CRYSTAL GROWTH & DESIGN
2007
VOL. 7, NO. 4
787−793

Protein Crystallization Using Room Temperature Ionic Liquids

Marc L. Pusey,*,† Mark Steve Paley,‡ Megan B. Turner,# and Robin D. Rogers#

MI Research Inc., Suite 109, 515 Sparkman Drive, Huntsville, Alabama 35816, NASA/MSFC, AZ Technology, Huntsville, Alabama 35812, and Department of Chemistry, Center for Green Manufacturing, and Alabama Institute of Manufacturing Excellence, The University of Alabama, Tuscaloosa, Alabama 35487

Received October 10, 2006; Revised Manuscript Received January 25, 2007

ABSTRACT: The ionic liquids (ILs) 1-butyl-3-methylimidizolium chloride ([C_4mim]Cl), 1-butyl-3-methylimidizolium 2(2-methoxyethoxy)ethylsulfate ([C_4mim][MDEGSO4]), and 1-butyl-1-methylpyrollidinium dihydrogenphosphate ([p1,4][DHP]) were tested for their effects on the crystallization of the proteins canavalin, β-lactoglobulin B, xylanase, and glucose isomerase, using a standard high throughput screen. The crystallization experiments were set up with the ILs added to the protein solutions at 0.2 and 0.4 M final concentrations. Crystallization droplets were set up at three protein/precipitant ratios (1:1, 2:1, and 4:1), which served to progressively dilute the effects of the screen components while increasing the equilibrium protein and IL concentrations. Crystals were obtained for all four proteins at a number of conditions where they were not obtained from IL-free control experiments. Over half of the protein−IL combinations tested had more successful outcomes than negative outcomes, where the IL-free crystallization was better than the corresponding IL-containing outcome, relative to the control. One of the most common causes of a negative outcome was solubilization of the protein by the IL, resulting in a clear drop. In one instance, we were able to use the IL-induced solubilizing to obtain β-lactoglobulin B crystals from conditions that gave precipitated protein in the absence of IL. The results suggest that it may be feasible to develop ILs specifically for the task of macromolecule crystallization.

Introduction

Ionic liquids (ILs), defined generally as salts composed of separate cations and anions with melting points below 100 °C, are a class of compounds that form a unique state of matter that has been known, but not intensely studied, for over a century.[1−4] Only fairly recently[5−9] has the investigation of ILs seen a resurgence of interest, primarily due to favorable solvent properties (e.g., the low or negligible volatility and large liquidus range that many examples exhibit) and the growing social pressure for new "green" technologies.[10−17]

ILs are structurally distinct materials, differing from molecular solvents, as they possess cationic and anionic components that can be individually and independently tailored for various applications while retaining the desirable properties of the IL state of matter. Recent studies delving into the physical properties of ILs have shown a degree of localized structuring around each ion contradicting the previously accepted notion that the materials were composed of disassociated ions setting them apart from both partially ionized solvents and salt solutions.[18,19]

In 1888, Hofmeister established the strength of salts used to salt-out egg proteins, now commonly referred to as the "Hofmeister series", that reflects an ordering of the salts from kosmotropic to chaotropic.[20] ILs are typically chaotropic by design; that is, they have depressed melting points as a result of low-symmetry ions that contain charge delocalization and only weak intermolecular interactions. Such salts should be water-destructuring, and this is born out by experiments where IL examples can be "salted-out" when aqueous solutions are mixed with aqueous solutions of kosmotropic salts.[21,22]

Studies have shown that enzymes may retain their catalytic activity in IL solutions presumably as a result of structure conservation.[23−28] This has led to the development of ILs specifically for use with proteins.[28,29] Interestingly, several examples have been found to thermally stabilize proteins, in some instances to over 100 °C.[29,30] The effects of adding hydroxyls to short aliphatic chains have been found to increase protein stability in imidazolium-based ILs.[31] ILs have also been shown to be useful for refolding proteins.[32,33] It is important to note here, however, that other studies have suggested the denaturation and subsequent inability of enzymes to refold is a result of contact with IL solutions.[34] This potential range of variability in IL structure and solution properties makes them attractive materials for macromolecule applications in general and crystallization in particular.

Despite the growing number of detailed studies involving the interactions of ILs and proteins, there has been surprisingly little effort to utilize ILs for protein crystallization (or for crystallization of any kind for that matter[35]). In fact, to date only the crystallization of lysozyme from the IL ethyl ammonium nitrate has been reported.[36] One reason for this may be that, until recently, many ILs have not been readily available commercially, and those that have been available were relatively expensive. The rapid expansion of the field has now resulted in a number of ILs becoming commercially available at reasonable prices.

Our own interest has been in the use of ILs for the crystallization of membrane proteins. However, preliminary studies have been carried out using model soluble proteins to test if they would be at all useful for macromolecule crystallization. This report looks into the utility of the ILs 1-butyl-3-methylimidizolium chloride ([C_4mim]Cl), 1-butyl-3-methylimidizolium 2(2-methoxyethoxy)ethylsulfate ([C_4mim][MDEGSO4]), and 1-butyl-1-methylpyrollidinium dihydrogenphosphate ([p1,4][DHP]) for the crystallization of the model proteins canavalin, β-lactoglobulin B, glucose isomerase, and xylanase.

Experimental Procedures

Chemicals. Crystallization screening solutions employed were from the Crystal Screen HT kit (cat. no. HR2-130, Hampton Research). The

* Corresponding author. Phone: (256) 585-0182. Fax: (256) 704-6002. E-mail: marc@mi-research.com.
† MI Research Inc..
‡ NASA/MSFC.
# The University of Alabama.

