Stability of β-Galactosidase, a Model Protein Drug, Is Related to Water Mobility as Measured by ^{17}O Nuclear Magnetic Resonance (NMR)

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Received June 22, 1992; accepted July 21, 1992

The inactivation of freeze-dried β-galactosidase during storage was studied, focusing on the effect of water mobility as measured by the spin-lattice relaxation time, T_1, of water using ^{17}O NMR. Inactivation of β-galactosidase lyophilized from phosphate buffer solution was studied as a function of water content, which in turn affected the T_1 of water. An increase in the water content of freeze-dried β-galactosidase brought about an increase in the T_1 of water, as well as a rise in pH. For the freeze-dried enzyme with sufficient water content to be dissolved, the inactivation rate was related to the T_1 of water rather than to the pH change. It is suggested that as the water content increases, the mobility of water around the enzyme increases, resulting in enhanced enzyme inactivation. The freeze-dried samples with limited moisture showed inactivation rates faster than those expected from the pH and water mobility, suggesting that the inactivation mechanism is different from that for the freeze-dried enzyme with a larger amount of water. Inactivation of β-galactosidase in solutions was also studied as a function of phosphate buffer and sodium chloride concentrations, which in turn affected the T_1 of water. Because the inactivation rate increased with increasing salt concentrations and the rate extrapolated to zero concentration was negligible, inactivation of the freeze-dried enzyme was apparently induced by the salts used as additives for lyophilization. The enhancing effect of phosphate buffer components, however, was reduced at higher concentrations, an effect related to the decrease in the T_1 of water. This result may be ascribed to the decrease in water mobility caused by phosphate buffer components and is consistent with the observation that the inactivation rate of the freeze-dried enzyme with a relatively large amount of water decreased with decreasing T_1 of water.

KEY WORDS: β-galactosidase; protein stability; water mobility; lyophilization; ^{17}O nuclear magnetic resonance.

INTRODUCTION

Formulation of proteins for pharmaceutical purposes is a challenge because proteins are likely to undergo chemical and physical degradation and lose biological activity during pharmaceutical processing and storage (1). A number of papers have dealt with degradation of proteins in solutions (2–5) and with that of freeze-dried proteins (6–10). In many cases, degradation of proteins is ascribed to irreversible polypeptide chain unfolding, molecular aggregation, and/or chemical modification of proteins via bond formation or cleavage.

Some proteins are found to be extremely thermostable in the dry state, as reported for trypsin and ribonuclease by Mullaney (11). This suggests participation of water in degradation processes of proteins, which has been confirmed by recent studies. For example, aggregation of lyophilized bovine serum albumin, ovalbumin, glucose oxidase, and lactoglobulin was induced by moisture (10). Inactivation rates of freeze-dried ribonuclease A were found to be directly related to the amount of residual moisture remaining in the sample (7). Likewise, the denaturation rate of soybean protein increased with the water content (12,13).

The effect of water on degradation of smaller molecular drugs has been studied with respect to water mobility, that is, the dynamic properties of water around drugs. We have reported that the degradation rate of drugs in gelatin gels is related to the mobility of water molecules, as measured by ^{17}O NMR, via a decrease in the diffusion rates of reactants (14). It is of interest to extrapolate the relationship observed in degradation of smaller molecular drugs to macromolecules and, specifically, to examine the effect of the mobility of water around protein molecules on their degradation.

We therefore studied the degradation of β-galactosidase, a model protein, in the presence of water of different mobilities. Mobilities were measured as spin-lattice relaxation times, T_1, of water using ^{17}O NMR. Inactivation of β-galactosidase lyophilized from phosphate buffer solution was studied as a function of water content, which in turn affected the T_1 of water. Inactivation of the enzyme in phosphate buffer solutions was also followed as a function of concentrations of buffer components and sodium chloride, which in turn affected the T_1 of water. Furthermore, the effect of the pH of freeze-dried β-galactosidase on the inactivation rate was also studied since the pH of the enzyme lyophilized from phosphate buffer solution may vary with the water content.

