International Journal of Biological Macromolecules 87 (2016) 563–569

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Charge state of arginine as an additive on heat-induced protein aggregation.

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ARTICLE INFO

Article history:
Received 29 January 2016
Received in revised form 8 March 2016
Accepted 9 March 2016
Available online 14 March 2016

Keywords:
Arginine
Charge state
Protein aggregation
Solvent additive
Lysozyme

ABSTRACT

Arginine (Arg) is one of the most versatile solvent additives, such as suppressing protein aggregation, increasing solubility of small aromatic compounds and peptides, and preventing protein binding on solid surfaces. In this study, we investigated the role of the charged state of α-amino group of Arg for the prevention of protein aggregation. As expected, Arg effectively suppressed thermal aggregation of hen egg-white lysozyme at neutral pH, whereas the suppression effect diminished at and above pH 9.0, which corresponds to the p*K* of Arg's α-amino group. The pH dependence of Arg as an aggregation suppressor was confirmed by additional experiments with neutral proteins, bovine hemoglobin and bovine γ-globulin. Interestingly, *N*-acetylated arginine, which lacks the α-amino group, showed a weaker suppressive effect on protein aggregation than Arg, even at neutral pH. These results indicate that both positively charged α-amino group and guanidinium group play important roles in suppressing heat-induced protein aggregation by Arg. The elucidated limitation of Arg at alkaline pH provides new insight in the application as well as the mechanism of Arg as a solvent additive.

1. Introduction

Arginine (Arg) is one of the most commonly used additives for preventing protein aggregation without altering or destabilizing the tertiary structure of the protein [1–3]. Since Arg was found to be a chemical chaperone, as reported by Buchner and Rudolph [4], various applications of Arg as a solvent additive have been reported, including the aggregation suppression of chemically reduced [5,6] and thermally denatured [6–8] proteins, reduction of the viscosity of concentrated protein solution [9,10], solubilization of poorly soluble compounds [11–13], and improvement of column chromatography performance [14,15]. To date, Arg is one of the most versatile additives used to improve the stability and solubility of proteins, peptides, and chemicals [16].

What is the mechanism behind these effects of Arg? The main driving force has been considered to be the interactions of the guanidinium group of Arg with aromatic or charged residues through cation-π interactions [17–19]. In addition, it was reported that the aliphatic moiety of the Arg side chain interacts with the aromatic ring of 4-mercaptoethylpyridine [20]. Thus, multimodal interactions seem to occur between Arg and the target solutes. Moreover, we have previously reported that both the guanidinium and α-amino groups of Arg play an indispensable role in protein stabilization [21]. The depletion of the negative charge by the chemical modification of the carboxyl group improved the effect of Arg in stabilizing against the thermal aggregation of proteins [22–24]. In this study, we focused on the role that the α-amino group of Arg plays in suppressing protein aggregation. The results presented here are the first reported about the effects of the α-amino group of Arg on protein aggregation.

2. Materials and method

2.1. Materials

Ultra-pure water was obtained from a Milli-Q SP TOC system water purifier (Millipore Corp., Bedford, Mass., USA). L-arginine (Arg base), L-arginine hydrochloride (ArgHCl), L-lysine hydrochloride (Lys), glycine (Gly), guanidine hydrochloride (Gdn), hydrochloric acid (HCl), and sodium hydroxide (NaOH) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nα-acetyl-L-arginine (Ac–Arg), hen egg-white lysozyme, hemoglobin from bovine blood, and γ-globulin from bovine blood were obtained from Sigma–Aldrich Corp. (St Louis, Mo., USA). Sodium chloride (NaCl) and sodium phosphate were obtained from

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http://dx.doi.org/10.1016/j.ijbiomac.2016.03.015
0141-8130/

Nacalai Tesque Inc. (Tokyo, Japan). All chemicals used were of reagent grade and used as received.

2.2. Turbidity measurements

The heat-induced aggregation of proteins was measured as follows. Stock protein solutions containing 1.0 mg/ml protein in 50 mM sodium phosphate, 50 mM glycine, and specified amounts of additives were prepared and adjusted to pH 7.0–10.0 by the addition of NaOH or HCl. A 2 mL aliquot of the stock solution was introduced into a 1 cm path-length glass cell and incubated at indicated temperatures using a Jasco temperature controller ETC-550T (Japan Spectroscopic Co., Ltd., Tokyo, Japan). For lysozyme experiments, the sample solutions were incubated at 90 °C and the light scattering intensity at 400 nm was monitored using a Jasco spectrophotometer model V-550 (Japan Spectroscopic Co., Ltd., Tokyo, Japan). For hemoglobin, the experimental conditions were modified to 70 °C and 500 nm; note that hemoglobin has an absorbance peak at 400 nm.

2.3. Analysis of protein aggregation kinetics curves

To analyze the protein aggregation kinetics curves measured by the increase in light scattering intensity (I) as a function of time (t) and characterize the effect of Arg on protein aggregation, the lag period was calculated by fitting the section of the sharply increase of light scattering intensity. We use the Eq. (1) as below,

$$I = I_0 + \nu(t - t_0)^2, \quad (1)$$

where I_0 is the initial value of the light scattering intensity, t_0 is the length of the lag period, and ν is a parameter characterizing the initial rate of aggregation. The empirical Eq. (1) was proposed after the inspection of the initial parts of the kinetic curves [25]. When the additive suppresses protein aggregation, the lag period t_0 will become longer than that in the absence of an additive. In this study, we investigated the length of the lag period to evaluate the suppression effect of the additives.

