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Protein renaturation by the liquid organic salt ethylammonium nitrate

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Abstract

The room-temperature liquid salt, ethylammonium nitrate (EAN), has been used to enhance the recovery of denatured-reduced hen egg white lysozyme (HEWL). Our results show that EAN has the ability to prevent aggregation of the denatured protein. The use of EAN as a refolding additive is advantageous because the renaturation is a one-step process. When HEWL was denatured reduced using routine procedures and renatured using EAN as an additive, HEWL was found to regain 75% of its activity. When HEWL was denatured and reduced in neat EAN, dilution resulted in over 90% recovery of active protein. An important aspect of this process is that renaturation of HEWL occurs at concentrations of 1.6 mg/mL, whereas other renaturation processes occur at significantly lower protein concentrations. Additionally, the refolded-active protein can be separated from the molten salt by simple desalting methods. Although the use of a low-temperature molten salt in protein renaturation is unconventional, the power of this approach lies in its simplicity and utility.

Keywords: hen egg white lysozyme; ethylammonium nitrate; differential scanning calorimetry

Genetic engineering has made the production of proteins routine using prokaryotic expression systems (Marston, 1986; Hochuli et al., 1988; Georgiou & Clark, 1991). Although expression in *Escherichia coli* and other hosts is a convenient method for the production of large amounts of protein, the absence of the proper refolding machinery in the host may lead to nonnative conformations and the formation of inclusion bodies (Armstrong et al., 1999). Complications arise when trying to obtain active, refolded protein from the unfolded, aggregated state.

Due to revolutionary advances in genetic expression processes, more general renaturation strategies are required. Commonly, the renaturation of inactive protein starts with the isolation of the inclusion bodies followed by dissolution of the proteins promoted with a chemical denaturant, often urea or guanidine hydrochloride. Dialysis or dilution of the denaturant then initiates refolding of the protein. Unfortunately, it is during this dilution process that the protein may reform inactive aggregates that further complicate isolation and purification. Therefore, it is essential to optimize the conditions that minimize the formation of aggregates during refolding. Aggregation occurs upon the exposure of the hydrophobic surfaces of a protein, and this phenomenon is the major reason for the failure of protein refolding (Georgiou & Bowden, 1991; Clark et al., 1998).

Another cause of aggregation is the reshuffling of disulfide bonds (Thomas & Baneyx, 1996). Topologically distant, but spatially close cysteine residues typically form disulfide bonds that stabilize the active conformation of proteins. During the refolding of reduced proteins, the formation of disulfide bonds between incorrectly matched cysteine residues (intra- and intermolecular) can lead to nonnative structures. As a result, the misfolded protein is trapped in a nonnative state that leads to aggregation.

Dissolution of aggregates or prevention of protein aggregation can be promoted utilizing small molecule additives. These additives reduce aggregation of the peptide segments and, therefore, promote protein folding. Dilution additives include detergents (Tandon & Horowitz, 1986), amphiphiles (Goldberg et al., 1996), cyclodextrins (Karuppiah & Sharma, 1995), and polyethylene glycol (Cleland & Wang, 1990). One diverse group of proteins, termed "chaperones," can also aid in the proper refolding of proteins. Chaperones bind to the target protein to promote proper folding and inhibit competing aggregation (Gething & Sambrook, 1992; Hendrick & Hartl, 1993). A detailed mechanism of this process is not yet unavailable (Hartl, 1996).

Inspired by the protein chaperone process, a novel strategy referred to as "artificial chaperone-assisted refolding" was recently developed by Gellman and colleagues (Rozema & Gellman, 1995, 1996a, 1996b; Daugherty et al., 1998). This strategy involves two steps: (1) protein capture by detergent, and (2) removal of the

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Abbreviations: HEWL, hen egg white lysozyme; EAN, ethylammonium nitrate; BEAS, bis(ethylammonium) nitrate; BAN, N-butylammonium nitrate; EAP, ethylammonium phosphate; DSC, differential scanning calorimetry; DTT, dithiothreitol; GSH, glutathione; GSSG, oxidized glutatione; CD, circular dichroism; UV, ultraviolet.

detergent by cyclodextrins. This two-step protocol results in high yields of correctly folded and active protein. This process is efficient at low protein concentrations, but at concentrations of 1 mg/mL aggregation begins to dominate, and the yield of recovered active protein decreases (Raman et al., 1996).

