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Correlation between thermal aggregation and stability of lysozyme with salts described by molar surface tension increment: an exceptional propensity of ammonium salts as aggregation suppressor

Atsushi Hirano,[1] Hiroyuki Hamada,[1] Tatsunori Okubo,[2] Takumi Noguchi,[2] Hiroki Higashibata,[3] and Kentaro Shiraki[1]

[1] Institute of Applied Physics, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan

[2] Institute of Material Science, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan

[3] Faculty of Life Science, Toyo University, 1-1-1 Izumino Itakura-machi, Gunma 374-0193, Japan

Running title

Protein aggregation in various salts solutions

Correspondence

K. Shiraki, Institute of Applied Physics, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan.

Fax: +81-29-853-5215

Tel: +81-29-853-5306

E-mail: shiraki@bk.tsukuba.ac.jp

Abbreviations

ATR, attenuated total reflection; T_m, midpoint temperature of thermal unfolding; UV, ultraviolet; MSTI, molar surface tension increment; Arg, arginine; Phe, phenylalanine; Ile, isoleucine; Ser, serine; Gly-Gly, glycylglycine.

Abstract

Protein aggregation is a critical problem for biotechnology and pharmaceutical industries. Despite the fact that soluble proteins have been used for many applications, our understanding of the effect of the solution chemistry on protein aggregation still remains to be elucidated. This paper investigates the process of thermal aggregation of lysozyme in the presence of various types of salts. The simple law was found; the aggregation rate of lysozyme increased with increasing melting temperature of the protein (T_m) governed by chemical characteristics of additional salts. Ammonium salts were, however, ruled out; the aggregation rates of lysozyme in the presence of the ammonium salts were smaller than the ones estimated from T_m. Comparing with sodium salts, ammonium salts increased the solubility of the hydrophobic amino acids, indicating that ammonium salts adsorb the hydrophobic region of proteins, which leads to the decrease in aggregation more effectively than sodium salts. The positive relation between aggregation rate and T_m was described by another factor such as the surface tension of salt solutions. Fourier transform infrared spectral analysis showed that the thermal aggregates were likely to form β-sheet in solutions that give high molar surface tension increment. These results suggest that protein aggregation is attributed to the surface free energy of the solution.

Keywords

thermal aggregation; lysozyme; ammonium; thermal stability; surface tension

1. INTRODUCTION

Protein aggregation has been a critical problem in biotechnology (Fink, 1998; Kopito, 2000; Tsumoto *et al*., 2003) as well as in living cells (Bucciantini *et al*., 2002; Dobson, 2003; Stefani and Dobson, 2003). In order to avoid unfavorable side reactions *in vitro*, researchers have explored the solution conditions that suppress protein aggregation. Guanidine hydrochloride and urea, known as denaturants, are classical additives in protein solutions (Buchner and Rudolph, 1991; Rudolph and Lilie, 1996). Denaturants, at non-denaturing concentration, interact with polypeptide chains via multiple hydrogen bonds, leading to the increase of the protein solubility in aqueous solution (Dunbar *et al.*, 1997; Bhuyan, 2002). A calorimetric analysis has roughly estimated that 12 and 26 molecules of denaturants adsorb the native and unfolded structures of a small model protein, cytochrome *c* (Makhatadze and Privalov, 1992).

Although non-denaturing additives, such as proline, glycerol, sugar, glycine and ethylene glycol, have been used as additives in protein solutions (Rudolph and Lilie, 1996), not all of these additives can solve the problems of protein aggregation. It has been recently reported that arginine (Arg) prevents various types of protein aggregation, which is often observed during protein refolding (Tsumoto *et al.*, 1998; Umetsu *et al*., 2003; Ishibashi *et al*., 2005; Chow *et al*., 2006; Ejima *et al.*, 2006), solubilization of inclusion bodies (Tsumoto *et al*., 2003; Umetsu *et al*., 2005) and protein purification with chromatography (Ejima *et al.*, 2005a,

b). While an effect of Arg on protein stability is not significant (Arakawa and Tsumoto, 2003; Ishibashi *et al*., 2005), it considerably enhances the solubility of aggregation-prone molecules (Shiraki *et al*., 2002) by interfering non-specific protein interactions (Tsumoto *et al*., 2005). Other chemicals have also been reported as additives that increase protein solubility. Polyamines, especially spermine and spermidine, prevent thermal aggregation and inactivation of lysozyme (EC 3.2.1.17) (Kudou *et al*., 2003). A systematic investigation has thereafter shown that diamines, rather than monoamines or diols, are essential structure as prominent additives to prevent lysozyme aggregation (Okanojo *et al*., 2005). Corresponding to this, spermidine and spermine, which are diamines, effectively prevent heat-induced aggregation of lysozyme with relatively low concentration (typically 0.1 M). We found that arginine ethylester (Shiraki *et al*., 2004), amino acid esters (Shiraki *et al*., 2005) and amidated amino acid (Hamada and Shiraki, in press; Matsuoka *et al*., in press) are more effective additives than polyamine and Arg.

We believe that a study of protein solubility would give us better idea to understand the correlation of proteins and chemical properties of solution (Green, 1931; Melander and Horvath, 1977). The solubility of protein increases in the presence of low concentrations of salts (salting-in), whereas it decreases in the presence of high concentration (salting-out). In early studies on the effect of salts on protein solubility, Hofmeister discovered that various salts alter the protein solubility, and ordered salts depending on their effectiveness on protein

solubility, which is known as Hofmeister series (Kunz *et al.*, 2004). Hofmeister series is almost identical to the order of molar surface tension increment (MSTI); i.e., kosmotrope has high MSTI and, inversely, chaotropes has low MSTI. For example, a typical kosmotrope, $(NH_4)_2SO_4$, decreases protein solubility while it stabilizes the native state of protein (Lee and Timasheff, 1981; von Hippel and Wong, 1964).