10.1021/cg060696t CCC: $37.00
Published on Web 03/21/2007

Table 1. Summary of the Effects of Added IL on Crystallization Outcomes[a]

	0.2 M IL improved			0.2 M IL worse				0.4 M IL improved			0.4 M IL worse			
protein + IL	1:1	2:1	4:1	1:1	2:1	4:1	precip → clear	1:1	2:1	4:1	1:1	2:1	4:1	precip → clear
CAN + [C_4mim]Cl	6	8	17	3	5	5	9	3	14	20	4	5	6	10
CAN + [C_4mim][MDEGSO4]	2	0	1	6	12	16	3	0	3	3	8	12	15	3
CAN + [p1,4][DHP]	1	1	1	8	10	15	4	0	2	1	8	11	15	11
BLB + [C_4mim]Cl	2	2	0	0	1	0	1	3	2	1	0	1	0	0
BLB + [C_4mim][MDEGSO4]	2	3	0	1	1	0	0	1	0	1	0	1	1	0
BLB + [p1,4][DHP]	6	4	1	1	1	0	0	4	4	0	1	1	0	0
XLN + [C_4mim]Cl	19	30	22	3	1	7	0	19	16	5	2	2	7	3
XLN + [p1,4][DHP]	4	4	0	2	2	7	5	5	0	1	2	2	7	3
GI + [C_4mim]Cl	11	15	6	13	18	21	37	4	10	11	13	19	20	36

[a]The numbers given are with respect to the corresponding IL-free control solutions. The precip → clear column is the number of outcomes where the control was precipitated, but the corresponding IL+ outcome was a clear solution.

composition for each precipitant solution is as given with the list accompanying the screen kit, also available at http://www.hamptonresearch.com. Specific salts and the polymers (poly(ethylene glycol) (PEG), polyethylene glycol monomethyl ether (PEGMME), and polyoxyalkyleneamine (Jeffamine)) are described for noted crystallization conditions.

PEG-4000 (cat. no. 33136, Serva) was prepared as a 50% w:v solution in distilled water. A working solution of 5-(and-6)-carboxyrhodamine 6G, succinimidyl ester (CR, cat. no. C-6157, Invitrogen) was prepared by dissolving the contents of a 5 mg bottle in 1 mL of reagent grade dimethylformamide (Sigma, cat. no. D-8654). This solution was stored at −20 °C when not in use.

Proteins. Canavalin (CAN) was purified as previously described.[37,38] β-Lactoglobulin B (BLB, cat. no. L-8005) was purchased from Sigma and used without further purification. Glucose isomerase (GI, cat. no. HR7-100) and xylanase (XLN, cat. no. HR7-104) were purchased from Hampton Research and used without further purification. All proteins were trace fluorescently derivatized with carboxyrhodamine (Molecular Probes, cat. no. C-6157) as previously described.[39] Concentrated derivatized and underivatized protein solutions were prepared by centrifugal ultrafiltration, and these were used to prepare stock solutions for crystallization trials.

Ionic Liquids. 1-Butyl-3-methyl imidazolium chloride ([C_4mim]Cl) was prepared as previously described.[40] 1-Butyl-3-methylimidazolium 2(2-methoxyethoxy)ethylsulfate ([C_4mim][MDEGSO4]) was obtained from Solvent Innovations (ECOENG 41M, cat. no. 99,200-1, Köln, Germany). 1-Butyl-1-methylpyrrolidinium dihydrogenphosphate ([p1,4]-[DHP]) was prepared by published methods. Stock solutions, 3.0 M, of each IL in deionized H_2O were prepared and used to make the IL-containing protein solutions.

Crystallization Trials. Crystallization plates were set up as previously described. Briefly, the reservoir solution for all wells was 0.1 mL of 50% PEG-4000.[41] Greiner crystallization plates were used, with the three crystallization drops for each well comprising a 1:1, 1:2, and 1:4 v:v ratio of precipitant and protein solution. After sealing was performed, the plates were stored in an incubator at 20 °C. Crystallization control solutions were prepared by dilution of stock higher protein concentration solutions with distilled water to the following final concentrations: CAN, 10 mg mL^{-1}; BLB, 20 mg mL^{-1}; GI, 15 mg mL^{-1}; and XLN 15 mg mL^{-1}. Where IL was added, part of the dH_2O was replaced by the appropriate volume of stock IL solution to give the desired final IL concentration with the protein at the same final concentrations as the controls.

Scoring of Results. Scoring of all wells was carried out using the numerical system accompanying the Hampton Screen kit. While distinctions can be made between granular or microcrystalline and amorphous precipitate, the analysis presented only considers those wells having a score of 6 (needles) or higher. Each crystallization drop is scored separately, for a total of 288 drops/plate, with comparisons being made between the IL-containing drops and the corresponding IL-free drop in the control plate.

As there were typically one control (IL-free) and two experimental (0.2 and 0.4 M IL) results, a case where the IL-free droplet had a higher scoring outcome than both test droplets resulted in a score of 2. Each test droplet that was higher scoring than the corresponding control received a score of 1. There were a number of instances where the control scored higher in one set of corresponding droplets, but the IL-containing test scored higher in another. Conditions where the outcomes were equivalent received no score. In cases where both wells had equivalent crystals, but one drop had a higher scoring precipitated phase than the other(s), that drop received a score of 1. Where one drop had a clear single outcome, such as just having crystals present, and a competing drop had crystals plus precipitant, than the single outcome drop was scored as having the better outcome.

Results

Proteins were selected on the basis of their ready availability. Crystallization droplets were set up in three different ratios of protein to precipitant solution, and the precipitant progressively diluted while the concentration of the protein solution and components within it were increased to eventual equilibrium. When IL was present in the protein solution, its concentration was also progressively increased relative to the precipitant solution; that is, the IL becomes a progressively more dominant component of the crystallization droplet. Monitoring where crystals are obtained in these IL-containing solutions thus may give some insight into whether the ILs are acting as additives, here defined as solutes that improve on a precipitated state, i.e., amorphous or granular precipitate to crystals, or as the precipitating species. No attempts were made to further optimize any of the positive outcomes with respect to IL concentration or other solution conditions.

The data for positive and negative outcomes, relative to the IL-free control droplets, for all experiments are summarized in Table 1. This table also gives the number of droplets where a precipitated outcome in the control solution yielded a clear drop in the IL+ condition. Figure 1 shows the number of cases where the positive outcomes were not due to an improvement in a crystal in the control but due to an improvement from a less well-structured state (spherulites, microgranular precipitate, amorphous precipitate, phase change, or clear solution). The number of droplets where crystals were obtained in IL+ solutions, which had clear solutions in the controls, is given in Table 2.

