Small Amine Molecules: Solvent Design Toward Facile Improvement of Protein Stability Against Aggregation and Inactivation

Kentaro Shiraki[1,*], Shunsuke Tomita[2] and Naoto Inoue[1]

[1]*Faculty of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan;* [2]*Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan*

Kentaro Shiraki

Abstract: Proteins are prone to inactivation in aqueous solutions because chemical modification and aggregation usually occur, particularly at high temperature. This review focuses on the recent advance in practical application with amine compounds that prevent the heat-induced inactivation and aggregation of proteins. Coexistence of amine solutes, typically diamines, polyamines, amino acid esters, and amidated amino acids decreases the heat-induced inactivation rate of proteins by one order of magnitude compared with that in the absence of additives under low concentrations of proteins at physiological pH. The amine compounds mainly suppress chemical modification, typically the β-elimination of disulfide bond and deamidation of asparagine side chain, thereby preventing heat-induced inactivation of proteins. Polyamines do not improve the refolding yield of proteins, owing to decrease in the solubility of unfolded proteins. In contrast, arginine is the most versatile additive for various situations, such as refolding of recombinant proteins, solubilized water-insoluble compounds, and prevention of nonspecific binding to solid surfaces; however, it is not always effective for preventing heat-induced aggregation. Amine compounds will be a key to prevent protein inactivation in solution additives.

Keywords: Amine compound, arginine, polyamine, amino acid derivative, protein aggregation, protein inactivation, solvent additive.

1. INTRODUCTION

Proteins are unstable in aqueous solution. The instability of proteins poses a serious problem for their application in purification, shipping, storage, and handling. The causes of protein inactivation can be classified into physical and chemical processes; the former involves structural unfolding and aggregation, whereas the latter involves chemical modification [1]. Various kinds of low molecular weight additives have been developed to avoid protein inactivation and aggregation [2].

This review focuses on the amine compounds that prevent the heat-induced inactivation and aggregation of proteins as solvent additives and provides the following information: 1) We summarize a systematic property of amine compounds that prevents the heat-induced inactivation and aggregation of proteins, such as amino acids, amino acid alkylesters, amidated amino acids, diamines, and naturally occurring polyamines; 2) We introduce the major causes for chemical modifications of proteins, deamidation of Asn residue, β-elimination of disulfide bond, and disulfide exchange; 3) We describe the prevention of such chemical modification by amine compounds from heat treatment. The inhibition of chemical modifications results in the prevention of heat-induced inactivation of proteins; 4) We introduce the most

well-known additive Arg. Arginine prevents the heat-induced aggregation of proteins as well as increases the solubility of aromatic compounds and aggregation-prone unfolded proteins; 5) We discuss the additives regarding the distinction between heat-induced aggregation and refolding-induced misfolding. Finally, we discuss why amine compounds from living cells prevent the heat-induced degradation of proteins.

1.1. Amine Compound Prevents Heat-induced Inactivation and Aggregation of Proteins

Several studies have reported on amine compounds as solvent additives for the prevention of heat-induced inactivation and aggregation under similar conditions, namely heat treatment at 98°C at neutral pH (pH 6.5-7.5) and relatively low protein concentration (0.2-1.0 mg/ml) for model proteins such as hen egg white lysozyme and bovine ribonuclease A (RNase A). The chemical structures of the reported amine additives and related compounds are shown in (Fig. **1**). It should be noted that additives that prevent heat-induced inactivation and aggregation have amine groups. Firstly, we briefly summarize the existing data of heat-induced inactivation and aggregation with additives as follows:

i) Arg achieves a stronger effect in preventing the heat-induced aggregation of proteins than the other naturally-occurring amino acids for lysozyme and other model proteins such as *Candida rugosa* lipase, bovine trypsin, bovine α-chymotrypsinogen, bovine RNase A, horse myoglobin,

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*Address correspondence to this author at the Faculty of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan; Tel/Fax: +81-29-853-5306; E-mail: shiraki@bk.tsukuba.ac.jp

bovine cytochrome c, and concanavalin A [3]. The additives were used at 0.2 M and 0.5 M. Arg has commonly been used as a protein refolding additive since a quarter of a century [4]; however, Shiraki et al. pointed out a new application of Arg as additive that prevents the heat-induced aggregation of proteins [3].
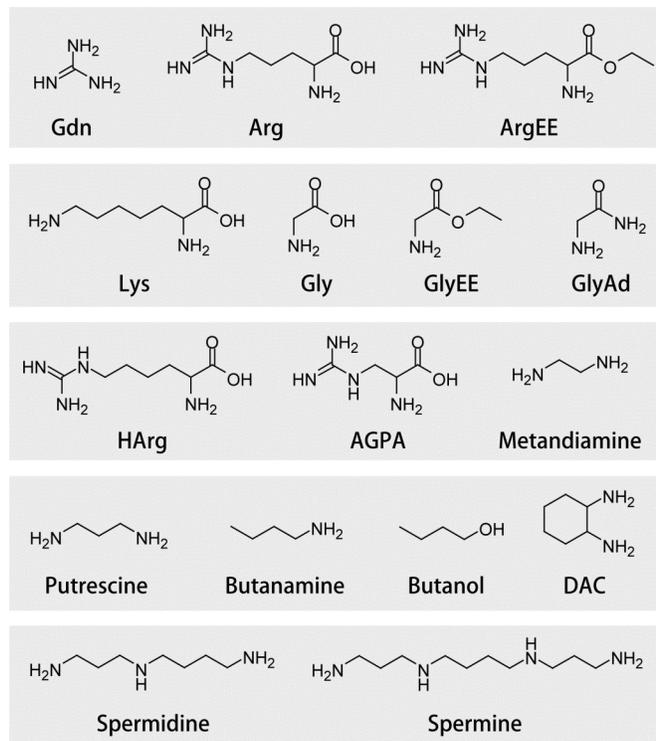

