Application of Combined Reagent Solution to the Oxidative Refolding of Recombinant Human Interleukin 6

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Human interleukin 6 (hIL-6), which is a cytokine involved in diverse biological activities, consists of a four-helix bundle with two disulfide bonds. For the clinical use of hIL-6 in cancer therapy, designing of commercial-scale production systems of recombinant hIL-6 (rhIL-6) expressed by *E. coli* has been attempted. Since rhIL-6 has been produced as inclusion bodies in the expression systems reported to date, establishment of a strategy to achieve a high yield of refolding of this recombinant protein is quite desirable. It has been reported that oxidation of rhIL-6 under a completely denaturing condition suppresses aggregation during the refolding process [Ejima *et al.*, *Biotechnol. Bioeng.*, 62, 301—310 (1999)]. In this protocol, however, small but significant amounts of unidentified by-products unavoidably arose, which might be problematic in the therapeutic use of rhIL-6. In the present study, detailed characterization of the individual by-products has been performed on inspection of peptide maps, and the by-products found to originate from improperly formed disulfide bonds, most of which are disulfide-linked dimers. In order to minimize these by-products, combined solutions of urea and LiCl were used for oxidative refolding of rhIL-6. It was demonstrated that combined use of 1—2 M urea and 1—3 M LiCl effectively suppresses the formation of the by-products as well as aggregates. We propose that the use of the combined reagents can be an alternative method for refolding of rhIL-6 for clinical purposes.

Key words protein refolding; interleukin 6; combined refolding solution; disulfide bond

Human interleukin-6 (hIL-6), which is a cytokine involved in diverse biological activities,[1,2] consists of a four-helix bundle with two disulfide bonds. Since its clinical use has been of great interest, designing of commercial-scale production systems of recombinant hIL-6 (rhIL-6) expressed by *Escherichia coli* has been attempted.[3—7] However, large-scale production of rhIL-6 has provided inactive aggregates called inclusion bodies.[3—7] In order to restore its bioactivity, it is necessary to dissolve aggregated protein by denaturant and to refold it into an intrinsic three-dimensional structure. It has been reported that 1) almost all of the Cys residues in rhIL-6 extracted from inclusion bodies exist in the form of free thiol,[3] and 2) the propensity of reduced rhIL-6 to aggregate lowered the refolding yield.[2,8] Therefore, the Cys residues in rhIL-6 must be disulfide-bonded correctly during the refolding procedure.

We earlier reported that two disulfide bonds of rhIL-6 were almost correctly oxidized with high yield even in 6 M guanidinium chloride (GdmCl) solution.[3] The success of such an exceptional method in which oxidation is carried out at the denaturing concentration of GdmCl is probably due to the close proximity in the amino acid sequence of the cysteine residues to be paired: Cys 44–Cys 50 and Cys 73–Cys 83. This protocol has a great advantage, especially in refolding at high protein concentrations, owing to minimization of aggregation caused by intermolecular interaction during the oxidation process. In this protocol, however, there were small but significant amounts of unavoidable unidentified by-products, which might be problematic in the therapeutic use of rhIL-6. Therefore, it is of vital importance to seek an improved refolding solution that can increase the selectivity as well as the yield.

There are many reagents that would influence various intra- and inter-molecular interactions. Using adequate reagents, we could modulate conformational change, folding, and aggregation of protein. To carefully control these processes, it is desirable to use a reagent whose effect on various interactions is as specific as possible. GdmCl employed previously is thought to possess a multiple effect on protein conformation, that is, it affects hydrogen bonds, hydrophobic interactions and ionic interactions as well. It has been proposed that the effect of GdmCl can be separated into the effects of urea and LiCl, which act mainly on hydrophobic interactions and hydrogen bonds and on ionic interactions, respectively.[9,10] Although the quantitative integrity of this idea is not yet confirmed, it is likely that the effects of urea and LiCl are more specific than that of GdmCl. Thus, the combined use of these reagents instead of GdmCl will provide us with more options in designing the refolding solution by optimizing the concentration of each reagent independently. Furthermore, in a large-scale production, it would be increasingly valuable that the disadvantages of GdmCl of high expense and corrosion be avoided by application of this combination system.

In the present study, we clarified that the refolding by-products of rhIL-6 are formed mainly by intermolecular disulfide bonding, and explored an appropriate refolding solution that can increase the selectivity and yield under suppression of the by-product formation as well as aggregation.

