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STUDIES ON ASPARTASE

V. DENATURANT-MEDIATED REACTIVATION OF ASPARTASE, WHICH HAS BEEN OTHERWISE IRREVERSIBLY INACTIVATED BY VARIOUS CAUSES

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Summary

Aspartase (L-aspartate ammonia lyase, EC 4.3.1.1) of *Escherichia coli*, inactivated by heat-treatment at 55°C or above in the presence of 10 mM 2-mercaptoethanol does not recover the activity at all upon simple chilling and remains to be seriously denatured resulting in the formation of insoluble aggregates. When the heat-denatured enzyme is further treated with 6 M guanidine-HCl followed by 51-fold dilution at 25°C and pH 6.8, the enzyme activity is gradually restored and reaches almost 40% that of the native enzyme in 20 min. The secondary and the quaternary structures of the reactivated enzyme exhibit a close similarity to those of the native enzyme, as revealed by circular dichroism and electron micrograph, respectively. A similar reactivation is attained, when proton-inactivated aspartase at acidic pH is treated with 6 M guanidine-HCl followed by dilution.

As previously reported, aspartase requires the sulfhydryl group for its activity (Mizuta, K. and Tokushige, M. (1975) Biochim. Biophys. Acta 403, 221—231) and is readily inactivated by sulfhydryl reagents. Although the inactivated enzyme can usually be reactivated with sulfhydryl compounds, the reactivation becomes impossible, when a large excess of the reagent is applied. Chemically-inactivated aspartase with a large excess of 5.5'-dithiobis(2-nitrobenzoic acid) is, however, reactivated by way of the reduction of fully exposed polypeptide side chains with dithiothreitol in the presence of 6 M guanidine-HCl.

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Abbreviations: ORD, optical rotatory disperison; CD, circular dichroism; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

These results are compatible with the possibility that a number of proteins denatured and inactivated by various causes in an irreversible fashion resulting in the formation of entangled polypeptide chains can be renatured by way of extensive unfolding with a potent denaturant.

Introduction

Aspartase (L-aspartate ammonia lyase, EC 4.3.1.1) purified from *Escherichia coli* W cells has a molecular weight of 193 000 and is composed of four subunits of seemingly identical molecular weight (48 000) [1]. The enzyme contains 9 sulfhydryl groups per subunit, 1.2 of which was found to be essential for the activity [2]. The enzyme exhibits, in its substrate saturation profile, a marked negative cooperativity at the neutral pH and a marked positive cooperativity at the alkaline pH [1,3]. Although the enzyme is activated by 10—20% glycerol to a considerable degree under the conditions where the sigmoidal kinetics are observed, the subunit interaction of the enzyme does not seem to be altered by the polyol [3]. In contrast, aspartase, which has been 3-fold activated by a limited proteolysis with trypsin exhibits a higher degree of cooperativity than the native enzyme as reported from this laboratory [4,5]. Our recent observation on the reversible denaturation showed that the enzyme denatured in 4—6 M guanidine-HCl is readily renatured upon dilution concomitant with restoration of the activity [6]. As determined by ORD of the renaturation processes, more than 90% of the ordered structure was recovered in 1 min, while the restoration of the activity proceeded much more slowly. Various environmental factors, such as temperature, pH and protein concentration exhibited a profound influence on the rate and the extent of the reactivation. Available evidence suggests that the subunit assembly is an essential prerequisite, though not adequate, for acquisition of the enzyme activity. In this communication we wish to report that aspartase, which had been inactivated by various causes leading to serious denaturation was readily reactivated by way of extensive unfolding with 6 M guanidine-HCl.

Experimental procedures

Monosodium L-aspartate was a product of Kyowa Hakko (Tokyo). Guanidine-HCl for routine use was a product of Nakarai (Kyoto) and that for optical measurements (ultrapure) was obtained from Mann. Dithiothreitol was from Sigma. All other chemicals were of reagent grade.

