Is Stability Prediction Possible for Protein Drugs? Denaturation Kinetics of β-Galactosidase in Solution

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Denaturation and aggregation kinetics of *Aspergillus oryzae* β-galactosidase in solution were studied in order to determine whether the stability of protein drugs can be predicted. Denaturation of β-galactosidase, monitored by measuring enzyme activity, conformed to first-order kinetics, whereas aggregation of the denatured form, monitored by high performance size exclusion chromatography, showed a reaction order higher than 1. Denaturation of β-galactosidase was irreversible and exhibited a biphasic kinetic pattern which could be explained by assuming that two isoenzymes denatured irreversibly at different rates. Linear Arrhenius plots were obtained for the estimated rate constants, and $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were estimated according to the Eyring equation. The estimated $\Delta H^{\ddagger}$ was much larger than $\Delta H^{\ddagger}$ observed in usual chemical reactions. The present study suggests that the denaturation of protein drugs can be analyzed by the Eyring equation in the same manner as chemical degradation, contradicting the general consensus that accelerated testing can not be used to predict the stability of protein formulations.

KEY WORDS: protein drug; denaturation; aggregation; kinetics; Eyring equation.

INTRODUCTION

Accelerated stability testing is generally considered to be invalid when applied to protein preparations intended for therapeutic use. The reason is that protein degradation is thought not to conform to the Arrhenius relationship in the same manner, as does the chemical degradation of small molecules.

Protein drugs can undergo chemical and physical degradation via various pathways (1,2). The Arrhenius behavior of chemical degradation, that is, covalent degradation, has been reported for several small peptides and proteins. Deamidation of an asparaginyl residue in hexapeptides (3,4) and calcitonin (5), and hydrolysis of gonadorelin (6), triptorelin (6), cholecystokinin-B reactor antagonist (7) and gonadorelin releasing hormone (8) all exhibit linear Arrhenius plots with an activation energy of 60 to 140 kJ/mol. Linear Arrhenius plots have also been reported for the degradation of various protein drugs in solution and in the solid state which degradation mechanisms were not elucidated. Examples include inactivation of horse serum cholinesterase (9), pancreatic lipase (10), human interferon-β (11), urokinase (12), α-chymotrypsin (13,14), bromelain (13) and kalikrein (13,14).

In contrast to chemical degradation, little has been reported on the Arrhenius behavior of protein denaturation, that is, noncovalent degradation involving conformational changes. Protein denaturation has been analyzed mainly by equilibrium theory from the viewpoint of thermodynamics. Limited information on denaturation kinetics has led to the conclusion that stability prediction of protein formulations by accelerated testing is impossible. It is of great interest to elucidate the denaturation kinetics of protein drugs from the viewpoint of stability prediction of protein formulations, since denaturation is one of the main degradation pathways for protein formulations, resulting in aggregation and precipitation (15–18). The present paper describes the kinetics of denaturation and aggregation of *Aspergillus oryzae* β-galactosidase in solution, and discusses the possibility of stability prediction for protein drugs based on kinetic studies.

MATERIALS AND METHODS

Materials

β-Galactosidase from *Aspergillus oryzae* was purchased from Toyobo Co. (Osaka) and used without further purification. 2-Nitrophenyl-β-D-galactopyranoside was purchased from Wako Pure Chemical Industry Co. (Osaka).

Kinetic Study on Denaturation of β-Galactosidase

β-Galactosidase was dissolved in a 50 mM phosphate buffer (pH 7.4) at protein concentrations of 0.05 to 2.0 mg/mL. The sample solutions were stored at constant temperatures between 45 and 55°C and removed at appropriate intervals for high performance size exclusion chromatography (HPSEC) and enzyme activity measurement.

The stored solutions were diluted with distilled water to yield 1 μg/mL protein solutions, and the activity of β-galactosidase was determined by using 2-nitrophenyl-β-D-galactopyranoside as the substrate, as described previously (19).

High Performance Size Exclusion Chromatography

The sample solutions containing 0.5 to 2.0 mg/mL of protein stored at various temperatures were diluted with 200 mM of phosphate buffer (pH 6.2) to yield 0.1 mg/mL protein solutions. The sample solutions of 0.05 and 0.1 mg/mL were diluted to 25 and 50 μg/ml, respectively. The solutions were injected through a 20-μl loop to a column (Tosoh G3000SW, 30 cm × 7.5 mm, Tokyo) maintained at 30°C after filtration (0.45 μm). The mobile phase was a 200 mM phosphate buffer (pH 6.2) delivered at a rate of 1 ml/min. The column eluate was monitored at 230 nm (UV detector, Model L4000, Hitachi Ltd., Tokyo).