2.4. Circular dichroism spectroscopy

Circular dichroism (CD) spectroscopy was performed using a Jasco CD spectropolarimeter model J-720W with a Peltier cell holder model PTC-348W. Heat-induced unfolding was monitored at 288.5 nm with a heating rate of 1 °C/min using a 1 cm path-length cell. The native lysozyme was solubilized in 50 mM glycine and 50 mM sodium phosphate buffer at pH 8.0 or 9.5 containing 200 mM additive. The final protein concentration was adjusted to 0.1 mg/ml.

3. Results

We investigated the effects of ArgHCl on heat-induced protein aggregation as a function of pH in the range 7.0–10.0, which causes the charge state of Arg to vary. To the best of our knowledge, lysozyme is the best model protein for this experiment, because lysozyme aggregates under a broad range of pH, from neutral to alkaline, owing to its high isoelectric point of 11.0 [26]. Fig. 1 shows representative light scattering intensity kinetic data for a 1.0 mg/ml lysozyme solution incubated at 90 °C in the absence or presence of ArgHCl at various pH. Fig. 1A shows the results at pH 7.0. In the absence of ArgHCl (solid line), turbidity sharply increased with incubation time, with nearly no lag period. When 100 mM ArgHCl was added, the turbidity increase was still sharp (dotted line) but with a slightly increased lag period. Upon further increasing ArgHCl concentration to 500 mM (broken line), the lag period greatly increased and turbidity increase became less sharp. At 1000 mM ArgHCl (broken and dotted line), the lag period drastically increased and turbidity increase was much shallower. Fig. 1B shows the results at pH 8.0. The lag period was short and the turbidity increase was sharp in the absence of ArgHCl (solid line), similar to the results obtained at pH 7.0. A similar trend to the results at pH 7.0 was observed at pH 8.0 in the presence of ArgHCl. Increasing ArgHCl concentration resulted in a longer lag period and a shallower turbidity increase. These results clearly demonstrate the suppressive effects of Arg on the thermal aggregation of lysozyme at these pH. The results at pH 9.0 (Fig. 1C) and pH 10.0 (Fig. 1D) are in sharp contrast to those at pH 7.0 and 8.0. The turbidity increase was sharp regardless of the ArgHCl concentration. Only a small increase at pH 9.0 and a negligible increase at pH 10.0 were observed in the lag period. Thus, Arg exhibited little suppression of the heat-induced aggregation of lysozyme at pH 9.0 and 10.0. The lag periods were determined by fitting the data in Fig. 1 to Eq. (1) and are plotted versus pH in Fig. 2A for different ArgHCl concentrations. The lag periods thus determined were 214, 112, 79, and 30 s at pH 7.0, 8.0, 9.0, and 10.0, respectively, slowly decreasing with increasing pH (closed circles). Fig. 2A also shows the pH dependence at different ArgHCl concentrations. At any ArgHCl concentration, the lag periods decreased with increasing pH, demonstrating that higher pH accelerates aggregation regardless of ArgHCl concentration. It is evident in Fig. 2A that a strong ArgHCl concentration dependence in the lag period at pH 7.0 and 8.0 exists, but such dependence appears to diminish at pH 9.0 and 10.0. All data points at different ArgHCl concentrations converge into each other. Such an ArgHCl concentration dependence in the lag period is more clearly seen in Fig. 2B, wherein the relative lag period is plotted against ArgHCl concentration. At pH 7.0 (closed circles), the lag period sharply increased with ArgHCl concentration, as is the case at pH 8.0 (closed squares). Such ArgHCl concentration dependence is weak at pH 9.0 (open circles) and 10.0 (open squares). The small increase in relative lag period at pH 9.0 and 10.0 may be due to the suppressive effects of the guanidinium group on aggregation. Arg suppressed the protein aggregation at pH 7.0 and 8.0, but it did not at pH 9.0 and 10.0. Arg has three functional groups, an α-carboxyl group (pK 1.8), a guanidinium group (pK 12.5), and an α-amino group (pK 9.0) [27]. Thus, the pH-dependence data show that the charge state of the α-amino group of Arg plays an important role in the inhibition of protein aggregation.

We examined the lag period of the thermal aggregation of lysozyme in the presence of 200 mM NaCl, Gly, Lys, Gdn, and ArgHCl at different pH; note that 200 mM ArgHCl exhibited strong suppressive effects at pH 7.0 and 8.0 (see Fig. 2B). Fig. 3A shows the results at pH 8.0. It is evident that NaCl is ineffective and Gly, Lys, and Gdn are effective but to a much lesser extent than ArgHCl. NaCl increases the ionic strength of the solution, thus indicating that higher ionic strength neither suppresses nor enhances aggregation. Gly is dipolar at pH 8.0 and thereby may weakly suppress aggregation by dipole–dipole interaction with lysozyme. Lys is positively charged at pH 8.0, indicating a contribution of net positive charge to aggregation suppression, which is weaker than ArgHCl. The data for Gdn are similar to those of Gly, perhaps owing to the 200 mM concentration being too low to be effective; the effects of Gdn as a protein denaturant normally occur above 2 M. Interestingly, the effects of additives at 200 mM concentration are far weaker at pH 9.5 (Fig. 3B), as seen with ArgHCl at pH 9.0 and 10.0 (Fig. 2). NaCl showed no effects at pH 9.5 as well as at pH 8.0, and Gly and Lys were marginally effective. Both Gdn and ArgHCl showed small suppressive effects with identical magnitude, suggesting that their guanidinium groups play a major role at pH 9.5, where the contribution of the positively charged α-amino group of Arg diminishes, as indicated earlier in Fig. 2B. These results suggest that both the guanidinium and positively charged α-amino groups of Arg play an important role in suppressing protein aggregation.