Appl Biochem Biotechnol (2011) 164:957–967
DOI 10.1007/s12010-011-9187-1

Protein Refolding by N-Alkylpyridinium and N-Alkyl-N-methylpyrrolidinium Ionic Liquids

Etsushi Yamamoto · Satoshi Yamaguchi · Teruyuki Nagamune

Received: 27 October 2010 / Accepted: 23 January 2011 /
Published online: 8 February 2011

Abstract An important property of ionic liquids consisting of cations and anions is that the chemical structures can be easily tuned. To expand the repertoire of effective ionic liquid-based refolding additives, we focused on this tunable property and investigated the effects of new candidates such as N-alkylpyridinium chlorides and N-alkyl-N-methylpyrrolidinium chlorides on protein refolding. Denatured lysozyme (30 mg/mL) was used as a model protein and refolded by 30-fold dilution with various refolding buffers containing different ionic liquids consisting of a systematic variety of alkyl chains. Compared with the refolding yield without additives (lower than 10%), less hydrophobic ionic liquids such as N-ethyl, N-butyl and N-hexylpyridinium chlorides, and N-butyl-N-methylpyrrolidinium chloride were effective in enhancing the refolding yields (46–69%), because they primarily suppressed aggregation because of their chaotropic properties. N-alkylpyridinium cations were more hydrophobic than N-alkyl-N-methylpyrrolidinium cations according to the calculated log *P* values and prevented aggregation at lower concentrations because of their hydrophobicity. The results provide a range of new effective ionic liquid-based additives for higher protein refolding yields and the knowledge of the effect of chemical structures of additives on protein refolding.

Keywords N-Alkylpyridinium chloride · N-Alkyl-N-methylpyrrolidinium chloride · Refolding · Aggregation · Protein stability · Ionic liquids

Electronic supplementary material The online version of this article (doi:10.1007/s12010-011-9187-1) contains supplementary material, which is available to authorized users.

E. Yamamoto · S. Yamaguchi (✉) · T. Nagamune
Department of Chemistry and Biotechnology, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
e-mail: yamaguchi@bio.t.u-tokyo.ac.jp

T. Nagamune
Department of Bioengineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

T. Nagamune (✉)
Center for NanoBio Integration (CNBI), The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
e-mail: yamaguchi@bio.t.u-tokyo.ac.jp

Introduction

The development of recombinant DNA technologies has enabled the production of several commercially important proteins by recombinant cells. Among recombinant bacteria used, *Escherichia coli* is widely employed as a host cell because it grows rapidly and the expression levels of the target protein are invariably high [1]. However, recombinant proteins produced by *E. coli* often form biologically inactive aggregates in the cytoplasm called inclusion bodies [2]. To obtain biologically active protein, the aggregates are separated from cell debris and are solubilized using a strong chemical denaturant. Reducing the concentration of the denaturant by dilution or dialysis can initiate refolding from the unfolded state to the native state [3, 4].

During the refolding process, the formation of aggregates competes with the correct folding pathway [5, 6]. As a unimolecular process, protein folding is a first-order reaction with respect to the protein concentration. On the other hand, aggregation is a second- or higher-order reaction caused by intermolecular interactions [7]. From an industrial applications viewpoint, the refolding process must be conducted under high protein concentrations because reducing solution volumes leads to time and economic benefits [8]. However, when refolding is performed at high protein concentrations, unproductive aggregation predominantly occurs due to the aforementioned kinetic competition [9], and as a consequence the refolding yield is significantly diminished. Therefore, effective refolding methods for obtaining high yields of correctly folded protein are required.

To improve the refolding yield, chemical compounds that can suppress protein–protein interactions are employed as refolding additives. Refolding additives such as detergents, low concentrations of chaotropes, amino acids, and amino acid derivatives have been shown to suppress intermolecular aggregation and improve the yield of biologically active proteins [10–15]. However, these molecules cannot act as universal refolding agents, and thus the expansion of the repertoire of effective additives is very important.

Ionic liquids generally consist of organic cations such as tetra-alkylated ammonium, N,N′-substituted imidazolium, N-substituted pyridinium, and N,N-substituted pyrrolidinium, and either organic or inorganic anions. These compounds exhibit a diverse range of physicochemical properties because it is easy to vary the cation and anion combinatorial patterns [16]. Currently, some ionic liquids have been shown to suppress aggregation and improve refolding yields [17–19]. However, whereas the influence of seven anions on protein refolding was analyzed in detail [16], only two cation types including tetra-alkylated ammonium and N,N′-substituted imidazolium have been often studied. Therefore, to expand the repertoire of effective additives, we have focused on other cations such as N-substituted pyridinium and N,N-substituted pyrrolidinium, and examined the effects of these two cations on refolding. We have investigated cations with different alkyl chain lengths and analyzed their effects on protein aggregation, refolding and stability.