Fig. (1). List for the chemical structures of additives. Gdn (guanidine hydrochloride), Arg (arginine), ArgEE (arginine ethylester), Lys (lysine), Gly (glycine), GlyEE (glycine ethylester), GlyAd (glycine amide), HArg (homo-arginine), AGPA (2-amino-3-propionic acid), and DAC (1,2-diaminocyclohexane).

ii) Arg ethylester (ArgEE) was more effective in preventing both the heat-induced inactivation and aggregation of lysozyme than Arg [5]. Heat-induced inactivation and aggregation of 1.0 mg/ml lysozyme at pH 7.1 was apparently a single-exponential process even in the absence and presence of additives; the rate constants of heat-induced inactivation were 7.0×10^{-3} s^{-1} (no additive), 4.2×10^{-3} s^{-1} (in 0.1 M Arg), and 1.2×10^{-3} s^{-1} (in 0.1 M ArgEE). The inactivation results from both irreversible unfolding of the tertiary structure and chemical modification on the catalytic site, though the cited study shows the difference in the inactivation rate without further discussion.

iii) It was serendipitously found that all varieties of amino acid alkylesters, as well as ArgEE, prevented both heat-induced inactivation and aggregation of lysozyme more effectively than amino acids themselves [6]. Glycine ethylester (GlyEE) was more effective in the heat-induced inactivation and aggregation of lysozyme than ethylene glycol monoester, guanidine, and Gly. Similarly, amidated amino acids, including glycineamide (GlyAd), prevented the heat-

induced inactivation and aggregation of lysozyme [7]. These data indicate that the modification of the carboxyl group of the amino acids is favorable for the heat-induced degradation of proteins.

iv) Arg derivatives (homo-arginine, Arg amide, and 2-amino-3-guanidinopropionic acid) increased the refolding yield of lysozyme from a reduced and denatured state compared to Arg [8]. A comparative analysis of amino acid derivatives as co-solutes revealed that amino acid alkylesters and amidated amino acids act as favorable additives for preventing the heat-induced degradation of proteins [9], while Arg derivatives are favorable for increasing the refolding yield of lysozyme. It is interesting that heat-induced inactivation is a different mechanism from the refolding-induced misfolding. This viewpoint will be discussed later.

v) Linear aliphatic diamines (1,3-diaminopropane, 1,4-diaminobutane, and 1,5-diaminopentane) at 0.1 M prevented the heat-induced inactivation and aggregation of 0.2 mg/ml lysozyme [10]. Note that the diols (i.e., 1,3-propanediol, 1,4-butanediol, and 1,5-pentanediol) did not prevent the heat-induced inactivation and aggregation of lysozyme. Arrhenius plots showed linear correlation between the temperature of the heat treatment and residual activity after the heat treatment in the presence or absence of diamines [10], meaning that a dominant single rate-limiting step controls the early stage of the heat-induced inactivation of lysozyme, which is possibly a chemical modification.

vi) Other amine compounds, such as the naturally-occurring polyamines of putrescine, spermidine, and spermine [11], and the synthetic compounds of 1,2-diaminocyclohexane, 1,3-diaminocyclohexane, and 1,4-diaminocyclohexane [12] were found to be superior suppressors of the heat-induced inactivation and aggregation of lysozyme and RNase A. The rate constants of heat-induced inactivation and aggregation at 98°C of lysozyme were one order of magnitude higher than those in the presence of 0.1 M diamines. The pH-dependent experiment showed that the charged state of diamines plays an important role for the prevention of thermal degradation [12].

vii) Inorganic ammonium salts prevented heat-induced aggregation more effectively than sodium salts [13]. For example, 0.6 M NH$_4$Cl and (NH$_4$)$_2$SO$_4$ as co-solutes decreased the aggregation rate of lysozyme by one-order magnitude compared with NaCl and Na$_2$SO$_4$. Aggregation rates of lysozyme in various salts were in good correlation with the molar surface tension increment of solvents, except for ammonium salts; thus, it was concluded that ammonium salts possess a function that prevents the thermal aggregation of proteins, rather than surface tension with the Hofmeister effect.

The above-mentioned data indicate that amine compounds, including ammonium ions, prevent the heat-induced inactivation and aggregation of proteins. As described below, the molecular mechanisms of the amine compounds acting as additive are mainly triggered by the prevention of chemical modifications, which would lead to the prevention of the irreversible inactivation of proteins.