Aspartase and assay of its activity

Aspartase was extracted and purified to homogeneity from *E. coli* W cells as described previously [1]. The enzyme activity was routinely determined spectrophotometrically by measuring the formation of fumarate following the increase in absorption at 240 nm at 30°C. The standard assay mixture contained sodium L-aspartate (100 mM, pH 7.4), $MgCl_2$ (2 mM), Tris · HCl (100 mM, pH 7.4) and the enzyme in a total volume of 1.0 ml. The molar extinction coefficient of fumarate, 2530 $mol^{-1} \cdot cm^2$ reported by Emery [7] was used. One unit

of enzyme was defined as the amount producing 1 μmol of fumarate per min under the standard assay conditions. Specific activity was expressed as units per mg of protein at 30° C.

CD measurements

CD measurements were carried out with a JASCO automatic recording spectropolarimeter, model J-20 with a CD attachment by using either 0.1- or 1.0-mm light path cells. Mean residue weight was assumed to be 128 based on the amino acid composition [1]. The unit of the molecular ellipticity, $[\theta]$ is degrees square cm per decimol. The CD spectra of the enzyme were measured at a protein concentration of 0.1—5 mg/ml. All recordings were made at 25° C.

Electron microscopy

The sample solutions for electron microscopy were prepared by diluting the stock enzyme solutions with potassium phosphate/KCl buffer (50 mM potassium phosphate, pH 6.8, containing 0.1 M KCl, 5 mM 2-mercaptoethanol and 1 mM EDTA) to designated concentrations. The buffer mixture was prepared by using double glass-distilled water. The enzyme molecules were negatively stained by a modification of the method described in a previous paper [6]. A JEM-7A electron microscope (JEOL, Tokyo), fitted with an anticontamination device, was used at an accelerating voltage of 80 kV. Micrographs were taken at an instrumental magnification of 50 000 or 60 000.

Other determinations

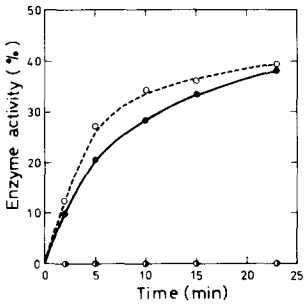
All spectrophotometric determinations were carried out in a Hitachi 124 recording spectrophotometer equipped with a constant temperature cell housing. Aspartase concentration was determined by the use of $E^{1\%}_{1cm}$ value of 5.9 at 280 nm [6].

Results and Discussion

Heat inactivation

As reported previously [6], aspartase denatured in 4 or 6 M guanidine-HCl is readily renatured in vitro by simple dilution. When the native enzyme (I) was heated at 55° C or above under weakly acidic conditions * (pH 5—6), the enzyme activity was completely lost in a few min and successive chilling did not lead to reactivation of the enzyme at all. Heat-treatment at higher protein concentrations resulted in the formation of white insoluble aggregates even in the presence of 2-mercaptoethanol. When 6 M guanidine-HCl was added to the heat-denatured enzyme (II), however, these aggregates readily dissolved to yield transparent solution (III). After extensive denaturation in 6 M guanidine-HCl for 30 min, III was 51-fold diluted with potassium phosphate/KCl buffer at 25° C and pH 6.8. The diluted enzyme (IV) progressively restored the activity to attain almost 40% that of the original enzyme in about 20 min. The activity

* Although the heat-treatment was carried out under slightly acidic conditions in order to prevent the oxidation of the sulfhydryl groups in the enzyme protein, an additional experiment revealed that nearly the same degree of reactivation was attained when the heat-treatment was carried out at the neutral pH.