Canavalin. Initial analysis of the results for CAN indicated that the ILs affected the outcomes in the order [C_4mim]Cl (96 drops) > [C_4mim][MDEGSO4] (78 drops) > [p1,4][DHP] (73 drops). When scored for positive outcomes in the IL containing drops vs the IL-free control, the same trends were noted, with the ratios being [C_4mim]Cl (68 drops) > [C_4mim][MDEGSO4] (9 drops) > [p1,4][DHP] (6 drops). There was not always overlap in the positive outcomes for the three ILs. [p1,4][DHP] gave positive outcomes for conditions F6 and F7 (10% Jeffamine M-600, 0.01 M $FeCl_3 \cdot 6H_2O$; 2.5 M hexanediol, respectively, both in 0.1 M sodium citrate buffer, pH 5.6). However, for condition F7 most of the outcomes for this IL were negative, while for [C_4mim][MDEGSO4] condition F6 had one positive and for F7 two negative outcomes, and [C_4mim]Cl had six

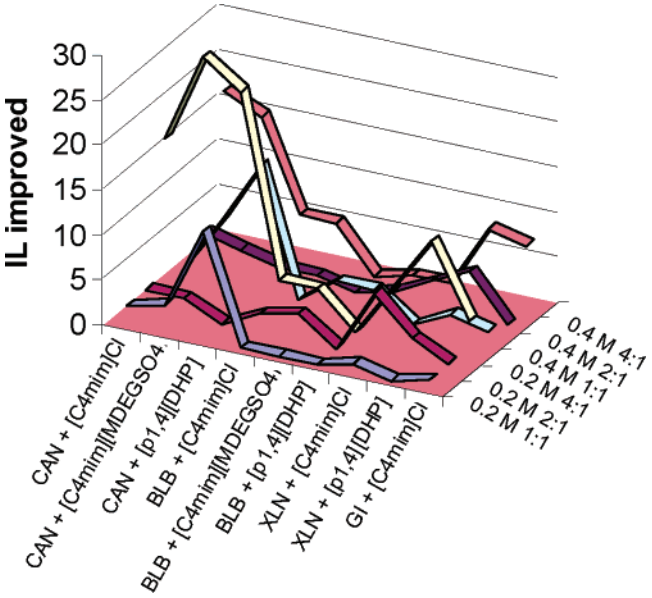

Figure 1. Number of crystals obtained for each protein + IL combination where the control outcomes had scores less than 6 (needles). The X axis is the protein−IL combination, the Y axis is the IL concentration in the protein with the protein/precipitant drop ratio, and the Z axis is the number of conditions where the IL+ outcomes had scores >6 (needles).

Table 2. Number of Positive IL+ Outcomes where the Corresponding Controls Were a Clear Solution

	0.2 M IL improved, control score < 6			0.4 M IL improved, control score <6		
protein + IL	1:1	2:1	4:1	1:1	2:1	4:1
CAN + [C_4mim]Cl	1	0	6	0	0	4
CAN + [C_4mim][MDEGSO4]	1	0	6	3	1	3
CAN + [p1,4][DHP]	2	0	8	2	0	2
BLB + [C_4mim]Cl	0	0	2	0	0	2
BLB + [C_4mim][MDEGSO4]	0	2	0	1	0	0
BLB + [p1,4][DHP]	0	0	0	0	0	0
XLN + [C_4mim]Cl	0	1	0	0	0	0
XLN + [p1,4][DHP]	0	0	0	1	2	0
GI + [C_4mim]Cl	0	0	0	1	0	0

negative outcomes for condition F7. Conditions E12 (30% PEG-400, 0.1 M $CdCl_2 \cdot 2.5H_2O$, 0.1 M HEPES, pH 7.5), F6, G2 (30% PEGMME 5000, 0.2 M $(NH_4)_2SO_4$, 0.1 M MES, pH 6.5), G7 (20% Jeffamine M-600, 0.1 M HEPES, pH 7.5), and H10 (20% PEGMME 550, 0.1 M NaCl, 0.1 M bicine, pH 9.0) were the only conditions where the outcomes from [C_4mim][MDEGSO4] and [p1,4][DHP] were unique compared to [C_4mim]Cl, i.e., changes from the control were not observed with [C_4mim]Cl in these cases, with F6, G7, and H10 being unique positives.

Generally, the improved outcomes for CAN were approximately evenly split between the 0.2 and 0.4 M IL conditions. Figure 2 shows the results for conditions A10c, precipitant condition A10 (30% PEG-4000, 0.2 M $(NH_4)OAc$, 0.1 M NaAc, pH 4.6) where the protein/precipitant v:v ratio was 4:1. This shows a progressively better outcome with increased IL concentration. In other instances, the better outcomes for the IL-containing conditions occurred with the 0.2 M IL conditions.

All conditions where a change in outcome resulted were characterized by having either a small organic molecule (methylpentanediol, 1,6 hexanediol, dioxane, isopropanol) or polymer (PEG, PEGMME, Jeffamine) as the dominant precipitating species. No correlation was obvious between precipitating species and whether a positive or negative outcome was achieved. In many cases, there were mixed outcomes, where, for example, in droplets at a 1:1 mixing ratio the control had a better outcome, but at 2:1 and/or 4:1 ratios the IL-containing solution had the better outcome. While there are insufficient data for CAN with [C_4mim][MDEGSO4] and [p1,4][DHP], the positive outcomes for CAN are generally weighted toward the higher protein/precipitant ratio drops. This was also the case for the negative outcomes.

β-Lactoglobulin B. BLB was soluble in all three ILs tested. All ILs gave more positive than negative outcomes in the crystallization screening trials. There were fewer overlaps in the positive outcomes for BLB, with only one precipitant solution, A6 (30% PEG-4000, 0.2 M $MgCl_2 \cdot 6H_2O$, 0.1 M Tris-HCl, pH 8.5), showing a change from the control for all three ILs. Only two other conditions, B5 and B10 (30% PEG-4000, 0.2 M $Li_2SO_4 \cdot H_2O$; 30% PEG-4000, 0.2 M $NaOAc \cdot 3H_2O$, respectively, both in 0.1 M Tris-HCl buffer, pH 8.5), showed effects from two ILs ([C_4mim][MDEGSO4] − [p1,4][DHP] and [C_4mim]Cl − [C_4mim][MDEGSO4], respectively). In only one condition, G10 (1.0 M $NaOAc \cdot 3H_2O$, 0.05 M $CdSO_4 \cdot 8/3H_2O$, 0.1 M HEPES, pH 7.5), shown in Figure 3, was the precipitant a salt and not a small organic molecule or higher MW PEG-based solution. The distributions of effects with respect to the protein/precipitant ratio predominately favored the 1:1 and 2:1 ratios for positive outcomes, with [C_4mim]Cl being 4:4:0, [C_4mim][MDEGSO4] being 1:3:1, and [p1,4][DHP] being 6:7:1 for ratios of 1:1, 2:1, and 4:1, respectively.