Analog signals from the photometer were amplified (Type 3131 amplifier, Yokogawa), and collected by an A/D converter with 12-bit resolution (EC-2325, Elmec). The low-pass cut-off was 10 Hz and the sampling interval was 40 ms. The digitized data point readings were transferred to disk files (PC-9801, NEC, Tokyo) for manipulation.

Calculation of Kinetic Parameters for Aggregation of β-Galactosidase

The HPSEC chromatogram of the β-galactosidase sample solutions was divided into three portions based on reten-

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tion volume (4.8–5.9, 5.9–6.6, and 6.6–8.3 mL). The area of each portion was calculated by integrating the digitized absorbance data, and the fraction of each portion was calculated from the ratio of each area to the total area.

The time course of each fraction of the HPSEC chromatogram was analyzed kinetically according to a consecutive reaction model, which assumes that native protein (N) is converted to its denatured form (D) at a first-order rate constant, k_1', then the denatured form is converted to aggregate (A) at a first-order rate constant, k_2', as shown in scheme 1. The rate constants, k_1' and k_2', were estimated by nonlinear regression analysis.

$$N \xrightarrow{k_1'} D \xrightarrow{k_2'} A$$

Scheme 1

Calculation of Kinetic Parameters for Denaturation of β-Galactosidase

The enzyme activity of the β-galactosidase solutions, which was determined as a function of time as described above, was analyzed using the model described in scheme 2, where two isoenzymes (N_1 and N_2) denature irreversibly at different rates.

$$N_1 \xrightarrow{k_1} D_1$$

$$N_2 \xrightarrow{k_2} D_2$$

Scheme 2

The total activity (A) of the mixture N_1 and N_2 changes with time according to equation 1.

$$A = A_1 \exp(-k_1 t) + (1 - A_1) \exp(-k_2 t) \qquad (1)$$

where A is the ratio of the activity to the initial activity, and A_1 is the ratio of the activity of N_1 to the total activity of the mixture. If the two native isoenzymes have the same specific activity, then A_1 represents the weight ratio of N_1.

RESULTS AND DISCUSSION

Aggregation Kinetics of β-Galactosidase in Solution

Figure 1 shows the HPSEC chromatograms obtained from the 0.05 and 2.0 mg/mL solutions (pH 7.4) of β-galactosidase stored at 55°C for various lengths of time. Before storage, the β-galactosidase solution yielded a peak representing intact β-galactosidase at a retention volume of 7.2 mL. As storage time increased, a larger peak was observed at a retention volume near the void (5.3 mL), while the main peak arising from intact β-galactosidase became smaller. For the 0.05 mg/mL solution, the increase in the leading peak during storage was less than that for the 2.0 mg/mL solution, while the decrease in the main peak was similar for both solutions. At the 0.05 mg/mL concentration, unresolved absorbance activity between the two peaks was larger in contrast to the smaller increase in the leading peak.

The chromatograms were divided into three portions based on retention volume (F_1:4.8–5.9 mL; F_2:5.9–6.6 mL, and F_3:6.6–8.3 mL), as shown in Fig. 1. We had previously found that F_1 was converted to F_2 in the presence of guanidine, and that the conversion needed no reductant. On the other hand, F_2 was formed when intact β-galactosidase (F_3) was dissolved in guanidine solution (16). It was suggested that β-galactosidase in aqueous solution was converted to the denatured form (F_2), from which aggregation products (F_1) were formed through a noncovalent interaction.

