The Effect of Salts on the Stability of β-Galactosidase in Aqueous Solution, as Related to the Water Mobility

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The effect of salts (KI, KBr, NaCl, KCl, KF, phosphate, and Na_2SO_4) on the stability of β-galactosidase in aqueous solution was studied from the aspect of changes in water mobility. At salt concentrations up to 200 m*M*, the inactivation rate of β-galactosidase in all the salt solutions studied increased with increasing salt concentration. At higher concentrations, those salts which had little effect on the spin-lattice relaxation time, T_1, of water (KI, KBr, and KCl) continued to increase the inactivation rate of β-galactosidase with increasing concentration, while those salts which decreased the T_1 of water (KF, phosphate, and Na_2SO_4) decreased the inactivation rate. It appeared that the decrease in water mobility caused by KF, phosphate, and Na_2SO_4 resulted in stabilization of β-galactosidase. The results indicate that water mobility is an important factor in the denaturation rate of proteins.

KEY WORDS: β-galactosidase; stability in solution; water mobility; spin lattice relaxation time.

INTRODUCTION

Proteins tend to denature in high-salt environments, although some salts prevent denaturation by causing protein precipitation (1,2). Since salts are often used in the formulation of proteins in pharmaceutical products, an understanding of the salt effect on protein stability is crucial. The effect of salts on the structure and properties of proteins has been studied extensively (1–7). Ions that increase the solubility of proteins are likely to cause their denaturation (salting-in), while ions that precipitate proteins tend to prevent denaturation (salting-out). The "salting-in" effect has been interpreted in terms of salt–peptide group interactions, which stabilize the denatured form of proteins by reducing its free energy. The "salting-out" effect, on the other hand, has been ascribed to the salt effects on nonpolar groups in proteins. These salt effects, however, have been studied only from thermodynamic aspects. Kinetic studies to clarify the effect of salts on denaturation rates of proteins have not been reported.

The inactivation of β-galactosidase (a process involving denaturation followed by aggregation) was enhanced by phosphate buffer components and sodium chloride both in aqueous solution and in the freeze-dried state (8,9). The inactivation rate was increased with increasing concentration of NaCl. The inactivation-enhancing effect of phosphate, however, was found to decrease at higher concentrations as a result of decreased water mobility, as measured by the spin-lattice relaxation time, T_1, of water. These results suggested that water mobility is one of the factors determining the denaturation rate of proteins.

We have subsequently determined the inactivation rate of β-galactosidase in the presence of various ions that have been demonstrated to increase or decrease the mobility of surrounding water molecules (10). The present paper describes the relationship between the salt-induced inactivation rate of β-galactosidase and the water mobility, as measured by the T_1 of oxygen-17 H_2O.

MATERIALS AND METHODS

Materials

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

Inactivation of β-Galactosidase in Aqueous Salt Solutions

Inactivation of β-galactosidase was studied in phosphate buffer solutions (pH 7.4, 50 m*M*) containing various concentrations (0–3 *M*) of KI, KBr, NaCl, KCl, KF, Na_2SO_4, and $CaCl_2$ at 45°C. A mixture of 50 m*M* Na_2HPO_4 and 50 m*M* KH_2PO_4, containing equimolar concentrations of the salt under study, was used to achieve a pH 7.4 solution. Inactivation was also followed in various concentrations (50–850 m*M*) of phosphate buffer solution (pH 7.4). In all experiments, the concentration of β-galactosidase was 0.1 mg/mL. Enzyme activity was determined as a function of time using 2-nitrophneyl-β-D-galactopyranoside as the substrate, as described previously (8).

^{17}ONMR Measurement

^{17}ONMR of the buffer solutions containing various concentrations of salts was measured by operating a Varian spectrometer (VXR-400S) at 54.2 MHz. The sample tubes were kept at 45°C. The inversion recovery method was employed to obtain the T_1 of $H_2{}^{17}O$, using a 90° ^{17}O pulse width of 50 μsec and a recycling time of 250 msec. The measurement was repeated three times and the standard deviation of the measured T_1 was less than 3%.

The T_1 of water in salt solutions containing β-galactosidase (0.1 mg/mg) was compared with that in salt solutions without β-galactosidase. No significant difference in the T_1 was observed as shown in Table I.