1.2. Chemical Modifications of Protein by Heat Treatment

Chemical modifications of protein by heat treatment have been well documented during the 1980s. Dr. Klivanov et al. examined the chemical modifications of hen egg white lysozyme and bovine RNase A as models when treated at high temperature [14-17]. As shown in (Fig. **2**), the chemical modifications of proteins were classified into five kinds: 1) deamidation of Asn and Gln side chains; 2) β-elimination of disulfide bond; 3) disulfide bond exchange of Cys; 4) oxidation of Met and Cys side chains; and 5) hydrolysis of the peptide bond with Asp.

Deamidation of the side chains of Asn and Gln (Fig. **2A**) frequently occurs among chemical modifications at broad pH ranges [18, 19], which is one of the main problems hampering the production of pharmaceutical antibody byproducts [20] and sample preparation for mass spectroscopy [21]. The molecular mechanism of deamidation was well investigated using a hexapeptide as a model [22]. Under the neutral or weak alkaline pH (typically pH 7-11), peptide deamidation occurs via a cyclic imide intermediate, while under the acidic pH, the Asn side chain directly undergoes hydrolysis. The deamidation rate of Asn is one order of magnitude faster than that of Gln.

Disulfide bond-linked Cys side chains stabilize the thermodynamic structure of the native state by decreasing the conformation entropy of the unfolded state. However, the disulfide bond is prone to cause β-elimination (Fig. **2B**) from neutral to alkaline pH ranges [16], such as monoclonal antibody during storage [23] and sample preparation for mass spectroscopy [24]. The occurrence of β-elimination triggered the deprotonation of Cα-H bonds by base catalyst, followed by the formation of thiocysteine and dehydroalanine [25]. Thiocysteine further degrades other sulfur compounds. Subsequently, the product dehydroalanine is prone to link with the Lys side chain.

The thiolate ion on Cys side-chain is an active nucleophile; thus, the sulfur atom of the dislfide bond is subjected to nucleophilic attack by thiolate ion. The disulfide bond exchange also occurs under a high concentration of protein at weak alkaline pHs [26, 27]. Such disulfide bond exchange causes protein aggregation by intermolecular cross-linking under high concentrations of proteins (> 1 mg/ml) around weak alkaline pH (around approximately the isoelectric point of the thiol side chain, pH 8.2).

Other chemical reactions are also known; the oxidation of sulfur containing amino acids (Met and Cys) (Fig. **2C**) and hydrolysis of peptide bond occur during heat treatment in aqueous solutions. Met and Cys in polypeptide are oxidized in aqueous solution [28, 29]. The C-terminus of Asp residue is prone to hydrolyze under weak-acidic condition [30].

The main causes of chemical modification at approximately neutral pHs at high temperature are deamidation, β-elimination, and disulfide exchange. As described below, β-elimination and deamidation are dominant causes at low protein concentration (typically < 1.0 mg/ml), while disulfide bond exchange is the dominant cause with increasing concentrations of proteins. The practical application of amine compounds as additives that prevents such chemical modifications is described below.
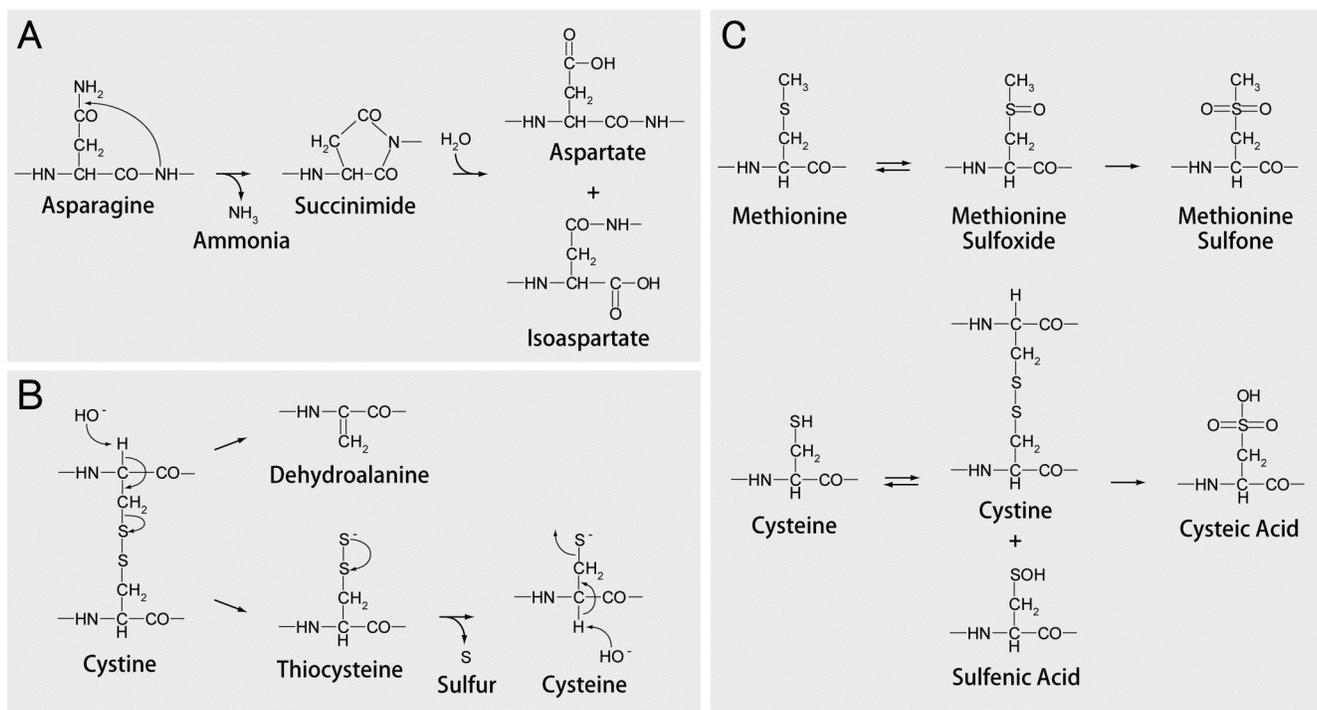