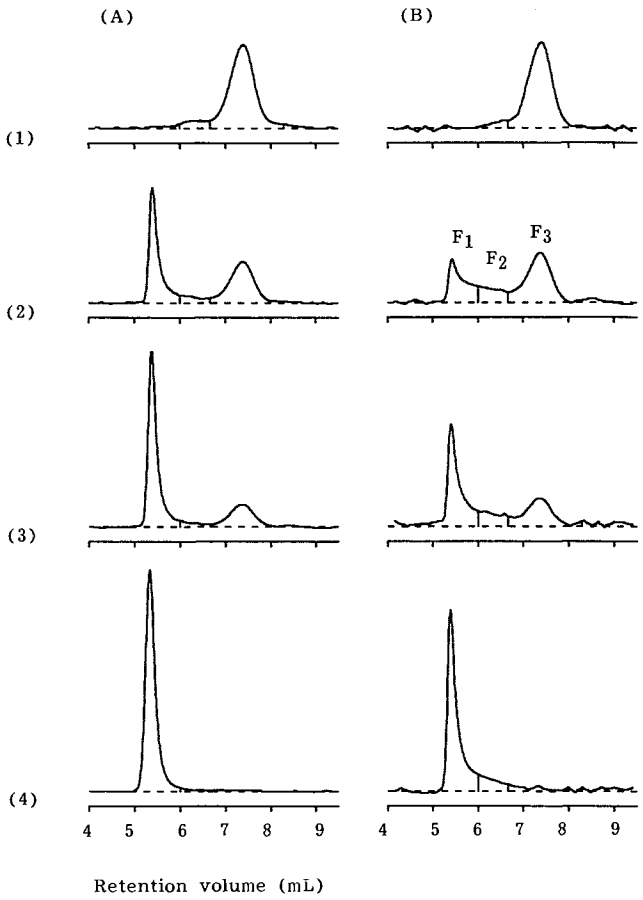

Fig. 1. High performance size exclusion chromatograms of the β-galactosidase solution stored at 55°C for 0 (1), 10 (2), 20 (3), and 75 min (4). Protein concentration: 2 mg/mL (A); 0.05 mg/mL (B).

All the HPSEC chromatograms obtained in the present study exhibited no significant change in the total absorbance activity with storage time. This indicates that no insoluble protein was formed during storage of the 0.05 to 2 mg/mL β-galactosidase solutions. The F_1, F_2 and F_3 fractions were calculated from the ratio of each area to the total area, and the time profiles of these fractions at concentrations of 2.0 and 0.05 mg/mL are shown in Figs. 2 and 3, respectively. These profiles support that F_1 is formed from F_3 via F_2. The solid lines in Figs. 2 and 3 were obtained by nonlinear regression analysis according to a consecutive reaction model, where both the conversion of F_3 to F_2 and that of F_2 to F_1 are first-order, as shown in scheme 1. Figure 4 shows the estimated first-order rate constants, k_1' and k_2', plotted against the protein concentration. The k_1' was independent of the concentration, whereas k_2' increased with increasing concentration. This indicates that first-order kinetics is capable of describing the transfer of F_3 to F_2, but not the transfer of

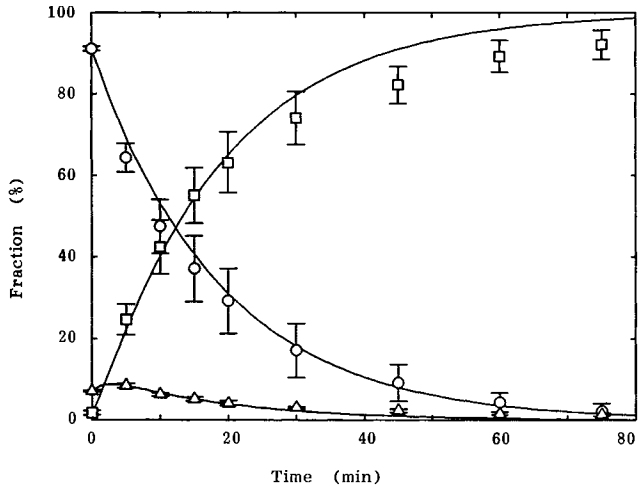

Fig. 2. Time courses of the fractions of F_3 (○), F_2 (△), and F_1 (□) for the β-galactosidase solution of 2 mg/mL stored at 55°C. Error bars: ±SD (n = 5).

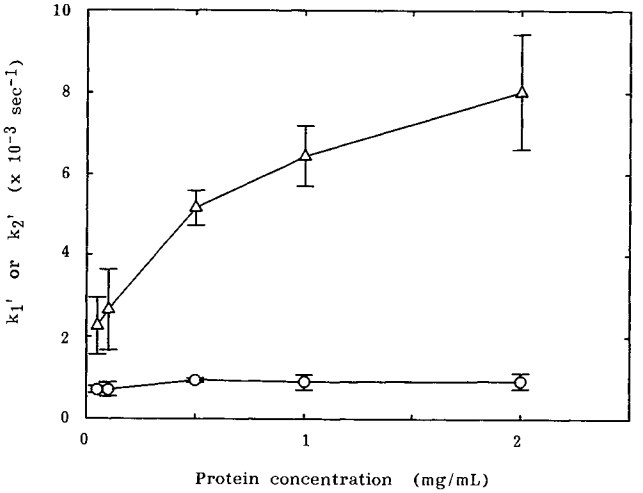

Fig. 4. Apparent first-order rate constants, k_1' (○) and k_2' (△), for the β-galactosidase solution stored at 55°C, as a function of protein concentration.