Materials and Methods

Materials

Hen egg white lysozyme was purchased from Sigma and was used without further purification. Native and denatured/reduced lysozyme concentrations were determined by absorbance at 280 nm using an extinction coefficient ε_{280} of 2.63 and 2.37 mL mg^{-1} cm^{-1}, respectively [20, 21]. Absorbance measurements were measured using a UV–vis

spectrophotometer model ND-1000 (NanoDrop Technologies, Inc.). Dried *Micrococcus lysodeikticus* was purchased from Sigma. Dithiothreitol (DTT), guanidine hydrochloride (GuHCl), oxidized glutathione (GSSG), and reduced glutathione (GSH) were from Wako Pure Chemical Industries Ltd. A series of N-alkylpyridinium chlorides and N-alkyl-N-methylpyrrolidinium chlorides, listed in Fig. 1, were obtained as described below. N-Ethyl (C2PyCl), N-butyl (C4PyCl) and N-hexyl (C6PyCl) pyridinium chlorides, and N-butyl- (C4PrCl), N-hexyl- (C6PrCl), and N-octyl- (C8PrCl) N-methylpyrrolidinium chlorides were purchased from Merck KGaA (Germany). N-Dodecylpyridinium chloride (C12PyCl) was obtained from Tokyo Kasei Kogyo Co. Ltd. All reagents were analytical grade.

N-octylpyridinium chloride (C8PyCl) was essentially synthesized according to a conventional heating method. Briefly, equimolar amounts of pyridine anhydrous and 1-chlorooctane were refluxed in ethyl acetate for 96 h. The ethyl acetate was decanted and the residual viscous liquid (C8PyCl) was washed with fresh ethyl acetate. After washing, residual ethyl acetate was removed by evaporation.

Preparation of Denatured and Reduced Lysozyme

Native lysozyme was denatured and reduced for at least 2 h at room temperature in denaturation buffer (6 M GuHCl, 100 mM DTT, 1 mM EDTA, 0.1 M Tris–HCl, pH 8.5). The solution was acidified to pH 4.0 with 1 M HCl to prevent disulfide bond formation and then dialyzed two times for at least 4 h each at 4 °C against 100× volume of a stock buffer (4 M GuHCl, 1 mM EDTA, 50 mM acetic acid-NaOH, pH 4.5).

Oxidative Refolding of Lysozyme

A solution of denatured and reduced lysozyme (30 mg/mL) was then diluted by 30-fold in a refolding buffer (100 mM Tris–HCl, pH 8.2, 3 mM GSH, 0.3 mM GSSG, 1 mM EDTA) including N-alkylpyridinium chlorides and N'-alkyl-N-methylpyrrolidinium chlorides at various concentrations. Prior to use, we confirmed all the refolding buffers were transparent and homogeneous. These diluted samples are the refolding solutions. Refolding samples were incubated at 25 °C for 24 h before the enzymatic activity assay. For kinetic analysis, aliquots of the refolding samples were withdrawn at sampling intervals.

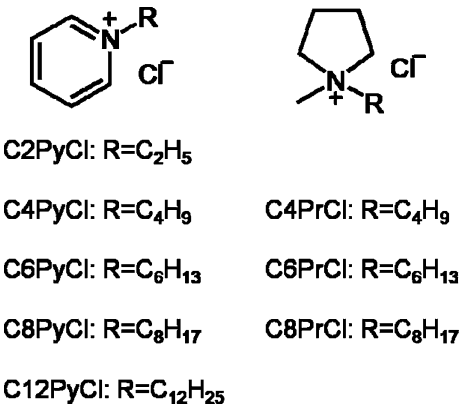

Fig. 1 Chemical structures of the series of N-alkylpyridinium chlorides and N-alkyl-N-methyl-pyrrolidinium chlorides

Aggregation Assay

The aggregation of lysozyme was semi-quantitatively assayed by measuring the light scattering intensity [19]. Briefly, a solution of denatured and reduced lysozyme was diluted 30-fold in various refolding buffers and incubated at 25 °C for 1 h in wells of 96-well plates. After the incubation, the turbidities of the samples were measured at 450 nm using a model 550 microplate reader (Bio-Rad).

Enzymatic Activity Assay

A high throughput assay for enzymatic activity was carried out according to a previously reported protocol [19]. Briefly, aliquots of refolding samples were loaded into the 96-well plates and a substrate suspension (1.35 mg/mL *M. lysodeikticus*, 66 mM sodium phosphate, pH 6.2) was added to each well. The initial rate of enzymatic lysis of the *M. lysodeikticus* at 25 °C was obtained by measuring the decrease in absorbance at 450 nm using the microplate reader. The refolding yield was expressed as the percentage of the amount of native lysozyme in the refolding solution relative to the amount of the initial denatured lysozyme.

