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STUDIES ON ASPARTASE

IV. REVERSIBLE DENATURATION OF *ESCHERICHIA COLI* ASPARTASE

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Summary

Aspartase (L-aspartate ammonia lyase, EC 4.3.1.1) of *Escherichia coli*, denatured in 4 M guanidine-HCl, was renatured in vitro by simple dilution with a concomitant restoration of the activity. While the native enzyme exhibited a marked negative Cotton effect centered at 233 ± 1 nm in optical rotatory dispersion, the enzyme denatured in 4 M guanidine-HCl retained little optical activity. Upon dilution of the denatured enzyme, however, more than 90% of the ordered structure was recovered in 1 min, while the restoration of the activity proceeded much more slowly. Estimation of molecular weights by gel permeation chromatography indicated that the tetrameric enzyme is subject to reversible dissociation into monomeric subunits under the experimental conditions.

Various environmental factors such as temperature, pH and protein concentration exhibited profound influence on the rate and extent of the reactivation. In order to examine the correlation between the restoration of the activity and the quaternary structure, electron microscopic inspection of the kinetic processes of reversible denaturation was attempted. Upon dilution of the denatured enzyme at 4°C, neither the activity nor tetrameric images were detected over several min. Upon the temperature shift up to 25°C, however, the activity regain was rapidly proceeded concomitant with the appearance of tetrameric molecules. These results are compatible with the possibility that the subunit assembly is an essential prerequisite, thought not sufficient, for enzyme activity.

Abbreviation: ORD, optical rotatory dispersion.

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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 [1]. The enzyme exhibits, in its substrate saturation profile, a marked negative cooperativity at the neutral pH and conversely a positive cooperativity at the alkaline pH [1,2]. The enzyme contains about 9 sulfhydryl groups per subunit, 1.2 of which were found to be essential for the activity [3]. Although the enzyme is activated by 10–20% glycerol to a considerable extent under conditions where the sigmoidal kinetics are observed, the subunit interaction of the enzyme does not seem to be altered by the polyol [2]. In contrast, aspartase, which has been activated 3-fold by a limited proteolysis with trypsin exhibits a higher degree of cooperativity than the native enzyme, as recently reported from this laboratory [4,5]. To elucidate the activity-subunit interaction relationship, the present study on the reversible denaturation has been undertaken.

Materials and Methods

Monosodium L- and D-aspartate were products 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. Proteins used as standards in gel permeation chromatography were obtained from the following sources: cytochrome *c*, myoglobin, chymotrypsinogen A, ovalbumin and bovine serum albumin from Schwartz/Mann as a set; yeast alcohol dehydrogenase and beef liver catalase from Boehringer. 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 100 μmol of sodium L-aspartate (pH 7.4), 2 μmol of $MgCl_2$, 100 μmol of Tris · HCl (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 [6] 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.

Denaturation

Aspartase was denatured by incubation of the enzyme in a solution of 4 M guanidine-HCl, which contained 25 mM potassium phosphate, pH 6.8, 50 mM KCl, 2.5 mM 2-mercaptoethanol and 0.5 mM EDTA for 30 min to 2 h at room temperature. Enzyme-guanidine-HCl solutions were prepared by diluting 1 : 1 a solution of the native enzyme into 8 M guanidine-HCl.

Renaturation

For determination of the activity regain, the enzyme denatured in 4 M guani-

dine-HCl was renatured by a rapid 100 : 1 dilution of the above mixture into potassium phosphate-KCl buffer (50 mM potassium phosphate, pH 6.8, 0.1 M KCl, 5 mM 2-mercaptoethanol and 1 mM EDTA) at 25°C, unless otherwise specified.