Fig. (2). Main chemical modifications of proteins in aqueous solution. (**A**) Deamidation of the Asn side chain via a cyclic imide intermediate. (**B**) β-elimination of the disulfide bond. (**C**) Oxidation of Met and Cys side chains.

1.3. Amine Compounds Prevent β-elimination of the Disulfide Bond and Deamidation of the Asn Side Chain

To investigate amine co-solutes in general, inactivation rates were compared with lysozyme and RNase A in the presence of eight kinds of additives, namely Arg, Gly, Lys, guanidine (Gdn), GlyAd, spermidine, NaCl, and NH$_4$Cl [31]. The half-life time of the enzyme activity (τ_1) treated at 98°C was 3.6 min under the condition at 1.0 mg/ml RNase A in 50 mM Na–phosphate buffer (pH 7.0; Fig. **3A**). In the presence of additives, the τ_1 values varied from 4.5 min (NaCl) to 41.1 min (GlyAd). As expected, the order is similar to that previously reported; amine compounds decreased the inactivation rate of the enzyme. GlyAd was the most favorable additive to prevent the heat-induced inactivation of both enzymes; the order of the effects of additives was GlyAd > spermidine > NH$_4$Cl > Arg = Lys > Gly = Gdn = NaCl. A similar order was obtained for the inactivation rate of lysozyme from 2.6 min (Gdn) to 19.5 min (GlyAd).

From the results, it was concluded that the suppressive effects of additives for heat-induced inactivation are because of their abilities to suppress the intrinsic chemical modifications of proteins. The most important point is the clear correlation between the inactivation rate of RNase A and lysozyme with the correlation coefficient of 0.98 in the presence of eight kinds of additives (Fig. **3B**). Moreover, the deamidation rate of the Asn side chain of RNase A and lysozyme decreased in the order GlyAd > spermidine > Lys > Arg, which is correlated to the inactivation rate of both proteins in the presence of these additives (Fig. **3C**). Similarly, the β-elimination of the proteins decreased in the almost same order (Fig. **3D**). Thus, these results indicate that amine compounds prevent the chemical modifications such as deamidation and β-elimination, leading to the prevention of thermal degradation of proteins.

In addition, the question of why amine co-solutes prevent such chemical modifications of proteins is interesting. Amine compounds are also cations; for example, polyamines
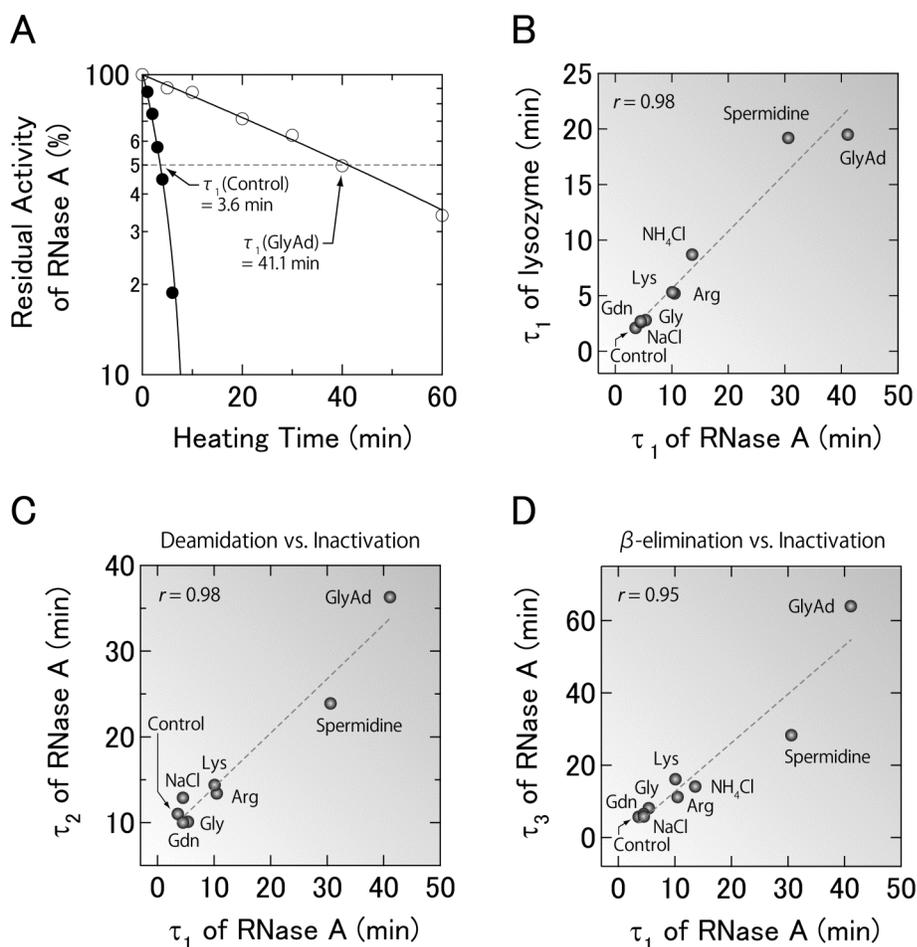