Calculation of Log *P*

The partition coefficient, log *P* of organic compounds in the 1-octanol/water system, was calculated by the ChemAxon Marvin software using the default values [22]. Log *P* of cation parts of N-alkylpyridinium chlorides and N-methylpyrrolidinium chlorides were calculated (Table 1).

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) experiments were carried out on a VP-DSC instrument (Microcal). For the measurement, a native lysozyme solution (30 mg/mL) was diluted 30-fold with buffer (100 mM Tris–HCl, pH 8.2, 1 mM EDTA, 500 mM ionic liquid). Temperature scans were performed from 10 to 100 °C with a scan rate of 90 °C/h.

Table 1 Calculated log *P* values of cations of ionic liquids

Cation	log *P*
$C2Py^+$	−2.83
$C4Py^+$	−1.86
$C6Py^+$	−0.97
$C8Py^+$	−0.09
$C12Py^+$	1.69
$C4Pr^+$	−2.24
$C6Pr^+$	−1.35
$C8Pr^+$	−0.46

Results and Discussion

Effect of Ionic Liquids on the Suppression of Protein Aggregation

Hen egg white lysozyme was used as a model protein because its refolding pathway has been well characterized [7, 13, 15, 23–25]. In the refolding pathway, the refolding intermediate of lysozyme (molten globule) forms within milliseconds. Consequently, the formation of the intermediate can be taken to be an instantaneous reaction [7]. Thus, the refolding of lysozyme can be represented by a simplified competition model between correct folding and unproductive aggregation. This model suggests that suppression of unproductive aggregation is required to obtain a high refolding yield.

In the first screening, we examined the abilities of ionic liquids to suppress aggregation. An increase in the turbidities of samples has often been used to semi-quantitatively indicate protein aggregation [21]. The turbidities of the refolding samples were measured by a microplate reader after incubation for 1 h.

The results of Fig. 2 show that the ionic liquids inhibit protein aggregation and this inhibition increases as the concentration of the ionic liquid increases. As the alkyl chain length of both cations increased, the median inhibitory concentration against aggregation (IC_{50}) decreased (Table 2). In other words, cations with a longer alkyl chain suppressed aggregation more effectively. As shown in Fig. S1, a good linear correlation between the calculated log P values of cations of ionic liquids and IC_{50} ($y=-0.18\ x+0.25$, $R^2=0.88$) indicates that the hydrophobic property of the cations plays an important role in reducing protein aggregation. Compared with cation head groups carrying the same length alkyl chain, N-alkylpyridinium chlorides were slightly more effective in suppressing aggregation than the N-alkyl-N-methylpyrrolidinium chlorides. This is probably because N-alkylpyridinium cations are more hydrophobic than N-alkyl-N-methylpyrrolidinium cations (Table 1). These results indicate that aggregation was suppressed by increasing the hydrophobicity of the refolding additives because the additive blocked intermolecular hydrophobic interactions.

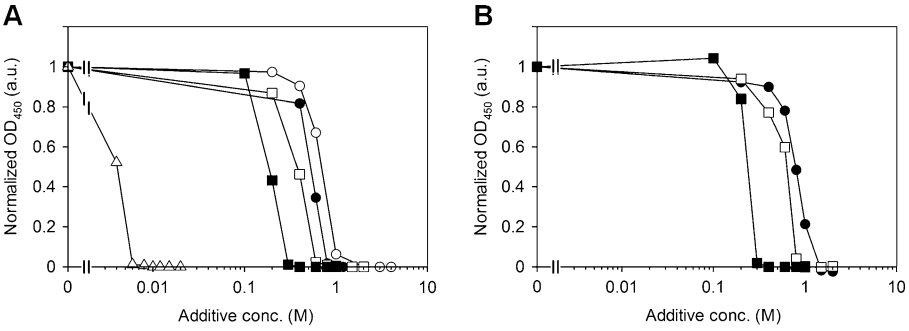

Fig. 2 Turbidities of the refolding buffers containing various ionic liquids as a function of their concentrations. The turbidities of lysozyme solutions containing **a** N-alkylpyridinium chlorides, namely, C2PyCl (*open circles*), C4PyCl (*closed circles*), C6PyCl (*open squares*), C8PyCl (*closed squares*) and C12PyCl (*open triangles*), and **b** N-alkyl-N-methylpyrrolidinium chlorides, namely, C4PrCl (*closed circles*), C6PrCl (*open squares*), and C8PrCl (*closed squares*) were measured at 450 nm after incubation for 1 h. Data points are the average of three replicates