F_2 to F_1. This explains why the experimental data deviates from the lines calculated in the regression analysis where both processes are assumed to be first-order (Figs. 2 and 3). The observed reaction order of more than one suggests that conversion of F_2 to F_1 is an intermolecular reaction, and supports the mechanism previously suggested that polymolecular aggregation of β-galactosidase occurs via a denatured form.

Denaturation Kinetics of β-Galactosidase in Solution

Denaturation of β-galactosidase at concentrations of 2 and 0.05 mg/mL was monitored by measuring enzyme activity. Figure 5 shows the time course of the activity remaining and the fraction of F_3 at 55 and 45°C. The decrease in the activity was consistent with that in the fraction of F_3 at both concentrations and both temperatures. Furthermore, no significant difference was observed in the time course between the two different concentrations. These results confirm that native β-galactosidase (F_3) is converted to the denatured form (F_2) in a concentration-independent manner.

Figure 6 shows the time course of β-galactosidase denaturation at constant temperatures between 45 and 55°C. At lower temperatures, denaturation was clearly biphasic. The biphasic profiles can be analyzed by the general model proposed for thermal inactivation of enzymes (20, 21, 22):

$$N \rightleftarrows U \rightarrow I$$

where N is the native enzyme, U is the reversibly unfolded (denatured) inactive enzyme and I is the irreversibly inactivated enzyme. The first step is reversible and the enzymic activity should recover when the enzyme is cooled down. Enzyme activity recovery is prerequisite in order to apply this model to denaturation of β-galactosidase. Figure 7 shows the enzyme activity and the F_3 fraction of β-galactosidase solution stored at 45°C after a 15 minute-storage at

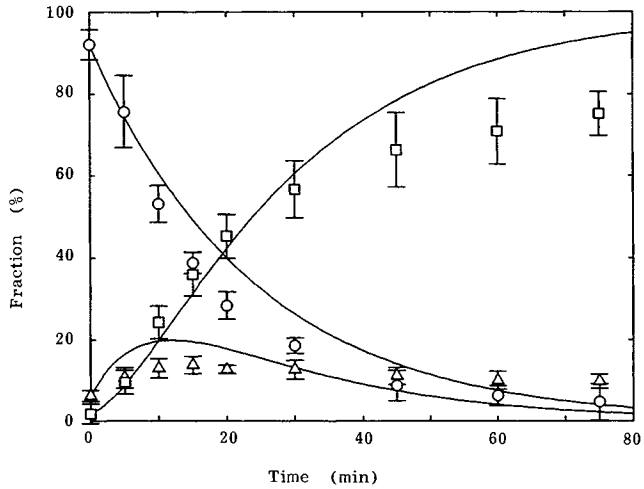

Fig. 3. Time courses of the fractions of F_3 (○), F_2 (△), and F_1 (□) for the β-galactosidase solution of 0.05 mg/mL stored at 55°C. Error bars: ± SD (n = 5).

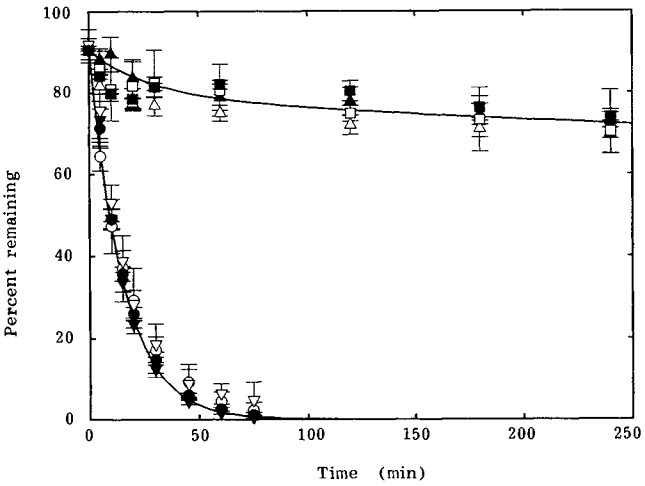

Fig. 5. Time courses of the enzyme activity remaining (●, ▼, ▲, ■) and the fraction of F_3 (○, ▽, △, □) for the β-galactosidase solution stored at 55 (○, ●, ▽, ▼) and 45°C (△, ▲, □, ■). Protein concentration: 2 mg/mL (○, ●, △, ▲); 0.05 mg/mL (▽, ▼, □, ■). Error bars: ±SD (n = 5).

