JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 100, No. 5, 556–561. 2005
DOI: 10.1263/jbb.100.556

Diamines Prevent Thermal Aggregation and Inactivation of Lysozyme

Masahiro Okanojo,[1] Kentaro Shiraki,[2]* Motonori Kudou,[1]
Shingo Nishikori,[1] and Masahiro Takagi[1]

School of Materials Science, Japan Advanced Institute of Science and Technology, 1-1 Asahidai, Nomi, Ishikawa 923-1292, Japan[1] *and Institute of Applied Physics, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan*[2]

Received 1 June 2005/Accepted 2 August 2005

Protein aggregation is a major obstacle in both biological applications and biomedical fields involving proteins. In this study, we investigated the essential structure of small additives that function as chemical chaperones. Aggregation-suppressing competent additives were 1,3-diaminopropane, 1,4-diaminobutane, and 1,5-diaminopentane, which suppressed aggregation in the given order; whereas no diols or monoamines prevented the thermal aggregation and the inactivation of lysozyme. The heat-inactivation rate of lysozyme with 1,3-diaminopropane was almost identical to that of lysozyme with spermine and arginine ethylester, which are the most prominent additives reported yet.

[**Key words:** lysozyme, thermal aggregation, polyamine, diamine, monoamine, diol]

Protein aggregation is an unproductive phenomenon in biotechnology. To reduce aggregation *in vitro*, various techniques have been developed, such as the use of a molecular chaperone and protein mutagenesis, as well as control of pH, temperature, protein concentration, and ionic strength. A simple, practical approach to solving the aggregation problem is the utilization of small molecular additives that act as aggregation suppressors. Many types of additive, such as guanidine (1, 2), proline (3), arginine (1, 4, 5), arginine ethylester (6), amino acid derivatives (7), and polyamines (8) have been reported.

Arginine is a well-used additive that prevents the aggregation and inactivation of proteins in various situations (1, 4, 5, 9–11). Arginine increases the refolding yield of various proteins from unfolded states, such as immunotoxin (12), antibody fragment (4, 13), and lysozyme (9), due to the suppression of aggregation, while arginine slightly affects the protein structure and stability (6, 14). Although the availability of arginine has been widely elucidated, little has been reported on the molecular mechanism of arginine regarding protein folding and aggregation, except for the aromatic-guanidium interaction (10) and the electrostatic interaction (6) between a protein and arginine.

Recently, we have shown that naturally occurring polyamines, such as putrescine, spermidine, and spermine, are prominent additives for preventing the heat-induced aggregation and inactivation of lysozyme (8). Arginine is not effective at a concentration below 1 M in suppressing protein aggregation, while polyamines are completely effective at a concentration of one order of magnitude lower than arginine. In particular, putrescine (1,4-diaminobutane) has a fairly simple structure, but it is more effective in suppressing the thermal aggregation and the inactivation of lysozyme than arginine and guanidine. To identify the basis of the structure of additives, we investigated the effects of several types of additive on the thermal aggregation and inactivation of lysozyme. Monoamines, diamines, and diols were selected to clarify the effects of multivalent amine, chain length, and charge on the suppression of protein aggregation.

MATERIALS AND METHODS

Materials Hen egg-white lysozyme was obtained from Sigma Chemical (St. Louis, MO, USA). 1,3-Diaminopropane was obtained from Kanto Kagaku (Tokyo). 1,4-Diaminobutane, 1,5-diaminopentane, 1-aminopropane, 1-aminobutane, 1-aminopentane, 1,3-propanediol, and 1,5-pentanediol were obtained from Wako Pure Chemical Industries (Osaka). 1,4-Butanediol was obtained from Lancaster (Morecambe, UK). *Micrococcus lysodeikticus* was obtained from Nacalai Tesque (Kyoto). All other chemicals and buffer salts used were of analytical grade.

Thermal aggregation All stock solutions containing 500 mM additives (monoamines, diamines, diols, amino acids, and NaCl) were dissolved in 50 mM sodium-phosphate buffer and adjusted to pH 6.5 by the addition of NaOH or HCl before sample preparation. Samples were prepared by properly mixing the stock solution of lysozyme with the additive solution. After heat treatment at 98°C for specific periods, the samples were centrifuged at 15,000×*g* for 30 min. The protein concentration of supernatants was determined by absorbance at 280 nm with Jasco spectrophotometer model V-550 (Japan Spectroscopic Company, Tokyo) using an extinction coefficient of 2.63 cm^{-1} per $mg \cdot ml^{-1}$.

Enzymatic activity The enzymatic activity of lysozyme after heat treatment was estimated by bacteriolysis. A total of 1.5 ml of

* Corresponding author. e-mail: shiraki@bk.tsukuba.ac.jp
phone: +81-(0)29-853-5306 fax: +81-(0)29-853-5205