Table 2 The effects of ionic liquids and conventional refolding additives on the aggregation and refolding of lysozyme

Additive	IC_{50} against aggregation (M)[a]	Optimal conc. (M)[b]	Maximum refolding yield (%)
C2PyCl	0.68	1.0	54
C4PyCl	0.54	0.80	46
C6PyCl	0.38	0.60	57
C8PyCl	0.19	0.30	31
C12PyCl	0.004	0.0040	9.4
C4PrCl	0.79	1.5	69
C6PrCl	0.6	1.0	57
C8PrCl	0.23	0.30	2.9
L-ArgHCl	nd[c]	1.0[d]	46[d]
Triton X-100	nd[c]	0.040[d]	19[d]
Tween 20	nd[c]	0.20[d]	8.4[d]

[a] IC_{50}=the concentration required to inhibit 50% aggregation. Assuming the two-state mechanism, the IC_{50} values were determined by fitting the following equation to the data shown in Fig. 2: $OD_{450}=1/(1+\exp(m(C_{additive}-C_{50})))$, where $C_{additive}$ and C_{50} are the concentrations of additive and the IC_{50}, respectively, and m is the partial differential coefficient of the transition free energy with respect to $C_{additive}$

[b] The optimal concentration was defined as the concentration that produced the maximum refolding yield

[c] *nd* not determined

[d] The data are from a previous study [19]

Effect of Ionic Liquids on Protein Refolding

To assess the effect of ionic liquids on the refolding yield, a renaturation experiment for lysozyme was conducted. The amounts of reactivated lysozyme were quantified after 24 h incubation. The refolding yield was only ~5% in the absence of ionic liquids. As shown in Fig. 3 and Table 2, addition of ionic liquids increased refolding yields by different amounts (~69%). Therefore, these ionic liquids functioned as refolding agents. Refolding yields increased with increasing concentrations of ionic liquids up to optimal concentrations which showed the maximum refolding yield. However, as the concentrations of ionic liquids were further increased, refolding yields decreased again. The increase in the refolding yield started at almost the same concentration as the observed decrease in aggregation (Fig. 3). In addition, Fig. 3 shows that all tops of the bell-shaped curves were observed near the concentration which fully suppressed aggregation. These results indicate that the increase in the refolding yields were due to the ionic liquids suppressing protein aggregation.

With respect to the influence of the length of the alkyl chain, both cations with relatively short and middle length alkyl chains (C2PyCl, C4PyCl, C6PyCl, C4PrCl, and C6PrCl) significantly elevated refolding yields. C4PrCl was the most effective refolding agent (Table 2). C2PyCl, C6PyCl, C4PrCl, and C6PrCl were superior to L-ArgHCl which has been widely used as a refolding additive. However, cations with longer alkyl chains (C8PyCl, C12PyCl, and C8PrCl) did not enhance refolding yields because these hydrophobic cations interacted strongly with refolding intermediates and prevented both aggregation and refolding, according to the refolding kinetic analysis (Fig. S2–S10 and Table S1, see Supplementary Material). Comparison of cations with the same alkyl chain

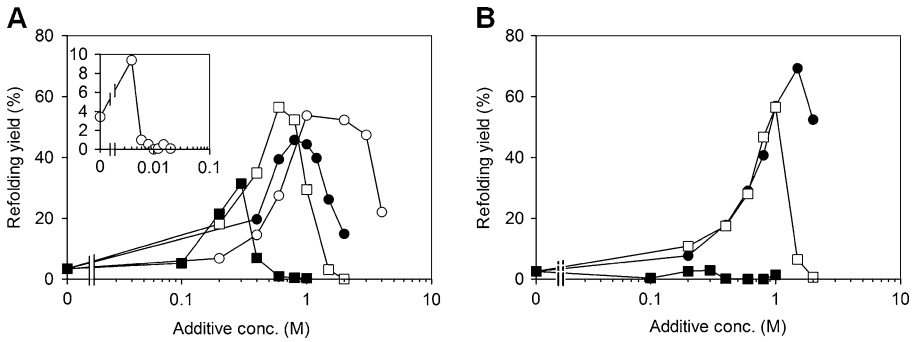

Fig. 3 Refolding yields of denatured and reduced lysozyme obtained by dilution with refolding buffers containing various N-alkylpyridinium chlorides and N-alkyl-N-methylpyrrolidinium chlorides as a function of their concentrations. The recovered activities of the refolded samples containing **a** N-alkylpyridinium chlorides, namely, C2PyCl (*open circles*), C4PyCl (*closed circles*), C6PyCl (*open squares*), C8PyCl (*closed squares*) and C12PyCl (*inset*, *open circles*), and **b** N-alkyl-N-methylpyrrolidinium chlorides, namely, C4PrCl (*closed circles*), C6PrCl (*open squares*), and C8PrCl (*closed squares*) were determined by measuring the bacteriolytic activities after incubation for 24 h. Data points are the average of three replicates

length showed that N-alkylpyridinium cations obtained the maximum refolding yield at lower additive concentrations. This is because more hydrophobic N-alkylpyridinium cations can fully prevent aggregation at lower concentrations.

