PREPARATION OF CHARACTERISTIC DIAGRAM FOR REFOLDING OF LYSOZYME

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The characteristic diagram for refolding of denatured reduced lysozyme was prepared in terms of recovered activity by employing urea and LiCl concentrations as two axes of rectangular coordinates. The diagram obtained will serve as a new tool not only for the optimum design of refolding media but also for the study of the refolding mechanism.

KEY WORDS protein refolding; lysozyme; urea; lithium chloride; refolding diagram

The three-dimensional structure of a protein is formed by various interactions such as hydrogen bond, ionic interaction, hydrophobic interaction, and disulfide linkage, excluding the covalent bond in the primary sequence of amino acids. A quantitative understanding of the effect exerted by each interaction may be indispensable for mechanistic study of protein unfolding/refolding. For evaluation of the effect, the idealized reagent that influences only a specific interaction-- i.e., the "monofunctional" reagent-- would be desired. However, in practice, it is convenient necessary to choose a real reagent as close to the monofunctional one as possible.

The authors reported previously[1] that the multiplex effect of guanidinium chloride(GdmCl) on protein unfolding can tentatively be separated into two effects that urea and LiCl exert individually mainly on hydrogen bonding or hydrophobic interaction and on ionic interaction, respectively, as shown in Fig. 1. On the basis of this concept, they proposed the characteristic unfolding diagram of lysozyme for semi-quantitative understanding of each interaction. It was also reported[2] that in the renaturation of fully reduced lysozyme, much higher recovered activity could be achieved in the presence of urea at 2 to 3 M than in the absence of urea.

In the present paper, we depict the characteristic refolding diagram of lysozyme in terms of the recovered activity by a method similar to that applied to preparation of the unfolding diagram.[1]

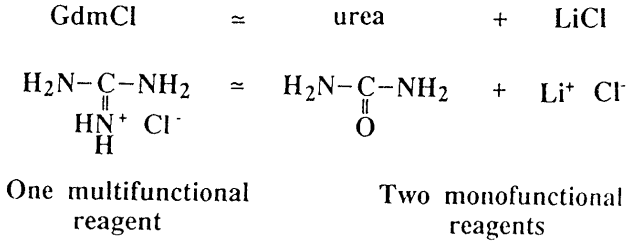

Fig. 1. Displacement of One Multifunctional Reagent with Two Monofunctional Reagents

MATERIALS AND METHODS

Hen egg-white lysozyme recrystallized three times, and *Micrococcus lysodeikticus* dried cell walls as substrate,[3] were purchased from Sigma. Urea, dithiothreitol, reduced glutathione(GSH), and oxidized glutathione(GSSG) were obtained from Nacalai

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Tesque. Protein concentrations were determined photometrically using the extinction coefficients, 37,600 and 33,890 cm^{-1} M^{-1} at 280 nm, for native lysozyme and fully reduced lysozyme, respectively.[4] The buffer used was 0.1 M TrisHCl buffer at pH 8.0.

Fully reduced lysozyme was prepared by the method described previously.[2] The reduced lysozyme was dissolved in 6 M GdmCl solution to a concentration of 1 mM. Such reduced and denatured lysozyme was diluted 99-fold with the solution prepared at various sets of concentrations of urea and LiCl. At 1 min after the dilution, reoxidation was initiated at room temperature by the addition of a mixed solution of GSH and GSSG prepared separately. The final concentrations of lysozyme, GSH, GSSG, and EDTA in the renaturation solution were 0.01, 3, 0.3, and 1 mM, respectively. The recovered activity was assayed by use of the substrate photometrically at 450 nm.

RESULTS AND DISCUSSION

Figure 2 shows the effects of urea and LiCl concentrations on refolding of the reduced lysozyme. The recovered activity represents the percentage of the activity exhibited by the native lysozyme solution at the corresponding concentration. The solution containing urea at A molar and LiCl at B molar concentration is expressed here as A M urea plus B M LiCl. The renaturation was evaluated in this paper by the yield or the recovered activity measured after 48 h. The renaturation of lysozyme in 3 M urea solution containing various amounts of LiCl is exemplified in Fig. 2(a). In the solution containing neither urea nor LiCl, the yield never exceeded 20%, and visible aggregation was observed. In the cases of 1 and 3 M LiCl, the initial rates of renaturation were the largest, and the