One common protein that has been found to renature successfully at low concentration in the presence of additives is hen egg white lysozyme (HEWL) (Maeda et al., 1996). The refolding pathway of HEWL is understood well (Goldberg et al., 1991; Fischer et al., 1993). While dilution-assisted renaturation of denatured HEWL with disulfide the bonds intact produces correctly folded active protein, renaturation of reduced HEWL is inefficient due to competition between aggregation and refolding (Raman et al., 1996). Although renaturation of reduced HEWL at low concentrations is possible, refolding becomes problematic at high protein concentrations (>1 mg/mL) because of the propensity for aggregation. The development of a renaturation additive that prevents aggregation and enables the recovery of active protein at high concentrations would be advantageous.

Herein we report a novel strategy that utilizes a liquid organic salt, ethylammonium nitrate (EAN), to prevent aggregation of denatured protein during the refolding process. Addition of EAN to chemically denatured-reduced HEWL leads to high yields of active protein. Moreover, EAN enables the recovery of active protein at relatively high concentrations (1.6 mg/mL). This technique provides a unique and expedient process for the recovery of large amounts of active protein.

Results and discussion

Ethylammonium nitrate is a clear, colorless room-temperature liquid salt. The preparation of EAN was first described in 1929 (Sudgen & Wilkens, 1929), but it did not attract much interest until the 1980s. Evans and coworkers found that EAN is a highly associated substance with many properties similar to water (Evans et al., 1981, 1982, 1983). We began our studies with the hypothesis that EAN may provide a suitable nonaqueous medium for proteins. We found that a variety of proteins were soluble in the anhydrous salt. During our initial studies, we discovered that thermally denatured HEWL did not precipitate from EAN solutions after it was heated to temperatures of 100 °C, while the thermally inactivated enzyme in buffer precipitated out of solution. We were unable to use many traditional spectroscopic techniques such as UV-Vis and CD spectroscopies for examining protein structure because the EAN absorption masked the characteristic protein peaks.

Calorimetry offers another method for investigating the influence of additives on the stability of protein structure. We employed differential scanning calorimetry (DSC) to examine the effect of EAN on the thermal properties of the enzyme. Figure 1 compares the DSC thermograms of HEWL without EAN (Fig. 1A) and with 5% EAN (Fig. 1B). When the HEWL without EAN is cooled in buffer, precipitation occurs, and no refolding is evident in subsequent DSC runs (Fig. 1D). Surprisingly, addition of 5% EAN to the HEWL solution in buffer appears to stabilize the enzyme against irreversible thermal denaturation. Integration of the thermogram of