MATERIALS AND METHODS

Materials

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

Inactivation of Freeze-Dried β-Galactosidase

Freeze-dried samples were prepared by lyophilizing β-galactosidase from 500 m*M* phosphate buffer solution (pH 7.4). The buffer solution was prepared by adding an adequate volume of 500 m*M* Na_2HPO_4 solution (ionic strength, $\mu = 1.5$) to 500 m*M* KH_2PO_4 solution containing 1 *M* NaCl ($\mu = 1.5$). β-Galactosidase was dissolved in the buffer solution to make 1 mg/ml protein solution. Sample tubes (14-mm diameter) containing 200 μl of the solution were immersed in liquid nitrogen for 1 min, and the frozen samples were dried at 5 Pa for 15 hr in a lyophilizer (Type 1CFS, Tozai Tsusho Co., Tokyo). The activity of the freeze-dried samples was 90% that of the solution to be lyophilized.

The freeze-dried samples were stored at 60°C and 11% relative humidity (RH) (adjusted with LiCl-saturated solu-

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0724-8741/93/0100-0103$07.00/0

tion), 21% RH (KF-saturated solution), or 50% RH (NaBr-saturated solution). The samples were removed at appropriate intervals for activity assay and water content measurement. Water content was determined by the Karl Fisher method (684 KF Coulometer, Switzerland).

For the experiments to examine the effect of larger amounts of water than that adsorbed at 50% RH, various amounts (5 μl to 2 ml) of distilled water were added to a freeze-dried sample in a tube, so that samples contained 0.31 to 122 mg of water/mg of freeze-dried sample. After the addition of water the samples were kept at 4°C for 15 hr to make moisture homogeneous. Then the samples were stored at 55 or 50°C and removed at appropriate intervals for activity assay.

Effect of pH on Inactivation of Freeze-Dried β-Galactosidase

The pH of freeze-dried samples with 3.1, 6.1, and 12.2 mg/mg of water content was determined at 50°C with a pH meter (HM-50AT, TOA, Tokyo). In order to determine the pH of freeze-dried samples with less water content, freeze-dried samples were prepared from 200 μl of 500 m*M* phosphate buffer (pH 7.4) containing one of the following mixed indicators (0.6%, v/v). Mixed indicators used were a mixture of 0.1% bromcresol green ethanol solution and 0.02% methyl orange aqueous solution (1:1), a mixture of 0.1% bromcresol green ethanol solution and 0.1% methyl red ethanol solution (3:1), a mixture of 0.1% methyl red ethanol solution and 0.1% methylene blue ethanol solution (1:1), a mixture of 0.1% chlorphenol red ethanol solution and 0.1% aniline blue ethanol solution (1:1), a mixture of 0.1% bromcresol green ethanol solution and 0.1% chlorphenol red ethanol solution (1:1), a mixture of 0.1% bromcresol purple ethanol solution and 0.1% bromthymol blue ethanol solution (1:1), a mixture of 0.1% neutral red ethanol solution and 0.1% methylene blue ethanol solution (1:1), and a mixture of 0.1% neutral red ethanol solution and 0.1% bromthymol blue ethanol solution (1:1). The pI's of these mixed indicators were reported to be 4.3, 5.1, 5.4, 5.8, 6.1, 6.7, 7.0, and 7.2, respectively (15). Five and 10 μl of distilled water were added to the freeze-dried samples containing the mixed indicators to make 0.31 and 0.61 mg/mg water contents, respectively, and the color was compared with that of pH standard solutions of pH 4.0 to 7.4 (500 m*M* phosphate).