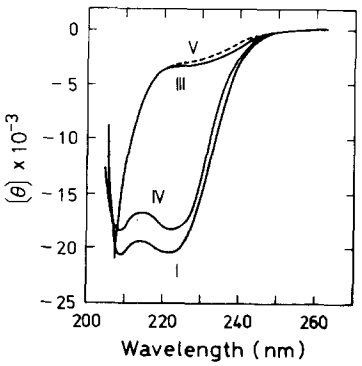


Fig. 1. Reactivation of heat-inactivated aspartase. Curve 1 (●———●), the enzyme (0.20 mg protein) was heated at 55°C for 5 min in a solution (50 μl) containing 50 mM acetate buffer, pH 5.0 and 10 mM 2-mercaptoethanol. The enzyme activity was completely lost by this treatment. The heat-inactivated enzyme was 4-fold diluted with 8 M guanidine-HCl and incubated for 30 min at room temperature (final concentration of guanidine-HCl was 6 M). The denatured enzyme was then 51-fold diluted with potassium phosphate/KCl buffer at 25°C and the restored activity was determined at designated time intervals by using small aliquots of the sample under the standard assay conditions. Curve 2 (○- - - - -○), the enzyme (0.20 mg protein), denatured in 6 M guanidine-HCl was renatured under the same conditions as for Curve 1, except that the heat-treatment was omitted. ◑———◑, the heat-inactivated enzyme was diluted without the guanidine-HCl treatment.

Fig. 2. CD spectra for native, denatured and renatured aspartase. The stock enzyme solution was diluted with potassium phosphate/KCl buffer to designated concentrations. I, the native enzyme (0.93 mg protein/ml); IIII, the enzyme denatured in 6 M guanidine-HCl (the native enzyme was 4-fold diluted with 8 M guanidine-HCl) following the heat-treatment at 55°C for 5 min (2.5 mg protein/ml); IV, the enzyme renatured by 26-fold dilution of III (0.10 mg protein/ml); V, the enzyme denatured in 6 M guanidine-HCl (2.5 mg protein/ml). CD spectra for III and V were measured 1 h after addition of the denaturant and that for IV was measured 30 min after dilution. IV showed 50% activity regain.

regain of the control enzyme denatured in 6 M guanidine-HCl followed by dilution without the heat-treatment proceeded in a similar fashion. These results are shown in Fig. 1.

The CD spectra of I, III and IV are shown in Fig. 2 together with the spectrum of the enzyme denatured in 6 M guanidine-HCl without heat treatment. The CD spectrum of I shows negative troughs at 222 and 209 nm. The molecular ellipticity at 222 nm was approximately $-2 \cdot 10^4$, indicating that the content of α-helix in the enzyme protein is close to 50% in comparison with that of poly-L-lysine. The CD spectrum of II could not be measured due to turbidity. The result of the guanidine-HCl treatment following the heat-treatment suggests that the denaturation process leads to the formation of random coils, devoid of long-range, non-covalent interactions as exemplified by Tanford and his collaborators [8—12]. Upon removal of the denaturant from III by dilution, a marked degree of renaturation was attained in a few min to restore more than 80% of the Cotton effect of the native enzyme at 222 nm.

Quaternary structure of the above enzyme preparations was examined by electron microscopy. As described in a previous paper [6], negatively stained aspartase appeared as tetrameric molecules with a diameter of 75—100 Å (Fig. 3A). In contrast, II appeared as an amorphous entity as shown in Fig. 3B (low protein concentration) and B′ (high protein concentration). In B′, enzyme aggregates of higher molecular sizes appeared to be linked with each other