Although correlation between the aggregation rate of amyloid-β and surface tension of solution was reported (Kim *et al.*, 2003), no quantitative analysis between protein aggregation and the surface tension of solution has been performed. In this paper, we focus on protein aggregation from the point of view of the surface tension of the solution. Investigating the process of thermal aggregation of lysozyme in the presence of either inorganic ions (Na_2SO_4, NaCl, $NaClO_3$, NaSCN, NaI, K_2SO_4, KCl, KSCN CsCl, CsI, $(NH_4)_2SO_4$, NH_4Cl and NH_4I) or organic ions (Na_3-citrate, Na_2-tartrate and K_3-citrate, and ArgHCl), we show the correlation between the protein stability and the surface tension of solution.

2. MATERIALS AND METHODS

2.1. Materials

Hen egg white lysozyme and ArgHCl were from Sigma Chemical Co. (St. Louis, USA). NaCl, Na_2HPO_4, and NaH_2PO_4 were from Nacalai Tesque (Kyoto, Japan). Na_2SO_4,

Na_3-citrate, Na_2-tartrate, $NaClO_3$, NaI, NaSCN, K_2SO_4, KCl, KSCN, CsCl, CsI, $(NH_4)_2SO_4$, NH_4Cl, NH_4I, K_3-citrate, and *Micrococcus lysodeikticus* were from Wako Pure Chemical Indus., Ltd. (Osaka, Japan). All chemicals used were of high-quality analytical grade.

2.2. Time course of thermal aggregation and inactivation

Heat-induced aggregation and inactivation of lysozyme was performed as follows. A stock solution containing 1.0 mg/ml lysozyme, 50 mM Na-phosphate buffer, and 0-1.35 M salts was prepared and adjusted to pH 7.4 by NaOH or HCl. A total of 200 μl of the stock solution was added to micro disposal tubes. The solutions were heated at 98˚C for various periods using a temperature control system, PC-880 (Astec, Fukuoka, Japan). After the heat treatment, the samples were centrifuged at 15,000 *g* for 20 min at 20˚C, and then the concentrations of soluble protein and residual activity were measured.

2.3. Measurements of protein concentration and residual activity

Concentration of soluble protein was determined by absorbance change at 280 nm using a ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Inc. Wilmington, Del, USA). The residual activity of the soluble fraction was determined as follows (Kudou *et al.*, 2003; Shiraki *et al.*, 2004). A total of 1.5 ml of 0.5 mg/ml *M. lysodeikticus* solution in 50 mM

sodium-phosphate buffer (pH 7.4) was mixed with 10 μl of the protein solution. The decrease in the light-scattering intensity of the solution was monitored by absorbance at 600 nm by Jasco spectrophotometer model V-550 (Japan Spectroscopic Company, Tokyo, Japan). The residual activity was estimated by fitting the data to a linear extrapolation.

2.4. Circular dichroism

Thermal unfolding of lysozyme was measured by circular dichroism (CD), with a Jasco spectropolarimeter model J-720W. Samples containing 1.0 mg/ml lysozyme, 600 mM additive, and 50 mM sodium-phosphate buffer (pH 7.4) or 0.2 mg/ml lysozyme, 600 mM additive, and 50 mM sodium-acetate (pH 4.5) were prepared. The thermal unfolding was measured by CD 288.5-nm intensity change with an increasing temperature rate of 0.5˚C/min. To determine the apparent melting temperature (T_m) for unfolding, the unfolding transition curve was analyzed from the van't Hoff plot, assuming a two-state transition and linear baselines for native and unfolded states (Pace, 1986, Kamal *et al*., 2002).

2.5. Measurement of solubility of amino acids

Solutions containing 50 mM sodium-phosphate buffer (pH 7.4), 0 or 1.5 M NaCl, NH_4Cl, Na_2SO_4 or $(NH_4)_2SO_4$, and excess concentrations of Gly-Gly, Phe, Ile or Ser were shaken and

incubated at 37˚C for one day. After the incubation, the samples were centrifuged for 20 min and the soluble concentrations of amino acids were determined by absorbance change at 280 nm using a ND-1000 UV-Vis spectrophotometer.

2.6. Fourier transform infrared spectra

Fourier transform infrared (FTIR) spectra of either solution or aggregate forms of lysozyme were measured on a Bruker IFS-66/S spectrophotometer (Bruker Optik GmbH, Ettlingen, Germany) equipped with an MCT detector (InfraRed D316/8) using the attenuated total reflection (ATR) method as described previously (Okubo and Noguchi, 2006). Protein solutions containing 5.0 mg/ml lysozyme and 600 mM additives in 50 mM Na-phosphate buffer (pH 7.4) were heated for 10 min using Astec a thermal incubator PC-880. The samples were then centrifuged at 15,000 *g* for 20 min at 20˚C. The protein aggregates were washed with same buffer to remove non-precipitated proteins. The protein solutions or aggregates were placed on the surface of a three reflection silicon prisms (3 mm in diameter) on an ATR accessory (DuraSampl*IR II*, Smiths Detection). For each spectrum, 40 interferograms were recorded at a spectral resolution of 4 cm^{-1} with a scanning mode of double-sided fact return. Spectra of the buffers were recorded under the same conditions and subtracted from the spectra of the protein.

3. RESULTS

3.1. Thermal inactivation and aggregation of lysozyme in the presence of additives

Figure 1A shows representative data for the thermal aggregation of lysozyme in the presence of either NaCl, Na_2SO_4, NH_4Cl or $(NH_4)_2SO_4$. Under the condition at 1.0 mg/ml lysozyme in 600 mM NaCl at 98˚C, there is an initial time lag of a few minutes before protein aggregation formed. The observation of the time lag was consistent with previous reports that thermal aggregation of lysozyme was described by a single rate-limiting step in the presence of all tested additives (Shiraki *et al*., 2004; Kurganov, 2002). Although the growth of protein aggregates is also a high-order process, they may be experimentally found in a first-order kinetics during refolding and thermal aggregation process (Kudou *et al*., 2003; Shiraki *et al*., 2004; Kurganov, 2002; Wang and Kurganov, 2003; Kudou *et al*., 2005). After the heat treatment for 10 min, almost all of lysozyme were aggregated even in the presence of 600 mM NaCl or Na_2SO_4, whereas ~60% proteins were retained as soluble in solution containing either NH_4Cl or $(NH_4)_2SO_4$ compared with the same concentration of sodium salts. The rate constants of aggregation of lysozyme in the presence of sodium salts such as NaCl or Na_2SO_4 were one order of magnitude larger than ones in the presence of ammonium salts such as NH_4Cl and $(NH_4)_2SO_4$ (NaCl: 0.074 min^{-1}, Na_2SO_4: 0.083 min^{-1} , NH_4Cl: 0.47, $(NH_4)_2SO_4$: 0.88 min^{-1}). The thermal inactivation processes of lysozyme (Fig. 1B) apparently showed similar tendency to those of the aggregation (Fig. 1A). Lysozyme was inactivated by heat