In order to examine the effect of pH on the inactivation rate, freeze-dried samples were prepared from phosphate buffer solutions of pH 7.3, 5.9, and 5.5 in a similar way to the freeze-dried samples lyophilized from pH 7.4 buffer solution. The pH's of these phosphate buffers were adjusted by adding adequate volumes of 500 m*M* Na_2HPO_4 solution to 500 m*M* KH_2PO_4 solution containing 1 *M* NaCl. After the water content was adjusted to be 12.2 mg/mg by adding distilled water to the freeze-dried samples, inactivation was followed at 50°C (pH 7.4 and 7.3) or 55°C (pH 5.9 and 5.5).

Further, freeze-dried samples of pH 7.4 with 6.1 mg/mg water content were prepared by adding an adequate volume of distilled water to the samples lyophilized from 1 *M* phosphate buffer solution of pH 7.4. The pH of the phosphate buffer was adjusted by adding an adequate volume of 1 *M* Na_2HPO_4 solution to 1 *M* KH_2PO_4 solution containing 2 *M* NaCl. Inactivation of the freeze-dried sample was followed at 50°C.

Inactivation of β-Galactosidase in Solution

Degradation of β-galactosidase in solution was followed at pH 7.4 and 50°C as a function of phosphate buffer concentration and sodium chloride concentration. Phosphate buffer solutions of 10 to 900 m*M* were prepared by mixing Na_2HPO_4 solution and KH_2PO_4 solution of specified concentrations. Buffer solutions used to examine the effect of sodium chloride were prepared from 50 m*M* Na_2HPO_4 solution and 50 m*M* KH_2PO_4 solution, both of which contained 0 to 1.5 *M* NaCl. The protein was dissolved in the solutions to make 0.1 or 1 mg/ml protein solution. Enzyme activity was measured as a function of time.

Activity Measurement

The samples were dissolved or diluted with distilled water to make 1 μg/ml protein solutions. The activity of β-galactosidase was determined by using 2-nitrophenyl-β-D-galactopyranoside as a substrate, as described previously (16).

^{17}O NMR Measurement

Freeze-dried samples for ^{17}O NMR measurement were prepared without β-galactosidase in a similar way as for degradation measurement. One milliliter of 500 m*M* phosphate buffer (pH 7.4) in a tube (16-mm diameter) was freeze-dried. Various amounts (50 μl to 2 ml) of distilled water were added to the freeze-dried samples, so that the water contents were 3.1 to 122 mg of water/mg of freeze-dried sample. ^{17}O NMR of the samples was measured by operating a Varian spectrometer (VXR-400S) at 54.2 MHz. The sample tubes were kept at the same temperature (55 or 50°C) as for degradation measurements. Spin-lattice relaxation times, T_1, of $H_2{}^{17}O$ were obtained using the inversion recovery method. A 90° ^{17}O pulse width of 50 μsec and a recycling time of 250 msec were used.

T_1 was also determined for $H_2{}^{17}O$ in 0 to 900 m*M* phosphate buffer solutions (pH 7.4) and in 50 m*M* phosphate buffer solutions (pH 7.4) containing 0 to 1.5 *M* sodium chloride.

NMR measurement was carried out with freeze-dried samples and solutions in which β-galactosidase was not added. The effect of the protein on the measured T_1 was considered to be negligible since the ratio of the protein to the phosphate buffer components and sodium chloride was very low.

RESULTS

Inactivation of Freeze-Dried β-Galactosidase

Figure 1 shows the time courses of inactivation of β-galactosidase lyophilized from 500 m*M* phosphate buffer solution of pH 7.4, when stored at 60°C and various humidities. The enzyme activity revealed a rapid decrease during the initial period (within several hours), followed by a slower decrease depending on the humidity. The latter decrease could be described by first-order kinetics. The lines in the figure were calculated from the parameters obtained by nonlinear regression analysis.