Thermodynamic and Kinetic Stability of Native Lysozyme in the Presence of Ionic Liquids

The results presented in Fig. 3 suggest that low refolding yields were obtained above optimal concentrations of ionic liquids, particularly hydrophobic compounds. To investigate the denaturing effect of ionic liquids, we carried out thermodynamic and kinetic stability measurements of native lysozyme in the presence of ionic liquids. Thermodynamic stability represents the resistance of the native conformation to the unfolded conformation while kinetic stability means the resistance to irreversible inactivation. The irreversible inactivation of many proteins can be described by the following reaction scheme:

$$N \leftrightarrow U \rightarrow X \tag{1}$$

where N represents the native state, U the unfolded state, and X the inactive state such as small aggregates [26].

Some effective refolding additives such as L-arginine, ethylammonium nitrate, and N′-substituted N-methylimidazolium chlorides decrease the melting temperatures (T_m) of proteins [16–18, 21, 27], which indicates that these additives decrease the thermodynamic stability of proteins ($N \leftrightarrow U$). In this study, all 500 mM N-alkylpyridinium and N-alkyl-N-methylpyrrolidinium chlorides also lowered the T_m values (Fig. 4 and Table 3). N-alkylpyridinium cations and cations with longer alkyl chains strongly decreased the T_m values, indicating that more hydrophobic ionic liquids strongly destabilize the native protein. In particular, in the presence of C8PyCl, C12PyCl and C8PrCl, we could not measure the T_m (Fig. 4 and Table 3), because these hydrophobic cations denatured lysozyme even at room temperature.

To understand the kinetic stability ($U \rightarrow X$ or $N \rightarrow X$) of native lysozyme in the presence of ionic liquids, we measured the residual activity of native lysozyme incubated for 24 h with refolding buffers. Although C2PyCl, C4PyCl, and C4PrCl decreased the refolding

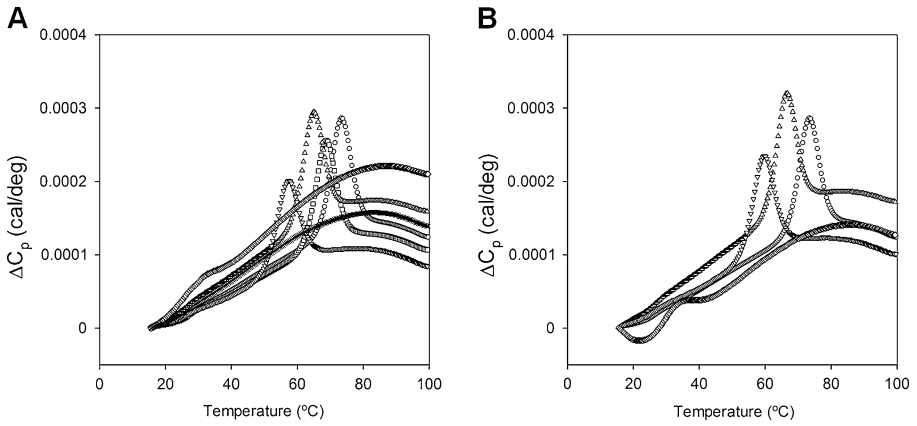

Fig. 4 The thermal unfolding transition of lysozyme. The thermal unfolding of lysozyme was measured by DSC after dilution with refolding buffer containing **a** no additive (*open circles*), C2PyCl (*open squares*), C4PyCl (*open triangles*), C6PyCl (*open inverted triangles*), C8PyCl (*open diamonds*) and C12PyCl (*crosses*), and **b** no additive (*open circles*), C4PrCl (*open triangles*), C6PrCl (*open inverted triangles*), and C8PrCl (*open diamonds*)

yields with increasing their concentrations above 1 M due to kinetic inhibition on protein refolding (Fig. 3 and Fig. 10, see Supplementary Material), the results in Fig. 5 show that C2PyCl, C4PyCl, and C4PrCl did not inactivate lysozyme above 1 M. In addition, residual activities in the presence of these additives were slightly higher compared with that in the absence of the additives. These results imply that the formation of the inactive state (X) was suppressed due to the inhibition of hydrophobic interactions between unfolded proteins ($U \rightarrow X$). Consequently, although these ionic liquids slightly decrease thermodynamic stability, they slightly increase the kinetic stability against the inactive state. Eventually, the refolded state was stabilized and the recovered enzymatic activity (refolding yield) was retained. Recently, Zhao et al. showed that enzymes are stabilized by the N-butylpyridinium cation [28, 29]. This finding is in good agreement with our results. However, the use of excessively high concentrations of ionic liquids with longer alkyl chains decreased the residual activity of the protein (Fig. 5) because these hydrophobic cations might displace the point of equilibrium to unfolded state ($N \rightarrow U$), in other words, destabilize the native state [28, 29].

