

Reversible Denaturation and Reactivation of Aspartase from *Hafnia alvei*

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Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible conversion of L-aspartate to fumarate and ammonium ion.¹ Aspartase is a catabolic enzyme in both bacteria and plants, but the reaction is reversible and favors aspartate formation with $\Delta G^0 = 3.2$ kcal/mol for aspartate deamination.² The aspartase from *Hafnia alvei*, that is important for aspartate synthesis in industrial purpose, is a tetramer composed of four apparently identical subunits of molecular weight 55,000 Da.³ Although many aspects of *H. alvei* aspartase reactions are well studied,^{4,6} the role of subunit-subunit interaction in this oligomeric protein is not known yet. As the relationship between subunit assembly and function are very important for elucidation of the protein functions at molecular basis,^{7,8} we have studied the activity-subunit interaction relationship and effect of environmental factors, such as temperature and pH, on reversible denaturation and reactivation of the *H. alvei* aspartase.

As shown in Figure 1(a) at < 1 M Gu-HCl, the activity was gradually decreased suggesting the existence of the native tetrameric form with denaturation intermediates such as dimeric and monomeric forms of the enzyme. At ≥ 1 M Gu-HCl, the enzyme completely lost the activity, suggesting that the enzyme structure was completely denatured. To reveal whether denaturation is reversible or not, the renaturation of the enzyme was investigated. As in Figure 1(b), the 100% activity recovery was observed when the enzyme was renatured by dilution at < 1 M Gu-HCl. In contrast, the renaturation yield at > 1 M Gu-HCl was 40%.

On a temperature scale (0-40 °C), the optimum temperature for reactivation was attained at 25 °C (Figure 2a). There was no reactivation between 0-10 °C, progressed with linearly increase at 10-25 °C, with a level off between 25-35 °C and completely stopped at 35 °C. The optimum pH for reactivation was 7.0 and little degree of reactivation was attained at the acidic or alkaline pH, presumably due to charge interaction of polypeptide chains (Figure 2b). When the temperature of renaturation was shifted from 4 to 25 °C, a rapid increase in the rate of reactivation was observed with ~45 min lag phase (Figure 3), reasoning need of time for enzyme to remove the Gu-HCl by dilution. These results attributed a temperature-dependent process, as a rate-determining step in the reactivation of *H. alvei* aspartase. This indicates that the enzyme reached to restructured form during renaturation process and reactivated at the suitable temperature.

Many cases are known, actually in which enzymes are not

able to be readily reactivated simply by removal of the causes due to partial or serious denaturation of the polypeptides. However, the fact that the native confirmation of most proteins is uniquely determined by the amino acid sequence in the protein,⁹ any protein to which this generalization applies should always be able to recover its native conformation, provided that its primary sequence remains intact. Sachiko *et al.* identified an active dimeric form of aspartase as a denaturation intermediate of *E. coli*.¹⁰ We have proposed the intermediate forms of *H. alvei* aspartase during reversible denaturation (Scheme 1). As at ≥ 1 M Gu-HCl, aspartase dissociates to monomers and largely unfolds,¹⁰ in the present investigation, the denaturation process at < 1 M Gu-HCl can be explained in terms of a two-state transition. The 100% renaturation below 1 M Gu-HCl known out