Fig. (3). Thermal inactivation of RNase A and lysozyme in the presence of additives. The protein of 1.0 mg/ml with 200 mM additives and 50 mM Na-phosphate (pH 7.0) were heated at 98°C, and then the residual activity, deamidation, and β-elimination were determined. (**A**) The residual activity of RNase A after the heat treatment at respective time. The value of τ_1 is defined as the half-life time of the residual activity of enzyme. (**B**) The correlation between τ_1 of RNase A and τ_1 of lysozyme after the heat treatment in the presence of additives. (**C**) The correlation between τ_1 and the time for deamidation of one Asn residue per a protein (τ_2) of RNase A after the heat treatment in the presence of additives. (**D**) The correlation between τ_1 and the time for β-elimination of 0.2 disulfide bond per a protein (τ_3) of RNase A after the heat treatment in the presence of additives. The r values in figures are correlation coefficients. The raw data were obtained from a previous study [31].

interact with the negatively charged region on proteins as a polycation that may protect the chemical modification. However, the binding between proteins and small amine compounds is too weak to identify the binding assay by surface plasmon resonance (SPR) and the structural biology by x-ray crystallography and nuclear magnetic resonance (NMR). Molecular dynamics simulation suggests weak binding between the enzyme chymotrypsin and polyamines [32]. Thus, in the future, the precise chemical mechanism may be described by quantum mechanics by calculation combined with various spectroscopic techniques.

From an industrial and experimental application point of view, 50-100 mM GlyAd or polyamine is recommended as an additive in protein solution when the prevention of chemical degradation and protein inactivation during storage and shipping at high temperature is preferred. NH$_4$Cl will be required at high concentration, such as 500 mM. In addition, Arg at 100-500 mM is a good condition for a solution when noncovalent interactions between proteins and/or proteins and solid surfaces are the driving force for the inactivation and aggregation of the proteins, as described below.

1.4. Arginine is a Versatile Additive that Prevents Physical Aggregation Rather than Chemical Inactivation

Arg is currently a well-known additive within biotechnology and pharmaceutics, as demonstrated in a comprehensive review by Dr. Arakawa and co-worker [33]. Arg as an additive appeared for the first time in the application of the refolding method on recombinant proteins. During the 1980s, Dr. Rudolph discovered Arg as a refolding additive on the recombinant full-length tissue-type plasminogen activator, as described in a well-documented review article for refolding of recombinant proteins [34], fab-fragment refolding [35], and suppression of aggregation [36]. The pioneering work using Arg as an additive outlined the refolding of a recombinant Fab-fragment from the inclusion body to the native state [4]. After the discovery, Arg has been use for the refolding of many medical proteins, including immunotoxin from monoclonal antibody fused with *Pseudomonas* exotoxin A [37], truncated tissue-type plasminogen activator [38], rabbit heterotetrameric casein kinase II [39], interleukin-6 receptor [40], *Psedomonas fluorescens* lipase [41], human neurotrophin-3 [42], human and mouse single-chain Fv fragments [43], human interleukin-21 [44], and human interleukin-10 [45]. Furthermore, Arg-assisted refolding technology has been practically enhanced for "step-wise dilution" [46], the synergistic effect of Arg with kosmotropic ions [47] or a weak detergent of lauroyl-L-glutamate and Arg [48] and on the column refolding of a monomeric noncollagenous domain of Type IV collagen [49].

It has been pointed for over two decades that the guanidino group of Arg stacks parallel with aromatic side chains [50], although the aromatic group is prone to being bound to the amino group [51] and cation [52]. Moreover, Arg increases peptide and protein solubility, such as an aromatic-rich insulin segment of FFYTP [53], gluten [54], reduced carboxymethyl lysozyme [9], and porcine myosin [55], several times higher than those in the absence of any additives. Thermodynamic analyses revealed that Arg does not decrease the melting temperature of lysozyme as a model pro-

tein [3, 5]. Arg is not a protein denaturant; however, it perturbs local structure around the aromatic amino acid of proteins [56, 57]. Moreover, x-ray crystallographic analyzes of hen egg lysozyme showed that three Arg molecules as additives are observed on an aromatic surface [58] and hydration water molecules are increased with increasing concentration of Arg molecules [59].