Figure 2 shows the time courses of inactivation of

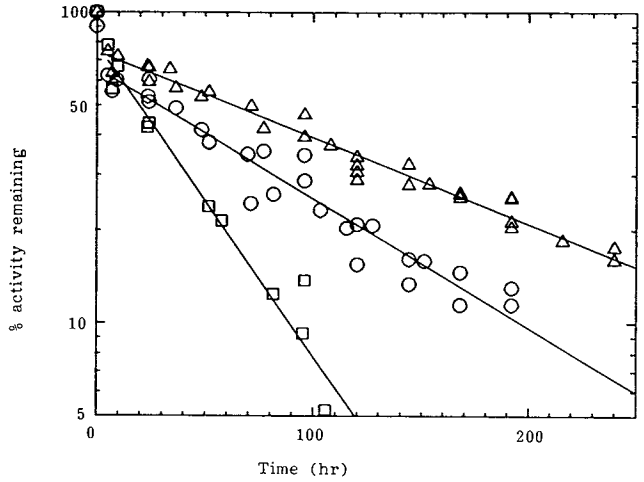

Fig. 1. Inactivation of freeze-dried β-galactosidase at 60°C as a function of humidity: (△) 11% RH; (○) 21% RH; (□) 50% RH.

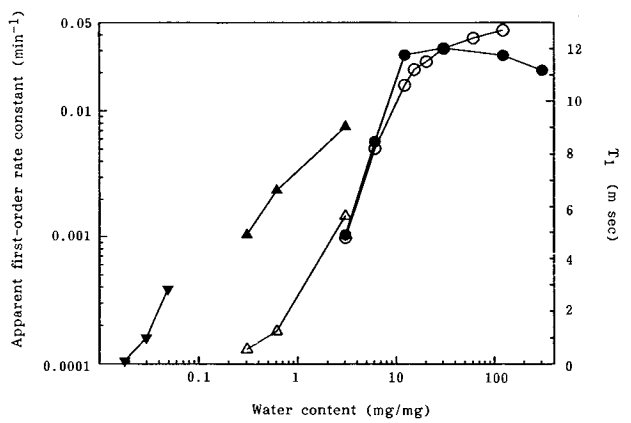

Fig. 3. The effect of water content on inactivation rate of freeze-dried β-galactosidase (●, ▲, ▼) and relaxation time T_1 of water ^{17}O (○, △). Apparent first-order rate constant was determined at 50°C (●), 55°C (▲), or 60°C (▼). T_1 was measured at 50°C (○) or 55°C (△).

freeze-dried β-galactosidase with larger amounts of water. A rapid decrease in the activity at the initial stage was observed in this range of water content as well. The inactivation rate at the latter stage depended on the water content. The solid lines were calculated from the parameters obtained by nonlinear regression analysis of the data observed at the latter stage according to first-order kinetic expression.

The effect of water content on inactivation of β-galactosidase is shown in Fig. 3. The apparent first-order rate constants calculated are plotted against the water content. The water contents of the samples stored under various humidities were determined by the Karl Fisher method. Since no significant change in the water content was observed with storage time after 24 hr, the water content at 100 hr of storage time was plotted in Fig. 3. The water contents of the samples with larger amounts of water were calculated according to the amount of water added. The ratio of the amount of water to the freeze-dried sample without water added (mg water/mg sample) was used to represent the water content. The sample with a water content of 12.2 mg/mg appeared to be a solution and can be considered to correspond to the buffer solution of β-galactosidase prior to lyophilization if the water content of the freeze-dried sample was assumed to be negligible before the addition of water. Over the wide range of water contents studied, from the solid state to the solution state, the rate of inactivation increased with the water content. The maximum inactivation rate, however, was observed at about 30 mg/mg water content.

The spin-lattice relaxation times, T_1, of $H_2{}^{17}O$ present in the freeze-dried samples of various water contents are also shown in Fig. 3. A marked decrease in T_1 was observed below 20 mg/mg water content, and this was related to the decrease in the inactivation rate.