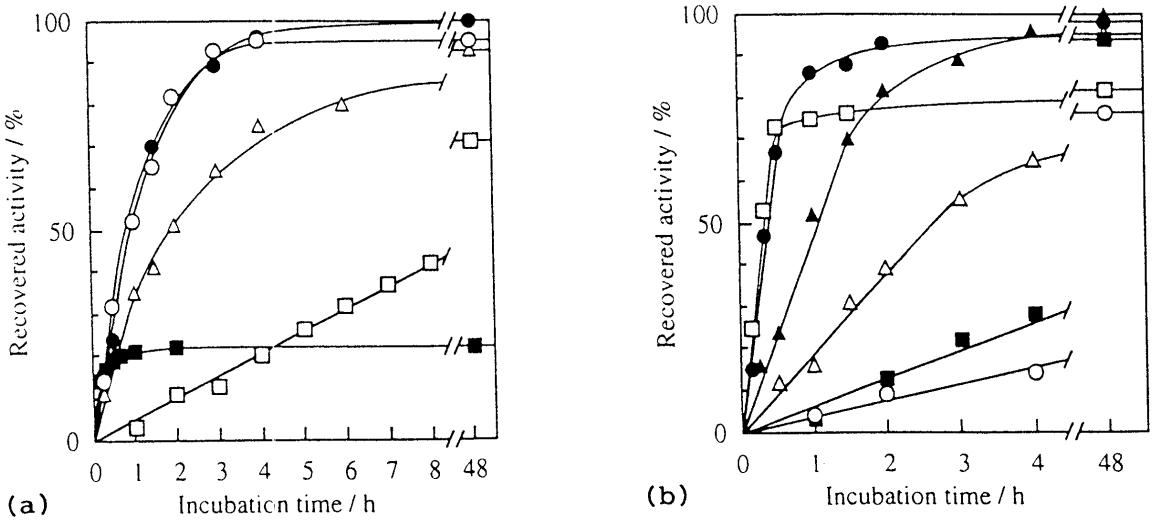

Fig. 2. Effects of Urea and LiCl Concentrations on Refolding of the Reduced Lysozyme

(a) ■ : 0M urea + 0M LiCl
△ : 3M urea + 0M LiCl
● : 3M urea + 1M LiCl
○ : 3M urea + 3M LiCl
□ : 3M urea + 5M LiCl

(b) □ : 1M urea + 1M LiCl
● : 2M urea + 1M LiCl
▲ : 3M urea + 1M LiCl
△ : 4M urea + 1M LiCl
■ : 5M urea + 1M LiCl
○ : 6M urea + 1M LiCl

Lysozyme conc.: 0.01 mM; Each renaturation buffer contains 3 mM GSH and 0.3 mM GSSG (0.1 M TrisHCl buffer at pH 8.0).

yields attained ca. 100%, whereas both the rate and the yield decreased considerably with 5 M LiCl.

Next, Fig. 2(b) shows the renaturation in 1 M LiCl solution containing various amounts of urea. Although the rate decreased with increase in the urea concentration, the yield attained ca. 100% after 48 h at the 2 to 5 M urea concentrations. In the case of 1 M urea, the yield reached a plateau in the short time of 30 min, probably due to aggregation competing with correct pairing of disulfide linkages. On the other hand, at higher urea concentrations such as 5 and 6M, the correct pairing would be delayed though the aggregation could be suppressed. This is probably because the access of sulfhydryl groups(-SH) in close vicinity for reoxidation is obstructed by the coexistence of urea at such high concentrations.

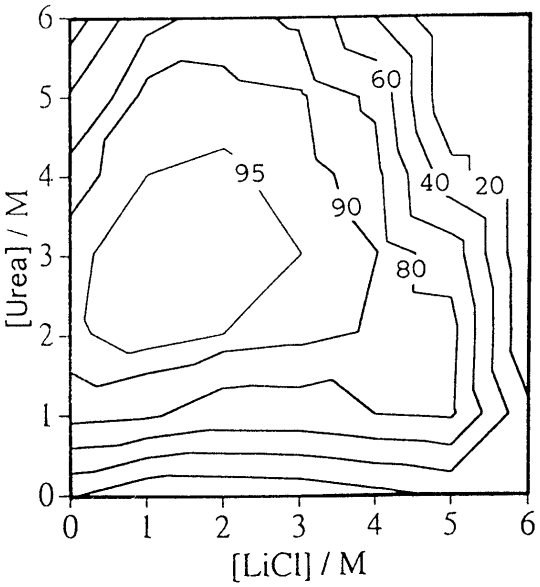

Fig. 3. Recovered Activity of Lysozyme Obtained at Various Sets of Concentrations of LiCl and Urea (Refolding Diagram of Lysozyme with LiCl and Urea)

The value illustrated on each contour line represents the recovered activity (%) after 48 h.

The contour lines are depicted in Fig. 3 based on 49 points of the recovered activity after 48 h obtained in the solutions prepared by various combinations of urea and LiCl concentrations. It can be seen that almost complete renaturation could be achieved in the relatively wide area enclosed by 2 to 4 M urea and 0.5 to 3 M LiCl. It is noted that the present refolding diagram is prepared for a protein concentration of 0.01 mM during refolding. The yield changed drastically depending on the protein concentration.[2] The higher concentration will cause reduction of the area where almost complete renaturation can be attained.

Displacement of a multifunctional reagent, GdmCl, with two monofunctional reagents such as urea and LiCl will serve for the quantitative discussion on the individual interaction described above. The preparation of such a refolding diagram will contribute as a new tool for any protein, not only to the optimum design of refolding media but also to the insight into the refolding pathway and mechanism. At the present stage of the research, it is still difficult to differentiate the effect on the hydrogen bond from that on the hydrophobic interaction. For further detailed discussion, it is still necessary to identify another adequate candidate for the specific monofunctional reagent.

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