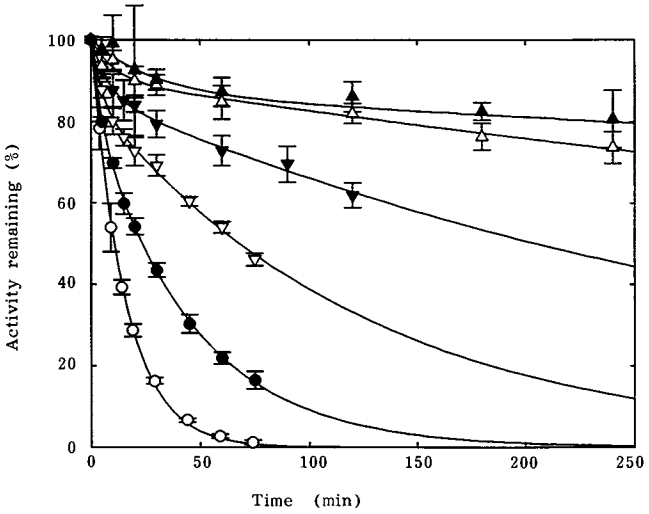

Fig. 6. Time courses of the enzyme activity remaining for the β-galactosidase solution of 2 mg/mL stored at 45 (▲), 47 (△), 49 (▼), 51 (▽), 53 (●) and 55°C (○). Error bars: ±SD (n = 5).

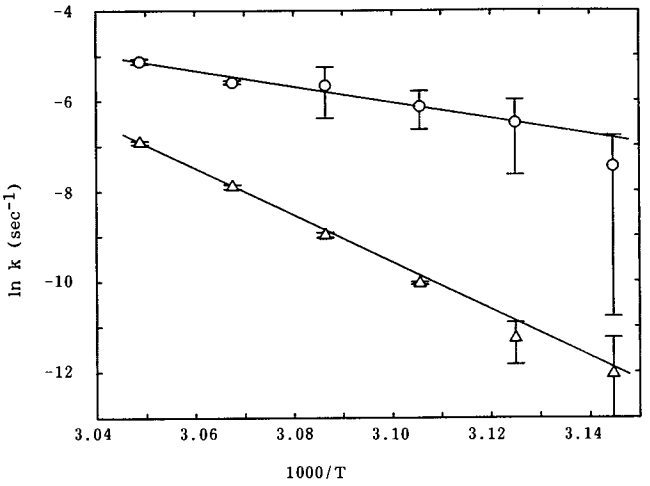

Fig. 8. Arrhenius plots for the denaturation rate constants at β-galactosidase estimated according to a model of two isoenzymes. ○ and △ represent the rate constants for the isoenzymes showing rapid and slower denaturation, respectively.

55°C. Both enzyme activity and the F_3 fraction did not recover after the temperature was lowered from 55°C to 45°C. This indicates that denaturation of β-galactosidase is irreversible, and therefore can not be analyzed by this model.

The biphasic pattern of irreversible denaturation can be explained by the model shown in scheme 2, where two isoenzymes denature at different rates. The rate constants (k_1 and k_2) and the ratio of the initial activity of N_1 (A_1) were estimated using equation 1. As shown in Fig. 8, linear Arrhenius plots were obtained for both of k_1 and k_2. There was no significant variation in the A_1 estimate as a function of temperature, as shown in Table 1. The solid lines in Fig. 6 were obtained by nonlinear regression analysis using equation 1, and those in Fig. 7 were calculated from the rate constants obtained separately at 55 and 45°C. The results suggest that the denaturation of β-galactosidase can be explained by the two-isoenzyme model.