with first-order kinetics at rate constants 0.56 or 0.95 min^{-1} in the presence of either NaCl or NH_4Cl, which were 1.1 times faster than those of the process of aggregation. The difference between the rate constants of inactivation and those of aggregation may be caused by that not all of the inactivated molecules were removed by our experimental condition of centrifugation.

3.2. Rate constants of thermal aggregation in the presence of salts

Figure 2 shows the rate constant of the aggregation process of lysozyme in various concentrations of different types of salts. Without salts, the rate constant of thermal aggregation was 0.88 min^{-1}. Our results showed tendency that the rate constants decreased with an increase of salt concentration. The minimum rate constant of aggregation in the presence of Na_3-citrate was 0.74 min^{-1}, and was observed around 100 mM (Fig. 2A). With further increasing concentration of Na_3-citrate, the aggregation rate steeply increased. At the concentration higher than 600 mM Na_3-citrate, the aggregation rate was too fast to determine the precise rate constant under our experimental system. In the presence of either Na_2SO_4 or Na_2-tartrate, similar curves were observed with a minimum at around 400 mM with a rate constant of 0.62 min^{-1} or 0.58 min^{-1}, respectively (Fig. 2A). NaCl, $NaClO_3$, KCl and CsCl showed similar profiles; the aggregation rates decreased with increasing concentrations of additives, while they nearly remained constant values with the further addition of salts. On

the other hand, the presence of either NaI or CsI decreased the aggregation with increasing concentrations up to 1.35 M (Fig. 2B,C).

Ammonium salts like NH_4Cl, $(NH_4)_2SO_4$ and NH_4I showed different profiles from the other salts: the aggregation rate sharply decreased by the addition of 100 mM salts (Fig. 2D). Even with further increasing salt concentrations, the aggregation rate did not increase, indicating that ammonium ion possesses a high propensity to prevent protein aggregation. Table 1 lists the aggregation rates of lysozyme at 600 mM of each additive. The aggregation rates of lysozyme varied from 0.074 min^{-1} for NH_4Cl to 2.1 min^{-1} for Na_3-citrate.

3.3. Rate constants of thermal inactivation in the presence of salts

Without salts, the thermal inactivation rate of lysozyme was 1.3 min^{-1}, which was 1.5 times faster than the rate of aggregation. All plots of the inactivation rate (Fig. 3) showed similar patterns to those of the aggregation rates (Fig. 2). However, the thermal inactivation rates were higher than those of thermal aggregation, indicating that not all of the inactivated lysozyme might not be able to detect by method we employed here.

The inactivation rates were proportional to the aggregation rates with the inclination of 0.88 with a correlation coefficient of 0.99 (data not shown). This was supported by the fact that the both aggregation and inactivation processes followed first-order kinetics, suggesting

that the rate-limiting step of the both processes is the irreversible unfolding of monomeric obligate steps (Kudou *et al.*, 2003; Shiraki *et al.*, 2004; Kurganov, 2002).

3.4. Thermal unfolding analysis of lysozyme in the presence of salts

The fact that protein denaturants prevent protein aggregation is well known (Buchner and Rudolph, 1991; Rudolph and Lilie, 1996; Umetsu *et al.*, 2003; Ishibashi *et al.*, 2005; Shiraki *et al.*, 2004; Shiraki *et al.*, 2005). In this context, we investigated whether salts destabilize the native structure, by means of monitoring the thermal unfolding by near-UV CD. In the absence of any additives, ellipticity of lysozyme steeply decreased at around 80˚C. With increasing temperature above 85˚C, noise was too much to determine T_m under the solution condition because visible aggregations were happened in the cuvette (data not shown). In order to obtain the precise T_m to avoid aggregation, we performed the same experiment under an acidic condition at pH 4.5 and a low protein concentration of 0.2 mg/ml lysozyme (Fig. 4). No aggregation was observed during the thermal unfolding experiment: T_m of lysozyme at pH 4.5 in the absence of additive was 80.1˚C. The unfolding curves of lysozyme at pH 4.5 were well fitted by two-state unfolding and T_m was successfully determined in the presence of salts as well. Table 1 lists T_m of lysozyme at pH 4.5 in the presence of 600 mM of each additive. The T_m of lysozyme in the presence of Na_2-tartrate gave the maximum T_m of 83.9˚C, while

$NaClO_3$ gave the minimum T_m of 76.1°C, which is thought to be correlated with the aggregation and inactivation rate, as discussed in Fig. 6A.