ORD measurements

ORD measurements were carried out in a JASCO spectropolarimeter, model ORD/UV-5 with either 10 or 2 mm light path cells at 25°C. Mean residue rotations were calculated assuming that the mean residue weight is 128 based on the amino acid composition [1]. Protein concentrations ranged from 0.1 to 2 mg per ml of solutions. Enzyme denatured in guanidine-HCl was renatured by diluting 0.1 ml of the denatured enzyme into 1.0 ml of 10 mM potassium phosphate, pH 7.4, containing 1 mM dithiothreitol and 1 mM EDTA. Changes in optical rotation as a function of time were measured at 233 nm within 1 min after the dilution of the enzyme. The unit of mean residue rotation was expressed as degrees square centimeter per decimol.

Electron microscopy

The sample solutions were prepared by diluting the stock enzyme solution with potassium phosphate-KCl buffer to a final concentration of 20 μg/ml. The buffer mixture was prepared by using double glass-distilled water. The enzyme molecules were negatively stained as follows. A drop of the sample solution was applied to a carbon-coated and ionized collodion grid (400 mesh). The excess liquid was removed with a piece of filter paper and immediately a drop of 2% potassium phosphotungstate (pH 7.2) or 2% uranyl acetate (pH 4.2) was added and then withdrawn with a piece of filter paper. The grid was dried at room temperature. 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. Protein concentration was determined according to the method of Lowry et al. [7], except that aspartase concentration was determined by the use of $E^{1\%}_{1\,cm}$ value of 5.9 at 280 nm as determined by a preliminary heating of a desalted enzyme solution at 80°C followed by heating at 110°C until a constant weight was attained.

Results

Reversible denaturation

Aspartase denatured in 4 M guanidine-HCl restored the ordered structure by simple dilution followed by regain of the activity. The native enzyme displayed a marked negative Cotton effect in the ORD curve with a minimum trough at 233 ± 1 nm. Mean residue rotation at this wavelength, $[m]_{233}$ was −4015. The dispersion curve for the denatured enzyme exhibited an optical activity as low as −1308. The dispersion curve for the renatured enzyme exhibited a close

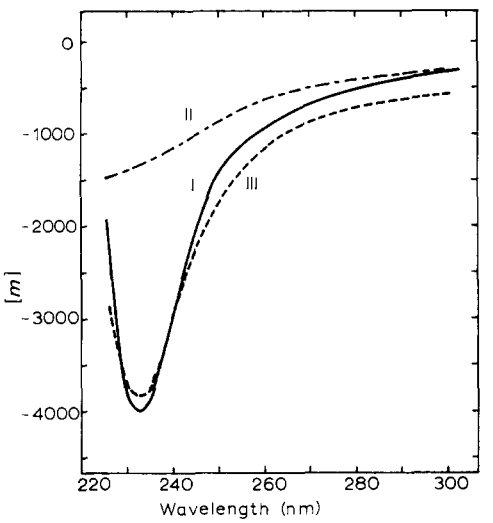


Fig. 1. ORD spectra for native, denatured and renatured enzyme species. Solutions of native (1.65 mg protein/ml), denatured in 4 M guanidine-HCl (1.65 mg protein/ml) and renatured (0.15 mg protein/ml) enzyme species were prepared as described in Materials and Methods. ORD spectrum for denatured enzyme was measured 1 h after addition of the denaturant and that for renatured enzyme was measured 30 min after dilution. I, native enzyme; II, denatured enzyme; III, renatured enzyme.

similarity to that of the native enzyme with a $[m]_{233}$ value of −3840, indicating that more than 90% of the ordered structure was restored. A small discrepancy observed in the ORD curves of the native and renatured enzymes beyond 240 nm may be ascribed to a minor conformational difference of the two molecular species. These results are shown in Fig. 1.

Simultaneous determinations of the restoration of ORD at 233 nm and of the activity revealed that the backbone structure of the enzyme polypeptides is formed in 1 min, while the activity regain needed almost 1 h to reach a maximal level.

