TRYPTIC DIGESTION OF IMMOBILIZED D-AMINO ACID OXIDASE

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Received 20 December 1977

1. Introduction

The activity of an enzyme is dependent on its conformation and on the structure of its active site. Chemical modification and specific labelling of an active site are useful techniques for the analysis on its structure; however, they often result in undesirable changes in enzyme conformation. It was observed that D-amino acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating) EC 1.4.3.3) was protected from its denaturation by urea and by storage, when immobilized to agarose [1]. We report here that the immobilized enzyme can reserve its conformation essential to the activity, even though some parts of the protein moiety are digested off by trypsin.

2. Materials and methods

D-Amino acid oxidase was purified from pig kidney as in [2]. To protect the substrate binding site, the enzyme was immobilized to agarose (Sepharose) or Sephadex in the form of the enzyme–benzoate complex. After immobilization, benzoate was expelled by substrate, D-alanine and then the coenzyme, FAD was liberated from the immobilized enzyme by washing with KBr as in [1]. The tryptic digestion of the immobilized enzyme was carried out at room temperature in the following reaction mixture: 0.63 μmol enzyme (15 ml beads) added to 15 ml 0.2 M sodium borate buffer (pH 7.0) containing 0.01 M $CaCl_2$ and 7.5 mg trypsin.

The enzymatic activity was measured as in [2] and the bound protein concentration as in [3] after alkaline hydrolysis [4]. The release of peptides was followed by measuring amino group with fluorescamine, 4-phenylspiro-(furan-2(3H)-1′-phthalan)-3.3′-dione [5]. At several time intervals, 0.1 ml reaction mixture was taken from the supernatant, and its pH was adjusted to 2.0 by addition of 1 N HCl. To the solution, 1.9 ml 0.2 M borate buffer (pH 10.0) and 0.5 ml 0.03% fluorescamine in acetone were added. Fluorescence intensity at 490 nm was measured in a corrected recording fluorospectrophotometer, Shimadzu RF 502, with the excitation wavelength of 390 nm. The remaining peptides after tryptic digestion were released from Sephadex with dextranase (1,6-α-D-glucan 6-glucanohydrolase, EC 3.2.1.11), obtained from Sigma Chemical Co. St Louis, at 34°C for 2 h.

3. Results and discussion

When the immobilized apoenzyme of D-amino acid oxidase was digested by trypsin, peptides were released with time of incubation, but the enzymatic activity was not changed (fig.1). It should be emphasized that the apoenzyme immobilized to agarose or Sephadex kept its complete activity, even after the bound protein concentration was reduced from 1.65–0.70 mg/ml. A further addition of trypsin did not increase the digestion of protein, nor affect the enzymatic activity.

On the other hand, when the free apoenzyme was digested by trypsin, peptides were liberated with the reduction of the enzymatic activity. After 4 h tryptic digestion, the enzymatic activity was reduced to 1/3rd original. Most of the activity was lost after 24 h (fig.2).

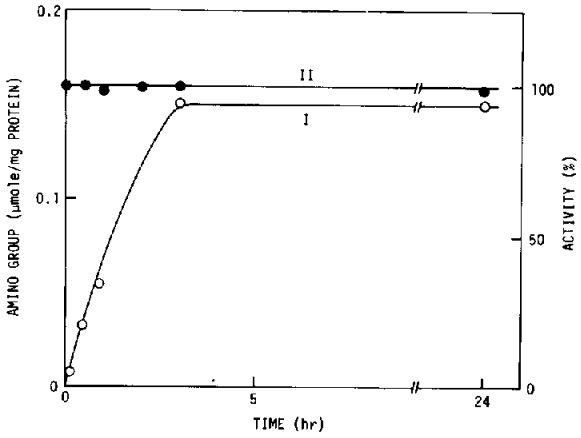

Fig.1. Tryptic digestion of the apoenzyme immobilized to CNBr-activated agarose. The immobilized apoenzyme (1.65 mg protein/ml beads, 15 ml) was digested with 0.025% trypsin. The enzymatic activity of the beads suspension was measured in the following reaction mixture: 5 μl beads suspended in 3.0 ml 0.1 M pyrophosphate buffer (pH 8.3) containing 0.1 M D-alanine and 10 μM FAD. Curve I, amounts of amino group liberated in the solution; curve II, enzymatic activity.