3.5. FTIR spectra of lysozyme aggregates

Fourier transform infrared (FTIR) spectroscopy has been used to investigate the secondary structure of protein aggregates (Byler and Susi, 1986; van Stokkum *et al.*, 1995). We investigated the secondary structure of heat-induced aggregates occurred in each salt solution (Fig. 5). FTIR spectra of native lysozyme before heat treatment showed two peaks with maxima at 1654 and 1544 cm^{-1} corresponding to the amide I and amide II modes, respectively (Fig. 5A). The amide I bands, which originate from the C=O stretching vibrations of peptide chains, have been used to investigate the secondary structures of proteins (van Stokkum *et al.*, 1995; Martinez *et al.*, 1996; Smeller *et al.*, 2006; Meersmon and Heremans, 2003; Okuno *et al.*, 2006). The maximum frequency at 1654 cm^{-1} of soluble lysozyme (Fig. 5A) is characteristic for the α-helix dominant structure (Susi and Byler, 1986). After the heat treatment at 98°C for 30 min, FTIR spectra in the amide I region dramatically changed; the peak was shifted from 1654 cm^{-1} to 1630 cm^{-1}, which corresponds to β-sheet structure (Fig. 5B) (Smeller *et al.*, 2006; Dong *et al.*, 2000; Frare *et al.*, 2006). Figure 5C and 5D show the second-derivative spectra that normalized on the basis of the maximal intensities of the amide I bands. The spectra of aggregates showed two peaks at around 1655 and 1620 cm^{-1}, most

probably assigned as the random coil and β-sheet structures, respectively (Dong *et al.*, 2000). Interestingly, the aggregates occurred in the solution containing K_3-citrate showed lower random coil content than those in the solution containing NaSCN, suggesting that the additives could affect the secondary structure of aggregates. Table 1 lists the intensity ratio of the random coil to β-sheet content of lysozyme aggregates calculated based on the amide I bands in second-derivative spectra. The ratios of random coil to β-sheet in aggregates depended on the chemical properties of additives and ranged from 0.197 to 0.497.

3.6. Relation between melting temperature and aggregation

Figure 6A shows a plot of the relation between rate constant of thermal aggregation and T_m in the solution containing 600 mM each salt listed in Table 1. The aggregation rates increased along with the increase of T_m. For examples, aggregation rates in the presence of organic ions, such as Na_3-citrate and Na_2-tartrate, are plotted on the top and right-hand region of the graph, i.e. a high T_ms and high aggregation rates. In contrast to other salts, ammonium salts and Arg effectively suppresses the thermal aggregation irrespective of the T_m (Fig. 6A).

Figure 6B shows a relationship between the secondary structures of aggregates and T_m in the various solutions. The vertical axis shows the peak intensities at around 1653-1648 cm^{-1} range (random coil) divided by those in the 1624-1618 cm^{-1} range (β-sheet), which indicates the intensity ratios of random coil to β-sheet in aggregates. This revealed that the

additive increasing T_m lowers the content of random coil in aggregates. The negative correlation was observed in all additives tested here, including ammonium salts and Arg.

3.7. Surface tension increment

In order to understand the relationships shown in Fig. 6 by property of solution, we introduced the third parameter, molar surface tension increment (MSTI), which defines the increment of the surface tension of solution per mol concentration (Jarvis and Scheiman, 1968; Morgan and Bole, 1913; Young and Harkins, 1928). Stability of native state and the aggregation rate of lysozyme increased along with the increase of surface tension of solution (Fig. 7A and 7B). Interestingly, ammonium ions exceptionally lowered the rate constant of aggregation, suggesting that ammonium salts prevented heat-induced aggregation of lysozyme irrespective of thermal stability. Figure 7C shows the correlation between secondary structure of aggregates and MSTI. The aggregates possess high β-sheet content in solution having high surface tension. These data suggest that surface tension of the solution affects T_m, the aggregation rates and secondary structure of aggregates

3.8. Solubility of amino acids in salt solution

Solutions containing ammonium salts greatly prevented protein aggregation comparing to solutions containing either inorganic or organic additives. To understand the uniqueness of ammonium salts, the solubility of amino acids and a dipeptide in solutions containing 1.5 M of each salt was measured (Table 2). In the presence of 1.5 M NaCl, the solubility of a hydrophobic amino acid, Ile, was decreased from 1.85 x 10^{-1} M to 1.50 x 10^{-1} M. On the other hand, in the presence of 1.5 M NH_4Cl, the solubility of Ile was almost identical to the absence of salt additives, and increased 1.2-fold with that of the presence of the same concentration of NaCl. Similar data were obtained from sulfuric salts, Na_2SO_4 and $(NH_4)_2SO_4$. In the presence of 1.5 M Na_2SO_4, the solubility of Ile was 0.928 x 10^{-1} M, while in the presence of 1.5 M $(NH_4)_2SO_4$, the solubility of Ile increased 1.6-fold comparer to that seen in the presence of the same concentration of Na_2SO_4. These data suggest that ammonium ions increase the solubility of hydrophobic amino acid compared to sodium ions. An aromatic amino acid, Phe, showed similar results to Ile (Table 2). Contrary to a hydrophobic amino acid, a hydrophilic amino acid, Ser, showed almost identical solubility in the presence of any type of salts. Similarly, glycylglycine, a model of the peptide bond, showed almost identical solubility in all tested salt solutions. These data suggest that the presence of ammonium ions increases the solubility of the hydrophobic side chain of amino acids, which may lead to a decrease in the aggregation rate.

4. Discussion

One of the findings of this paper is representation of the positive relation between aggregation rate and T_m of lysozyme (Fig. 6A). This result suggests that thermal protein aggregation is also prevented under the condition where native protein structure is destabilized. This ambivalent effect is not unexpected because protein denaturants, such as urea, guanidine, carbonic acid amides and alcohols, are known to reduce protein aggregation when non-denaturing concentrations are used (Rudolph and Lilie, 1996; Orsini and Goldberg, 1978; Wetlaufer and Xie, 1995). As shown in Fig. 6A, T_m of lysozyme increased in the presence of salts in order of KSCN < NaSCN < $NaClO_3$ < CsCl < KCl < NaCl < K_2SO_4 < Na_2SO_4, which shows identical propensity of the salting-out effect described by the Hofmeister series (Kunz *et al.*, 2004).