Xylanase. XLN was not soluble in either 0.2 or 0.4 M [C_4mim][MDEGSO4]. Quite different results were obtained between [C_4mim]Cl and [p1,4][DHP]. The predominant crystal form obtained for XLN was plates, and the improvement typically observed was greater separation between the plates and/or thicker plates, as shown in Figure 4. A total of 133 crystallization droplets were affected by the presence of [C_4mim]Cl, 111 of them being improved, and the balance displaying inferior results. For [p1,4][DHP], only 14 out of 36 affected crystallization droplets were improved over the IL-free control. The distributions about the dilution ratios also differed between the two ILs, with [C_4mim]Cl having improvements in 38 (1:1), 46 (2:1), and 27 (4:1) droplets, and [p1,4][DHP] showing improvements in 9, 4, and 1 for the corresponding droplet ratios.

All XLN crystallization conditions affected by [C_4mim]Cl were also affected by [p1,4][DHP]. There were no instances where one IL gave improved results and the other negative results. Also, the general conditions affected were somewhat different than for the other proteins. Of the 62 precipitants where an effect, positive or negative, was observed, 22 were strictly salt-based conditions. Of these, only one was a negative effect, and two were mixed negative and positive effects. For [p1,4]-[DHP], of the 18 conditions where an effect was observed, 3 were salt-only conditions, of which 2 were negative and one mixed.

Glucose Isomerase. GI was only soluble in one of the three ILs, [C_4mim]Cl. The IL was found to affect the outcome in 163 wells, 55 positively and the rest negatively. Of the negative outcomes (108 wells), 24 of them involved conditions where a salt was the precipitant. In all these cases, the IL-containing solutions remained clear, indicating that the [C_4mim]Cl was solubilizing the protein in the presence of the precipitating salt.

Figure 5 shows the results for GI+ [C_4mim]Cl at screen condition H9 (20% PEGMME-2000, 0.01 M $NiCl_2 \cdot 6H_2O$, 0.1

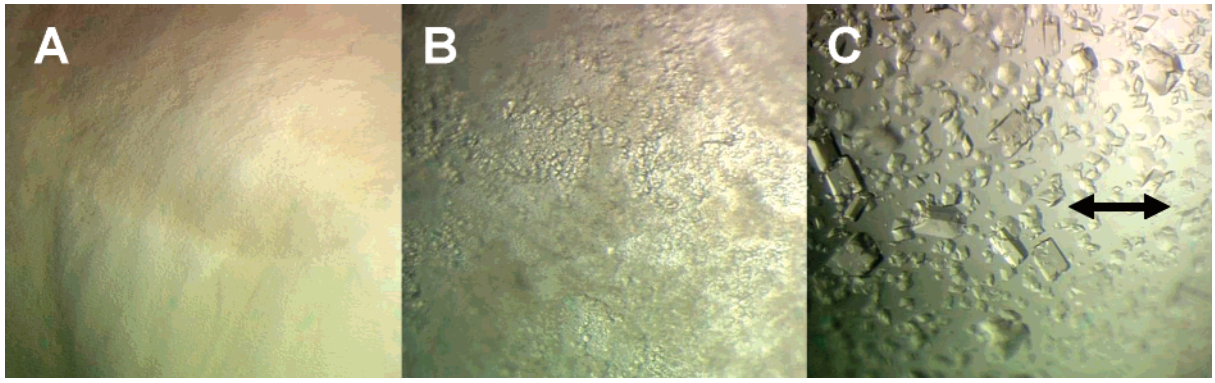

Figure 2. CAN crystallization in the absence (panel A) of IL and in the presence of 0.2 M (panel B) or 0.4 M (panel C) [C_4mim]Cl. The protein/precipitant ratio was 4:1, v:v. All panels are at the same scale, with the bar in panel $C = 200$ μm.

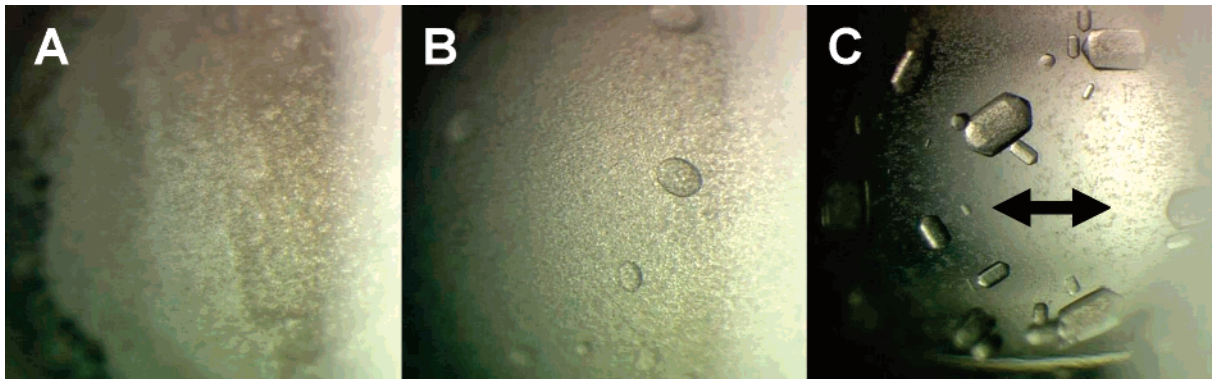

Figure 3. BLB crystallization in the absence (panel A) of IL and in the presence of 0.2 M (panel B) or 0.4 M (panel C) [C_4mim][MDEGSO4]. Screen solution G10 was used, and the protein/precipitant ratio was 4:1, v:v. The scale bar = 400 μm.