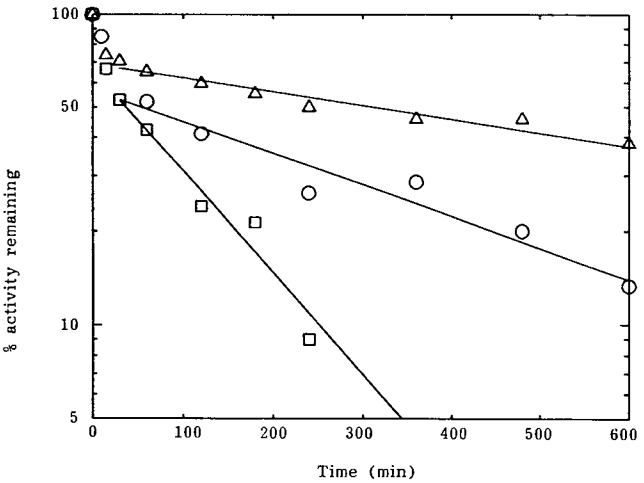

Fig. 2. Inactivation of freeze-dried β-galactosidase with 0.31 mg/mg (△), 0.61 mg/mg (○), or 3.1 mg/mg (□) of distilled water added, at 55°C.

Effect of pH on Inactivation of Freeze-Dried β-Galactosidase

The freeze-dried β-galactosidase prepared from 500 m*M* phosphate buffer solution of pH 7.4 exhibited pH values of 7.40, 7.37, and 7.32 at 50°C, when water contents were adjusted to 12.2, 6.1, and 3.1 mg/mg, respectively. Although the freeze-dried samples were completely dissolved in these amounts of water, a decrease in pH was observed with decreasing water content.

The pH values of the freeze-dried samples, the water contents of which were adjusted to be 0.61 and 0.31 mg/mg, were determined to be about 5.9 and 5.5, respectively, at 55°C by the mixed indicator method described under Materials and Methods. In this range of water contents, the freeze-dried samples appeared to be water-adsorbed solids and exhibited markedly decreased pH values.

Figure 4 shows a comparison of inactivation rates among the freeze-dried samples of the same pH but of different water contents. The freeze-dried samples of pH 7.4 and 7.3 showed significant decreases in inactivation rate when the water contents decreased from 12.2 to 6.1 and 3.1 mg/mg, respectively. In contrast, the inactivation rate of the freeze-dried samples of pH 5.9 and 5.5 increased when the water content decreased from 12.2 to 0.61 and 0.31 mg/mg, respectively.

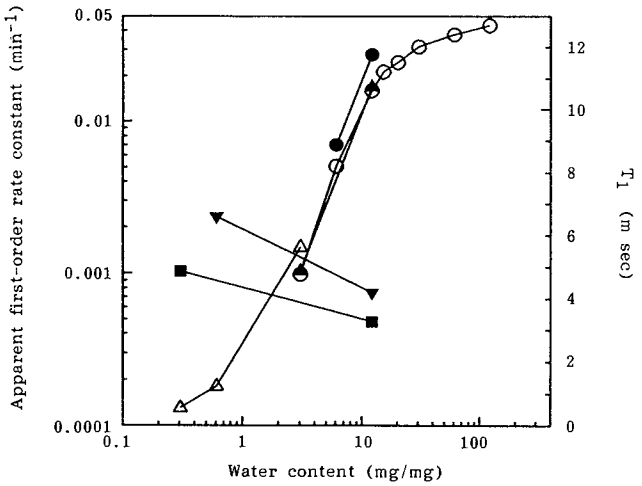

Fig. 4. The effect of pH on inactivation rate of freeze-dried β-galactosidase. Inactivation rates of freeze-dried samples of pH 7.4 (●), pH 7.3 (▲), pH 5.9 (▼), and pH 5.5 (■) with 12 mg/mg water were compared with rates of freeze-dried samples of the same pH with a smaller amount of water. Inactivation rate was determined at 50°C (pH 7.4 and pH 7.3) or 55°C (pH 5.9 and pH 5.5). (○, △) T_1 plotted in Fig. 3.