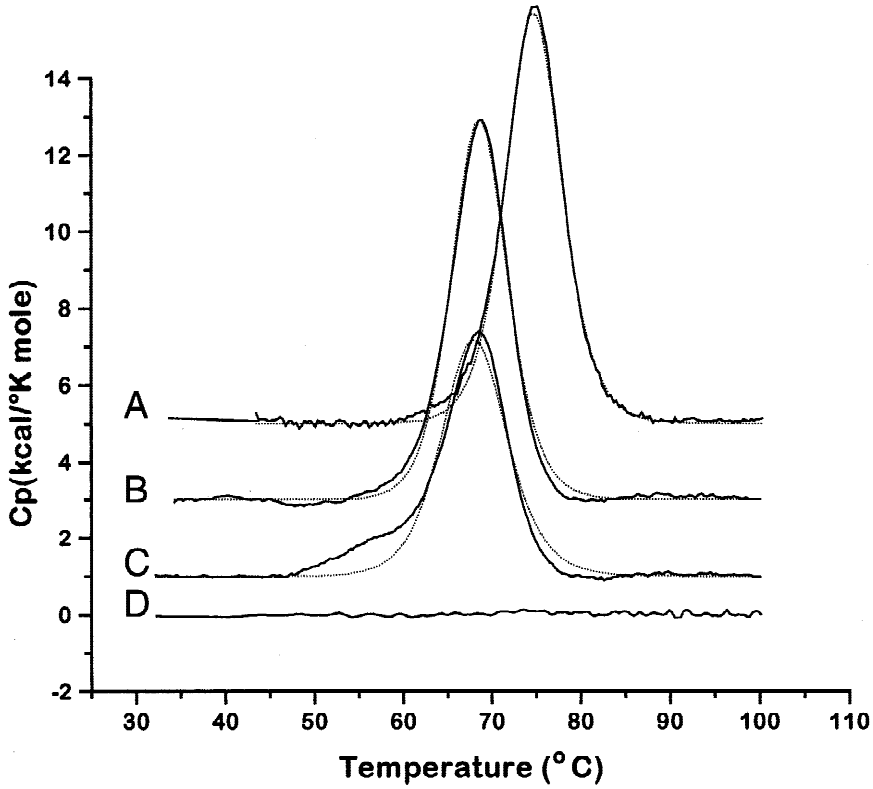


Fig. 1. Stacked plot of DSC profile of 0.069 mM HEWL in with 5% EAN solution of 0.1 M phosphate buffer, pH 6.24, vs. buffer alone. **A:** Initial thermal denaturation scan of HEWL in 0.1 M phosphate buffer solution, pH 6.24. **B:** Initial thermal denaturation scan of HEWL in 5% EAN solution. **C:** Second thermal denaturation scan of HEWL in 5% EAN solution. **D:** Second thermal denaturation scan of HEWL in buffer solution. Refolding occurred after initially heating to 100 °C, slowly cooling to RT, equilibrating for 1 h and then heating to 100 °C to examine refolding of the enzyme.

Table 1. *Differential scanning calorimetry of HEWL in buffer and in a 5% EAN buffer solution*[a]

Sample	T_m (initial)	ΔH (initial)	T_m (reheating)	ΔH (reheating)
Buffer	74.76 + 0.01 °C	89.8 + 0.3 kcal/mol	No refolding	No refolding
5% EAN	71.39 + 0.02 °C	87.2 + 0.4 kcal/mol	74.76 ± 0.01°C	76.1 ± 0.2 kcal/mol

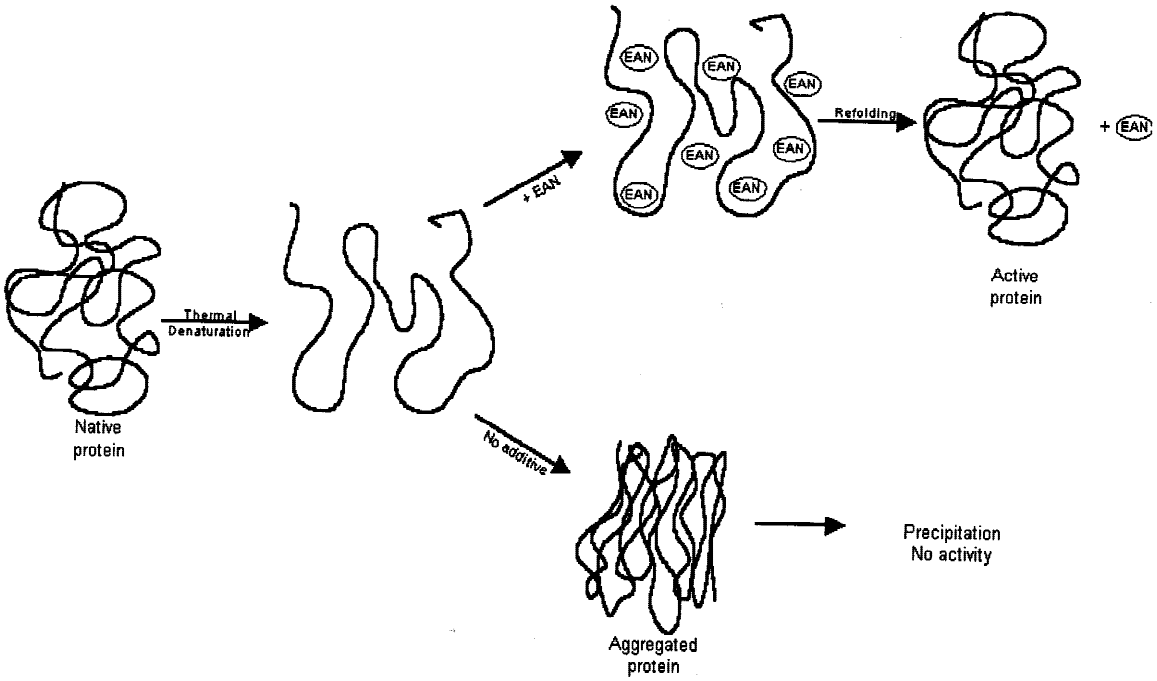
[a]The data have been fitted using the ORIGIN software (Microcal Inc.).

the EAN treated HEWL solution shows ~87% refolding (Fig. 1C). Careful inspection of Figure 1C also shows the appearance of a small shoulder indicating that the transition is no longer two state. The presence of this additional peak indicates that there may be a small structural change in the enzyme. This may be due to destabilization of one of the domains of the protein structure or a slight misfolding during the cooling process. Nonetheless, the sample retains activity. Standard activity assays of these samples showed that ~90% of the original activity was retained.