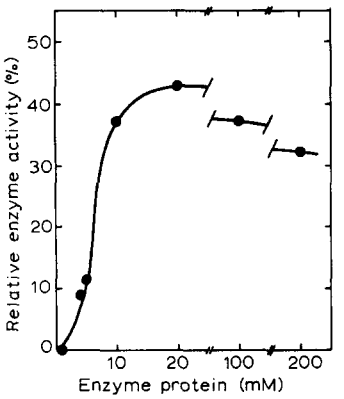
The molecular weights of the native, denatured and renatured enzyme species were estimated by gel permeation chromatography on a calibrated Sepharose 6B column (0.6 × 90 cm) in the presence of 4 M guanidine-HCl or in its absence *. The following proteins were selected as the standards (molecular weight given in parentheses): cytochrome *c* (12 400), myoglobin (17 800), chymotrypsinogen A (25 000), ovalbumin (45 000), bovine serum albumin (67 000), alcohol dehydrogenase (148 000) and catalase (240 000). The results showed that the tetrameric enzyme molecule reversibly dissociates into monomeric subunits in 4 M guanidine-HCl **.

Effects of environmental factors on reactivation

Reactivation of the enzyme denatured in 4 M guanidine-HCl was examined under various conditions. When the renaturation was carried out at various tem-

* 5 mM 2-mercaptoethanol was included throughout the column chromatography.

** Results of the molecular weight determination by sedimentation equilibrium method will be reported in a succeeding paper.



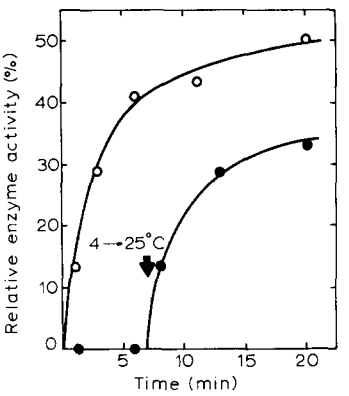


Fig. 2. Effects of protein concentration on reactivation. The enzyme at designated concentrations was denatured in 4 M guanidine-HCl and 101-fold diluted at 25° C into potassium phosphate-KCl buffer. The residual activity was determined at various time intervals by using aliquots of the above mixture under the standard assay conditions. The data in the figure represent the results of activity regain in 30 min.

Fig. 3. Effects of temperature shift-up on reactivation. The experimental conditions were the same as those described in Fig. 2, except that a 51-fold dilution of the denatured enzyme (2 mg protein/ml) was carried out at 4° C, followed by a shift-up to 25° C at the arrow (●———●), or at 25° C (○———○).

perature, the most efficient reactivation was attained at a temperature between 25—40° C. As the enzyme concentration increased, the optimum temperature for the reactivation shifted towards the lower one. When the temperature of renaturation was shifted up from 0—20° C to 25—40° C, a rapid increase in the rate of reactivation was observed (Fig. 3), indicating that some temperature-dependent process is involved as a rate-determining step in the reactivation processes.

When the reactivation was carried out at various pH values, both the initial rate and extent of reactivation were the highest at the neutral pH and little degree of reactivation was attained at the acidic or alkaline pH, presumably due to charge interaction of the polypeptide chains.

The effect of protein concentration on reactivation was examined. The results are shown in Fig. 2, which indicate that the degree of reactivation is a function of the protein concentration up to 20 nM, and thereafter it gradually decreased as the protein concentration increased. When the protein concentration was as high as 0.2—1.5 μM, the degree of reactivation markedly decreased, as the temperature of renaturation increased. When the denatured enzyme was diluted at high protein concentrations, the enzyme subunits readily formed inactive and turbid aggregates.

Effect of various compounds on reactivation

Effects of various compounds on reactivation were examined. The results are shown in Table I. The reactivation was somewhat inhibited under high salt concentration and divalent metal ions and L-aspartate * were without appreciable effect. In contrast, pyridoxal phosphate, which showed no influence on the

* Although L-aspartate at concentrations as high as 0.1—0.3 M occasionally accelerated the rate of activity regain to a considerable degree, the results were not reproducible and the reason for it remains to be elucidated.