Inactivation of β-Galactosidase in Buffer Solution

Figures 5 and 6 show the time courses of inactivation of β-galactosidase in phosphate buffer solutions as a function of concentrations of phosphate buffer and sodium chloride, respectively. Both salts enhanced the inactivation. The inactivation was fitted by first-order kinetics (solid lines in Figs. 5 and 6). In some cases, time courses deviated from first-order kinetics because of the relatively fast initial inactivation. The apparent first-order rate constants are plotted against the concentration of phosphate buffer or sodium chloride in Fig. 7. Enhancement of the inactivation by sodium chloride was observed at the concentrations up to 1.5 *M*, while phosphate buffer components exhibited a maximum enhancement at about 0.5 *M*. The results shown in Fig.

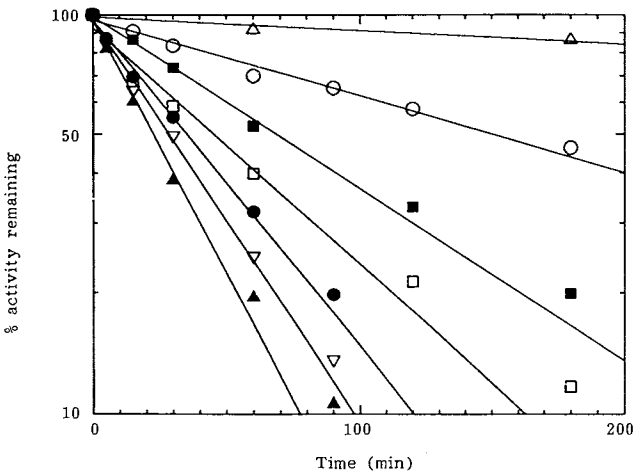

Fig. 5. Inactivation of β-galactosidase in pH 7.4 phosphate buffer solution at 50°C, as a function of phosphate buffer concentration: (△) 10, (○) 50, (□) 100, (▽) 200, (▲) 500, (●) 700, and (■) 900 m*M*. The concentration of β-galactosidase was 0.1 mg/ml.

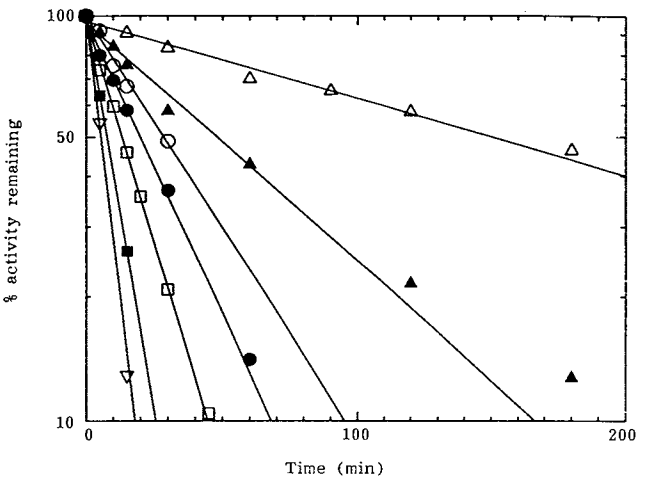

Fig. 6. Inactivation of β-galactosidase in pH 7.4 phosphate buffer solution (50 m*M*) at 50°C, as a function of sodium chloride concentration: (△) 0, (▲) 0.1, (○) 0.2, (●) 0.3, (□) 0.5, (■) 1.0, and (▽) 1.5 *M*. The concentration of β-galactosidase was 0.1 mg/ml.

7 were obtained for β-galactosidase solution at 0.1 mg/ml. A similar profile was obtained when the concentration of β-galactosidase was 1 mg/ml. The relaxation times, T_1, of $H_2{}^{17}O$ in phosphate buffer solution and sodium chloride solution are also shown in Fig. 7. The T_1 decreased with increasing concentrations of both salts. The decrease in T_1 was larger for the phosphate buffer solution than for the sodium chloride solution.