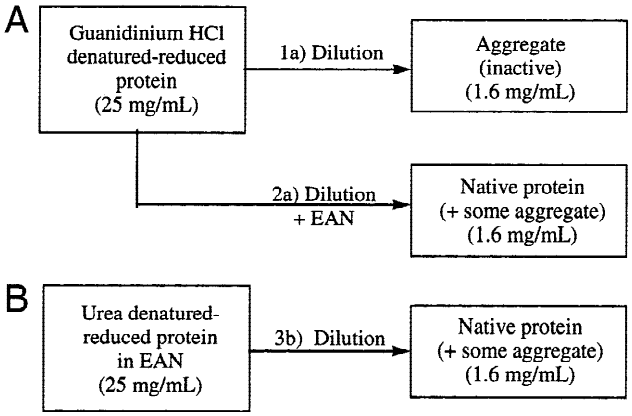
Table 1 shows the T_m of HEWL in buffer and in 5% EAN solutions after one heating cycle. There is a 3 °C drop in the T_m of HEWL upon the addition of EAN. This suggests that EAN is a denaturant. To further ascertain whether EAN is a denaturant, calorimetric experiments were carried out, and the T_m of HEWL was monitored as a function of EAN concentration. These experiments clearly show that EAN was acting as a denaturant (see Supplementary material in the Electronic Appendix).

During thermal denaturation of HEWL, the hydrophobic core of the protein is exposed, but the disulfide bonds remain intact (Khechinashvili et al., 1973; Privalov & Khechinashvili, 1974; Griko et al., 1995; Ibara-Molero & Sanchez-Ruiz, 1997). Intermolecular association of the hydrophobic core of proteins leads to aggregation and precipitation. The interesting feature of the EAN treatment is that no precipitation occurs even after extended heating (up to 6 h at 100 °C) of the HEWL solution. It is our supposition that the ethyl group of EAN interacts with the hydrophobic portion of the protein and protects it from intermolecular association while the charged portion of the salt stabilizes the electrostatic interactions of its secondary structure (Scheme 1) (Kohn et al., 1997). Because precipitation is a major problem in the production of active proteins via recombinant techniques, we decided to examine the influence of EAN on the refolding and aggregation of chemically denatured-reduced HEWL.

Lysozyme contains four disulfide bonds that are important in maintaining the tertiary structure of the protein. Renaturation of large quantities of chemically denatured-reduced HEWL by dilution is an inefficient process due to the propensity for aggregation (Goldberg et al., 1991; Fischer et al., 1993; Sundari et al., 1999). Reduced-denatured lysozyme was prepared by the procedure described by Gellman (Rozema & Gellman, 1996a). Renaturation of HEWL was attempted using EAN as an additive in the renaturation solution. Scheme 2 provides a detailed illustration of the proce-



Scheme 1. Illustration of the unfolding process during the thermal denaturation of protein in the presence and absence of EAN.



Scheme 2. A representation of the renaturation procedure for HEWL using EAN and an additive. HEWL was denatured with 6.0 M Gdm-HCl and 43 mM DTT in 0.100 M Tris-sulfate buffer, pH 8.5. Refolding of HEWL was achieved via (1a) dilution assisted protein refolding, (2a) dilution assisted protein refolding using EAN in the dilution buffer, and (3b) denaturation in EAN and dilution-assisted protein refolding.

dure, and the results are contained in Table 2. As the denatured-reduced HEWL was diluted with renaturation solutions containing increasing amounts of EAN, the activity of the enzyme increased, reaching a maximum activity at 75% recovery in the presence of 0.54 M EAN. Recovery of active protein declined at higher concentrations of EAN. The detergent cetyltrimethylammonium bromide (CTAB) was also used in the renaturation solution, but minimal activity was regained, as shown in Table 2. We conclude that at high concentrations EAN denatures the HEWL, but at lower concentrations it provides a high yield of active protein. Concentrations of EAN lower than 0.5 M do not successfully prevent aggregation of the protein, and therefore, do not support an environment conducive to proper refolding.