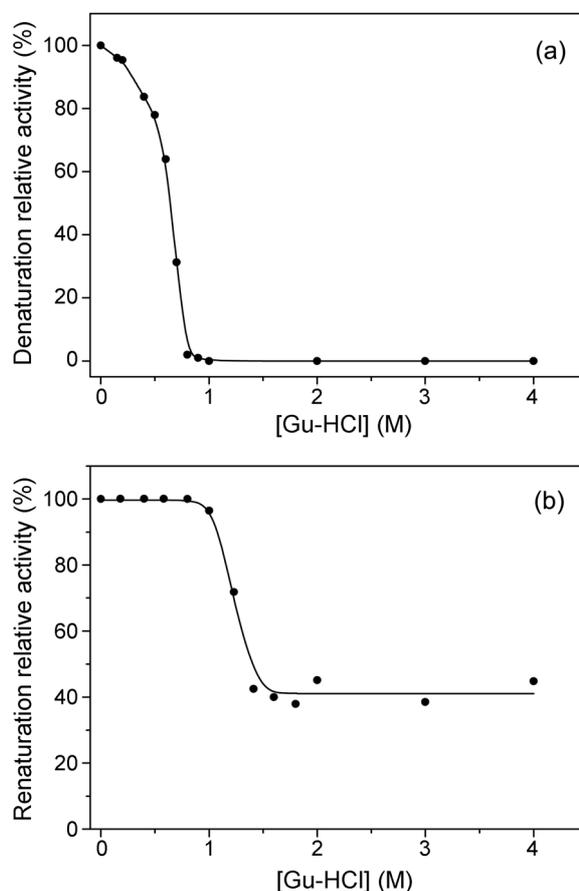


Figure 1. (a) Denaturation and (b) Renaturation activity of *Hafnia alvei* aspartase at various concentrations of Gu-HCl.

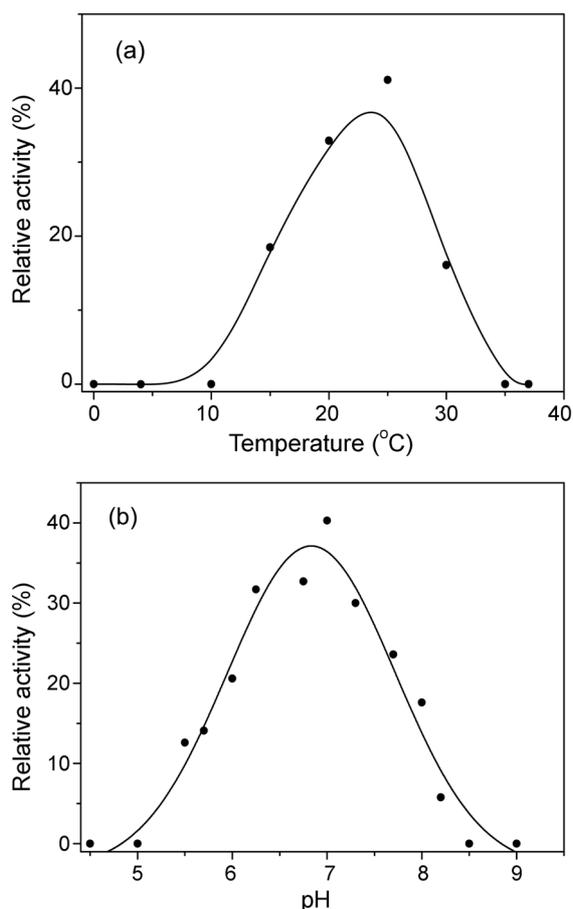


Figure 2. (a) Temperature effect and (b) pH effect on reactivation of denatured enzyme.

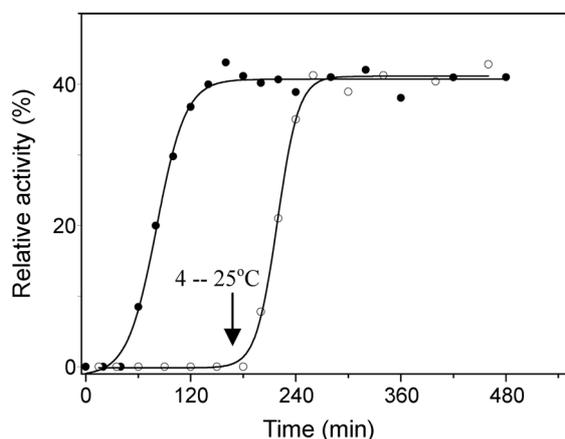
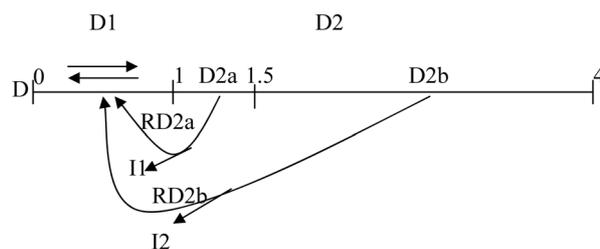


Figure 3. Effect of temperature shift-up on reactivation of aspartase. The renaturation was carried out at 25 °C (●) and at 4 °C (○) (~120 min) followed by a shift to 25 °C.

the possibility of presence of inactive dimer. In contrast, both active and inactive dimers were observed in denaturation of *E. coli* aspartase.¹⁰ In this study, the results suggest that the dissociation process from native tetramer to dimer is reversible but the dissociation process from dimer to monomer is not reversible.