DISCUSSION

β-Galactosidase lyophilized from phosphate buffer solutions showed an initial rapid inactivation followed by a slower inactivation during storage in the presence of limited moisture. The inactivation could not be fitted by first-order kinetics due to the rapid initial inactivation (Figs. 1 and 2). The initial rapid inactivation, however, was not observed during storage of β-galactosidase solutions. The inactivation in solutions appeared to conform to first-order kinetics.

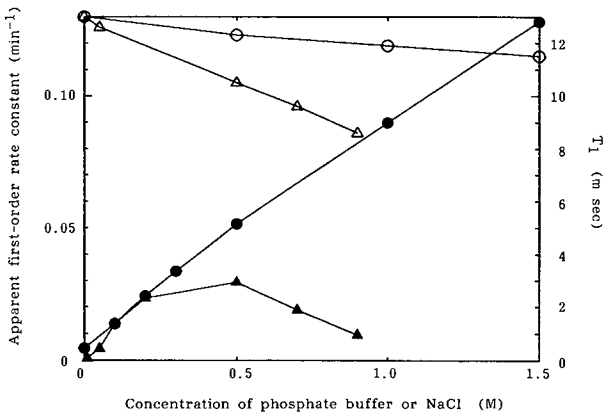

Fig. 7. The effects of phosphate buffer (▲, △) and sodium chloride (●, ○) on the inactivation rate of β-galactosidase (▲, ●) and the relaxation time T_1 of water ^{17}O (△, ○) in pH 7.4 solution at 50°C. Sodium chloride was dissolved in 50 m*M* phosphate buffer solution (pH 7.4).

Careful analysis of the inactivation vs time profiles, however, indicates a small deviation of the time profile from first-order kinetics, suggesting that the inactivation conforms to kinetic model other than first-order (Figs. 5 and 6). On the other hand, the initial rapid inactivation observed in the presence of limited moisture may be ascribed to changes in the physical state of the freeze-dried samples caused by rapid vapor adsorption and dissolution. Detailed kinetic analysis of β-galactosidase inactivation under these conditions should be accompanied by further studies to elucidate the mechanism of inactivation.

Inactivation rates of freeze-dried β-galactosidase depended largely on the water content (Fig. 3). A decrease in the water content was accompanied by a decrease in the T_1 of water as well as a decrease in pH. It is well-known that the T_1 of water measured by ^{17}O NMR depends on the average mobility of water molecules present in a system, and this parameter has been used to represent the mobility of water present around various compounds (17,18). The increase in T_1 with increasing water content observed for the β-galactosidase sample suggests an increase in the mobility of water molecules around the enzyme molecules. On the other hand, the decrease in pH resulting from decreased water content of the freeze-dried β-galactosidase may be ascribed to the solubility differences of the phosphate species of the buffer. During the freezing process of phosphate buffer solutions, Na_2HPO_4 preferentially precipitates because its solubility is lower than that of KH_2PO_4, causing a decrease in pH (19). The pH drop may contribute to the decrease in the inactivation rate caused by a decrease in water content, since β-galactosidase becomes more stable as pH decreases. Thus, variations both in water mobility and in pH, which result from a change in water content of the freeze-dried samples, should affect the inactivation rate of β-galactosidase.