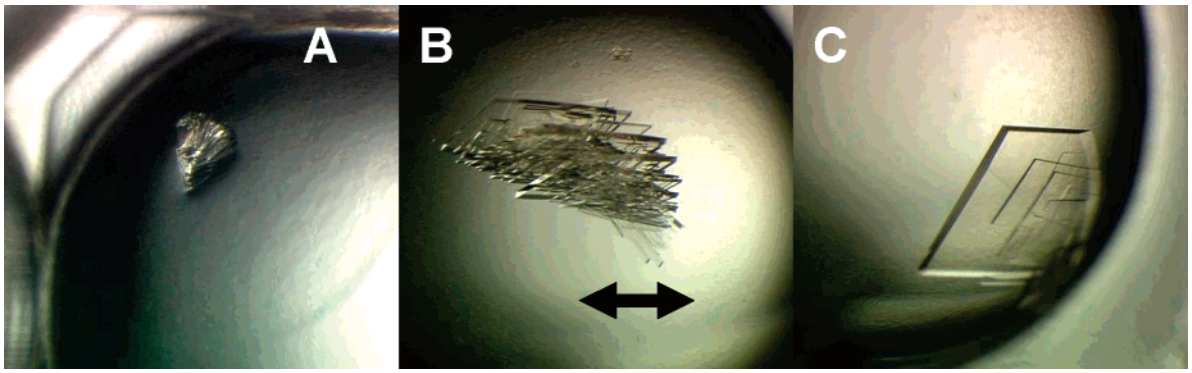

Figure 4. XLN crystallization in the absence (panel A) of IL and in the presence of 0.2 M (panel B) or 0.4 M (panel C) [C_4mim]Cl. Screen condition A6 was used, and the protein/precipitant ratio was 4:1, v:v. The scale bar = 400 μm.

M Tris-HCl, pH 8.5). The control is a granular precipitate, while the 0.2 M IL solution shows crystalline disks. The corresponding 0.4 M IL solution was clear, indicating that the IL had solubilized the protein. The rough edges of the disks in Figure 4 (panel B) suggest that the IL may be affecting particular protein−protein interactions, as chaotropes are known to do, leading to the rough edges observed. As with CAN and BLB, the listed precipitant for all other conditions where the IL was found to have an effect was either a small organic molecule or PEG-based polymer. The distribution of the IL effects was more even for [C_4mim]Cl with GI. Positive effects were found for 15, 25, and 17 wells at 1:1, 2:1, and 4:1 protein/precipitant ratios, respectively.

Protein Solubility. In a significant number of cases outcomes were obtained where addition of the IL increased the protein's solubility. However, these are not necessarily negative outcomes, and the frequency of occurrence (Table 1) led us to test whether this could be exploited to obtain crystallization conditions. Accordingly, we selected conditions from the data obtained for BLB and XLN where the control solution was a precipitate, not crystalline, and the corresponding outcome, under conditions containing 0.2 M IL, was a clear solution. In all cases, the IL was [C_4mim]Cl.

Sitting drop plates were set up with the IL at 25, 50, 75, 100, 125, and 150 mM in the protein solution for the eight conditions investigated. Within 2 days the results shown in Figure 6 were obtained for BLB with screen condition E5 (5% isopropanol, 2.0 M $(NH_4)_2SO_4$). Four other test conditions had precipitated protein at all IL concentrations, and the remainder all had clear solutions, indicating that the IL was keeping the protein soluble at even the lowest concentration employed (0.025 M). It is interesting to note that this ability of particular

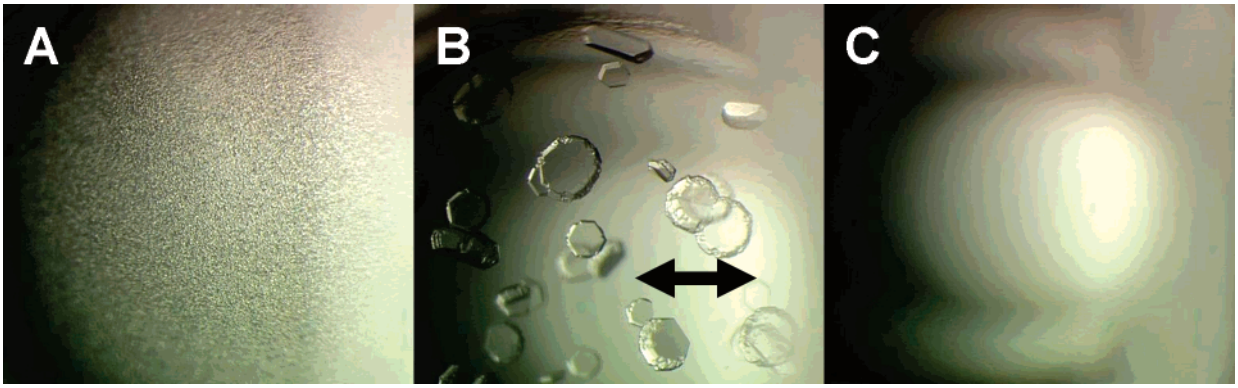

Figure 5. GI crystallization in the absence (panel A) of IL in the presence of 0.2 M (panel B) or 0.4 M (panel C) [C_4mim]Cl. Screen condition H9c was used, and the protein/precipitant ratio was 4:1, v:v. The scale bar = 400 μm.