RESULTS

Figures 1 and 2 show typical time courses of inactivation of β-galactosidase in 50 m*M* phosphate buffer solution (pH 7.4) containing NaCl and Na_2SO_4, respectively, as a function of salt concentration. The solid lines in the figures represent nonlinear regression curves determined according to first-order kinetics. Although the inactivation appeared to deviate from first-order kinetics at the latter stage, the ap-

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Table I. The T_1 of Water in Salt Solutions with and Without β-Galactosidase

Salt solution	T_1 (msec) at a β-galactosidase concentration of	
	0 mg/mL	0.1 mg/mL
50 m*M* phosphate	11.36	11.34
3 *M* KCl[a]	11.12	11.37
2 *M* Na_2SO_4[a]	5.55	5.51

[a] Containing 50 m*M* phosphate.

parent first-order rate constant was used as a measure of the inactivation rate. The inactivation rate increased with increases in the NaCl concentration. In Na_2SO_4 solutions, the inactivation rate increased at low Na_2SO_4 concentration, but in 1 *M* Na_2SO_4 solution it was negligible.

The apparent rate constants obtained for the inactivation of β-galactosidase in the presence of the various salts studied are plotted against salt concentration (Fig. 3). The rate at zero concentration represents the rate observed in 50 m*M* phosphate solution containing no other added ions. For phosphate (Na_2HPO_4 and KH_2PO_4), the rate constant is plotted against the value calculated after subtracting 50 m*M* from the total concentration so that the values can be compared to the results for the other ions studied. KI, KBr, NaCl, and KCl enhanced the inactivation with increasing concentration, as shown in Fig. 3A. The inactivation rate in the presence of KF, phosphate, or Na_2SO_4 increased with concentrations at lower concentrations, then decreased at higher concentrations, as shown in Fig. 3B. Maximum enhancement was observed around 200 m*M* for phosphate and Na_2SO_4 and around 500 m*M* for KF. The inactivation rates in phosphate and Na_2SO_4 solutions were similar to those in KI, KBr, and NaCl solutions at concentrations below 200 m*M*, while a striking difference in the inactivation rate at higher concentrations was observed between these two groups of salts. The inactivation rate in 1 *M* $CaCl_2$ solution was found to be too fast to determine the rate constant.

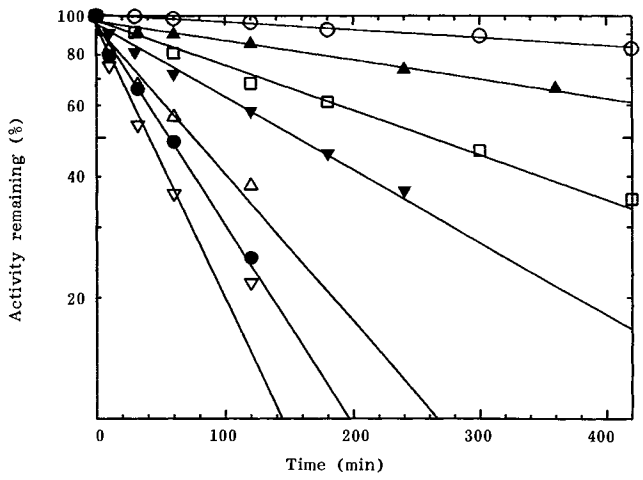

Fig. 1. The time courses of inactivation of β-galactosidase in NaCl solution at 45°C as a function of salt concentration. The pH was adjusted to 7.4 with 50 m*M* phosphate. NaCl concentrations were 0 (○), 0.1 (▲), 0.3 (□), 0.5 (▼), 1 (△), 2 (●), and 3 *M* (▽).

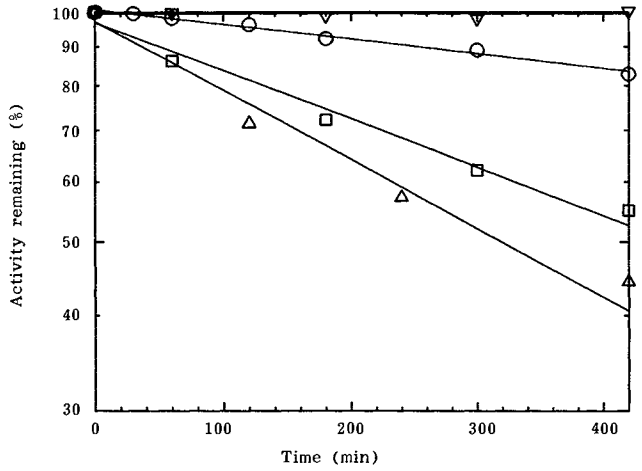

Fig. 2. The time courses of inactivation of β-galactosidase in Na_2SO_4 solution at 45°C as a function of salt concentration. The pH was adjusted to 7.4 with 50 m*M* phosphate. Na_2SO_4 concentrations were 0 (○), 0.1 (□), 0.2 (△), and 1 *M* (▽).