Even though the oligomeric state of aspartase between 1-4



Scheme 1. The denaturation (D) and renaturation (R) processes of the *Hafnia alvei* aspartase. 0, tetramer; D1, dimer; D2 monomer; D2a and D2b, differentially unfolded molecules depend upon Gu-HCl concentration; RD2a, renaturation state between D2a-D1; RD2b, renaturation state between D2b-D1; I1 and I2, incorrectly folded molecules during renaturation. The numbers on line represents concentration of Gu-HCl during de/renaturation process.

M Gu-HCl (*i.e.* monomer) is same, the renaturation yield between 1-1.5 M (RD2a) and 1.5-4 M (RD2b) is different with formation of incorrectly folded molecules more in later (I2). Tokushige *et al.* observed that inactive molecule renatured at 25 °C was proved to recover the native secondary structure and to have dimeric or monomeric structure.¹¹ In *H. alve* aspartase the temperature sensitive RD2b state is different from RD2a as % renaturation is more in later at 30 °C. From these results the dissociation process from tetramer (T) to dimer (D1) was almost completely reversible but not from tetramer (T) to monomer (D2 or D2a and D2b) in *H. alve* aspartase.

Experimental Section

Aspartase was purified from *H. alve* as described previously.³ Briefly, aspartase was obtained from a combination of diethylaminoethyl cellulose (DEAE cellulose), Red Agarose, and Sepharose 6B chromatography. The catalytic function of the purified enzyme which stored at -70 °C until use remained stable for at least one month at 4 °C without appreciable loss of enzymatic activity. Protein concentration was determined by the Bradford assay following the instruction manual (Bio-Rad, Hercules, USA).¹²

Denaturation was performed in phosphate-KCl buffer (100 mM potassium phosphate, pH 7.0, 1 mM β ME, 1 mM EDTA, and 0.1 M KCl) and various concentrations (0-4.0 M) of Gu-HCl at 25 °C for 30 min after addition of 0.5 mg aspartase. For determination of activity regain, the enzyme was renatured by a rapid 100:1 dilution of the above mixture into phosphate-KCl buffer at 25 °C for 5 h, unless otherwise specified. For investigation of temperature effect on reactivation, aspartase was denatured in above conditions with 4.0 M Gu-HCl and renatured by 100 fold dilution with phosphate-KCl buffer at various temperature (0-40 °C) for 5 h. The effect of temperature shift-up on reactivation was studied at 4 °C for every 20 min and shift-up to 25 °C. The experimental conditions for pH effect on reactivation were same as above, except 100 fold dilution of denatured enzyme was carried out at various pH 4.5-9.0. The pH was maintained using the following buffers at 100 mM concentrations: MES, 5.5-6.5; HEPES, 7.0-8.0 and CHES, 8.5-9.0.

All kinetic assay data were collected using Shimadzu UV-2101PC Diode-Array spectrophotometer in a 1 mL cuvette with 1 cm light path length. The temperature was maintained using circulating water in thermospacers of the cell compartment. The progress of reaction was monitored by measuring the formation of fumarate with increase in absorbance at 240 nm ($\epsilon_{240} = 2,255 \text{ M}^{-1} \text{ cm}^{-1}$).¹³ The aspartate concentration was corrected for complexation with divalent metal using the following dissociation constants obtained at 0.1 mM ionic strength; Mg-aspartate 4 mM. One unit of enzyme activity was defined as the amount of 1 μM of fumarate formed per minute under the standard assay conditions.

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