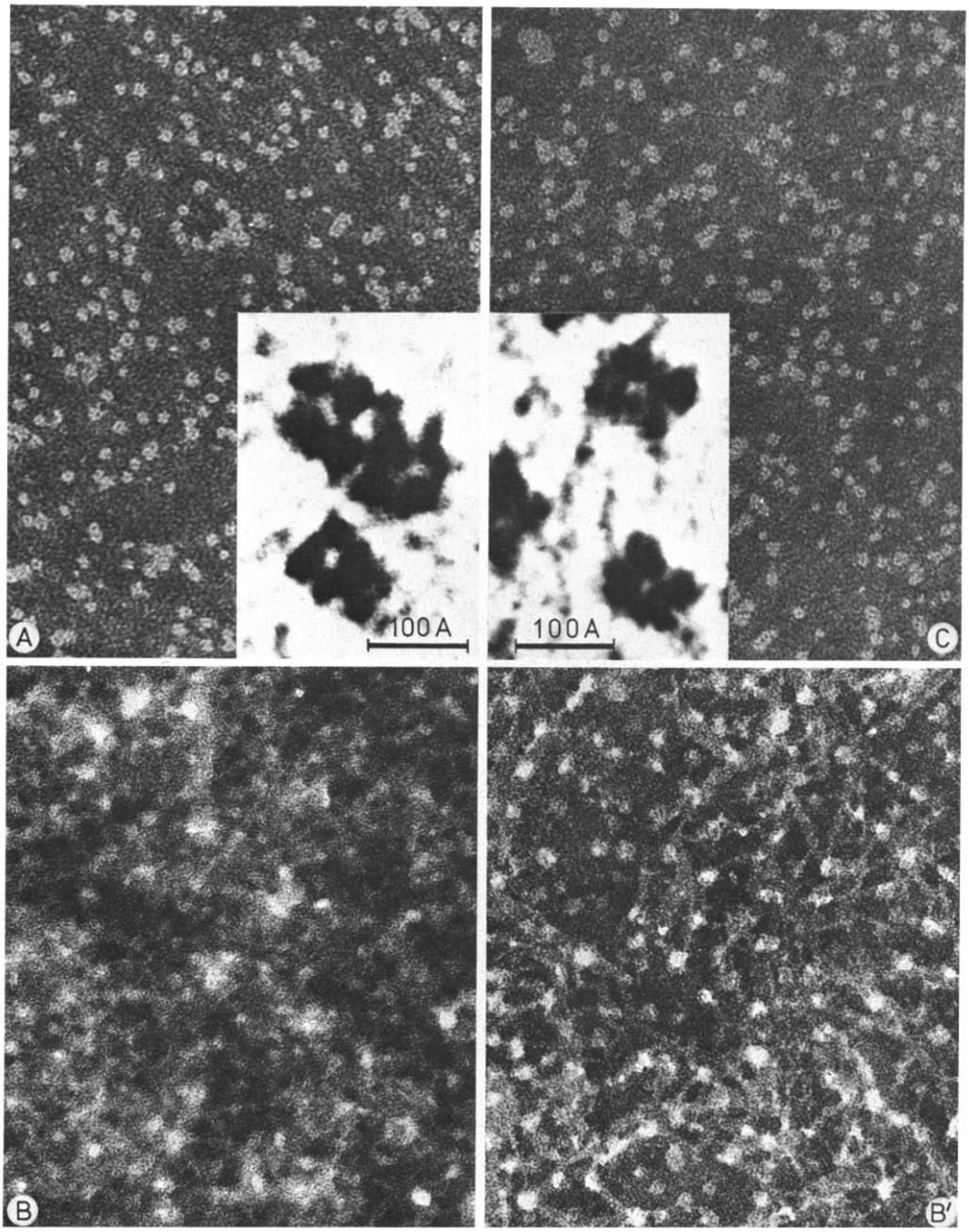


Fig. 3. Electron micrographs of negatively-stained aspartase with 2% sodium phosphotungstate. The experimental procedures were the same as those described in the text. A, the native enzyme; B and B′, the heat-inactivated enzyme at 55°C for 5 min; C, the renatured enzyme by 101-fold dilution of the denatured enzyme in 6 M guanidine-HCl following the heat-treatment as described in Fig. 1. Protein concentration in A, B and C was 20 μg/ml and that in B′ was 2.5 mg/ml. Magnification × 187 500. Typical tetrameric images are shown in the reversed and enlarged photographs taken from A and C.

forming entangled filaments. Upon dilution of III, however, tetrameric features appeared concomitant with restoration of the activity.

Acid inactivation

Similar to the heat denaturation described above, aspartase was seriously denatured under acidic conditions. When the enzyme was exposed to pH 3.5 in 50 mM acetate buffer containing 10 mM dithiothreitol, its activity was gradually lost and only 2% of the original activity remained after 1 h at room temperature, forming white aggregates. Upon treatment of the proton-inactivated enzyme with 6 M guanidine-HCl for 30 min followed by dilution, however, more than 20% of the original activity was restored in 20 min at 30°C and pH 6.8. When the proton-inactivated enzyme was incubated in a large excess of potassium phosphate/KCl buffer without the guanidine-HCl treatment, no appreciable reactivation was detected for 1 h.