The above results indicate that the immobilized apoenzyme retains its activity after losing some parts of its structure. To check further this point, the immobilized apoenzyme after the tryptic digestion

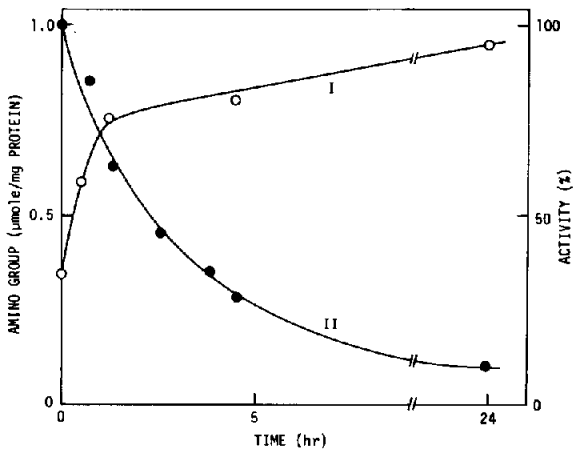

Fig.2. Tryptic digestion of the free apoenzyme. The free apoenzyme (2 mg/ml) was digested in the same way as in fig.1. Curve I, amounts of amino group liberated in the solution; curve II, enzymatic activity.

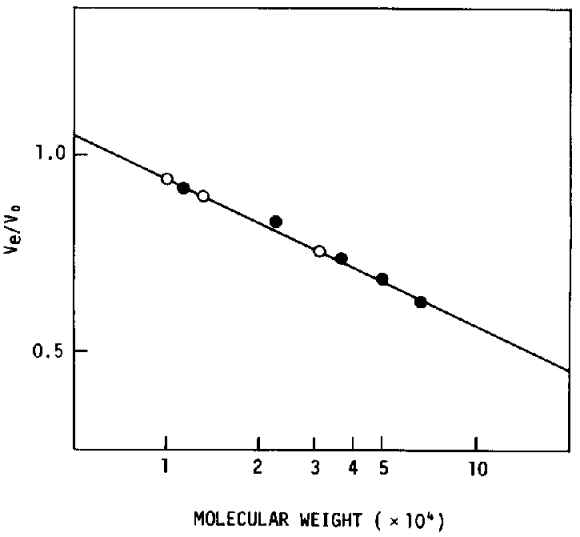

Fig.3. Molecular weight determination of the major peptides obtained from the Sephadex beads by dextranase. The three major peptides obtained from the Sephadex beads were expressed by open circles, and solid circles show the marker proteins, cytochrome *c*, trypsin, D-amino acid oxidase, γ-globulin (heavy chain) and bovine serum albumin from the left to the right.

was released from Sephadex with dextranase. The released preparation had no enzymatic activity. It was applied onto Sephadex G-200 column (26.4 × 1000 mm) and eluted with 10 mM Tris–HCl buffer (pH 8.0). Three major peaks were observed. Their molecular weights were estimated to be 30 000, 12 000 and 10 000 (fig.3). Considering that some amounts of sugar must be contained in these peptides, the molecular weights of their protein moieties should be somewhat smaller than these figures. The peak corresponding to the intact enzyme (mol. wt 39 000) was not observed in the elution pattern. Accordingly, it becomes clear that trypsin digested the immobilized enzyme and that the immobilization of the enzyme to agarose or Sephadex resulted in the stabilization of the enzyme conformation to keep the enzymatic activity.

The analysis of the peptides, which were obtained by releasing from the Sephadex by dextranase after the tryptic digestion, will give us useful information on the relation between the structure and function of this enzyme.

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