Table 3 T_m values of native lysozyme in the presence of various ionic liquids

Additive	T_m (°C)	ΔT_m [a] (°C)
No additive	73.4	
C2PyCl	68.8	−4.6
C4PyCl	65.1	−8.3
C6PyCl	57.4	−16
C8PyCl	nd[b]	nd
C12PyCl	nd	nd
C4PrCl	66.7	−6.7
C6PrCl	59.7	−13.7
C8PrCl	nd	nd

[a] $\Delta T_m = T_m$ (CnPyCl, CnPrCl) $- T_m$ (no additive)

[b] *nd* not determined

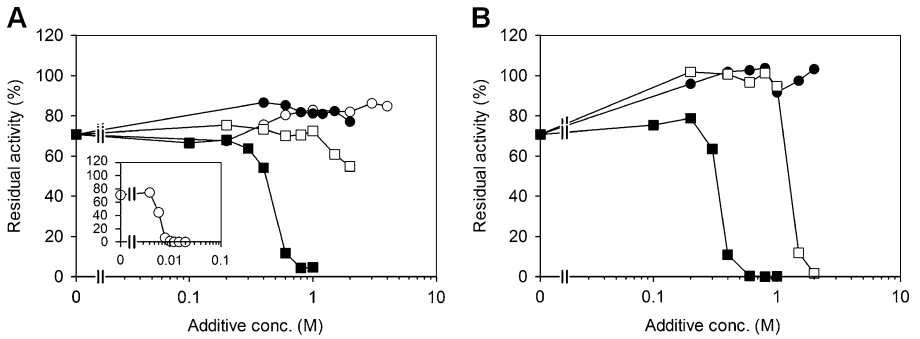

Fig. 5 Residual activities of native lysozyme by dilution with refolding buffers containing various N-alkylpyridinium chlorides and N-alkyl-N-methylpyrrolidinium chlorides as a function of their concentrations. The residual activities of the native samples containing **a** N-alkylpyridinium chlorides, namely, C2PyCl (*open circles*), C4PyCl (*closed circles*), C6PyCl (*open squares*), C8PyCl (*closed squares*) and C12PyCl (*inset, open circles*), and **b** N-alkyl-N-methylpyrrolidinium chlorides, namely, C4PrCl (*closed circles*), C6PrCl (*open squares*), and C8PrCl (*closed squares*) were determined by measuring the bacteriolytic activities after incubation for 24 h. Data points are the average of three replicates

In the previous work, we showed that the Hofmeister series and direct binding effect of refolding additives can describe refolding kinetics [19]. The Hofmeister series represents the relationship between ions and enzyme activity in aqueous solutions [30]. On the basis of salting-in or salting-out effects, ions in a series can be ranked. Ions that tend to break water structure and solubilize proteins are classified as chaotropes ("structure-breaker"), whereas the opposite group of ions are referred to as kosmotropes ("structure-maker") [31]. Recently, the Hofmeister series of ionic liquids has been investigated [28–30, 32]. According to these investigations, large and singly charged organic cations such as N-alkylpyridinium and N-alkyl-N-methylpyrrolidinium with shorter alkyl chains are chaotropes. Less hydrophobic cations are expected to solubilize aggregation-prone refolding intermediates partly due to their chaotropic property, and then prevent aggregation during refolding (Fig. 2). In contrast, the cation kosmotropicity increases as the cation hydrophobicity increases upon the lengthening of the alkyl chain [29]. However, hydrophobic ionic liquids, kosmotropes, destabilized native lysozyme (Figs. 4 and 5). This result suggests that the destabilizing effect was probably due to the direct binding of the ionic liquids to the protein [19]. Taking into consideration the chemical structure of ionic liquids, hydrophobic cations with longer alkyl chains are similar to cationic detergents. Cationic detergents such as cetyltrimethylammonium bromide suppress protein aggregation in protein refolding processes due to the hydrophobic interaction between the detergent molecules and the refolding intermediates [33, 34]. Therefore, hydrophobic cations might also bind to the hydrophobic surfaces of proteins, thereby leading to the prevention of refolding and thus destabilization of the native conformation. The influence of additives on protein refolding must be consisdered from the viewpoint of the Hofmeister series and direct binding effects.