Experimental

Materials According to the protocols by Ejima *et al.*,[3] rhIL-6 was expressed in *E. coli* strain HB101 and purified. Lysylendopeptidase was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of reagent grade.

Preparation of Reduced rhIL-6 Purified rhIL-6 was unfolded and reduced in the presence of 5.5 M GdmCl and 4 mM dithiothreitol (DTT) (pH

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8.0) at 25 °C for 90 min. Reduced rhIL-6 was desalted by application to a gel filtration column (Sephadex G-25, Amersham Pharmacia Biotech, Tokyo, Japan) equilibrated with 0.1 M acetic acid and subsequently lyophilized.

Circular Dichroism Measurement Circular dichroism (CD) measurements were performed in a 0.1 cm quartz cuvette at room temperature using a spectropolarimeter (J-720, JASCO, Tokyo). After subtraction of a solvent spectrum, data were represented as mean residue ellipticities.

Re-oxidation of Fully Reduced Denatured rhIL-6 in Various Refolding Solutions Lyophilized powder of reduced rhIL-6 was dissolved in degassed 8 M GdmCl (pH 3.0) or 9 M urea (pH 3.0). The protein concentration was adjusted to 5 mg/ml. Air oxidation of reduced rhIL-6 was carried out at 25 °C by shaking (75 rpm) in a thermoregulated bath for 24 h following 10-fold dilution of the dissolved sample into oxidative refolding solutions containing a given set of concentrations of urea and LiCl.

Analytical Reverse-Phase HPLC and Peptide Mapping by Lysylendopeptidase Analytical reverse-phase (RP) HPLC was carried out using a Vydac C4 column (214TP54, 4.6 mm internal diameter×250 mm, The Separations Group, Hesperia, CA, U.S.A.). Protein was eluted at a flow rate of 1 ml/min with a linear gradient of 32—60% acetonitrile in 0.1% trifluoroacetic acid (TFA) for 25 min. The elution was monitored by UV absorbance at 280 nm.

Peptide mapping of lysylendopeptidase digestion products was performed by analytical RP-HPLC. Protein samples were dissolved at a concentration of 0.2 mg/ml in 50 mM Tris–HCl buffer (pH 8.0) containing 4 M urea. For digestion, 90 μl of lysylendopeptidase solution (0.1 AU/ml) was added to 1 ml of the sample solution. The mixture was kept at 25 °C for 24 h and subsequently injected directly into a Vydac C4 column equilibrated with an aqueous solution containing 0.1% TFA. The peptides were eluted at a flow rate of 1 ml/min with a linear gradient of 0—52% acetonitrile in 0.1% TFA for 65 min. UV absorbance was monitored at 215 nm. Each peak arising from the digestion products of native rhIL-6 was assigned by matrix assisted laser desorption ionization-time of flight mass spectrometry with a Voyager Elite spectrometer (Perseptive Biosystems, Inc., Framingham, MA, U.S.A.) and by amino acid sequence analysis carried out on a protein sequencer (PE Applied Biosystems model 491).

SDS-PAGE SDS-PAGE was performed in 12.5% acrylamide gel on slabs as described by Laemmli.[11] The electrophoretogram was stained with Coomassie brilliant blue.

Results and Discussion

Oxidative Refolding of rhIL-6 in Various Concentrations of GdmCl and Urea Lyophilized reduced rhIL-6 was firstly dissolved in 8 M GdmCl, and then diluted 10-fold with various concentrations of GdmCl. The samples were incubated for air oxidation at 25 °C for 24 h. After removal of precipitates by centrifugation, soluble fractions of the samples were analyzed by RP-HPLC, which gave results corroborating these in the previous report.[3] Namely, oxidation at low concentrations (<3 M) of GdmCl resulted in low yields because an insoluble fraction arose. Aggregation did not take place at GdmCl concentrations above 4 M, where the yields reached to 87%. When using urea instead of GdmCl as a denaturant, similar tendencies were observed (data not shown). Thus, although oxidation at high concentrations of denaturants can adequately suppress aggregation, another device is needed to prevent the formation of complex by-products as described below.