A quantitative experiment showed the transfer free energies from water to 1.0 M Arg solution of small aromatic compounds; coumarin was 2 kJ/mol [60]; the nucleobases adenine, guanine, cytosine, thymine, and uracil were 1–2 kJ/mol [61-63]; alkyl gallates were 3 kJ/mol [64]; caffeic acid was 5 kJ/mol [65]. These data showed that Arg increases the solubility of aromatic compounds, resulting from cation-π noncovalent interaction. The molecular mechanism of Arg as an additive is briefly summarized as follows:

i) Prevention of chemical modification. In addition, Arg prevents the heat-induced chemical modifications of proteins, as described in the current review. However, the preventive effect of Arg on chemical modification is not extraordinary but normal, as shown in (Fig. **3**).

ii) Gap effect. The chemically inactive molecule of neutral crowder increased the activation energy for association between proteins, leading to a decrease in the binding energy. The "gap theory" is favorably described by the Arg function on macromolecules [66, 67], α-chymotrypsinogen A, and melittin [68]. Various sizes of self-associated Arg molecules may function as crowders to prevent protein aggregation [69].

iii) Specific interaction. Cation-π interaction of the amino acid residues is commonly observed in a protein tertiary structure and protein interface [70, 71]. Similarly, Arg molecules bind to proteins [58] and small compounds [61] with cation-π interaction. In addition, electrostatic interaction and hydrogen bonds occur. A cationic guanidium group can bind to an anion. Moreover, the binding free energy of the ion-pair between guanidium and phosphate are −4.1 kJ/mol from theoretical computational calculation [72] and −2.4 kJ/mol from experimental work [73]. Such enthalpic binding may promote aggregation, while guanidine–aromatic interaction solubilizes proteins [74].

iv) Solvent effect. It is to be noted that amino acids are preferentially excluded from protein surfaces [75], leading to the stabilization of the folded and compact state of proteins [76, 77]. Arg also increases the surface tension of water [78]; hence, Arg is basically excluded from protein surfaces. Thus, it may be concluded that Arg molecules bind to the protein surface with very low energy derived from cation-π and other specific interactions. In other words, this marginal effect of Arg additive on protein structures results in an outstanding versatility for troublesome protein solutions in any situations.

v) Salt effect. Arg often uses at neutral pHs in buffer solution. Arg solution is prepared by dissolution of the powder of arginine monohydrochloride (ArgHCl) or Arg base into buffer solution, followed by pH is adjusted with NaOH or HCl toward the desired neutral pH. Accordingly, the Arg solution is a kind of salts solution. Actually, Arg and Lys decrease the viscosity of high concentration of bovine (275

mg/ml) and human (305 mg/ml) serum albumin solutions as similar extent to the NaCl and guanidine hydrochloride solutions [79]. The decrease in viscosity results from the salt effect that decreases the repulsion between high concentration albumins. It is noted that ArgHCl specifically decreases the viscosities of high concentration of gamma globulins from human and bovine among amino acids and salts, including lysine hydrochloride and NaCl [80]. Taken together, Arg solution decreases the protein viscosity both salt effect and Arg-specific interaction effects.

These complex mechanisms of Arg with chemical reaction, specific interaction, and bulk effect result in the controversial appearance as a solvent additive that prevents the thermal aggregation of proteins [33]. For example, Arg did not prevent the heat-induced aggregation of human growth hormones and interferon-α2b [81] because Arg at low concentration (< 0.1 M) promotes aggregation by the suppression of electrostatic repulsion as salt effect. Similarly, Arg promotes heat-induced aggregation for the large immunoglobulin G1 protein [82] and the β-lactoglobulin (βLG) oligomeric protein [74]. Such structurally unstable proteins can be stabilized by the use of Arg with the acidic amino acids Glu and Asp [82] and the kosmotropic ions [47]. Thus, it is possible that Arg promotes the heat-induced aggregation of large and complex proteins at low concentration. It is noted that Arg does not prevent amyloid-like fibril aggregation of hen egg lysozyme induced by heat treatment at acidic pH [83]. The difference between thermal aggregation and amyloid fibrillization will be a key to understand the molecular mechanism of Arg on protein aggregation.

1.5. Difference between Heat-induced Degradation and Refolding-induced Misfolding

A deeper examination of the molecular mechanism of additives could facilitate a discussion of the difference between refolding-induced misfolding and heat-induced degradation. Figure **4** shows a simple scheme of protein refolding. Recombinant proteins usually form inclusion bodies (insoluble and inactive aggregates) when expressed in host cells. Thus, the refolding is an indispensable process necessary to recover the native-state protein from the inclusion body. Firstly, the inclusion body should be solubilized using a reducing reagent (ex. dithiothreitol, 2-mercaptoethanol, or glutathione) with a high concentration of a denaturant (ex. GdnHCl or urea). Then, the reduced and unfolded state protein (U_R) is diluted into a large amount of buffer. During this step, some proteins refold spontaneously into its native structure according to Anfinsen's thermodynamic dogma (process (2) in Fig. **4**), while the others form amorphous aggregates (process (3) in Fig. **4**).