Horowitz and coworkers studied the effects of concentration on the detergent-assisted refolding of denatured rhodanese (Tandon & Horowitz, 1987; Zardeneta & Horowitz, 1992). They found that regardless of the type of detergent, each had a specific concentration range that provided reasonable refolding yields. When the same detergents employed by Horowitz (CTAB, Z-3-14, Triton X-100) were used to refold denatured/reduced HEWL (Rozema & Gellman, 1996a) and carbonic anhydrase (Rozema & Gellman, 1996b) dilution of the protein–detergent solution did not result in renatured protein. Removal of the detergent from the protein solution was necessary for refolding to occur. Common detergents with long hydrophobic alkyl chains associate with the small protein to such an extent that their displacement upon dilution is difficult, and the addition of a β-cyclodextrin is necessary to remove the associated detergent from the protein and enable it to refold to its native conformation. It is likely that EAN has a lower affinity for the hydrophobic portion of the protein than detergent, so at lower concentrations it can be displaced more easily by the protein during refolding. Lower concentrations of EAN do not effectively prevent all aggregation, but higher concentrations of EAN may be harder for the protein to displace during the renaturation process.

The first question that comes to mind is, do other small chain alkyl ammonium salts display analogous behavior or is EAN a unique additive for protein renaturation? Renaturation of HEWL was attempted in the presence of the other salt additives and the results are shown in Table 3. Butylammonium nitrate (BAN) gave low yields of active protein compared to EAN. Surprisingly, ammonium nitrate (AN) provided modest yields of active enzyme. Comparison of EAN, BAN, and AN clearly show that EAN is the most effective renaturation additive. A series of additional alkyl ammonium and other salts were investigated using the Hofmeister series as a guide (von Hippel & Wong, 1964; Baldwin, 1996). Ethylammonium phosphate (EAP) provided low yields of active enzyme. One salt in particular, bis(ethylammonium) sulfate (BEAS), provided reasonable refolding yields of 45% at a concentration of 4.0 M. Out of all of the salts examined so far, EAN is superior for the prevention of aggregation and the renaturation of HEWL.

The success of using a salt additive for renaturation is likely to be dependent on the protein's surface charge and degree of hydrophobicity. The data in Table 3 clearly show that ammonium nitrate plays an important role in renaturation of HEWL, but the presence

Table 2. *Results for chemical renaturation of HEWL using EAN as an additive during renaturation-oxidation dilution process*

Sample[a]	Additive	% EAN	Aggregation	Activity ($\times 10^4$ U/mg protein)	% Activity
Control[b]	None	0%	No	1.5 + 0.1	100 + 10%
2	None	0%	Yes	0.01 + 0.01	1.0 + 0.1%
3	0.05 M EAN	0.5%	Yes	0.25 + 0.09	16 + 9%
4	0.16 M EAN	1%	Yes	0.34 + 0.03	22 + 3%
5	0.54 M EAN	5%	Yes	1.15 + 0.03	75 + 3%
6	1.01 M EAN	11%	Yes	0.33 + 0.09	22 + 9%
7	3.07 M EAN	33%	Yes	0.19 + 0.03	12 + 3%
8	5.09 M EAN	55%	Yes	0.42 + 0.03	27 + 3%
9	0.018 M CTAB	0%	Yes	0.08 + 0.02	5 + 2%
10	0.006 M CTAB	0%	No	0.06 + 0.01	4 + 1%

[a] Denaturation conditions involve 14 μL of a solution containing 8.7 M GdmHCl, 143 mM Tris buffer (pH 8.5), and 43 mM DTT, to which 6 μL of 83.5 mg/mL HEWL stock solution was added. The solution of the denatured-reduced HEWL was diluted in a 1:1 GSH:GSSG solution with Tris buffer (pH 8.5) and the concentration of EAN as indicated.

[a] Control solution was diluted with buffer only and not denatured or renatured with any of the respective solutions.

Table 3. *Results for chemical renaturation of HEWL using other salts as additives during renaturation-oxidation dilution process*

Sample[a]	Additive	Aggregation	Activity ($\times 10^4$ U/mg protein)	% Activity
Control[b]	None	No	1.5 + 0.1	100 + 10%
2	None	Yes	0.01 + 0.01	1 + 1%
11	0.06 M NaCl	Yes	0.3 + 0.01	19 + 1%
12	0.5 M NaCl	Yes	0.14 + 0.01	8 + 1%
13	5.0 M NaCl	Yes	0	0%
14	0.05 M KCl	Yes	0.25 + 0.01	17 + 1%
15	0.5 M KCl	Yes	0.22 + 0.01	14 + 1%
16	4.0 M KCl	Yes	0.02 + 0.01	2 + 1%
17	0.05 M BAN	Yes	0.11 + 0.01	8 + 1%
18	0.5 M BAN	Yes	0.27 + 0.02	18 + 2%
19	5.0 M BAN	No	0.01 + 0.01	1 + 1%
20	0.05 M BEAS	Yes	0.40 + 0.40	27 + 4%
21	0.5 M BEAS	Yes	0.44 + 0.08	26 + 8%
22	4.0 M BEAS	No	0.67 + 0.07	45 + 7%
23	0.06 M EAP	Yes	0.67 + 0.07	24 + 6%
24	0.5 M EAP	Yes	0.35 + 0.05	21 + 5%
25	2.2 M EAP	Yes	0.05 + 0.01	3 + 1%
26	0.07 M AN	Yes	0.44 + 0.01	29 + 1%
27	0.5 M AN	Yes	0.33 + 0.01	22 + 1%
28	5.1 M AN	Yes	0.27 + 0.01	18 + 1%