This equilibrium consideration called salting-in and salting-out effects may partly describe the kinetic process of our aggregation data. In early study of the effect of ions on protein stability, salting-out effect found at high salt concentration has been proved to relate to the surface tension (Melander and Horvath, 1977): the effect of salting-out on protein increased with increasing surface tension of the solution. Here, it would be hypothesized that the increase of both stability of native state and protein aggregation is driven by the change in the surface free energy of protein. The free energy (ΔG) for cavity formation given by Melander et al is function of the molecular surface area (A) of the solute and surface

tension (γ) of the solvent (Melander and Horvath, 1977). The change of the free energy caused by the change of the surface area is described by the following equation.

$$\Delta\Delta G = \gamma \Delta A \quad (1)$$

The free-energy change of native state ($\Delta\Delta G_N$) is also described by the equation,

$$\Delta\Delta G_N = \Delta G_N - \Delta G_U = \gamma (A_N - A_U) \quad (2)$$

where subscripts N and U show native and unfolded states, respectively. A_N is also smaller than A_U. Equation (2) indicates that both large γ and the increase of surface area accompanied by unfolding result in the increase of negative ΔG_N, leading to the stabilization of the native state (Fig. 7A).

Simple Equation (2) holds to a good approximation to describe protein aggregation. The free-energy change of aggregation ($\Delta\Delta G_{agg}$) is the following equation,

$$\Delta\Delta G_{agg} = \Delta G_{agg} - \Delta G_U = \gamma (A_{agg} - A_U) \quad (3)$$

where subscript agg shows aggregate states. Equation (3) shows that both large γ of the solution and decreasing surface area accompanied by aggregates increase negative $\Delta\Delta G_{agg}$, leading to the increase of the aggregation rate (Fig. 7B). Furthermore, A_{agg} must be described as a function of γ because the aggregates were prone to form β-sheet rich structure in the presence of kosmotrope (Fig. 7C); i.e., the thermal unfolded polypeptides associate as compactly as possible to increase the free-energy change of aggregates ($\Delta\Delta G_{agg}$) in the

presence of kosmotropic solution. Actually, Kim et al have shown that fibril assembly of a designed amyloid-β increases with increasing surface tension of solution (Kim *et al*., 2003).

Another our finding is that ammonium salts exceptionally prevented heat-induced aggregation of lysozyme irrespective of the surface tension of the solution (Fig. 7B). As shown in Table 2, ammonium salts increased the solubility of hydrophobic amino acids rather than hydrophilic amino acids or glycylclycine, which is a model of peptide bonds. These data suggest that ammonium ions adsorb the hydrophobic side-chains of amino acids resulting in the decrease of the aggregation rate irrespective of thermal stability. It should be noted that amine compounds, typically polyamines, are good suppressors of thermal aggregation of lysozyme (Kudou *et al*., 2003; Okanojo *et al*., 2005). On the other hand, chaotropes and denaturants show different characteristics from ammonium ions; they increase the solubility of the hydrophilic amino acid and peptide bond as well as hydrophobic regions (Nozaki and Tanford, 1965; Nozaki and Tanford, 1970).

Figure 8 summarizes a qualitative image of energy landscapes for thermal aggregation of lysozyme in solutions containing various salts. It is assumed that surface area of the unfolded state is larger than that of the transition state; similarly, that of the transition state is larger than that of aggregate state. In a kosmotropic solution, all states are destabilized by high surface tension although unfolded state is more destabilized than transition state (Fig. 8A). This means that the aggregation rate accelerated (Fig. 7B). On the other hand, in a

chaotropic solution, all states are stabilized by salting-in effect although unfolded state is more stabilized than transition state (Fig. 8B). Accordingly, the aggregation rate decelerated (Fig. 7B). However, ammonium ions possess the unique property that binds to the hydrophobic amino acids (Table 2) with medium MSTI. Due to the electrostatic repulsion, the adsorption of ammonium ions on the aggregated-prone molecules decreases in the chance of protein-protein interaction, which increases the energy barrier between aggregate and unfolded states (Fig. 8C). Therefore, in the presence of ammonium ions, the aggregation rate decelerated specifically (Fig. 7B).

In this paper we show that the correlation between stability and thermal aggregation of lysozyme in salt solutions can be described by surface tension but ammonium salts were ruled out. This fundamental idea will be necessary not only for the comprehension of protein aggregation but also for the application to the biotechnology and pharmaceutical industries.

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Hirano et. al., Table 1

Table 1. Aggregation rate constants, T_m and random coil to β-sheet content of aggregates in the presence of 600 mM additives. N.D., not determined.

Additive	Aggregation Rate Constant (min^{-1})	T_m (°C)	random/β-sheet	MSTI (dyn·g/cm·mol)
Na_3-citrate	2.1	83.6	0.207	N.D.
K_3-citrate	1.8	82.2	0.197	3.12[a]
Na_2-tartrate	0.73	83.9	0.302	2.35[a]
Na_2SO_4	0.88	82.7	0.231	2.73[a]
NaCl	0.47	78.7	0.414	1.64[a]
$NaClO_3$	0.42	76.1	0.413	0.55[a]
NaSCN	0.29	68.5	0.497	N.D.
NaI	0.25	N.D.	N.D.	1.02[a]
K_2SO_4	1.3	82.5	N.D.	2.58[a]
KCl	0.49	78.3	0.412	1.4[b]
KSCN	0.33	67.9	0.495	0.45[a]
CsCl	0.46	77.6	0.416	N.D.
CsI	0.25	N.D.	N.D.	1.39[a]
NH_4Cl	0.074	77.6	0.433	1.39[a]
$(NH_4)_2SO_4$	0.083	81.3	0.339	2.16[a]
NH_4I	0.054	N.D.	N.D.	0.74[a]
ArgHCl	0.12	76.7	0.442	N.D.
No Additive	0.88	80.1	0.390	—

[a]Data from (Melander and Horvath, 1977)

[b]Data from (Young and Harkins, 1928)

Hirano et. al., Table 2

Table 2. The solubility of amino acids in the presence of 1.5 M salt.