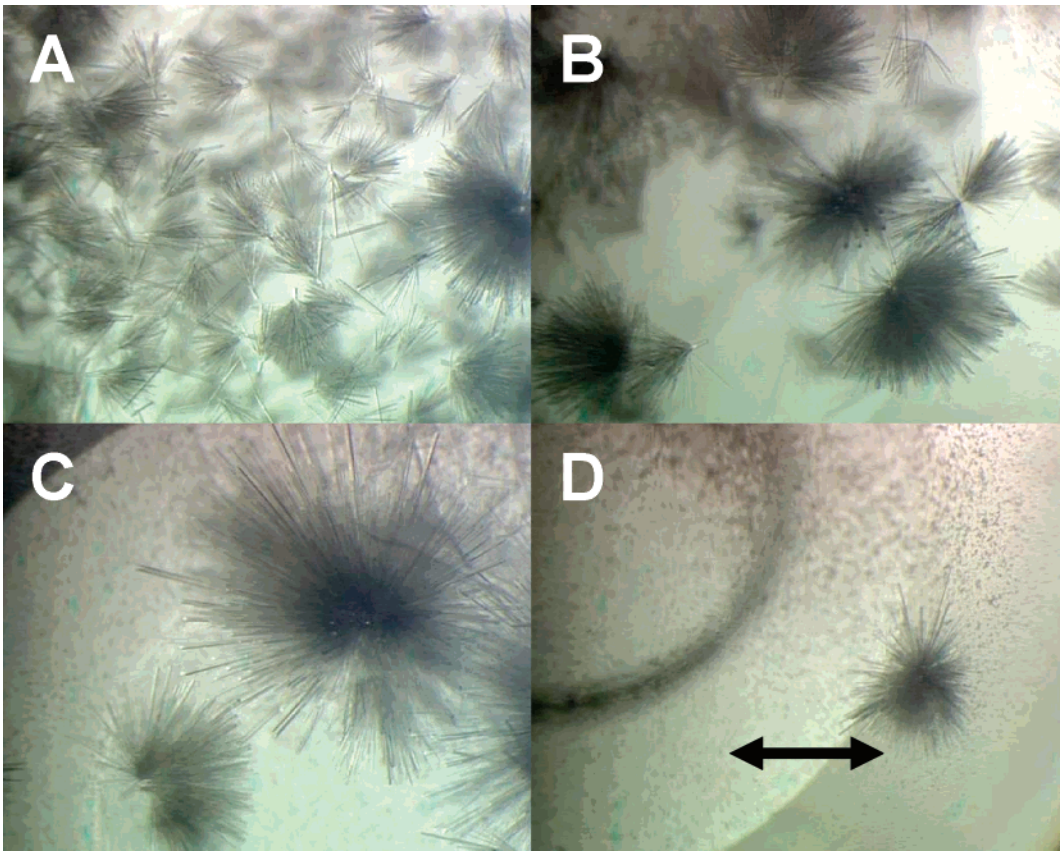

Figure 6. BLB sitting drop results, condition E5. The protein concentration is 20 mg/mL, and the [C_4mim]Cl concentration in the protein solution is panel A, 0.025 M; panel B, 0.05 M; panel C, 0.075 M; panel D, 0.1 M. The scale bar = 400 μm.

ILs to solubilize proteins at very low concentrations has been previously reported for cellulase in solutions of [C_4mim]Cl.[34]

Discussion

The use of fluorescently labeled protein was important for interpreting the results obtained. The ILs, particularly [C_4mim][MDEGSO4] and [p1,4][DHP], were found to readily crystallize out of solution, and it was often necessary to resort to fluorescence observation to distinguish whether individual crystals were protein or IL. Similarly, the ILs were also found to form lower scoring features such as spherulites. By using trace fluorescent labeling, it was possible to more accurately ascertain how the protein was responding to the presence of the screen conditions and added IL.

For the purposes of this work, additives are defined as those added solutes that improve on a precipitated state, such as amorphous or granular precipitate to crystal, needle crystals to plates or 3D crystals, etc. In carrying out this function, the additive presumably affects specific intermolecular interactions, such that those formed are more defined and a more ordered or higher dimensional solid state is attained. Precipitants are defined as those species that are able to precipitate a soluble protein. Presumably, these interactions are on a more global scale with respect to the protein. These definitions are used to define how the ILs affect the crystallization process.

The data do not indicate a single, clear mechanism for how the ILs affect the crystallization process. In some instances, they clearly act as additives, as shown in Figures 2−4. In others,

where crystals were obtained with conditions that gave clear solutions in the controls, the IL apparently acted as a precipitant. No attempts were made to further refine any of the crystallization conditions with respect to the ILs or other components. The results shown in Figure 5, GI crystallization in the presence [C_4mim]Cl, suggest that the IL interacted with specific sites on the protein affecting specific crystallographic contacts. Further evidence supporting specific IL−protein interaction has been described in previous experimentation noting that the protein, cellulase, becomes denatured (unfolded) when contacted with the IL [C_4mim]Cl and its inability to refold into its native structure upon reintroduction into an aqueous solution.

A kinetics argument for how the ILs may affect the crystallization process would be through the vapor equilibration rate. Adding salts to the protein solution will slow down the vapor transfer from the protein to the reservoir droplet. This mechanism would enable a slower approach to the nucleation and crystal growth regime resulting in crystals, whereas the IL-free solutions would rapidly transit the "crystallization slot",[42,43] resulting in precipitated protein. The data in Figure 2 provide some support for this argument; the 4:1 drop ratio experiments, those having a higher final concentration of IL at equilibrium, consistently gave more crystals than the 1:1 and 2:1 drop ratio experiments. However, the 0.2 and 0.4 M IL data are comparable, whereas we might expect some improvement with higher IL concentrations based upon this mechanism. One approach to determining this would be comparative control experiments where the IL cation is replaced by an inorganic cation. Such a series of experiments would also have to separate the IL anion and cation effects on the crystallization process as well. We chose to use dH_2O as the control, as for our goals it gave the most direct answer for the effects of ILs on the crystallization process.

Another potential mechanism for the ILs positively affecting the crystallization outcome is through increasing the protein solubility. Interestingly, BLB is known to bind hydrophobic ligands,[44] and an examination of the data in Table 1 shows that it was the only protein to consistently have more positive than negative outcomes. IL binding would replace a hydrophobic site with a salt, reducing the likelihood that site would subsequently participate in nonspecific interactions. If one views the formation of ordered crystals as a kinetic process, where the rate of amorphous precipitate formation is competing with that for ordered structure formation (crystallization), then raising the solubility, or reducing nonspecific binding between hydrophobic sites, would effectively slow down the self-association process. This would result in the solute being better able to form ordered structures before the kinetics of adding additional molecules traps the assembly in a disordered state. Partial support for this would come from the relatively large numbers of negative outcome results where the IL-containing solutions were clear drops, particularly for CAN with [C_4mim]Cl, XLN with [p1,4][DHP], and GI with [C_4mim]Cl.

This mechanism was tested directly with the experiment shown in Figure 6. Here, the working hypothesis was that if the screen conditions were sufficient to precipitate the protein, it might be possible to incrementally add IL such that the resulting outcome was crystalline and not precipitate formation. That was clearly the case in the example shown. Only one of the eight samples had such a positive outcome. The crystals obtained are not particularly suitable for structure determination; however, beyond varying the IL concentration no other adjustments were made to the solution conditions, so there is considerable room for further optimization. Note also that the condition illustrated, E5 is not buffered, and a first choice for any subsequent optimization would be to explore the effects of pH on the crystals obtained. Of the remaining seven samples, four were precipitated at all IL concentrations tested, and three remained clear. If the added IL is increasing the protein's solubility, then crystals could possibly be obtained in the clear outcomes by raising the protein concentration in the drop, further reducing the IL concentration, increasing the precipitant concentrations, or a combination of all three. Higher IL concentrations may be needed to solubilize the protein in the precipitated samples.