[a]Denaturation conditions involve 14 μL of a solution containing 8.7 M GdmHCl, 143 mM Tris buffer (pH 8.5), and 43 mM DTT, to which 6 μL of 83.5 mg/mL HEWL stock solution was added. The solution of the denatured-reduced HEWL was diluted in a 1:1 GSH:GSSG solution with Tris buffer (pH 8.5) and the concentration of EAN as indicated.
[b]Control solution was diluted with buffer only and not denatured or renatured with any of the respective solutions.

of an alkyl group significantly enhances the refolding process. This finding clearly shows the significance of the ethyl portion of the salt and suggests that specific combinations of salts and alkyl groups can be tuned to interact more favorably with specific proteins, depending on their structure.

It is often desirable to conduct refolding at as high of concentration as possible to recover a high yield of protein. Most reported studies on protein refolding are in the range of μg/mL (Raman et al., 1996; Yasuda et al., 1998). Gellman and colleagues examined HEWL refolding at concentrations of 1 mg/mL and recovered 57% of the activity (Rozema & Gellman, 1996a). In the present study, a final concentration of 1.6 mg/mL was examined, with 75% activity recovered using EAN as the refolding additive. This concentration is close to that used in protein crystallization studies.

An alternative denaturation of HEWL was carried out in anhydrous EAN containing DTT and urea (Scheme 2B) (Makhatadze

Table 4. *Results for chemical renaturation using EAN in the denaturation-reduction process*

Sample	Denaturation Conditions	Additive	Activity ($\times 10^4$ U/mg protein)	% Activity
Control[a]	None	None	1.5 + 0.1	100 + 10%
29[b]	Pure EAN	None	1.4 + 0.1	90 + 10%
30[b]	Pure EAN	0.54 M EAN	1.46 + 0.08	95 + 8%
31[b]	Pure EAN	No dilution	0.01 + 0.01	1 + 1%
32[c]	Pure BAN	0.5 M BAN	1.41 + 0.06	94 + 6%
33[c]	Pure BAN	None	1.32 + 0.01	88 + 1%

[a]Control solution was diluted with buffer only and not denatured or renatured with any of the respective solutions.
[b]Denaturation conditions involve mixing 14 μL of a solution containing 8 M urea, 43 mM DTT dissolved in pure EAN with 6 μL of 83.5 mg/mL HEWL stock solution. Solution was diluted with a 1:1 GSH:GSSG solution with Tris buffer (pH 8.5) to yield a final concentration of 1.6 mg/mL of HEWL, and if indicated, more additive. For solution 31, no renaturation buffer was added. For solution 29, no EAN was present in the renaturation solution.
[c]Denaturation conditions involve mixing 14 μL of a solution containing 8 M urea, 43 mM DTT dissolved in pure BAN with 6 μL of 83.5 mg/mL HEWL stock solution while being heated to 40 °C in a water bath. Solution was diluted with a 1:1 GSH:GSSG solution with Tris buffer (pH 8.5), to yield a final concentration of 1.6 mg/mL of HEWL, and if indicated, more additive. For solution 33, no BAN was present in the renaturation solution.

& Privalov, 1992; West et al., 1997; Vanzi et al., 1998). When the denatured sample in EAN was assayed, no activity was present, indicating that the protein was completely denatured (Table 4, sample 31). Surprisingly, when the EAN denatured-reduced solution was diluted with the renaturation solutions, minimal precipitation was present and over 90% of the activity was regained. Similar results were obtained using another alkyl ammonium nitrate salt, butylammonium nitrate (BAN). These data are contained in Table 4. There may be a difference in the denatured state of the protein because the denaturant was changed from urea to GdmHCl. This may explain the increase in recovery of active protein when HEWL was denatured in EAN instead of buffer.

Another advantage of this procedure is that EAN is easily removed from the protein solutions by simple desalting techniques. Figure 2 contains the fluorescence spectra of the native enzyme, the enzyme in the presence of EAN, and the desalted enzyme. The results indicate that the presence of EAN in the solution significantly quenches the fluorescence of the tryptophan residues in HEWL, and once desalted, the intrinsic fluorescence spectrum of the native protein is obtained.