Figure 4 shows the T_1 of water in salt solutions containing 50 m*M* phosphate (pH 7.4) as a function of salt concentration. The T_1 was determined at the same temperature as the inactivation rate measurements (45°C). A marked decrease in T_1 with increasing salt concentration was observed for Na_2SO_4 and phosphate solutions. An increase in NaCl and KF concentrations was also accompanied by a decrease in the T_1 of water, but the decrease was much less than those observed for Na_2SO_4 and phosphate solutions. The T_1 was little affected by salts such as KI, KBr, and KCl, though the T_1 of KI tended to increase with increasing salt concentration.

DISCUSSION

All salts studied enhanced the inactivation of β-galactosidase at concentrations below 200 m*M*, when added to the 50 m*M* phosphate solution (Fig. 3). This destabilization effect of the salts has been explained by salt–peptide group interaction (3–5,7). At higher concentrations, however, these salts can be classified into two groups: salts that continued to increase the inactivation rate with increasing salt concentration up to 2 or 3 *M* (KI, KBr, NaCl, and KCl) and salts that decreased the inactivation rate with increasing concentration (KF, Na_2SO_4, and phosphate). The difference in the inactivation rate vs salt concentration profile observed between these two groups appears to be related to the T_1 of water in the salt solutions (Fig. 4). Phosphate and Na_2SO_4, which caused marked decreases in T_1 with increasing concentration, also decreased the inactivation rate markedly with increasing concentration. A moderate decrease in the inactivation rate with increasing salt concentration was observed for KF, which decreased the T_1 of water moderately with increasing concentration. Salts that enhanced the inactivation even at high concentrations (KI, KBr, KCl, and NaCl) revealed no significant decrease in the T_1 of water, though NaCl showed a tendency to decrease the T_1 at high concentrations.

The T_1 of water has been used as a parameter to repre-

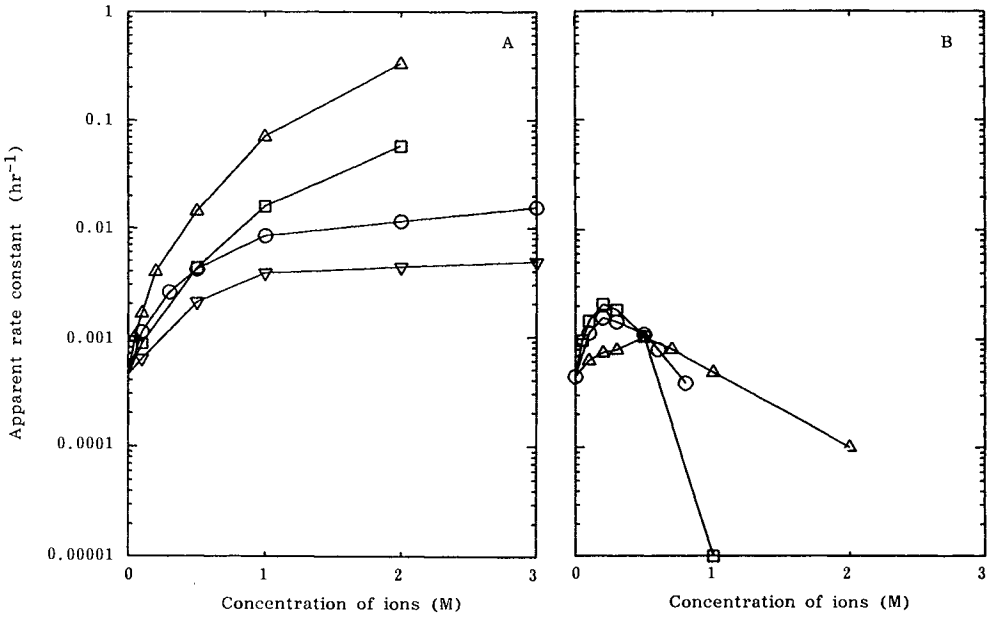

Fig. 3. The effect of salts on the apparent rate constant of β-galactosidase inactivation. (A) KI (△), KBr (□), NaCl (○), and KCl (▽). (B) KF (△), phosphate (○), and Na_2SO_4 (□). The plot at zero concentration represents the rate constant observed in 50 m*M* phosphate solution.

sent the average mobility of water molecules (10–12). A decrease in the T_1 of water in a system indicates a decrease in the amount of freely mobile water. The T_1 of water in the salt solutions determined in the present study can be used to evaluate the water mobility in the salt solutions. The parallel decrease in the inactivation rate with increasing concentrations of Na_2SO_4, phosphate, and KF and the decrease in the T_1 of water suggest that decreased water mobility caused by these salts stabilized β-galactosidase.