TABLE I

EFFECTS OF VARIOUS COMPOUNDS ON REACTIVATION

The enzyme (1.9 mg/ml) was denatured in 4 M guanidine-HCl as described in Materials and Methods. 1 h later, the denatured enzyme was diluted 101-fold at 25°C with a solution which contained 10 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol and each compound as indicated. The restored activity was determined by using small aliquots of each mixture under the standard assay conditions. The following data represent the activity regain in 20 min.

Addition (mM)		Activity regained (%) *
None		41.0 (100)
KCl	(10)	41.0 (100)
	(100)	35.3 (86)
NaCl	(100)	32.4 (79)
NH_4Cl	(100)	35.0 (86)
$(NH_4)_2SO_4$	(100)	26.5 (65)
$MgCl_2$	(10)	38.0 (93)
Na L-aspartate	(1)	35.0 (86)
Pyridoxal phosphate	(1)	0 (0)

* Percent activity relative to the native enzyme activity.

native enzyme activity exhibited a complete cessation of the reactivation. Furthermore, effects of heterologous proteins on the reactivation were examined. When a 101-fold dilution of the denatured enzyme (1.0 mg/ml) was carried out with a buffer mixture, which contained bovine serum albumin (0.03 mg/ml) or *E. coli* sonic extract (0.3 mg/ml), the activity regain proceeded as efficiently as in the absence of these foreign proteins without an appreciable influence.

Effects of temperature shift-up on renaturation

As described earlier, the restoration of the secondary structure is completed within 1 min, whereas only a few percent of the activity are regained in this time period. To examine further the correlation between the reactivation and the restoration of the quaternary structure, the enzyme activity and the electron micrographic images of the enzyme molecules were simultaneously determined by using the temperature shift-up method. The enzyme denatured in 4 M guanidine-HCl was first diluted 101-fold at 4°C, and then the temperature of renaturation was shifted up to 25°C. As a control experiment, another aliquot of the denatured enzyme was renatured at 25°C. The results of activity regain are shown in Fig. 3. At 4°C the activity regain was not detected at all up to 6 min, while a rapid regain of the activity was initiated as soon as the temperature was shifted up to 25°C. When the renaturation was carried out at 25°C, the reactivation proceeded more efficiently.

The sampling for electron micrographs was carried out by using small aliquots of the above solutions at designated time intervals. The electron micrograph images of the native enzyme molecules negatively stained by phosphotungstate at pH 7.2 appeared as small particles of 75—100 Å in diameter, composed of 4 or several subunits. These images are compatible with our previous conclusion that the enzyme is composed of four subunits of seemingly identical molecular weight [1]. The use of uranyl acetate for negative staining resulted in a partial distortion of the subunit arrangement presumably due to the treatment effect under acidic conditions. Nevertheless, the staining of the sam-

