DIFFERENCE BETWEEN GUANIDINIUM CHLORIDE AND UREA AS DENATURANTS OF GLOBULAR PROTEINS: THE POSSIBILITY OF APPLICATION TO IMPROVED REFOLDING PROCESSES

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The difference in the effect of guanidinium chloride (GdnHCl) and urea on the unfolding of hen egg-white lysozyme was evaluated mostly by means of circular dichroism(CD) measurements at pH 6.2 and at room temperature. The CD spectrum of lysozyme in 6M GdnHCl revealed the unfolded state, but that in 8M urea was almost identical with that of the native lysozyme. The effectiveness of urea as a denaturant corresponding to GdnHCl was attained by increasing the ionic strength of the solution by introducing lithium chloride(LiCl). This suggests that GdnHCl affects hydrophobic and ionic interactions simultaneously, while urea affects almost solely the hydrophobic interaction. Knowledge that the bifunctional ability of GdnHCl can be divided into two independent reagent abilities will provide a new tool to assist with correct refolding of unfolded proteins.

KEYWORDS unfolding; refolding; guanidinium chloride; urea; hen egg-white lysozyme

One of the unanswered questions in biochemistry is how a polypeptide chain folds into its biologically active three-dimensional structure *in vivo*. Recently, with the advent of recombinant DNA technology, it has become possible to obtain large amounts of the desired proteins; however, they are often produced as inactive aggregates. Therefore, establishing a refolding procedure for these inactive aggregates *in vitro* is an urgent problem. As it is necessary to dissolve the aggregates, GdnHCl and urea have been widely used to obtain unfolded random coil states in the proteins. However, we lack convincing knowledge about the difference between these two important denaturants.

In the present paper, we disclose that the effect of 6M GdnHCl on the unfolding of hen egg-white lysozyme was almost equivalent to that of 6M urea plus 6M LiCl, which is a new combination of denaturants. If four interactions or bondings can be defined for stabilizing the three-dimensional structure of proteins, i.e., hydrophobic interactions, ionic interactions, hydrogen bonding and disulfide bonding, it appears that GdnHCl affects hydrophobic and ionic interactions simultaneously, while urea and LiCl separately affect hydrophobic and ionic interactions, respectively. Thus, the bifunctional ability of GdnHCl was divided into two independent reagent abilities, which corresponded to two interactions stabilizing the three-dimensional structure of proteins. This knowledge is expected to serve very usefully as a new tool to achieve correct refolding of globular proteins dissolved as random coils.

MATERIALS AND METHODS

Hen egg-white lysozyme recrystallized three times was purchased from Sigma. Lysozyme concentrations were determined spectrophotometrically by using the extinction coefficient($A^{1\%}_{280nm}$=26.3).[1] Protein solutions containing varied amounts of GdnHCl or urea were prepared with 50mM phosphate buffer at pH 6.2. Because of the low solubility of LiCl in the phosphate buffer solution, the solution containing LiCl was prepared as an aqueous solution whose pH was adjusted to 6.2 by use of a dilute HCl solution. The solution containing GdnHCl at A molar concentration or urea at B molar and LiCl at C molar concentrations, etc., is expressed hereafter as A M GdnHCl or B M urea plus C M LiCl, etc., respectively. The extent of unfolding was measured by CD as well as by an enzymatic activity which was determined by methods using the *Micrococcus lysodeikticus* cell wall.[2] The CD measurements were carried out by use of a Jasco J-600 spectropolarimeter furnished with a 1mm quartz cell, at room temperature. Lysozyme concentrations on CD measurements were approximately 25-30 μM. Protein solutions containing certain denaturants were incubated at room temperature for 1h before CD measurements. The CD spectra were expressed as the mean residue molar ellipticity (θ).

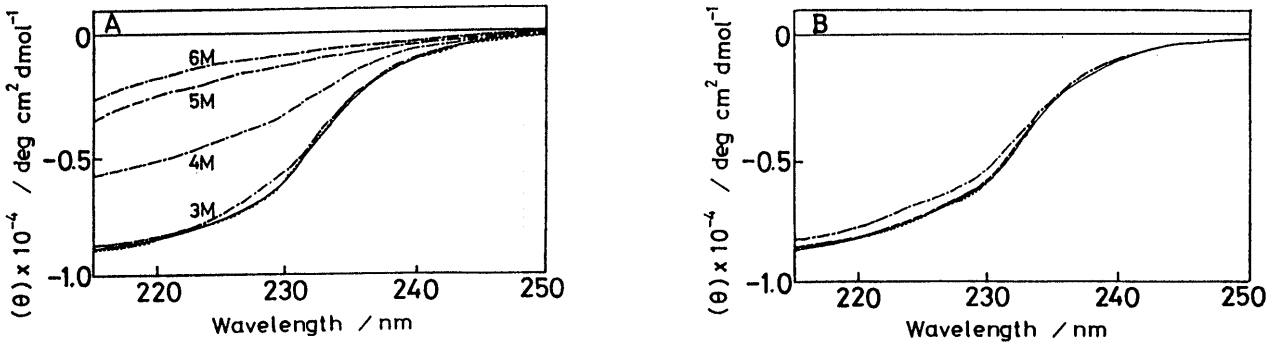

Fig.1. CD Spectra of Lysozyme Solutions Measured at Various GdnHCl or Urea Concentrations

Lysozyme concentration: approx. 30μM. All measurements were made in 50mM phosphate buffer solutions at pH 6.2 and at room temperature.
A. ——— : Native lysozyme, ····· : Refolded lysozyme (GdnHCl 6M→0.3M), —·— : in GdnHCl at indicated concentrations.
B. ——— : Native lysozyme, ····· : in 8M urea, – – – : in 9M urea, — — — : in 10 M urea.