To increase the refolding yield of proteins, various techniques have been developed. The early stage of the refolding methods has been well documented by Dr. Rudolph [34, 35] and Dr. Clark [84, 85]. The fundamental approach to increasing the refolding yield of proteins is to decrease the irreversible aggregation. For example, the denaturant of urea is gradually removed by controlled dialysis [86]; detergent solubilizes the aggregation-prone folding intermediate, and then the detergent is stripped off the protein surface, so called "artificial chaperones" [87]; nano-scale pore isolates

the aggregation-prone folding intermediate, followed by the controlled release by polyethylene glycol, which leads to a decrease in the probability of aggregation [88]. During the dilution step of the denaturant, the addition of 0.2-1.0 M Arg can prevent protein aggregation [89]. Arg is substituted for diluted denaturant that prevents intermolecular interaction between aggregation-prone folding and the intermediate state of proteins.
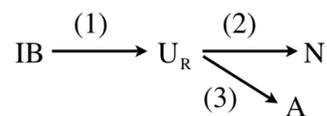

Fig. (4). Refolding process from the inclusion body (IB) of a recombinant protein. (1) The recombinant protein of the inclusion body is solubilized by the reducing reagent and a high concentration of denaturant. Following this, (2) the reduced and unfolded protein (U_R) is refolded to the native state (N) by various refolding methods. However, (3) the refolding process competes with aggregation (A). The refolding process of (2) is basically spontaneous folding; thus, refolding methods are designed to suppress the side reaction of (3).

By contrast, heat-induced inactivation is shown in (Fig. **5**). Amine compounds prevent chemical degradation during heat treatment (Process (2) in Fig. **5**). After the heat treatment, an unfolded molecule refolds spontaneously into the native state and the other unfolded molecule forms an aggregate. Moreover, the heat-induced aggregates of lysozyme possess higher β-sheet content than those of refolding-induced aggregates, as quantified by Fourier-transform infrared (FT-IR) spectroscopy [90]. It is noted that intermolecular disulfide bond exchanges occurred during heat treatment; however, this is not a crucial step when protein concentration is ≤ 1 mg/ml.
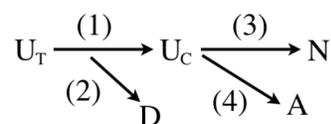

Fig. (5). Heat-induced inactivation and aggregation of protein. First, protein is thermally unfolded at high temperature (U_T). During the heat treatment of (1), the unfolded molecule gradually degrades chemically and structurally (D) during process (2). Subsequently, the samples are cooled down to room temperature (U_C). The U_C molecules are immediately refolded into the native state (N) or an irreversible aggregate (A). The solution additive affects the suppression of the processes (2) and (4). It is thought that amine compounds prevent chemical inactivation in the process of (2), while Arg mainly prevents the aggregation process of (4).

The difference between amine compounds (typically spermidine), Arg, and guanidine has been systematically investigated for refolding yield and for the heat-induced inactivation of lysozyme as a model [90]. The following results obtained were clear: 1) Guanidine and Arg increase the solubility of reduced and carboxymethylated (RCM)-

lysozyme, while spermidine does not; 2) spermidine suppresses the heat-induced inactivation and aggregation of lysozyme; and 3) guanidine and Arg increase the refolding yield of lysozyme. These data support the following hypothesis: guanidine and Arg primarily increase the solubility of an aggregation-prone molecule, leading to the prevention of the irreversible inactivation of proteins (process (4) in Fig. **5**). On the other hand, spermidine primarily suppresses the chemical modifications of proteins caused by heat treatment (process (2) in Fig. **5**), leading to the suppression of the heat-induced inactivation of proteins.

Furthermore, five kinds of amino acids (Arg, Lys, Glu, Ser, and citrulline) and their derivatives have been investigated for clarifying the indispensable chemical structure of additives for refolding and heat treatment [9]. The additives that prevent refolding-induced aggregation possess guanidium or ureido groups (Fig. **6**). Guanidine and urea are known as protein denaturants. These kinds of chemicals are bound to the hydrophobic surface that solubilizes aggregation-prone molecules, which indirectly increases the refolding yield of proteins as the aggregation suppressor. On the other hand, the additives that prevent heat-induced inactivation possess a principal amino acid chain (Fig. **6**). This is because the heat-induced inactivation mainly results from the chemical modifications of proteins. It is interesting to note that the suppressive effect of Arg on heat-induced aggregation of α-chymotrypsin retains constant when temperature different from 65°C to 85°C, while that of a small poly(ethylene glycol) of tetraethylene glycol (TEG) increases with increasing temperature [91]. This results from the increase in hydrophobic interaction between TEG and unfolded protein with increasing temperature.