The biphasic pattern of irreversible denaturation can also be explained by the consecutive reaction model shown in scheme 3, where one native enzyme (N_1) is converted to the denatured form (D) via an intermediate (N_2) having specific activity different from the native enzyme.

$$N_1 \xrightarrow{k_1} N_2 \xrightarrow{k_2} D$$

Scheme 3

The change in the total activity in the consecutive model can be described by equation 2,

$$A = \left(R_1 + \frac{R_2 k_1}{k_2 - k_1}\right) \exp(-k_1 t) - \left(\frac{R_2 k_1}{k_2 - k_1}\right) \exp(-k_2 t) \quad (2)$$

where R_1 and R_2 are the specific activities of N_1 and N_2, respectively. The pre-exponential term in equation 2, $R_1 + R_2k_1/(k_2 - k_1)$, should vary with temperature, whereas that in equation 1, A_1, is independent of temperature. This is useful to determine which model best describes the denaturation mechanism. In the present study, no significant change in the pre-exponential term was observed among the estimates obtained at different temperatures (Table 1), thus supporting the two-isoenzyme model (23).

The linear Arrhenius plots obtained (Fig. 8) may indicate the validity of the kinetic analysis for denaturation of β-galactosidase. The kinetic parameters, ΔH‡ and ΔS‡, at 45°C were estimated by nonlinear regression analysis ac-

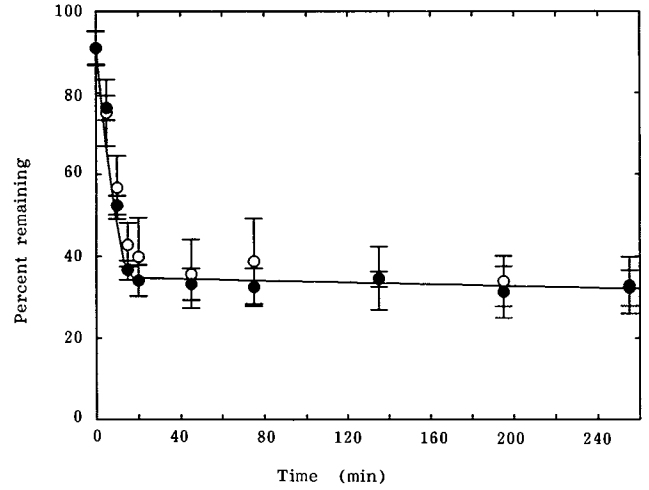

Fig. 7. Time courses of the enzyme activity remaining (●) and the fraction of F_3 (○) for the β-galactosidase solution of 2 mg/mL stored at 45 after a 15 minute-storage at 55°C. Error bars: ±SD (n = 5).

Table I. The Ratio of the Activity of N_1 to the Total Activity (A_1) Estimated by Two-Isoenzyme Model

Temperature (°C)	A_1	SD
45	13.1	3.1
47	11.4	4.0
49	14.1	2.1
51	15.8	0.9
53	16.0	1.5
55	9.0	8.7

cording to the Eyring equation. The transfer of N_1 to D_1 (k_1) exhibited a $\Delta H\ddagger$ of 149 kJ mole^{-1} and $\Delta S\ddagger$ of 176 J mole^{-1} deg^{-1}, and the transfer of N_2 to D_2 (k_2) indicated a $\Delta H\ddagger$ of 444 kJ mole^{-1} and $\Delta S\ddagger$ of 1.06 kJ mole^{-1} deg^{-1}. The estimates of activation energy were much larger than those commonly observed for chemical reactions.

CONCLUSION

The present kinetic data for denaturation and aggregation of β-galactosidase in solution, which were obtained by HPSEC and enzyme activity measurement, indicate that denaturation of β-galactosidase conforms to first-order kinetics, whereas the reaction order of aggregation of the denatured form is more than 1. Denaturation of β-galactosidase was irreversible and exhibited a biphasic kinetic pattern, which is assumed to result from two isoenzymes denaturing at different rates. The estimated rate constants provided linear Arrhenius plots, and the kinetic parameters, $\Delta H\ddagger$ and $\Delta S\ddagger$, estimated by the Eyring equation were reasonable compared to those already reported (20,24). These results indicate the validity of kinetic studies for analyzing the denaturation of β-galactosidase.

Protein denaturation usually exhibits a large activation energy for other proteins (20,24). Since the denaturation rate depends largely on temperature, it is usually difficult to obtain experimental data for judging the conformity of the denaturation rate to the Eyring equation. This has led to the conclusion that protein denaturation does not exhibit Arrhenius behavior, as does the chemical degradation of protein drugs. The present study, however, suggests that the denaturation of protein drugs can indeed be analyzed by the Eyring equation, and that stability prediction of protein formulations based on kinetic studies is possible in principle.

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