Figure 1 exemplifies RP-HPLC elution profiles of rhIL-6 reoxidized at 0.6 M and 6 M GdmCl, where rhIL-6 is in its native state and a fully denatured state, respectively.[12] Oxidation under non-denaturing condition gave rise to one main peak (M_1) and one sub peak (D_1, Fig. 1(b)), whereas additional sub-peaks (S, D_2 and D_3) emerged around a main peak (M_2) when oxidation was carried out under denaturing condition (Fig. 1(c)). The peak fractions indicated by arrows were collected and analyzed by SDS-PAGE and lysylendopeptidase peptide mapping, which are shown in Fig. 2 and Fig. 3,

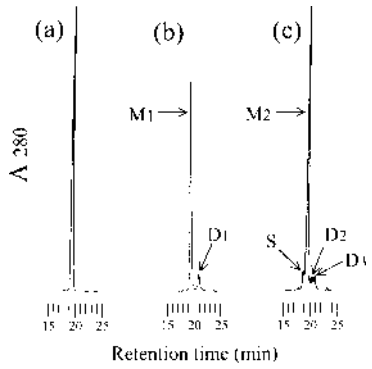

Fig. 1. RP-HPLC Profiles of rhIL-6 Reoxidized in the Presence of Non-denaturing or Denaturing Concentration of GdmCl

Denatured reduced rhIL-6 dissolved in 8 M GdmCl was diluted in a given concentration of GdmCl and subsequently oxidized by air at 25 °C for 24 h. The protein concentration was adjusted to 0.5 mg/ml. After removal of precipitate by centrifugation, soluble fractions were subjected to RP-HPLC. Samples were eluted by acetonitrile gradients at a flow rate of 1 ml/min. Elution was monitored by UV at 280 nm. (a), native rhIL-6; (b), oxidized in 0.6 M GdmCl; (c), oxidized in 6 M GdmCl.

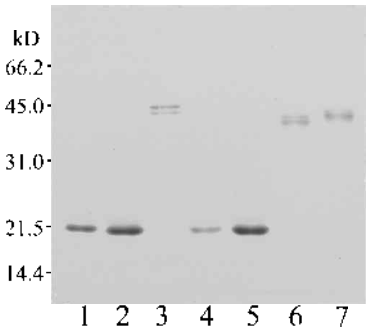

Fig. 2. SDS-PAGE of the Reoxidation Products of rhIL-6

Desalted samples reoxidized by air at 25 °C for 24 h at 0.6 M or 6 M GdmCl were injected into RP-HPLC. The peak fractions indicated by arrows in Fig. 1 were collected and lyophilized and subsequently subjected to non-reduced SDS-PAGE using 12.5% gel. The electrophoretogram was stained with Coomassie brilliant blue. Lane 1, native rhIL-6; lane 2, peak M_1; lane 3, peak D_1; lane 4, peak S; lane 5, peak M_2; lane 6, peak D_2; lane 7, peak D_3. Positions of molecular weight markers are indicated at the left periphery.

respectively. Both main peaks (M_1 and M_2) were comprised of correctly disulfide-bonded rhIL-6, which was confirmed by comparing their electrophoretic mobility (Fig. 2, lanes 2 and 5) and the peptide map with those of native rhIL-6 (data not shown). Samples corresponding to sub peaks D_1, D_2 and D_3 were electrophoresed at the twofold molecular weight of native rhIL-6 (Fig. 2, lanes 3, 6 and 7) and became monomeric with the addition of 10 mM DTT. On the basis of these data, D_1, D_2 and D_3 were identified as rhIL-6 dimers formed by intermolecular disulfide bonding. Next, we made peptide maps of these dimers in order to identify their intermolecular disulfide bonding sites. For D_1, only the peak K4+K5, which corresponds to the peptide including Cys 44 and Cys 50, disappeared while the rest of the peptide map was identical with that of native rhIL-6 (Fig. 3(b)). This indicates that it was dimerized *via* disulfide bonds involving Cys 44 and Cys 50, each existing in a different molecule. On the other hand, in the peptide maps of D_2 and D_3, the area of peak K8, which corresponds to the peptide including Cys 73 and Cys 83, was significantly reduced, suggesting that Cys 73 and/or Cys 83, in addition to Cys 44 and Cys 50, were involved in the dimerization of these by-products (Fig. 3(c) and

