REVERSIBLE DENATURATION AND ACTIVATION OF CYTOPLASMIC ASPARTATE AMINOTRANSFERASE

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1. Introduction

Pyridoxal-5′-phosphate can bind to cytoplasmic apo-aspartate aminotransferase (EC 2.6.1.1) in two ways. One mode of binding gives an enzymatically active holoenzyme with well defined chemical and spectral properties. The other mode of binding gives an enzymatically inactive holoenzyme characterized by an absorption maximum at 340 nm. The active mode of binding prevails in the less anionic subforms, the inactive mode in the more anionic subforms [1–5]. In various experiments of aging under mild conditions [2, 3, 6] "active" enzyme has been transformed into "inactive" enzyme and the less anionic have been converted into the more anionic subforms.

Attempts to convert "inactive" into "active" enzyme have previously failed.

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Table 1
Properties of aspartate aminotransferase fractions before and after reversible denaturation.

Chromatographic fraction	Before denaturation			After denaturation	After renaturation by dialysis***	
In order of emergence from the CM-Sephadex column	Specific activity*	Coenzyme bound in the inactive mode (%)**	Coenzyme irreversibly inactivated by reduction (%)	Specific activity*	Specific activity*	Activity (relative to that of the same fraction before denaturation) (%)
I	8	81	–	< 0.05	16	200
II	27	66	–	< 1.0	15	55
III	62	28	–	< 1.0	24	38
IV	100	< 7	–	< 1.0	37	37
II after reduction	2.1	66	34	< 0.01	9.7	460
IV after reduction	1.3	< 7	> 93	< 0.01	1.9	140

* Specific activities are expressed as % of the specific activity of fraction IV (300 μmoles of substrate modified per min per mg of protein under standard assay conditions).
** Calculated from spectral data according to Martinez-Carrion et al. [1].
*** In these experiments the activity was assayed after 16 hr of dialysis against the renaturing solution.

In the present paper, we show that a partial activation of enzyme species which initially contain the coenzyme bound in the inactive mode can be achieved by denaturation followed by renaturation under controlled conditions [7].

2. Experimental and results

The subforms of aspartate aminotransferase were separated by chromatography as described by Martinez-Carrion et al. [2] except that a less steep gradient (2 ℓ of each of the buffering systems) was used in the carboxymethyl-Sephadex elution step. The properties of the four main fractions thus obtained are reported in table 1.

Denaturation was performed by incubating the protein fractions (at concentrations ranging from 0.03 to 3 mg/ml) in a freshly prepared solution containing 6 M guanidine-HCl*, 0.1 M Tris-HCl buffer, pH 7.8, 10^{-3} M EDTA, and 10^{-3} M dithiothreitol, at 5–10° for 6 hr. Varying the time of incubation in the denaturing solution between 2 and 48 hr did not affect the results.

The above treatment caused the complete loss of enzymic activity and an extensive unfolding of the protein; the optical rotatory dispersion of the denatured protein thus obtained (fig. 1) approaches that of randomly coiled peptide chains [9].

Renaturation experiments were performed in two different ways: either by a 100-fold dilution of the denatured protein into the renaturing solution at room temp., or by dialysis of the denatured protein against two successive batches of renaturing solution (40 vol each) at 5–10°, for periods ranging from 2 to 48 hr. The renaturing mixture contained: 0.1 M Tris-HCl, pH 7.8, 10^{-3} M EDTA, 10^{-3} M dithiothreitol, 10^{-4} M pyridoxamine-5′-phosphate, 1.3×10^{-2} M L-aspartate.

The recovery of activity was measured as a function of the time of exposure to the renaturing solution. Activity was monitored spectrophotometrically by following the NADH consumption at 340 nm and 25° in a solution containing 0.1 M Tris-HCl, pH 7.8, 10^{-3} M EDTA, 10^{-3} M dithiothreitol, 10^{-4}M pyridoxamine-5′-phosphate, 1.3×10^{-2} M L-aspartate, 3.1×10^{-3} M α-ketoglutarate, 10^{-4} M NADH and 5 μg/ml malic dehydrogenase, to which aliquots of the enzyme solutions (final protein conc.: from 0.03 to 1 μg/ml) were added.

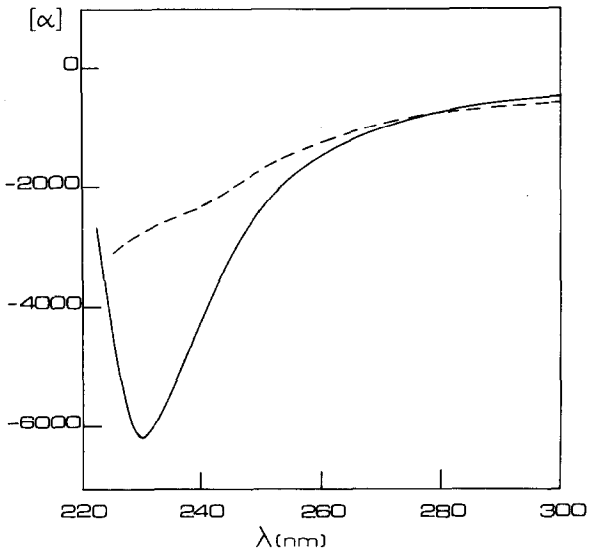

Fig. 1. ORD of native and denatured aspartate aminotransferase. (——) Native enzyme in 0.1 M Tris-HCl buffer, pH 7.8, 10^{-3} M EDTA, 10^{-3} M dithiothreitol. (----) Enzyme incubated for 2 hr in the denaturing solution (see text). Identical results were obtained after 6 hr incubation. The measurements were performed with a Cary 60 recording spectropolarimeter at 27°, in 0.1 cm path length cuvettes. Protein concentration was 3.25 mg/ml.