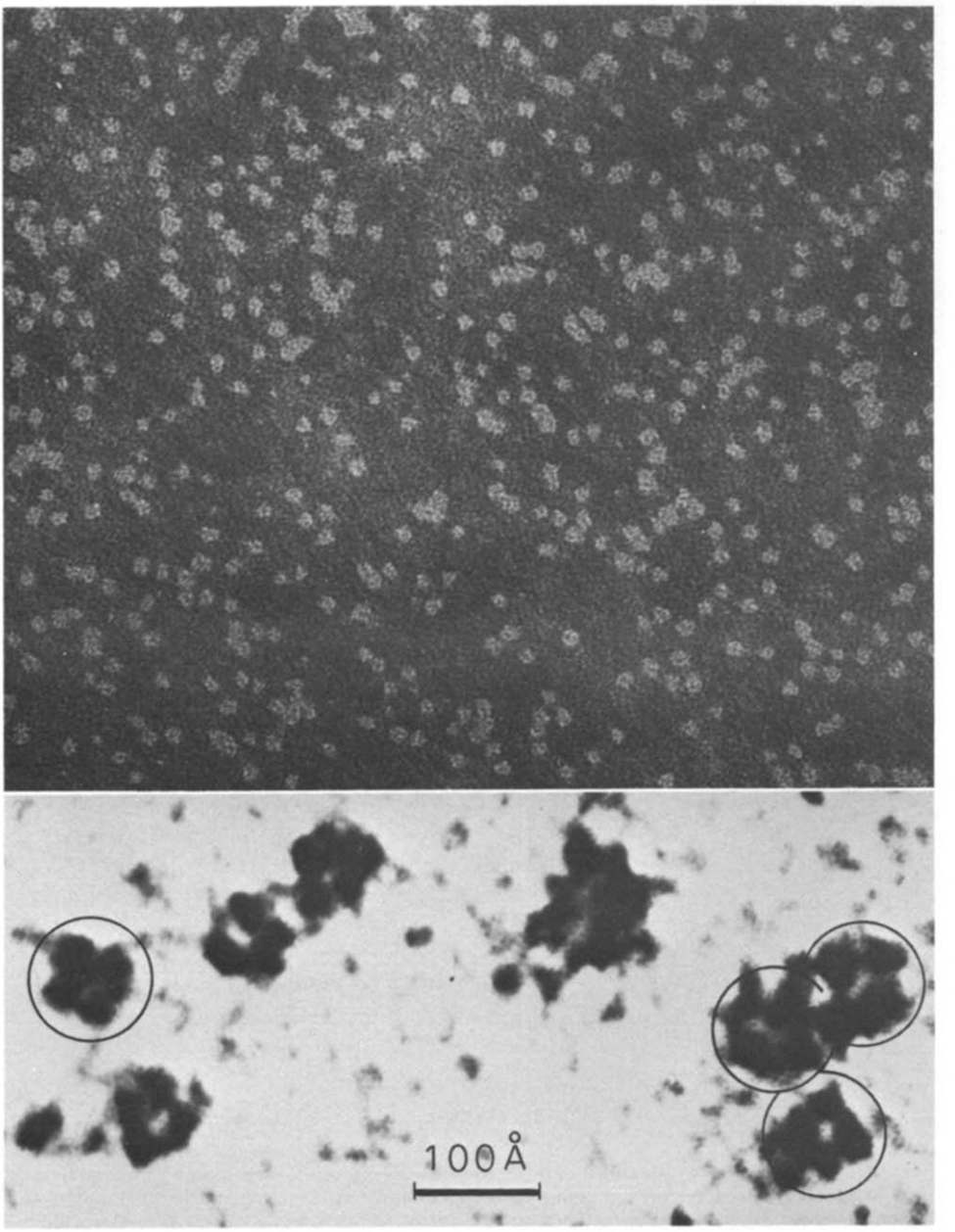


Fig. 4. Electron micrographs of the native enzyme. The enzyme molecules were negatively stained by phosphotungstate according to the method described in Materials and Methods. The final protein concentration was 20 μg/ml. The circles indicate typical tetrameric structure. Magnification of upper photograph: × 240 000.

ples, (A to F, in Fig. 5) was carried out with uranyl acetate in order to obtain a distinct contrast of the images along the course of reversible denaturation of the enzyme molecules. The results are shown in Fig. 5. In contrast to the images of the native enzyme (A), the enzyme denatured in 4 M guanidine-HCl (B) was observed as an amorphous entity. When the renaturation was carried