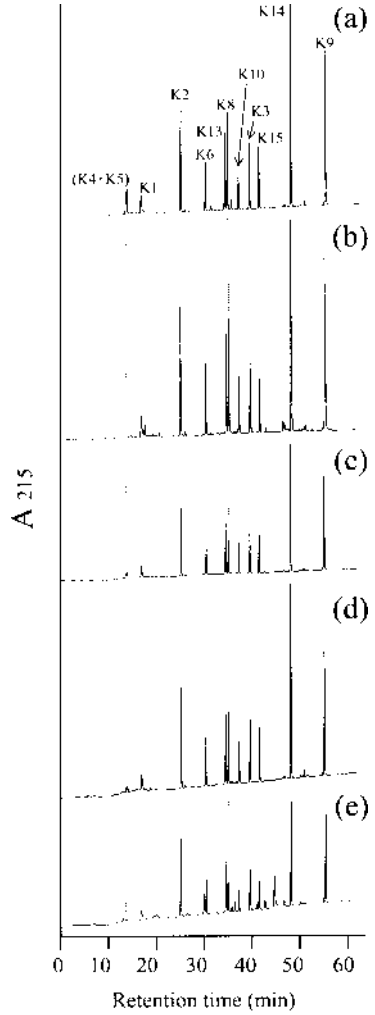

Fig. 3. Lysylendopeptidase Mapping of Reoxidized Products of rhIL-6

Peak fractions D_1—D_3 and S indicated by arrows in Fig. 1 were collected and concentrated by a centrifugal evaporative condenser. The samples were dissolved at a concentration of 0.2 mg/ml in 50 mM Tris–HCl buffer, pH 8.0, containing 4 M urea followed by addition of 90 μl of lysylendopeptidase solution (0.1 AU/ml) to 1 ml of the sample solution. The mixture was kept at 25 °C for 24 h and subsequently injected directly into RP-HPLC using a Vydac C4 column. The peptides were separated by acetonitrile gradient from 0% to 52% over 65 min. Flow rate was 1 ml/min and UV absorbance at 215 nm was monitored. (a), native rhIL-6; (b), peak D_1; (c), peak D_2; (d), peak D_3; (e), peak S. Note that peaks in (a) were coded in order of the fragment in the amino acid sequence of rhIL-6.

(d)). Preferential formation of disulfide-bonds involving Cys 44 and Cys 50 under non-denaturing condition can be reasonably explained by the fact that these Cys residues are located near the surface of the rhIL-6 molecule.[13,14]

Oxidation under denaturing condition gave rise not only to various disulfide-bonded dimers but also a monomeric by-product corresponding to peak S (Fig. 2, lane 4). The peptide map of S is quite different from those of the native and other by-products, suggesting that this by-product results from extensive modification of rhIL-6.

Application of Combined Reagent Solutions to the Oxidative Refolding of rhIL-6 To minimize the by-product formation, construction of a native-like conformation prior to disulfide oxidation is prerequisite in refolding of rhIL-6. In order to achieve high yield of refolding by suppressing aggregation, however, the addition of a denaturant at an appropriate concentration is needed. To satisfy both of these requirements, combined reagent solutions were applied to the

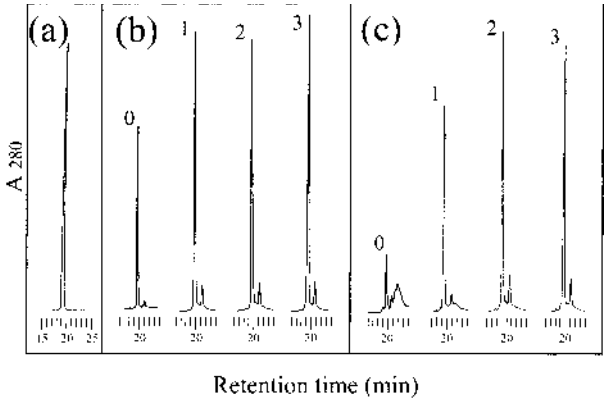

Fig. 4. Effect of Various Sets of Concentrations of Urea and LiCl on the Reoxidation of rhIL-6

Reduced rhIL-6 was firstly dissolved in 9 M urea followed by air oxidation in a given set of concentrations of urea and LiCl at 25 °C for 24 h. The protein concentration was adjusted to 0.5 mg/ml. Soluble fraction was subjected to RP-HPLC and analyzed as in Fig. 1. (a), native rhIL-6; (b), oxidized in 1 M urea plus 0—3 M LiCl; (c), oxidized in 3 M urea plus 0—3 M LiCl.

oxidative refolding of rhIL-6. In this study, we adopted a combination of urea and LiCl. It is thought that urea modulates mainly the hydrophobic interaction between refolding intermediates so they do not aggregate with each other, and that LiCl interferes with undesirable intra- and/or inter-molecular ionic interaction. Ahmad and Bigelow have reported that low concentrations of LiCl stabilize ribonuclease against denaturation by urea, even though the salt itself is a denaturant.[15] The urea–LiCl combination system has been shown to be very effective for the oxidative refolding of lysozyme.[10]