Additive	Ile ($x10^{-1}$ M)	Phe ($x10^{-1}$ M)	Ser (M)	Gly-Gly (M)
No Additive	1.85±0.07	1.79±0.02	3.42±0.06	1.58±0.02
NaCl	1.50±0.05	1.38±0.01	3.66±0.06	1.70±0.02
NH_4Cl	1.82±0.07	1.68±0.01	3.71±0.06	1.70±0.02
Na_2SO_4	0.588±0.016	0.584±0.005	3.65±0.06	1.73±0.02
$(NH_4)_2SO_4$	0.928±0.023	0.940±0.017	3.72±0.06	1.69±0.02

FIGURE CAPTIONS

Fig. 1. Representative data for heat-induced inactivation and aggregation of lysozyme in the presence of salts. The samples containing 1.0 mg/ml lysozyme, 50 mM Na-phosphate (pH 7.4) and 600 mM additives were heated at 98˚C for the respective periods. After the heat treatment, the concentration of soluble protein (A) and residual activity (B) were determined. NaCl, closed circles; Na_2SO_4, open circles; NH_4Cl, closed squares; $(NH_4)_2SO_4$, open squares. The continuous lines show the theoretical curves fitted to the data with single exponential equations.

Fig. 2. Rate constants of heat-induced aggregation of lysozyme in the presence of various salts. (A) Na_3-citrate, closed circles; Na_2SO_4, open circles; Na_2-tartrate, closed squares. (B) NaCl, closed circles; $NaClO_3$ open circles; NaI, closed squares. (C) KCl, closed circles; CsCl, open circles; CsI, closed squares. (D) $(NH_4)_2SO_4$, closed circles; NH_4Cl, open circles; NH_4I, closed squares.

Fig. 3. Rate constants of heat-induced inactivation of lysozyme in the presence of various salts. (A) Na_3-citrate, closed circles; Na_2SO_4, open circles; Na_2-tartrate, closed circles. (B) NaCl, closed circles; $NaClO_3$ open circles; NaI, closed squares. (C) KCl, open circles; CsCl, open circles; CsI, closed squares. (D) $(NH_4)_2SO_4$, closed circles; NH_4Cl, open circles; NH_4I, closed squares.

Fig. 4. Representative data for thermal unfolding curves of lysozyme in the presence of salts monitored by near-UV CD. The samples containing (A) 1.0 mg/ml lysozyme, 600 mM additive and 50 mM Na-phosphate buffer (pH 7.4) and (B) 0.2 mg/ml lysozyme, 600 mM additive and 50 mM Na-acetate buffer (pH 4.5) were heated at an increasing temperature rate of 0.5˚C/min. No additives, solid line; Na_2SO_4, broken line; NH_4Cl, dotted line. Solid lines indicate the theoretical curves calculated on the basis of the two-state thermal unfolding equation.

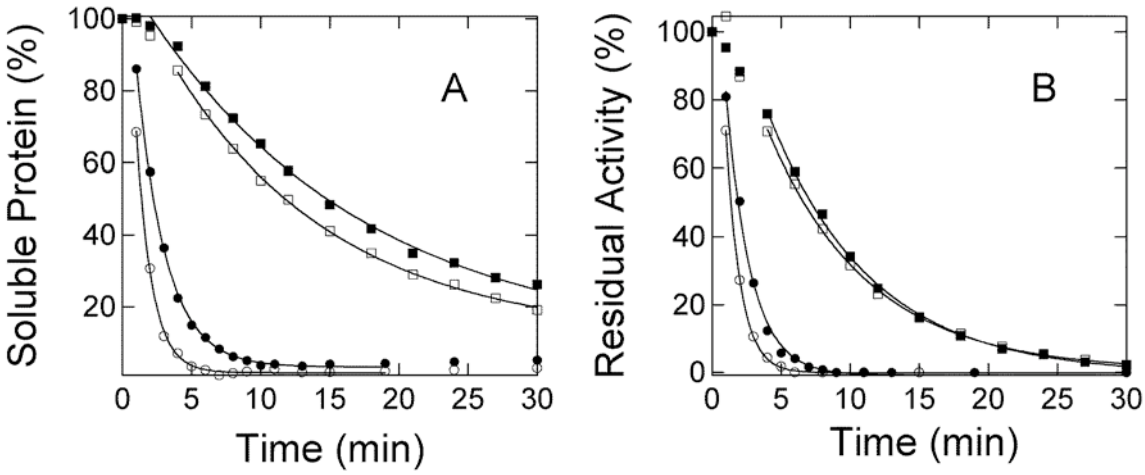
Fig. 5. FTIR spectra in the amide I and amide II regions of lysozyme. The samples containing 5.0 mg/ml lysozyme, 600 mM additive and 50 mM Na-phosphate buffer (pH 7.4) were measured before (A,C) of after (B,D) heat treatment at 98 °C. Original spectra (A,B) and second-derivative spectra (C,D) are shown. The spectra are normalized on the basis of the maximal intensity in the amide I bands. Solid line, no additives; dotted line, K_3-citrate; broken line, NaSCN.