In many cases, dilution of denatured proteins not only decreases the apparent concentration of the denaturant (urea, guanidinium hydrochloride), but also allows the protein to refold by reducing the probability of intermolecular interactions between polypeptide chains. Remarkably, renaturation using EAN in place of detergent does not require a second dilution because its presence in small amounts as an additive does not appear to inhibit refolding. The results described herein show that EAN prevents aggregation of the enzyme, and its easy removal allows proper refolding to an active state. The use of EAN as a renaturation additive is a novel approach to enhancing protein recovery. While one might suspect that this polar medium would be unsuitable for protein studies, our work shows that the unique physical characteristics of EAN make it a useful renaturation additive for protein studies. We have recently reported that EAN is also a suitable additive for protein crystallization (Garlitz et al., 1999). We are currently examining the use of EAN and other alkyl ammonium salts as renaturation additives in refolding studies of other denatured proteins. These findings will be presented in a forthcoming paper.

Materials and methods

Materials

HEWL, crystallized, dialyzed, lyophilized and aseptically filled, and *Micrococcus lysodeikticus* cells were purchased from Sigma (St. Louis, Missouri). Glutathione (95%), oxidized glutatione (99%), dithiothreitol (99%), and cetyltrimethylammonium bromide (CTAB) were obtained from Aldrich (Milwaukee, Wisconsin).

Denaturation reduction of lysozyme

A solution of 25 mg/mL lysozyme in 6 M GdmHCl, 100 mM Tris sulfate, pH 8.5, and 30 mM DTT was prepared by the addition of 6 μL of 83.5 mg/mL HEWL stock solution to 14 μL of 8.7 M GdmHCl, 143 mM Tris sulfate, and 43 mM DTT solution.

Denaturation in neat EAN was carried out by adding 6 μL of HEWL stock solution to 14 μL of 8 M urea and 43 mM DTT dissolved in EAN. After vortexing, the solution was stored overnight at room temperature.

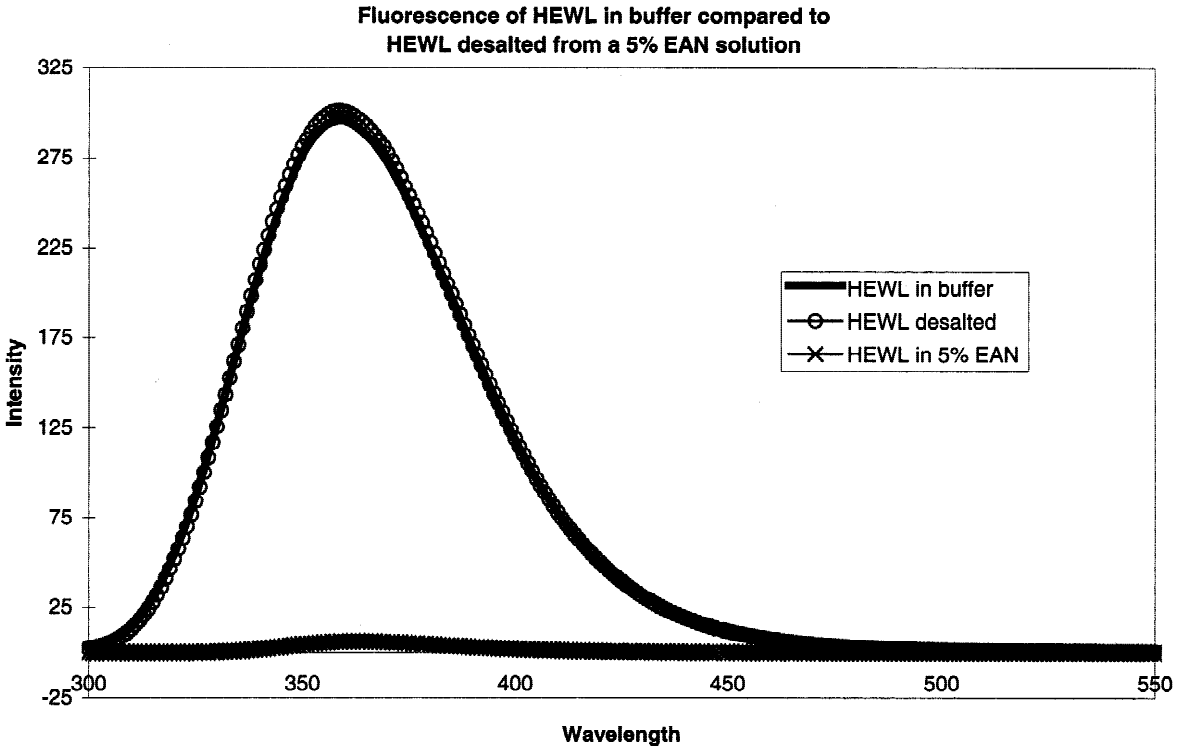


Fig. 2. Fluorescence spectra of HEWL in buffer (---), before desalting (×), and after desalting from 5% EAN (○).