All protein−IL combinations produced at least one crystal at precipitant conditions unique from where crystals were obtained in the IL-free controls. However, the four proteins tested all had different responses to the three ILs. Two of the proteins were insoluble in at least one IL, suggesting some utility for ILs as monoprecipitants for protein crystallization. Not surprisingly, the variety of response between just the four proteins used herein indicates that a given IL will not affect all proteins in a similar manner and that a range of ILs would be required for protein crystallization purposes. Preliminary experiments using the ILs directly (instead of NaCl) for the crystallization of lysozyme have been carried out (data not shown). While crystals were obtained, the lysozyme concentration had to be increased due to the increased protein solubility.

One advantage to the use of ILs is the wide range of possible structures. The three ILs tested, [C_4mim]Cl, [C_4mim]-[MDEGSO4], and [p1,4][DHP], all have a butyl group in common but differ in their anion and represent two different parent cations. Variations of the parent cation, the cation side chain alkyl group(s), or of the anion structure, are possible, and previous work has shown that protein stability can be affected by changes in the length of side chain groups on the cation, and that a protein's thermal stability can be significantly enhanced with specific IL structures. ILs are not limited to a few parent cations or anions and have been prepared from a variety of molecules of biological interest, such as aspartame,[45] amino acids,[46,47] and choline.[28,48] Di- or multivalent ILs have also been prepared,[47,49,50] which may be useful analogues to diacids for protein crystallization.[51]

Whether positively or negatively, the precipitant conditions most affected by the presence of the ILs tested were those that contained small molecule organics or polymers such as PEG or Jeffamine (polyoxyalkyleneamine). These results would suggest that these ILs may be best used as additives for crystallization conditions employing these types of precipitants, i.e., as a precipitant class specific additive. If this is the case, then it may be possible to design other ILs that are more suitable for use with salt-based precipitant conditions.

Many of the precipitant solutions that were affected by the presence of the IL also have salts present, although they are typically at concentrations of 0.2 M or lower. In only a few instances were IL effects found with solutions where a salt was the primary precipitant. In these cases, the IL was generally found to have a negative effect in that it solubilized the protein to the extent that no precipitation occurred. Although not explored herein, this effect could potentially be exploited, where the IL is used at much lower concentrations, potentially altering the precipitation to crystallization conditions. Alternatively, one could use an IL specifically designed for the task of solubilizing proteins. Such an approach would be particularly useful if the precipitated phase was as a microgranular precipitate.

It does not escape our attention that the potential range for variability of IL structure and properties may make this class

of compounds particularly useful for working with membrane proteins. Preliminary experiments for this application have been carried out (data not presented) and suggest that even some "off the shelf" ILs may be applicable for the solubilization and crystallization of this important class of protein. We used ILs in this work as an aqueous salt solution and are exploiting their properties as such. We speculate that ILs specific for the tasks of solubilizing, stabilizing, and manipulating membrane proteins may be possible.

In summary, this study was focused on the utility of ILs as a class of molecules for the crystallization of soluble proteins. The data indicate that they are. When employed, crystals were often obtained under conditions that had previously not resulted in crystallization. The results also suggest that in some instances the ILs may have been acting as the primary precipitant, with the standard screen components as the additive. Subsequent studies to explore the effects of IL structure on protein crystallization and their effects on diffraction resolution, as well as how they interact with proteins, are now needed.

Acknowledgment. This research has been supported by the U.S. Environmental Protection Agency's STAR program through Grant No. R-83143201. (Although the research described in this article has been funded in part by EPA, it has not been subjected to the Agency's required peer and policy review and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred.) The authors also thank Merck KGaA/EMD Chemicals for support of the Center for Green Manufacturing and acknowledge the generous support from NASA Grant No. NCC8-256, Task 7.