As may be noticed in fig. 2 and table 1, both renaturing treatments partially restore the activity; the percentage of recovery, however, varies for the different enzyme fractions; those which initially contain a larger amount of the coenzyme bound in the inactive mode are, in all cases, those which recover a higher percentage of the initial activity. This phenomenon is particularly evident in fraction I, which has a higher activity after the treatment than in the native state. This suggested that extensive denaturation followed by renaturation activates the enzyme species which were initially inactive.

To test this hypothesis samples of fraction IV and II were treated with sodium boronhydride. It is known that this treatment irreversibly inactivates the

* Guanidine hydrochloride was recrystallized according to Nozaki and Tanford [8].

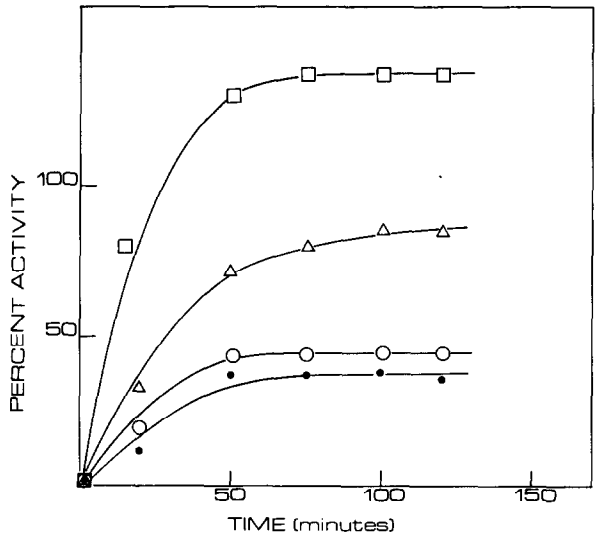

Fig. 2. Reactivation by dilution. Enzymic activity is expressed as percent of the specific activity of the same fraction before denaturation (see table 1). The protein concentration after dilution in the renaturing mixture was 0.5–1 μg/ml (for details, see text). (□—□—□) Fraction I, (△—△—△) fraction II, (○—○—○) fraction III, (●—●—●) fraction IV.

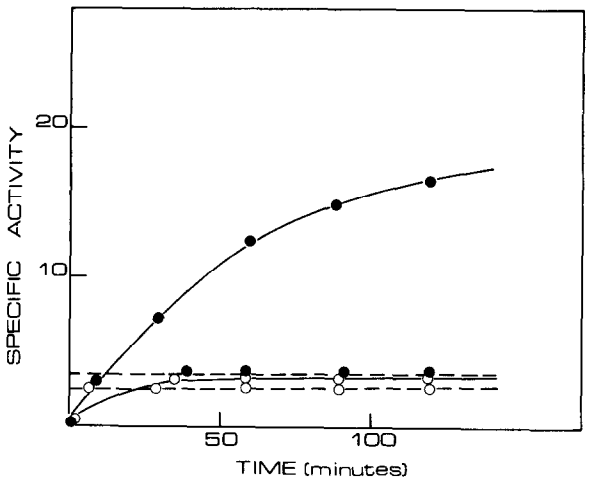

Fig. 3. Specific activity of the reduced fractions II (●) and IV (○) after dilution in the renaturing mixture. (——) Fractions submitted to denaturation before dilution in the renaturing mixture. (- - - -) Fractions directly diluted into the renaturing mixture. Specific activity is expressed as μmoles of substrate transformed per minute per mg of protein, under standard conditions (for details see text). Specific activity of non reduced fractions under the same conditions were: fraction II = 90; fraction IV = 300.

"active" species of aspartate aminotransferase by reducing the azomethinic linkage between the coenzyme and the apo-protein, but has no effect on the enzyme species which contain coenzyme bound in the inactive mode. The reduced enzymes, which had lost all but 1–3% of the initial activity, were then subjected to the usual denaturation–renaturation procedure.

As shown in fig. 3 and table 1, the specific activity of the boronhydride-treated fraction II (which contains a large amount of coenzyme bound in the inactive mode insensitive to reduction) increases more than four times after the denaturation–renaturation treatment; conversely, this treatment has a scarcely appreciable effect on the reduced fraction IV, in which practically all the coenzyme has been irreversibly inactivated by reduction.

These results show that it is indeed the portion of fraction II which initially contained the coenzyme bound in an inactive mode which is partially converted into an active form by the denaturation–renaturation cycle. The different extent of reactivation reported by Kaplan [10] for two subforms of chicken heart aspartate aminotransferase can probably be ascribed to the same phenomenon.

3. Conclusions

Present results demonstrate for the first time that the "inactive" species of cytoplasmic aspartate aminotransferase can be transformed into "active" enzyme.

The activation of the "inactive" enzyme by the denaturation–renaturation treatment could be explained by two, possibly concurrent, phenomena: i) denaturation promotes the breakdown of the bonds responsible for the inactive mode of coenzyme binding to the protein; ii) in a "physiological" medium the inactive mode of coenzyme binding is favoured in thermodynamically stable conformers formed by a slow transconformation from less stable, active, conformers; in concentrated guanidine the stable, inactive, conformers would lose their secondary, tertiary and quaternary structure and, upon exposure to the "renaturing" medium, they would refold forming kinetically favoured (though thermodynamically less stable) conformers, which are capable of binding the coenzyme in the active mode.

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