Conclusion

An important property of ionic liquids is that the structure of such compounds can be easily tuned. To expand the repertoire of effective ionic liquid-based refolding additives, we focused on this tunable property and investigated the effect of new candidates such as N-

alkylpyridinium chlorides and N-alkyl-N-methylpyrrolidinium chlorides on protein refolding. In addition to some tetra-alkylated ammonium and N,N′-substituted imidazolium salts, less hydrophobic ionic liquids such as C2PyCl, C4PyCl and C4PrCl were effective in enhancing the refolding yields, because they predominantly suppressed aggregation due to their chaotropic properties. Conversely, hydrophobic ionic liquids such as C8PyCl and C12PyCl fully prevented aggregation at lower concentrations; however, these compounds were not efficacious in improving refolding yields because they prevented protein refolding by directly interacting with the protein. Compared with N-alkylpyridinium and N-alkyl-N-methylpyrrolidinium cations, N-alkylpyridinium cations were found to be slightly more hydrophobic than N-alkyl-N-methylpyrrolidinium cations according to log *P* values. N-alkylpyridinium ionic liquids prevented aggregation of the unfolded protein at lower concentrations and slightly destabilized the protein conformation due to their hydrophobicity. Since the cations and anions used in ionic liquids can be easily tuned, their salts can potentially be optimized as refolding additives for any type of protein. We envisage that this work should offer new possibilities to expand the repertoire of ionic liquid-based refolding additives for various proteins.

Acknowledgments We thank Prof. Kouhei Tsumoto and Dr. Motonori Kudou for providing us with the opportunity to do DSC measurements. This work was supported in part by a Grant-in-Aid for Young Scientists (B) from the MEXT of Japan.

References

1. Baneyx, F. (1999). Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology, 10*, 411–421.
2. Baneyx, F., & Mujacic, M. (2004). Recombinant protein folding and misfolding in *Escherichia coli*. *Nature Biotechnology, 22*, 1399–1408.
3. Singh, S. M., & Panda, A. K. (2005). Solubilization and refolding of bacterial inclusion body proteins. *Journal of Bioscience and Bioengineering, 99*, 303–310.
4. Jungbauer, A., & Kaar, W. (2007). Current status of technical protein refolding. *Journal of Biotechnology, 128*, 587–596.
5. Goldberg, M. E., Rudolph, R., & Jaenicke, R. (1991). A kinetic study of the competition between renaturation and aggregation during the refolding of denatured-reduced egg white lysozyme. *Biochemistry, 30*, 2790–2797.
6. Clark, E. D. (2001). Protein refolding for industrial processes. *Current Opinion in Biotechnology, 12*, 202–207.
7. Hevehan, D. L., & Clark, E. D. (1997). Oxidative renaturation of lysozyme at high concentrations. *Biotechnology and Bioengineering, 54*, 221–230.
8. Yasuda, M., Murakami, Y., Sowa, A., Ogino, H., & Ishikawa, H. (1998). Effect of additives on refolding of a denatured protein. *Biotechnology Progress, 14*, 601–606.
9. Raman, B., Ramakrishna, T., & Rao, C. M. (1996). Refolding of denatured and denatured/reduced lysozyme at high concentrations. *The Journal of Biological Chemistry, 271*, 17067–17072.
10. Wetlaufer, D. B., & Xie, Y. (1995). Control of aggregation in protein refolding: a variety of surfactants promote renaturation of carbonic anhydrase II. *Protein Science, 4*, 1535–1543.
11. Bam, N. B., Cleland, J. L., & Randolph, T. W. (1996). Molten globule intermediate of recombinant human growth hormone: Stabilization with surfactants. *Biotechnology Progress, 12*, 801–809.
12. Orsini, G., & Goldberg, M. E. (1978). Renaturation of reduced chymotrypsinogen-a in guanidine Hcl-Refolding versus aggregation. *The Journal of Biological Chemistry, 253*, 3453–3458.
13. Dong, X. Y., Shi, J. H., & Sun, Y. (2002). Cooperative effect of artificial chaperones and guanidinium chloride on lysozyme renaturation at high concentrations. *Biotechnology Progress, 18*, 663–665.
14. Tsumoto, K., Umetsu, M., Kumagai, I., Ejima, D., Philo, J. S., & Arakawa, T. (2004). Role of arginine in protein refolding, solubilization, and purification. *Biotechnology Progress, 20*, 1301–1308.