References

(1) Wallach, O. *Chem. Ber.* **1883**, *16*, 534−547.
(2) Sudgen, S.; Wilkens, H. *Chem. Soc. J.* **1929**, *51*, 1291−1298.
(3) Walden, P. *Bull. Acad. Sci. St. Petersburg* **1914**, *8*, 405−422.
(4) Chan, B. K. M.; Chang, N. H.; Grimmett, M. R. *Aust. J. Chem.* **1977**, *30*, 2005−2013.
(5) Wilkes, J. S.; Levisky, J. A.; Wilson, R. A.; Hussey, C. L. *Inorg. Chem.* **1982**, *21*, 1263−1264.
(6) Hussey, C. L. *Adv. Molten Salt Chem.* **1983**, *5*, 185−229.
(7) Hussey, C. L. *Pure Appl. Chem.* **1988**, *60*, 1763−1772.
(8) Seddon, K. R. In *Molten Salt Forum: Proceedings of 5th International Conference on Molten Salt Chemistry and Technology*; Wendt, H., Ed.; Trans Tech Publication: Dresden, Germany, 1998; Vol. 5−6, pp 53−62.
(9) Chauvin, Y.; Olivier-Bourbigou, H. *CHEMTECH* **1995**, *25*, 26−30.
(10) Carter, M. T.; Hussey, C. L.; Strubinger, S. K. D.; Osteryoung, R. A. *Inorg. Chem.* **1991**, *30*, 1149−1151.
(11) Marsh, K. N.; Boxall, J. A.; Lichtenthaler, R. *Fluid Phase Equilib.* **2004**, *219*, 93−98.
(12) Forsyth, S. A.; Pringle, J. M.; MacFarlane, D. R. *Aust. J. Chem.* **2004**, *57*, 113−119.
(13) *Ionic Liquids; Industrial Applications to Green Chemistry*; Rogers, R. D.; Seddon, K. R., Eds.; ACS Symposium Series 818; American Chemical Society: Washington, DC, 2002.
(14) *Green Industrial Applications of Ionic Liquids*; Rogers, R. D.; Seddon, K. R.; Volkov, S., Eds.; NATO Science Series II. Mathematics, Physics and Chemistry, Vol. 92; Kluwer: Dordrecht, 2003.
(15) *Ionic Liquids as Green Solvents: Progress and Prospects*; Rogers, R. D.; Seddon, K. R., Eds.; ACS Symposium Series 856; American Chemical Society: Washington, DC, 2003.
(16) *Ionic Liquids IIIA: Fundamentals, Progress, Challenges, and Opportunities - Properties and Structure*; Rogers, R. D.; Seddon, K. R. Eds.; ACS Symposium Series 901; American Chemical Society: Washington, DC, 2005.
(17) *Ionic Liquids IIIB: Fundamentals, Progress, Challenges, and Opportunities - Transformations and Processes*; Rogers, R. D.; Seddon, K. R., Eds.; ACS Symposium Series 902; American Chemical Society: Washington, DC, 2005.
(18) Bowron, D. T.; Hardacre, C.; Holbrey, J. D.; McMath, J. E. J.; Soper, A. K. *J. Chem. Phys.* **2003**, *118*, 273−278.
(19) Cadena, C.; Zhao, Q.; Snurr, R. Q.; Maginn, E. J. *J. Phys. Chem. B* **2006**, *110*, 2821−2832.
(20) Hofmeister, F. *Arch. Exp. Pathol. Pharmakol.* **1888**, *24*, 247−260.
(21) Gutowski, E. K.; Broker, G. A.; Willauer, H. D.; Huddleston, J. G.; Swatloski, R. P.; Holbrey, J. D.; Rogers, R. D. *J. Am. Chem. Soc.* **2003**, *22*, 6632−6633.
(22) Bridges, N. J.; Gutowski, K. E.; Rogers, R. D. *Green Chem.* **2007**, *9*, 177−183.
(23) Lozano, P.; de Diego, T.; Guegan, J. P.; Vaultier, M.; Iborra, J. L. *Biotech. Bioeng.* **2001**, *75*, 563−569.
(24) Kragl, U.; Eckstein, M.; Kaftzik, N. *Curr. Opin. Biotechnol.* **2002**, *13*, 565−571.
(25) Sheldon, R. A.; Lau, R. M.; Sorgedrager, M. J.; van Rantwijk, F.; Seddon, K. R. *Green Chem.* **2002**, *4*, 147−151.
(26) Park, S.; Kazlauskas, R. *Curr. Opin. Biotechnol.* **2003**, *14*, 432−437.
(27) de Diego, T.; Lozano, P.; Smouh, S.; Vaultier, M.; Iborra, J. L. *Biomacromolecules* **2005**, *6*, 1457−1464.
(28) Fujita, K.; MacFarlane, D. R.; Forsyth, M. *Chem. Comm.* **2005**, *38*, 4804−4806.
(29) Fujita, K.; Forsyth, M.; MacFarlane, D. R.; Reid, R. W.; Elliot, G. D. *Biotech. Bioeng.* **2006**, *94*, 1209−1213.
(30) Baker, S. N.; McCleskey, T. M.; Pandey, S.; Baker, G. A. *Chem. Comm.* **2004**, *8*, 940−941.
(31) Turner, M. B.; Holbrey, J. D.; Spear, S. K.; Pusey, M. L.; Rogers, R. D. In *Ionic Liquids IIIB: Fundamentals, Progress, Challenges, and Opportunities*; Rogers, R.D., Seddon, K. R., Eds.; ACS Symp. Ser. 902; American Chemical Society: Washington, D.C., 2004; pp 233−243.
(32) Summers, C. A.; Flowers, R. A., II *Protein Sci.* **2000**, *9*, 2001−2008.
(33) Lange, C.; Patil, G.; Rudolph, R. *Protein Sci.* **2005**, *14*, 2693−26701.
(34) Turner, M. B.; Spear, S. K.; Huddleston, J. G.; Holbrey, J. D.; Rogers, R. D. *Green Chem.* **2003**, *5*, 443−447.
(35) Reichert, W. M.; Holbrey, J. D.; Vigour, K. B.; Morgan, T. D.; Broker, G. A.; Rogers, R. D. *Chem. Comm.* **2006**, 4767−4779.
(36) Garlitz, J. A.; Summers, C. A.; Flowers, R. A., II; Borgstahl, G. E. *Acta Crystallogr.* **1999**, *D55*, 2037−2038.
(37) Sumner, J. B.; Howell, S. F. *J. Biol. Chem.* **1936**, *113*, 607−610.
(38) Smith, S. C.; Johnson, S.; Andrews, J.; McPherson, A. *Plant Physiol.* **1982**, *70*, 1199−1209.
(39) Forsythe, E.; Achari, A.; Pusey, M. L. *Acta. Crystallogr.* **2006**, *D62*, 339−346.
(40) Huddleston, J. G.; Visser, A. E.; Reichert, W. M.; Willauer, H. D.; Broker, G. A.; Rogers, R. D. *Green Chem.* **2001**, *3*, 156−164.
(41) Newman, J. *Acta. Crystallogr.* **2005**, *D61*, 490−493.
(42) George, A.; Chiang, B.; Guo, A.; Arabshahi, A.; Cai, Z.; Wilson, W. W. *Methods Enz.* **1997**, *276*, 100−110.
(43) George, A.; Wilson, W. W. *Acta. Crystallogr.* **1994**, *D50*, 361−365.
(44) Collini, M.; D'Alfonso, L.; Molinari, H.; Ragona, L.; Catalano, M.; Baldini, G. *Protein Sci.* **2003**, *12*, 1596−1603.
(45) Carter, E. B.; Culver, S. L.; Fox, P. A.; Goode, R. D.; Ntai, I.; Tickell, M. D.; Traylor, R. K.; Hoffman, N. W.; Davis, J. H., Jr. *Chem. Comm.* **2004**, 630−631.
(46) Fukumoto, K.; Yoshizawa, M.; Ohno, H. *J. Am. Chem. Soc.* **2005**, *127*, 2398−2399.
(47) Tao, G. H.; He, L.; Sun, N.; Kou, Y. *Chem. Comm.* **2005**, 3562−3564.
(48) Abbott, A. P.; Capper, G.; Davies, D. L.; Rasheed, R. K.; Shikotra, P. *Inorg. Chem.* **2005**, *44*, 6497−6499.
(49) Anderson, J. L.; Ding, R.; Ellern, A.; Armstrong, D. W. *J. Am. Chem. Soc.* **2005**, *127*, 593−604.
(50) Tang, J.; Tang, H.; Sun, W.; Plancher, H.; Rodosz, M.; Shen, Y. *Chem. Comm.* **2005**, 3325−3327.
(51) McPherson, A. *Protein Sci.* **2001**, *10*, 418−422.

CG060696T