2. CELLULAR AMINES

Finally, we would like to mention amine compounds in living cells. Spermine, spermidine, and putrescine are universally distributed in all living materials [92]. Cellular polyamines electrostatically bind to negatively charged macromolecules, typically DNA and RNA; hence, they stabilize (or destabilize) their tertiary structure. The nonspecific binding to the negative region of polyamines on macromolecules are involved in many cellular functions, such as cell growth, survival, proliferation, metabolism regulation, drag response, and diseases [93, 94]. These compounds *in vivo* may prevent the chemical degradation of proteins [11] as well as the degradation of DNA and RNA. Their function to prevent the chemical degradation of macromolecules serves to remind us that organic solute "osmolyte" in living cells exists in an extreme environment [95]. The osmolytes reduce the structural and functional damages of macromolecules under stressful condition, such as trimethylamine-N-oxide, taurine, β-alanine, polyols, and sugars. It should be pointed out that these chemicals are amine compounds. The osmolytes play an important role in the extreme environment that resists osmotic water stress on macromolecules. Furthermore, these osmolytes may facilitate the prevention of chemical damage in living cells.

Hyperthermophiles exist in an environment at a temperature > 85°C; hence, hyperthermophilic proteins should have high conformational stability under high temperature as compared with that of their mesophilic counterpart proteins [96-98]. In general, high temperature accelerates the chemical reaction [99]. Thus, it is known that the amino acid composition of a hyperthermophilic protein is different from that of its mesophilic counterpart; moreover, Asn, Gln, Cys, and Met in hyperthermophilic proteins occur at lower contents than in mesophilic proteins [100, 101]. Although the tertiary-folded state of protein is characterized by a higher tolerance of deamidation than the disulfide-reduced and protease-digested state peptide [99], these heat-labile amino acids are also degraded by such high temperature environment. It is noted that hyperthermophiles have unique polyamines in the cells [102-105]. Thus, polyamines may prevent the thermal degradation of thermophilic proteins under extreme conditions.
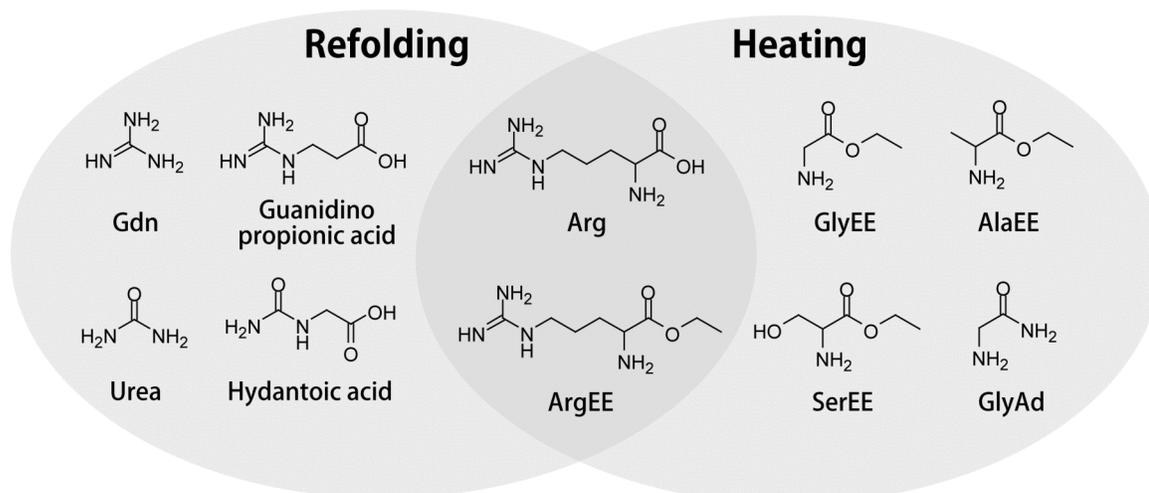

Fig. (6). Typical additives for refolding and heat-induced inactivation of lysozyme. Additives for refolding have the guanidium or ureido group, whereas additives for heat-induced inactivation have an amino acid backbone. Thus, ArgEE is, for example, a favorable additive for both refolding and heating.

CONCLUSION

This review summarizes the amine compounds that prevent the heat-induced inactivation of proteins by suppressing chemical modification. We practically recommend the amine co-solute (ex. 50 mM spermidine) in protein solutions when thermal degradation of proteins needs to be avoided. Note that chemical reaction depends on temperature. It is assumed by the Arrhenius equation that heat treatment at 90°C for 10 min results in full inactivation (< 10%) compared with that at 30°C for one day. Thus, from the application point of view, it is suggested that amine compounds completely prevent chemical degradation as a preservative additive under the condition of low protein concentration at neutral pH. The simple chemicals that are used as solvent additives will improve protein purification, shipping, storage, and heat treatment for biotechnology and pharmaceutics.

LIST OF ABBREVIATIONS

ArgHCl	=	Arginine monohydrochloride
ArgEE	=	Arginine ethylester
βLG	=	β-lactoglobulin
FT-IR	=	Fourier-transform infrared
GlyAd	=	Glycine amide
GlyEE	=	Glycine ethylester
Gdn	=	Guanidine
τ_1	=	Half-life time of the enzyme activity
τ_2	=	The time for deamidation of one Asn residue per a protein
NMR	=	Nuclear magnetic resonance
RCM	=	Reduced and carboxymethylated
RNase A	=	Ribonuclease A
SPR	=	Surface plasmon resonance
TEG	=	Tetraethylene glycol
τ_3	=	The time of β-elimination of 0.2 disulfide bond per a protein

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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