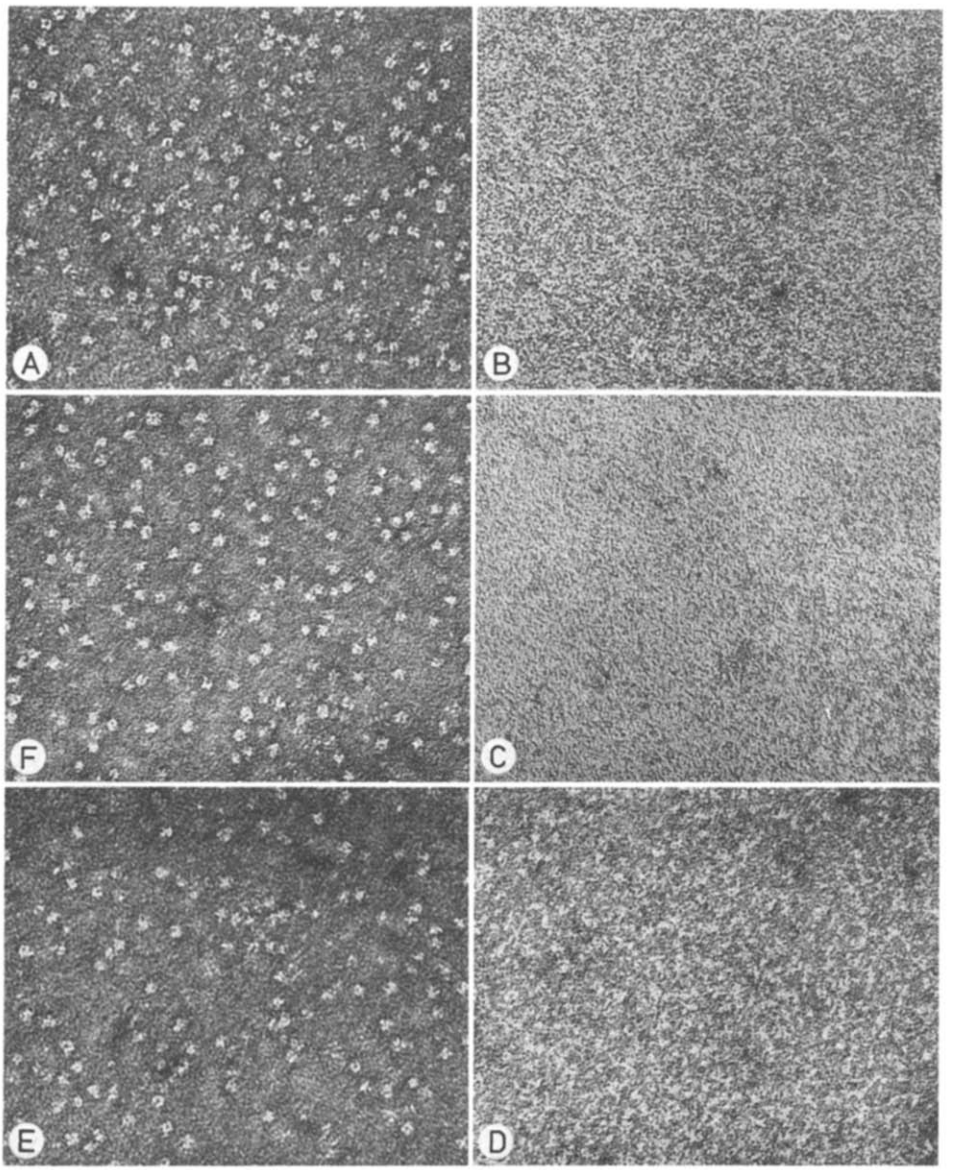


Fig. 5. Electron micrographs of aspartase during reversible denaturation. The enzyme molecules were negatively stained by uranyl acetate according to the method described in Materials and Methods. The final protein concentration was 20 μg/ml. A, native enzyme; B, enzyme denatured in 4 M guanidine-HCl at 25°C for 30 min; C, denatured enzyme (B) was renatured at 4°C and the sampling was made 1 min after a 51-fold dilution; D, the sampling was made 6 min after the dilution at 4°C; E, the sampling was made 13 min after a temperature shift-up from 4°C (D) to 25°C following renaturation for 7 min at 4°C; F, denatured enzyme (B) was renatured at 25°C and the sampling was made after 10 min. All samplings were made by using small aliquots of the enzyme solutions in Fig. 3. Magnification × 170 000.

out at 4°C, the subunit assembly did not proceed to an appreciable extent up to 6 min (C and D). When the temperature of renaturation was shifted up to 25°C, the molecular assembly rapidly proceeded, together with the appearance of the activity (E). The light (unstained) areas in B-D do not seem to be ascriba-

ble to overcrowded protein molecules, since the protein concentration in D was the same as that in E. When the renaturation was carried out at 25°C, the quaternary structure of the enzyme was readily completed, concomitant with the activity regain (F). These results are compatible with the possibility that the molecular assembly of the enzyme subunits is an essential prerequisite for appearance of the activity.