Sulfhydryl group modification

The above study was further extended to another attempt for renaturation of chemically modified enzyme. As reported previously [2], aspartase requires the sulfhydryl group for its activity. The available evidence suggests that the enzyme contains no disulfide linkage. Although the enzyme inactivated with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was readily reactivated upon addition of dithiothreitol following desalting with a small column of Sephadex G-50, the reactivation became impossible upon aging of the modified enzyme, when a large excess of the sulfhydryl reagent was applied, presumably due to partial denaturation of the enzyme conformation, making dithiothreitol inaccessible. However, upon extensive denaturation of the chemically modified aspartase with 6 M guanidine-HCl in the presence of 10 mM dithiothreitol for 30 min followed by 51-fold dilution, the enzyme readily restored the activity to a considerable degree. These results are shown in Fig. 4.

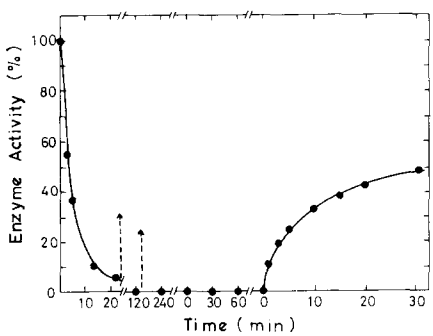


Fig. 4. Reactivation of chemically modified aspartase. To the desalted aspartase (0.3 mg protein) by a small column of Sephadex G-50 was added 1 mM DTNB at 4°C and the residual activity was determined at designated time intervals by using small aliquots under the standard assay conditions. After the enzyme activity, as well as the capability for reactivation with dithiothreitol (indicated by dotted arrows), was completely lost, the enzyme preparation was denatured in 6 M guanidine-HCl containing 10 mM dithiothreitol for 1 h. The denatured enzyme was then 51-fold diluted with potassium phosphate/KCl buffer at 25°C and the restored activity was determined under the standard assay conditions.

General Discussion

The instability of various enzymes may often be ascribed to fragility of the protein conformation in nature. In fact, tremendous cases are known, in which enzymes inactivated by various causes, such as heat, aging and chemical modifications are not able to be readily reactivated simply by removal of the causes due to partial or serious denaturation of the polypeptides. Anfinsen and his co-workers, however, demonstrated in their elegant experiments that the secondary, tertiary and quaternary structures of proteins are essentially dictated by the primary structure [13]. In addition, Tanford predicted in his extensive review [14] that the native conformation of most proteins is uniquely determined by the sequence of amino acids in the protein polypeptide chains; any protein to which this generalization applies should always be able to recover its native conformation, provided that its primary sequence remains intact. He also stated that whether the above conclusion applies to all proteins, to most proteins, or only to a few proteins, has not been ascertained. Only example he cited was the work of Westhead, in which yeast enolase that had been boiled at neutral pH for 20 min, yielding a "tough insoluble mass" recovered substantial activity by solubilization in 8 M urea at pH 1 [15]. Apparently the products of thermal transitions at low pH of a number of enzymes retain regions of ordered structure, susceptible to disruption by guanidine-HCl and the ordered regions which remain in heat-denatured proteins contain one-fourth to one-third as much ordered chains as the native molecules, as Tanford and his co-workers described [8—12]. Likewise, Brandts suggested that thermally denatured chymotrypsinogen may not be a completely disordered chain, because the reduced viscosity of the denatured protein could be further increased by the addition of urea [16].

The present investigation extended the above possibility to more general ones that enzymes, which have been irreversibly inactivated not only by heat, but also by proton, aging and chemical modification can be equally by way of extensive unfolding treatments. In the case of the proton-inactivated aspartase, the degree of the activity regain was considerably low and the reason may be due to alteration of the primary structure, such as deamidation of glutaminyl or asparaginyl residues under acidic conditions [17].

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