Reduced rhIL-6 was dissolved in degassed 9 M urea solution (pH 3.0) followed by 10-fold dilution into solutions containing various sets of concentrations of urea and LiCl. Samples oxidized by air at 25 °C for 24 h were analyzed by RP-HPLC after removal of precipitates. Figure 4 shows RP-HPLC profiles of reoxidized samples at 1 M or 3 M urea containing 0—3 M LiCl. When urea was used alone, the yields of oxidative refolding were not high because of precipitate formation. Addition of 1—3 M LiCl to the refolding solutions containing 1—3 M urea suppressed aggregation and remarkably improved the yield of oxidative refolding: Using a combination of 1 M urea with 1—3 M LiCl, the yields increased to about 90%. It should be noted that, by the combined use of urea and LiCl, by-product formation was effectively suppressed without additives such as redox reagents. For example, complex by-products that arose from oxidation in 3 M urea disappeared upon addition of LiCl (Fig. 4(c)). The residual by-product corresponding to peak D_1, which is the disulfide-bonded dimer involving Cys 44 and Cys 50, could be easily removed by a single step of conventional RP-HPLC. Figure 5 shows the far UV regions of the CD spectra of reduced rhIL-6 in solutions containing various combinations of concentrations of urea and LiCl. It was shown that the reduced form of rhIL-6 adopts a native-like secondary structure in the solutions containing 1—3 M urea and 1—2 M LiCl. It has been shown that thiols of Cys residues buried in the interior of protein molecules are not easily oxidized to disulfide.[16,17] The near UV region of the CD spectra of the reduced rhIL-6 in solution containing 1 M urea plus 1 M LiCl exhibited no negative peak at 280 nm, which is characteristic for the protein with native folding (data not shown). This

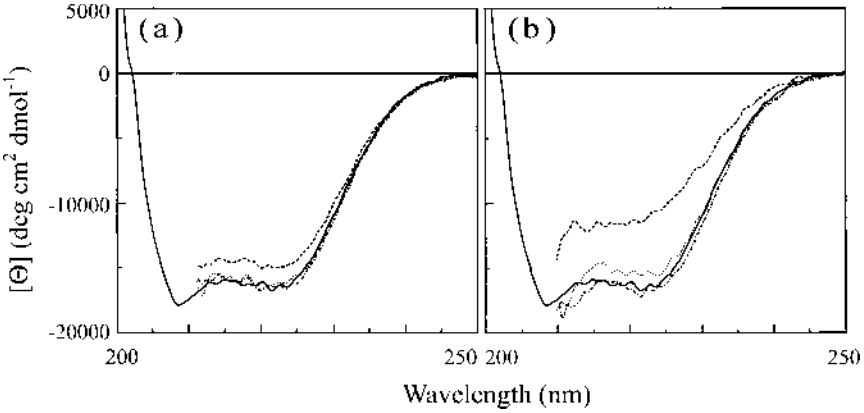

Fig. 5. CD Spectra of Reduced rhIL-6 in Solutions Containing Various Sets of Concentrations of Urea and LiCl

To preserve a reduced state, 1 mM DTT was added to the solutions. (a): 1 M urea (------), 1 M urea +1 M LiCl (·····), 1 M urea +2 M LiCl (–·–·–); (b): 3 M urea (– – –), 3 M urea +1 M LiCl (·····), 3 M urea +2 M LiCl (–·–·–). CD spectra of native rhIL-6 are plotted with a solid line in (a) and (b).

suggests that the side chains of rhIL-6 are not tightly packed under the above conditions, resulting in oxidation of Cys 73 and Cys 83, which are buried if the protein adopts the native conformation. On the basis of these observations, we suggest that the loose folding with the native-like secondary structure under the oxidation conditions leads to the correct disulfide formation, resulting in the high yield of rhIL-6.

Although the influence of each reagent on rhIL-6 conformation is not yet fully understood, acceleration or retardation of a particular process in the refolding, *i.e.*, hydrophobic core formation, formation of intramolecular ion bondings, *etc.*, could be performed by optimizing the concentration of each reagent independently. Some reagents other than urea or LiCl thought to have another specific effect on the refolding, for example, primarily influencing hydrogen bonding, should be surveyed using this combined reagent method. In conclusion, use of multiple reagents in combination can be powerful technique for designing appropriate refolding solutions of a variety of valuable recombinant proteins.

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