Renaturation oxidation of lysozyme

The 25 mg/mL solution of denatured-reduced lysozyme solution was diluted with 143 mM Tris sulfate buffer (pH 8.5) containing 4 mM GSH and 4 mM GSSG with varying concentration of salt to give a final lysozyme concentration of 1.6 mg/mL. Solutions were stored at room temperature for 24 h before being assayed for enzymatic activity.

Assay of enzymatic activity

The assay for enzymatic activity was adopted from a standard assay from published procedures (Jolles, 1962). A stock solution of 0.3 mg/mL *M. lysodeikticus* cell suspension was prepared in 0.1 M phosphate buffer, pH 6.24. To a 3 mL cuvette, 2.99 mL of suspension was added, followed by 13 μL of renatured-oxidized lysozyme solution. The cuvette was inverted once and then placed in a UV-Vis spectrophotometer. The decrease in the light scattering intensity of the solution was then measured by following the decrease in apparent absorbance of the solution at 450 nm.

Differential scanning colorimetry experiments

Protein and reference solutions were degassed for 15 min before data acquisition. HEWL (0.069 mM) and the reference solution each ~1.5 mL in volume were loaded into their respective cells in the MicroCal Differential Scanning Calorimeter. An external pressure of 30 psi was applied with nitrogen gas to both sample and reference cells. The sample was scanned relative to the reference solution over a temperature range of 15–100 °C at a rate of 90 °C/h.

Heat capacity (ΔC_p) plots were baseline corrected according to standard techniques (Haynie & Freire, 1994; Duguid et al., 1996). The calorimetric enthalpy (ΔH_{cal}) changes were obtained as areas under plots versus temperature of ΔC_p and $\Delta C_p/T$, respectively.

For experiments involving refolding after thermal denaturation, solutions were slowly cooled to room temperature and then re-equilibrated for another hour. After equilibration, another scan of the same solution was performed and data were collected. The data were fit as described above, and percent refolding was obtained from comparison of enthalpies of the initial peak and refolding peak.

Desalting of HEWL solution

A 5 mL solution of 0.32 mM HEWL (Sigma, Lot 16H6830) in a 50% EAN solution was placed in two Centriprep centrifugal concentrators, with a 3,000 molecular weight cutoff, and centrifuged for 4 h. The solutions were washed six times with 0.1 M phosphate buffer, pH 6.24. After washing, the remaining solution was diluted to 5 mL and examined using UV-Vis and fluorescence spectroscopies. The UV-Vis spectrum was monitored at 280 nm for evidence of denaturation (Raman et al., 1996). Changes in the tryptophan fluorescence spectra of HEWL were monitored at 360 nm.

Supplementary material in the Electronic Appendix

Differential scanning calorimetry experiments

Protein and reference solutions were degassed for 15 min before data acquisition. HEWL (0.069 mM) and the reference solution, each ~1.5 mL in volume, were loaded into their respective cells in the MicroCal Differential Scanning Calorimeter. The sample was scanned relative to the reference solution over a temperature range of 15–100 °C at a rate of 90 °C/h. The raw data were baseline corrected according to standard techniques (Haynie & Freire, 1994; Duguid et al., 1996). A stacked plot of increasing percentages of EAN in HEWL solutions is represented in Figure S1.

Repeated thermal denaturation of HEWL in a 5% EAN solution is shown in Figure S2. After the initial scan, the solution was slowly cooled to 5 °C and equilibrated. The heating scan was then repeated three times, and the data were collected. The data were then interpreted using standard techniques.

Desalting of HEWL solution

The presence of EAN in HEWL solutions was determined using UV-Vis spectroscopy. The analysis was performed in a 3 mL quartz cuvette using a Hewlett-Packard A3460 UV-Vis spectrometer. The UV-Vis spectra of the HEWL solution before (Fig. S3A) and after (Fig. S3C) desalting are shown in Figure S3. These results were compared to the HEWL spectrum in buffer (Fig. S3B).

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