000), soybean urease (M_r 483000), thyroglobulin (M_r 669000) and

glutamic acid dehydrogenase (M_r 1 015 000), all from Sigma were used as markers.

To estimate the thiol groups, urease was denatured with 8 M urea for 1 hr at 26° and the assay was conducted by the method of ref. [17] as modified in ref. [18] using *N*-ethylmaleimide as reagent. When indicated, denaturation of protein was achieved with 0.48 M sodium dodecyl sulphate (SDS), 5.8 M guanidine hydrochloride or a mixture of 1% SDS and 8 M urea, for 1 hr at 26° or, alternatively 4 M Na hypochlorite, 2% SDS or 2% β-mercaptoethanol at 100°.

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