The freeze-dried samples with a water content of more than 3 mg/mg appeared to be solutions at 50°C. A decrease in water content from 12.2 mg/mg (the solution prior to lyophilization) to 3.1 mg brought about a decrease in pH from 7.4 to 7.3. Comparison of the inactivation rates of the samples with water contents of 3.1 and 12.2 mg/mg, the pH's of which were both 7.3, suggests that a remarkable difference in inactivation rate is caused by some factors other than pH variation (Fig. 4). This is also suggested by comparison of the inactivation rates of the samples with water contents of 12.2 and 6.1 mg/mg, the pH's of which were 7.4 (Fig. 4). Therefore the decrease in the inactivation rate of β-galactosidase with decreasing water content, which is shown in Fig. 3, cannot be ascribed to only the pH drop. Since the decrease in the inactivation rate was related to a decrease in the T_1 of water, that is, a decrease in the amount of freely mobile water molecules (Fig. 3), it is suggested that freely mobile water molecules play an important role in the inactivation. As the water content increases, the mobility of water around the enzyme increases, resulting in enhancement of the enzyme inactivation. This is supported by the fact that the inactivation rate in solutions of pH 7.4 decreased with increasing phosphate concentration (with decreasing T_1 of water) at very high phosphate concentrations as discussed below (Fig. 7).

The freeze-dried samples with water contents of less than 1 mg/mg appeared to be water-adsorbed solids and exhibited much lower pH's than the solution prior to lyophilization. The pH's of the samples with water contents of 0.31 and 0.61 mg/mg were 5.5 and 5.9, respectively. However, the decreases in the inactivation rates of these samples were smaller than those expected from the pH drop. The inactivation rates of the samples with water contents of 0.31 and 0.61 mg/mg were larger than those of the samples of the corresponding pH's with 12.2 mg/mg water. This suggests that in the presence of limited moisture, the freeze-dried enzyme inactivates via a mechanism different from that in the presence of sufficient water to be completely dissolved. Further studies are required to elucidate the difference in the inactivation mechanism.

The inactivation rate of β-galactosidase in solutions increased with the concentration of phosphate buffer components and sodium chloride (Fig. 7). This suggests enhancing effects of both ions on the inactivation. Since the inactivation rate extrapolated to zero concentration was negligible, the inactivation can be considered to be salt induced. It has been reported that thermal stability of proteins is affected by particular salts (20,21). High-salt environments induced perturbations in protein structure (20) and resulted in a low heat of inactivation (21). Although the mechanism of the enhancing effect of phosphate buffer and sodium chloride on inactivation of β-galactosidase is not clear, electrostatical effects may be involved. For the freeze-dried sample with water added, a decrease in the inactivation rate was observed at the highest water contents (Fig. 3). This may be due to dilution of phosphate buffer and sodium chloride.

The enhancing effect of sodium chloride increased with the concentration up to 1.5 *M*, while that of phosphate buffer components decreased at concentrations above 0.5 *M* (Fig. 7). This may be interpreted by the difference in the mobility of water surrounding these two salts. Although the T_1 of water in the solutions decreased with increasing concentrations of both salts, phosphate buffer reduced the T_1 more intensely than sodium chloride. This indicates that phosphate buffer reduces the mobility of surrounding water molecules more intensely than sodium chloride. The enhancing effect of phosphate buffer components on inactivation may be counteracted by the effect of reducing water mobility. This can explain the decrease in the inactivation rate observed for the concentrated phosphate buffer solution.

In conclusion, an increase in the water content of freeze-dried β-galactosidase brought about an increase in the T_1 of water molecules, as well as a rise in pH. For the freeze-dried samples with a water content sufficient to be dissolved, inactivation rates were affected more by water mobility as measured by the T_1 of water than by pH variation. The freeze-dried samples with limited moisture showed inactivation rates faster than those expected from the pH and water mobility, suggesting that the inactivation mechanism is different from that for the freeze-dried samples with larger amounts of water. Further, it is suggested that inactivation of the freeze-dried enzyme was induced by phosphate buffer components and sodium chloride used as additives for lyophilization, since the inactivation rate in solutions increased with increasing concentrations of phosphate buffer components and sodium chloride and the rate extrapolated to zero concentration was negligible. The enhancing effect of phosphate buffer components, however, was reduced at very

high buffer concentrations, an effect which appears to be related to the decrease in the T_1 of water. This is consistent with the observation that the inactivation rate of the freeze-dried enzyme with a relatively large amount of water decreased with decreasing T_1 of water.

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