Thermodynamic studies of protein solubility in concentrated salt solutions (1–2 *M*) revealed that salts are arranged in the order (Na_2SO_4 = phosphate > KF > NaCl = KCl > KBr > KI) with respect to the "salting-out" effect (3–6).

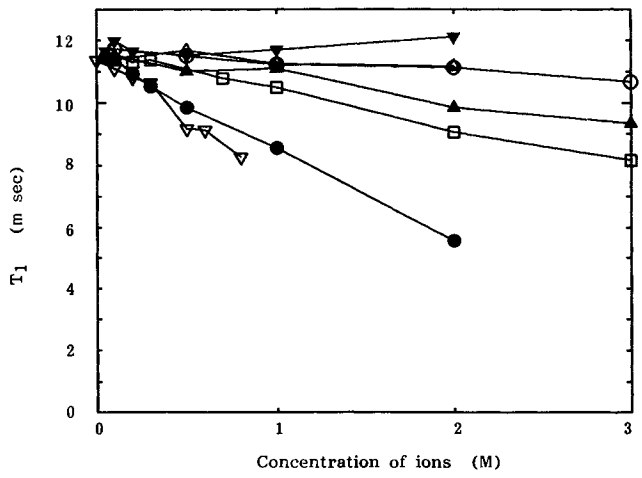

Fig. 4. The effect of salts on the T_1 of water in salt solutions: KI (▼), KBr (△), KCl (○), NaCl (▲), KF (□), Na_2SO_4 (●), and phosphate (▽). T_1 was determined at 45°C. The pH was adjusted to 7.4 with 50 m*M* phosphate. The plot represents the mean of three measurements.

This order is consistent with the order for salts to reduce the T_1 of water determined in the present study. This suggests that "salting-out" is related to the decreased water mobility induced by salts. As the availability of freely mobile water molecules decreases, protein solubility decreases, resulting in protein precipitation if the protein concentration is high. It is not clear whether β-galactosidase was precipitated in the Na_2SO_4, phosphate, and KF solutions at high concentration. The decreased water mobility induced by these salts may bring about stabilization of β-galactosidase even if the protein is still solubilized. The thermodynamic studies have also indicated that salts that decrease protein solubility can be used to isolate proteins primarily via precipitation without denaturation. The present study clarifies that salts known to be effective in "salting-out" at high concentrations can actually enhance protein denaturation at lower concentrations where the decrease in water mobility is insufficient to stabilize the protein.

The relationship between the inactivation rate of β-galactosidase and the T_1 of water, which was observed for KI, KBr, KCl, KF, Na_2SO_4, and phosphate, cannot be applied to the rate observed in the presence of $CaCl_2$. $CaCl_2$ decreased the T_1 of water markedly, but the T_1 decrease was not accompanied by a decrease in the inactivation rate. The inactivation rate of β-galactosidase in the presence of $CaCl_2$ was too fast to determine the rate constant at 45°C. The strong denaturation effect of the salt may be due to the strong interaction between salts and the peptide groups in proteins. The free energy of peptides is markedly decreased in the presence of $CaCl_2$ because of salt–peptide interaction (3–5,7). The strong denaturation effect of the salt via salt–peptide interaction may diminish the stabilization effect of the salts which results from the decrease in water mobility. Thus the stabilization effect of $CaCl_2$ was not observed even though the T_1 of water was decreased by the salt. LiCl and

$MgCl_2$, which like $CaCl_2$ decrease the T_1 of water, also enhance the denaturation of proteins.

In the present study, NaCl was found not to decrease the inactivation rate of β-galactosidase as much as expected from the decrease in the T_1 of water at high concentrations. Though the effect of NaCl on the T_1 of water was larger than that of KCl (Fig. 4), NaCl gave an inactivation rate-vs-concentration profile similar to that of KCl. No decrease in the inactivation rate at high concentrations was observed (Fig. 3). These results may be ascribed to the stronger salt–peptide interaction of NaCl than that of KCl.

In conclusion, the inactivation rate of β-galactosidase in all the salt solutions studied increased with increasing salt concentration, at concentrations below 200 m*M*. At higher concentrations, salts with little effect on the spin-lattice relaxation time, T_1, of water (KI, KBr, and KCl) continued to increase the inactivation rate of β-galactosidase with increasing concentration, while salts that decreased the T_1 of water (KF, phosphate, and Na_2SO_4) decreased the inactivation rate. It appeared that the decrease in water mobility caused by KF, phosphate, and Na_2SO_4 resulted in stabilization of β-galactosidase.

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