RESULTS AND DISCUSSION

The conformational changes of lysozyme were measured in the presence of various concentrations of GdnHCl or urea on the CD spectra. Fig.1A implies that lysozyme unfolds completely in 5M-6M GdnHCl and that refolding of lysozyme is attained by a 20-fold dilution of GdnHCl. On the contrary, as shown in Fig.1B, the CD spectrum of lysozyme in 8M urea appears to be identical with that of the native lysozyme. No significant change could be seen even in 10M urea. Almost the same CD spectra were obtained after an overnight incubation of the same solutions. The lysozyme becomes unfolded in 8M urea at a low pH range,[3),4)] but this is not the case at a neutral pH range. Results depicted in Fig.1 demonstrate that the effectiveness of GdnHCl and that of urea for the unfolding of lysozyme are quite different.

Examination of the enzymatic activity was performed for the same samples. When the lysozyme incubated in 8M urea for 1h or even for 1 day was diluted about 30-fold by introducing it into the substrate solution for an assay, the enzymatic activity was measured as almost 100 % of the native lysozyme. On the other hand, when the same examination was done by use of lysozyme incubated in 6M GdnHCl, the enzymatic activity was scarcely detected.

Fig.2A shows the CD spectra of lysozyme in B M urea plus C M LiCl. The CD spectrum of lysozyme in 6M LiCl is almost identical with that of the native lysozyme. Similar results were reported in the litera-

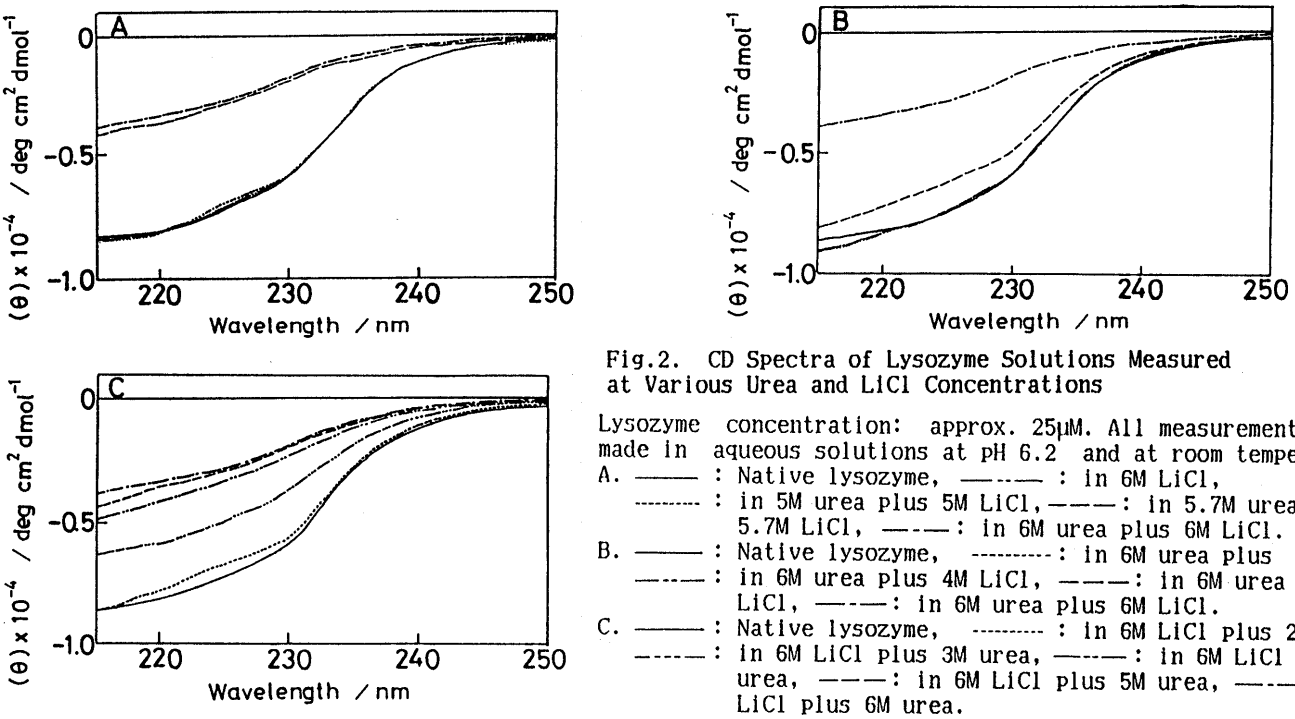

Fig.2. CD Spectra of Lysozyme Solutions Measured at Various Urea and LiCl Concentrations