Discussion

The activity regain of aspartase denatured in 4 M guanidine-HCl followed by dilution was markedly influenced by various environmental factors. Restoration of the activity closely associated with refolding and reassociation of the enzyme polypeptides proceeded most efficiently under near physiological conditions, except that the optimum concentration of the enzyme protein appeared to be rather lower than that assumed to be present in the bacterial cells. Taking our enzyme purification data into consideration, i.e., about 50 mg of a homogenous enzyme preparation from 50—100 wet cells with an overall yield of 30% [1], the intracellular concentration of aspartase in *E. coli* cells appears to be in the range of 8.3—16.5 μM (1.7—3.3 mg protein/ml). In contrast, the optimal concentration of the enzyme protein for the reactivation was found to be lower than 0.1 μM at 25°C. Although no information is available at present to explain this discrepancy, it is plausible to speculate that some ligand(s) or unknown environmental factor(s) contribute to accelerate the rate and to improve the extent of the acquisition of enzyme activity in the living cells.

It is of interest that pyridoxal phosphate, which has no influence on the native enzyme activity completely abolished the reactivation. Our preliminary experiments showed that not only pyridoxal phosphate, but also several other compounds such as pyridoxamine or nicotinic acid, which have no aldehyde group, were equally effective in preventing the reactivation. The mechanism for the effect of these compounds is, therefore, still unknown.

Like a number of other enzymes [8—10], upon dilution of the denatured aspartase, a major regain of the secondary structure occurred rapidly within 1 min, while the restoration of the activity proceeded much more slowly. The parallel restoration of the activity and quaternary structure as revealed by electron microscopy upon the temperature shift-up indicates that the molecular assembly of the enzyme subunits is an essential prerequisite for acquisition of the activity. However, the following preliminary experiment suggest that the subunit assembly is not adequate for acquisition of the activity. When the denatured enzyme was renatured at pH 4.5, a near native oligomeric structure was observed under electron microscope without an appearance of the activity. Jaenicke described in his study on the pH-dependent reversible denaturation of lactic dehydrogenase that the second-order kinetics of reactivation process prove reassociation to be the rate-limiting step in the process of reactivation and that the enzyme subunit does not represent the enzymatically active unit [11]. In the present case, however, the overall kinetics of reactivation appear to be rather complex and the possibility that either conformational alignment (maturation) of monomeric or oligomeric enzyme molecules is the rate-limiting step, still remains to be elucidated.

The present investigation indicates that electron microscopy is an excellent means for study of kinetic processes of the enzyme renaturation. Among several methods available for this purpose, the sedimentation analysis and light scattering measurement are most frequently employed. The former method, however, is not suitable for determination of fairly rapid kinetic processes. The latter method is useful for analysis of rate processes as rapid as one to several min. In fact, Teipel accomplished an elaborate study on kinetics of refolding, subunit reassociation and reactivation of aldolase mainly by means of Rayleigh light scattering [10]. In general, however, the light scattering measurements have some limitations in terms of sensitivity at reasonably low protein concentrations, as Frieden pointed out [12]. In contrast, electron microscopic inspection requires as low as 10—20 μg/ml protein concentration and only a few microliters for sampling. In addition, another advantage is that electron micrographs provide direct features of the molecular structure as long as careful sample preparation and scanning over wide areas are made as described in the pertinent reviews of Haschemeyer and his coworker [13,14]. In contrast, sedimentation analyses and light scattering measurements provide only average values, unless the rate of equilibrium is quite slow. Elucidation of the precise molecular structure including the number, shape and arrangement of enzyme subunits as well as the presence of intermediary molecular species, such as dimer or trimer in the process of renaturation are under investigation.

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