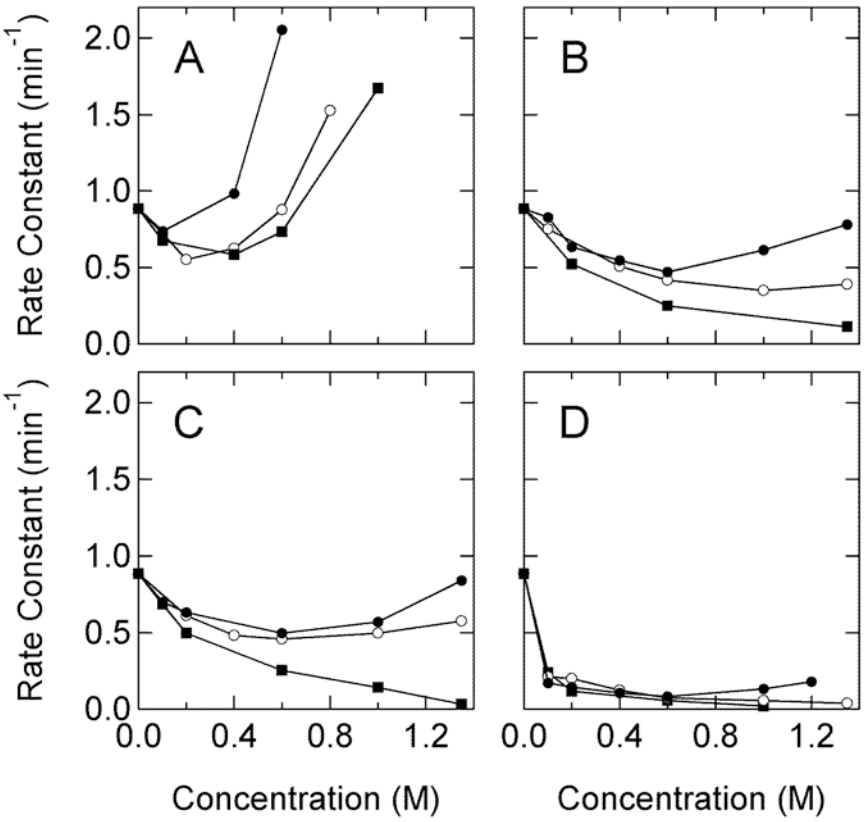
Fig. 6. The relationship between rate constant of aggregation and T_m (A) and between ratio of random coil to β-sheet of aggregates and T_m (B) in the presence of 600 mM additives. The parameters are shown in Table 1. Open circles, ammonium ions (NH_4Cl, $(NH_4)_2SO_4$ and NH_4I); closed triangles, Arg; open triangles, organic ions (Na_3-citrate, K_3-citrate and Na_2-tartrate); closed circles, other salts (Na_2SO_4, NaCl, $NaClO_3$, NaSCN, K_2SO_4, KCl, KSCN and CsCl); crosses, no additives.

Fig. 7. The relationship between T_m and MSTI (A), between rate constant of aggregation and MSTI (B), and between ratio of random coil to β-sheet content of aggregates and MSTI (C). The parameters are shown in Table 1. Open circles, ammonium ions (NH_4Cl, $(NH_4)_2SO_4$ and NH_4I); closed triangles, organic ions (K_3-citrate, and Na_2-tartrate); closed circles, other salts (Na_2SO_4, NaCl, $NaClO_3$, NaI, K_2SO_4, KCl, KSCN and CsI).

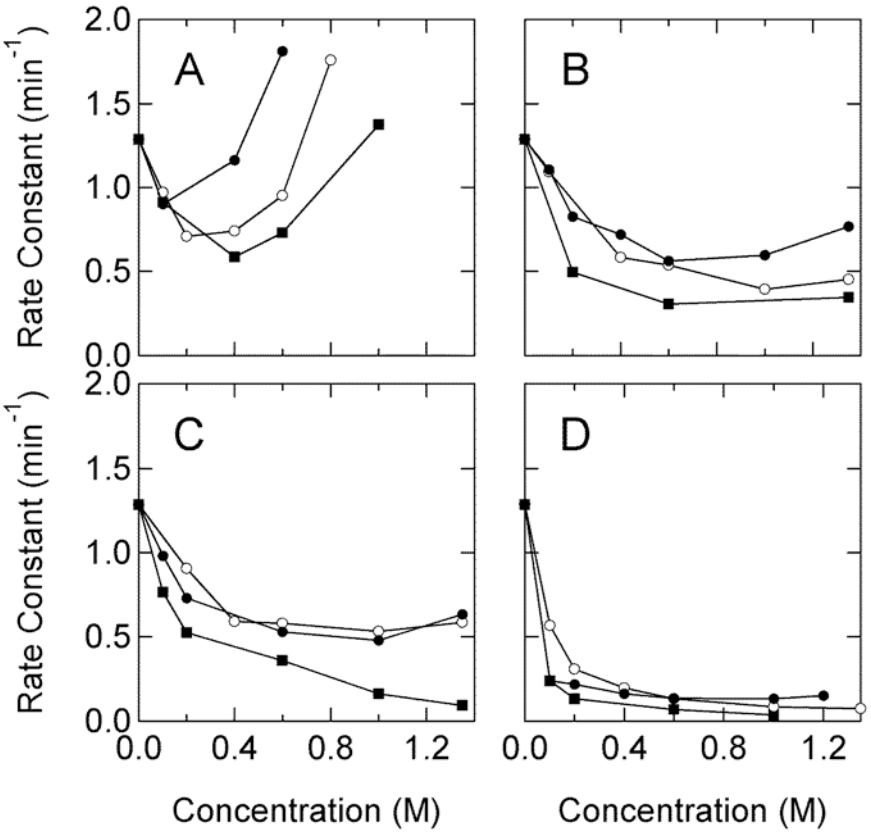
Fig. 8. Schematic representation of the energy sates of unfolded, transition and aggregates of lysozyme in kosmotrope, chaotrope and ammonium salt. The characters (U, $A^{\ddagger}$ and A_2) denote unfolded state, transition state, and aggregation state, respectively. The broken and solid lines show the states in the presence or absence of additives, respectively.