Lysozyme concentration: approx. 25μM. All measurements were made in aqueous solutions at pH 6.2 and at room temperature.
A. ——— : Native lysozyme, — — — : in 6M LiCl, ····· : in 5M urea plus 5M LiCl, – – – : in 5.7M urea plus 5.7M LiCl, — — — : in 6M urea plus 6M LiCl.
B. ——— : Native lysozyme, ····· : in 6M urea plus 3M LiCl, — — — : in 6M urea plus 4M LiCl, – – – : in 6M urea plus 5M LiCl, — — — : in 6M urea plus 6M LiCl.
C. ——— : Native lysozyme, ····· : in 6M LiCl plus 2M urea, — — — : in 6M LiCl plus 3M urea, – – – : in 6M LiCl plus 4M urea, — — — : in 6M LiCl plus 5M urea, — — — : in 6M LiCl plus 6M urea.

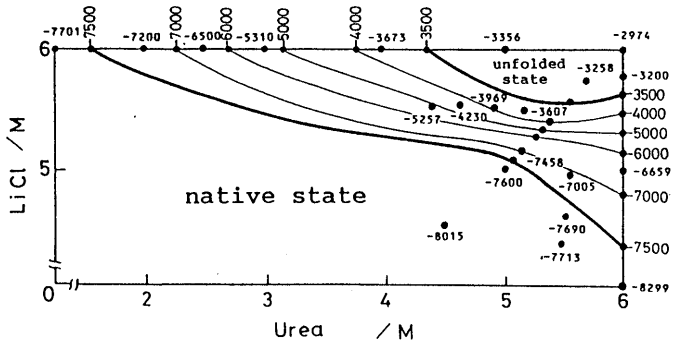

Fig.3.
Contour Lines of Molar Ellipticity of Lysozyme Measured at 222 nm in Various [Urea] Plus [LiCl] Solutions

Bold lines show borders of native or unfolded state to transient states. Other lines were drawn at every 1000 deg cm^2 $dmol^{-1}$.

ture.[5] In contrast, the CD spectrum of lysozyme in 6M urea plus 6M LiCl shifted to the unfolded state and quantitatively corresponded to that in 5M GdnHCl. This means that the addition of LiCl to urea brings about the capability of the unfolding of lysozyme similar to that of GdnHCl, clearly due to the increase in the ionic strength. Figs.2B and 2C show the CD spectra of lysozyme in 6M urea plus C M LiCl or in 6M LiCl plus B M urea. We also conducted refolding experiments in three different ways, initiated by the 10-fold dilution of the lysozyme unfolded in 6M urea plus 6M LiCl by introducing it to aqueous solution, 6M LiCl solution or 6M urea solution. All CD spectra of the lysozyme solutions diluted as mentioned above were similar to those of the native lysozyme. Refolding proceeded almost completely on dilution of these denaturants.

Fig.3 represents tentative contour lines of the ellipticity measured at 222 nm, which vary with concentrations of urea and LiCl. From such contour lines, we can see the conformational changes of lysozyme under various concentrations of the specific two denaturants. In other words, this will imply a specific curve indicating the stability of lysozyme against the specific denaturants. The result shown in Fig.3 reflects the fact that the conformation of lysozyme is stabilized by ionic interactions rather than by hydrophobic interactions, although sufficient data have not yet been obtained at present. This type of figure will be helpful in searching for better conditions for refolding of a specific protein in the future.

It must be noted that, when the lysozyme incubated in 6M urea plus 6M LiCl either for 1h or for 1 day is diluted about 30-fold by introducing it into the substrate solution for an assay, very little enzymatic activity will be detected, as in the case of lysozyme in 6M GdnHCl. The cause for these inactivities originates merely from the increase in ionic strength in the assay solution, as reported by Davies et al.[6] A similar conclusion was confirmed by ourselves, and we made a detailed presentation orally.[7] Ionic strength in the assay solution should be lower than 0.02M to obtain authentic lysozyme activity value.

In the present study, we demonstrated that the protein unfolding ability of GdnHCl could be replaced by that of urea plus LiCl. Quantitative knowledge about the possibility of replacing GdnHCl by urea plus LiCl will serve as a new tool for exploration of a suitable refolding pathway by varying the concentrations of urea or LiCl independently. We regret that it is very difficult now to discuss the different effects of the two denaturants on the hydrogen bonding of the protein structure. At present, we know that polyols (glycerine, sucrose, trehalose. etc.) and urea influence hydrogen bonding in different ways: the former stabilize the three-dimensional structure of proteins, while the latter destroy the structure. How these denaturating reagents affect the hydrogen bonding of the protein structure is one of the remaining problems for researchers in this field.

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(Received November 22, 1991)