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 Na2SO4) 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 mM, 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, T1, of water (KI, KBr, and KCl) continued to increase the inactivation rate of β-galactosidase with increasing concentration, while those salts which decreased the T1 of water (KF, phosphate, and Na2SO4) decreased the inactivation rate. It appeared that the decrease in water mobility caused by KF, phosphate, and Na2SO4 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, T1, 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 T1 of oxygen-17 H2O.

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 mM) containing various concentrations (0–3 M) of KI, KBr, NaCl, KCl, KF, Na2SO4, and CaCl2 at 45°C. A mixture of 50 mM Na2HPO4 and 50 mM K2HPO4, 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 mM) 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-nitrophenyl-β-D-galactopyranoside as the substrate, as described previously (8).

17ONMR Measurement

17ONMR of the buffer solutions containing various concentrations of salts was measured by operating a Varian spectrometer (VXR-400S) at 34.2 MHz. The sample tubes were kept at 45°C. The inversion recovery method was employed to obtain the T1 of H217O, using a 90° 17O 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 T1 was less than 3%.

The T1 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 T1 was observed as shown in Table 1.

RESULTS

Figures 1 and 2 show typical time courses of inactivation of β-galactosidase in 50 mM phosphate buffer solution (pH 7.4) containing NaCl and Na2SO4 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-
Table 1. The $T_1$ of Water in Salt Solutions with and Without β-Galactosidase

<table>
<thead>
<tr>
<th>Salt solution</th>
<th>0 mg/mL</th>
<th>0.1 mg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM phosphate</td>
<td>11.36</td>
<td>11.34</td>
</tr>
<tr>
<td>3 M KCl$^a$</td>
<td>11.12</td>
<td>11.37</td>
</tr>
<tr>
<td>2 M Na$_2$SO$_4$</td>
<td>5.55</td>
<td>5.51</td>
</tr>
</tbody>
</table>

$^a$ Containing 50 mM 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$_2$SO$_4$ solutions, the inactivation rate increased at low Na$_2$SO$_4$ concentration, but in 1 M Na$_2$SO$_4$ 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 mM phosphate solution containing no other added ions. For phosphate (Na$_2$HPO$_4$ and KH$_2$PO$_4$), the rate constant is plotted against the value calculated after subtracting 50 mM 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$_2$SO$_4$ increased with concentrations at lower concentrations, then decreased at higher concentrations, as shown in Fig. 3B. Maximum enhancement was observed around 200 mM for phosphate and Na$_2$SO$_4$ and around 500 mM for KF. The inactivation rates in phosphate and Na$_2$SO$_4$ solutions were similar to those in KI, KBr, and NaCl solutions at concentrations below 200 mM, 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 mM phosphate. NaCl concentrations were 0 (○), 0.1 (▲), 0.3 (□), 0.5 (▼), 1 (▲), 2 (●), and 3 M (▼).](image)

![Fig. 2. The time courses of inactivation of β-galactosidase in Na$_2$SO$_4$ solution at 45°C as a function of salt concentration. The pH was adjusted to 7.4 with 50 mM phosphate. Na$_2$SO$_4$ concentrations were 0 (○), 0.1 (□), 0.2 (▲), and 1 M (▼).](image)

DISCUSSION

All salts studied enhanced the inactivation of β-galactosidase at concentrations below 200 mM, when added to the 50 mM 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$_2$SO$_4$, 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$_2$SO$_4$, 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-