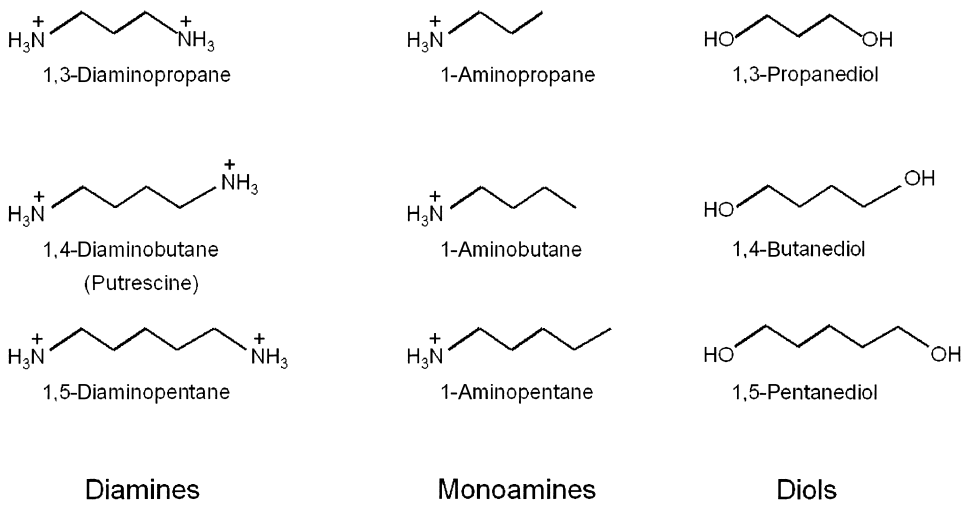

FIG. 1. Chemical structure and composition of diamines, monoamines, and diols.

0.5 mg·ml^{-1} *M. lysodeikticus* in 50 mM sodium-phosphate buffer (pH 6.5) was mixed with 20 μl of the heated samples. The decrease in the light scattering intensity of the solution was monitored by absorbance at 600 nm. The residual activity was estimated by fitting the data through linear extrapolation.

Thermal inactivation kinetics Thermal inactivation kinetics of lysozyme was investigated at temperatures ranging from 80°C to 98°C in the presence or absence of additives. Samples containing 0.2 mg·ml^{-1} lysozyme in the presence or absence of 100 mM additives in 50 mM sodium-phosphate buffer (pH 6.5) were incubated at 80°C–98°C. After heat treatment for various periods, residual activities were estimated. The inactivation rate constant (k_i) of each heating temperature was calculated from a plot of logarithmic residual activity *versus* heating time.

Calculation of thermodynamic parameters on thermal inactivation The effects of diamines on preventing the thermal inactivation of lysozyme were analyzed as follows. The temperature dependence of the inactivation rate constant was determined using the Arrhenius equation (15)

$$k_i = k_0 \exp(-E/RT) \quad (1)$$

where E, k_0, R, and T are the activation energy, frequency factor, gas constant, and absolute temperature, respectively. E was obtained from the slope of the Arrhenius plot in Fig. 4. The activation free energy change (ΔG^*) at each heating temperature was calculated by the Eyring Eq. 2

$$\Delta G^* = -RT \ln(k_i h/k_b T) \quad (2)$$

where h and k_b are the Plank and Boltzmann constants, respectively. The activation enthalpy change (ΔH^*) at each heating temperature was calculated using

$$\Delta H^* = E - RT \quad (3)$$

The activation entropy change ($T\Delta S^*$) was calculated using

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad (4)$$

Circular dichroism CD experiments were performed using Jasco spectropolarimeter model J-720 W equipped with a variable-temperature cell holder. Lysozyme (0.2 mg·ml^{-1}) in 50 mM sodium-acetic acid (pH 4.0) or sodium-phosphate buffer (pH 6.5) was prepared. In the presence or absence of 100 mM or 400 mM diamines, monoamines, and diols, the thermal unfolding of lysozyme was monitored by scanning from 30°C to 90°C at a rate of 1°C·min^{-1} with 2-mm-path-length cells. Loss in the secondary structure of lysozyme was monitored at 222 nm.

RESULTS

Figure 1 shows the chemical structures of the additives investigated in this study. It was previously shown that putrescine (1,4-diaminobutane) is one of the prominent additives that prevent the heat-induced aggregation and inactivation of lysozyme (8). In this paper, we focus on understanding the essential chemical structure that is involved with aggregation suppression.

Comparison of diamines, monoamines, and diols on the thermal aggregation of lysozyme Figure 2 shows the effects of additives on the thermal aggregation of lysozyme. After incubation at 98°C for 30 min, the amount of aggregates was determined by measuring the protein concentration of the supernatant as previously described (5–8). With increasing concentrations of 1,3-diaminopropane, 1,4-diaminobutane, and 1,5-diaminopentane, the amount of aggregates decreased (Fig. 2A). The aggregation-suppressing competent additives were 1,3-diaminopropane, 1,4-diaminobutane, and 1,5-diaminopentane, which suppressed the degree of aggregation in the order given. Conversely, no monoamines prevented the thermal aggregation of lysozyme (Fig. 2B), suggesting that the presence of a multivalent cation is essential for aggregation suppression. In addition, no diols prevented the thermal aggregation of lysozyme (Fig. 2C), suggesting that charged ends, rather than hydrophilic ends, play an important role in the prevention of the aggregation of lysozyme.

NaCl and glycine did not prevent the thermal aggregation of lysozyme, while arginine did (Fig. 2D). However, the prevention effect of arginine was weaker than those of 1,3-diaminopropane and putrescine.

Thermal inactivation kinetics of lysozyme Figure 3 shows the thermal inactivation kinetics of lysozyme at temperatures between 80°C and 98°C in the presence or absence of 100 mM additives. As seen in Fig. 3A–D, diamines prevented the thermal inactivation of lysozyme. The plots of residual activity versus heating time indicate that the ther-