Hirano et.al., Fig.1



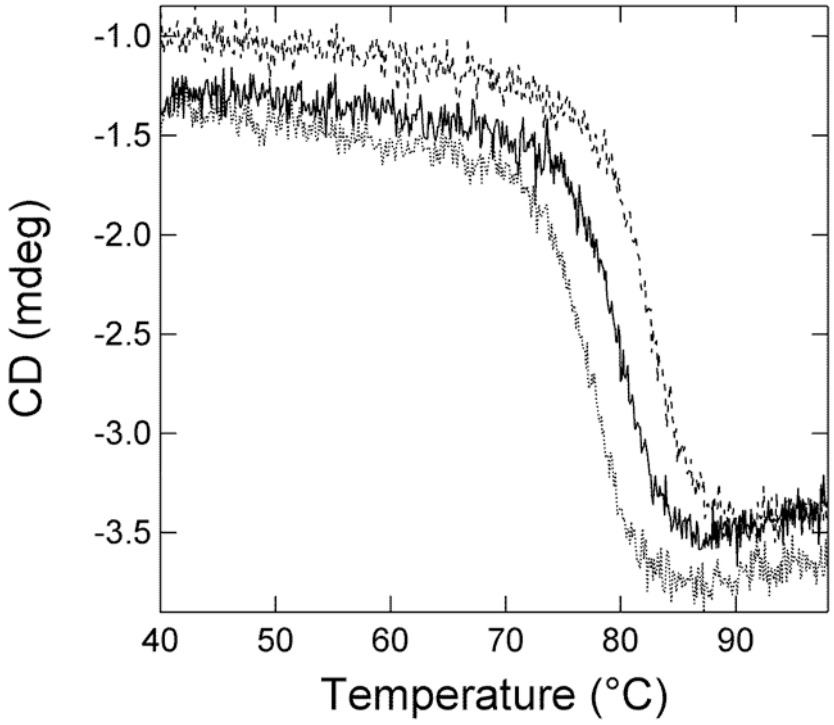


Hirano et.al., Fig.2

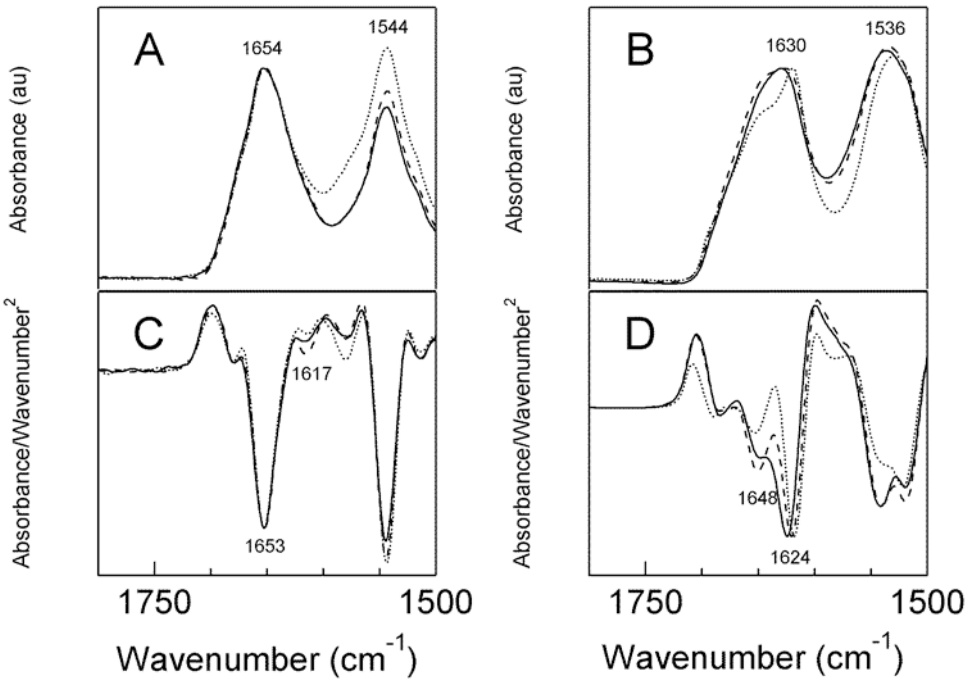


Hirano et.al., Fig.3

Hirano et.al., Fig.4



Hirano et.al., Fig.5



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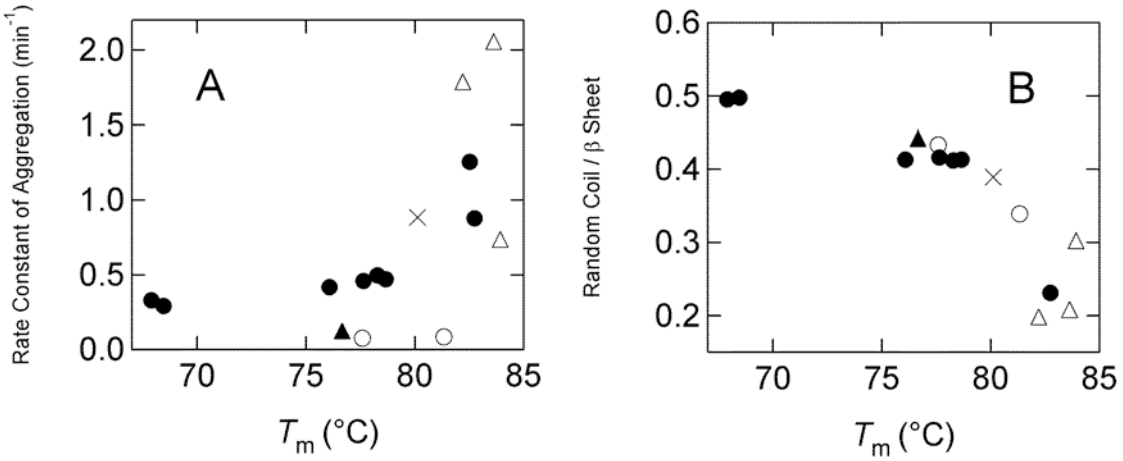
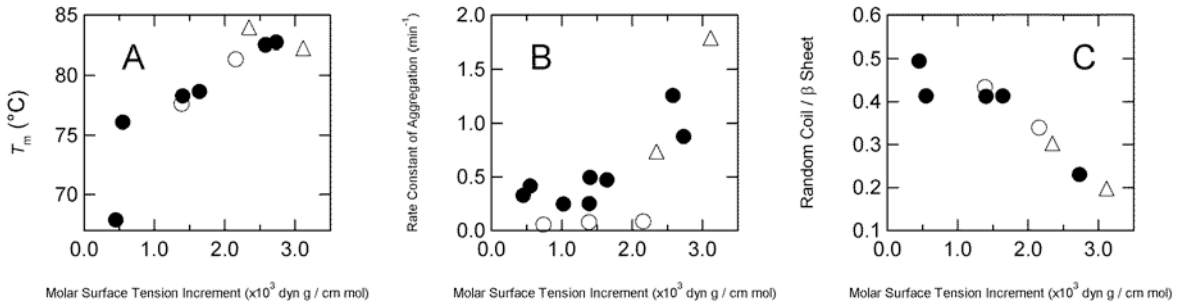


Hirano et.al., Fig.7



Hirano et.al., Fig.8