15. Hamada, H., & Shiraki, K. (2007). L-argininamide improves the refolding more effectively than L-arginine. *Journal of Biotechnology, 130*, 153–160.
16. Buchfink, R., Tischer, A., Patil, G., Rudolph, R., & Lange, C. (2010). Ionic liquids as refolding additives: Variation of the anion. *Journal of Biotechnology, 150*, 64–72.
17. Summers, C. A., & Flowers, R. A., 2nd. (2000). Protein renaturation by the liquid organic salt ethylammonium nitrate. *Protein Science, 9*, 2001–2008.
18. Lange, C., Patil, G., & Rudolph, R. (2005). Ionic liquids as refolding additives: N′-alkyl and N′-(omega-hydroxyalkyl) N-methylimidazolium chlorides. *Protein Science, 14*, 2693–2701.
19. Yamaguchi, S., Yamamoto, E., Tsukiji, S., & Nagamune, T. (2008). Successful control of aggregation and folding rates during refolding of denatured lysozyme by adding N-methylimidazolium cations with various N′-substituents. *Biotechnology Progress, 24*, 402–408.
20. Saxena, V. P., & Wetlaufer, D. B. (1970). Formation of three-dimensional structure in proteins. I. Rapid nonenzymic reactivation of reduced lysozyme. *Biochemistry, 9*, 5015–5023.
21. Reddy, R. C., Lilie, H., Rudolph, R., & Lange, C. (2005). L-Arginine increases the solubility of unfolded species of hen egg white lysozyme. *Protein Science, 14*, 929–935.
22. Kolkenbrock, S., Parschat, K., Beermann, B., Hinz, H. J., & Fetzner, S. (2006). N-acetylanthranilate amidase from Arthrobacter nitroguajacolicus Ru61a, an alpha/beta-hydrolase-fold protein active towards aryl-acylamides and -esters, and properties of its cysteine-deficient variant. *Journal of Bacteriology, 188*, 8430–8440.
23. Clark, E. D., Hevehan, D., Szela, S., & Maachupalli-Reddy, J. (1998). Oxidative renaturation of hen egg-white lysozyme. Folding vs aggregation. *Biotechnology Progress, 14*, 47–54.
24. MaachupalliReddy, J., Kelley, B. D., & Clark, E. D. (1997). Effect of inclusion body contaminants on the oxidative renaturation of hen egg white lysozyme. *Biotechnology Progress, 13*, 144–150.
25. Dong, X. Y., Huang, Y., & Sun, Y. (2004). Refolding kinetics of denatured-reduced lysozyme in the presence of folding aids. *Journal of Biotechnology, 114*, 135–142.
26. Wetzel, R., Perry, L. J., Baase, W. A., & Becktel, W. J. (1988). Disulfide bonds and thermal stability in T4 lysozyme. *Proceedings of the National Academy of Sciences of the United States of America, 85*, 401–405.
27. Matsuoka, T., Tomita, S., Hamada, H., & Shiraki, K. (2007). Amidated amino acids are prominent additives for preventing heat-induced aggregation of lysozyme. *Journal of Bioscience and Bioengineering, 103*, 440–443.
28. Zhao, H., Olubajo, O., Song, Z. Y., Sims, A. L., Person, T. E., Lawal, R. A., et al. (2006). Effect of kosmotropicity of ionic liquids on the enzyme stability in aqueous solutions. *Bioorganic Chemistry, 34*, 15–25.
29. Zhao, H., Campbell, S. M., Jackson, L., Song, Z. Y., & Olubajo, O. (2006). Hofmeister series of ionic liquids: Kosmotropic effect of ionic liquids on the enzymatic hydrolysis of enantiomeric phenylalanine methyl ester. *Tetrahedron Asymmetry, 17*, 377–383.
30. Zhao, H. (2005). Effect of ions and other compatible solutes on enzyme activity, and its implication for biocatalysis using ionic liquids. *Journal of Molecular Catalysis. B, Enzymatic, 37*, 16–25.
31. Broering, J. M., & Bommarius, A. S. (2005). Evaluation of Hofmeister effects on the kinetic stability of proteins. *The Journal of Physical Chemistry. B, 109*, 20612–20619.
32. Zhao, H. (2006). Are ionic liquids kosmotropic or chaotropic? An evaluation of available thermodynamic parameters for quantifying the ion kosmotropicity of ionic liquids. *Journal of Chemical Technology and Biotechnology, 81*, 877–891.
33. Wang, J., Lu, D., Lin, Y., & Liu, Z. (2005). How CTAB assists the refolding of native and recombinant lysozyme. *Biochemical Engineering Journal, 24*, 269–277.
34. Yamamoto, E., Yamaguchi, S., & Nagamune, T. (2011). Synergistic effects of detergents and organic solvents on protein refolding: Control of aggregation and folding rates. *Journal of Bioscience and Bioengineering, 111*, 10–15.