study with the specific derivatives.

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Characterization of Disulfide Bonds in Recombinant Proteins: Reduced Human Interleukin 2 in Inclusion Bodies and Its Oxidative Refolding

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ABSTRACT: Cloned cDNA of human interleukin 2 (IL-2) was expressed in *Escherichia coli* cells in which IL-2 formed insoluble inclusion bodies. Human IL-2 has three Cys residues, namely, Cys-58, Cys-105, and Cys-125, and native IL-2 has an intramolecular disulfide bond between Cys-58 and Cys-105. Since the formation of inclusion bodies was thought to be due to disorder in the oxidation state of these Cys residues, all intramolecular disulfide bond isomers of IL-2 were prepared by denaturation of native IL-2 to characterize the state of a disulfide bond in IL-2 in the inclusion bodies. These isomers can be separated from native IL-2, reduced IL-2, and IL-2's with intermolecular disulfide bonds by means of reversed-phase high-performance liquid chromatography. Human IL-2 produced in inclusion bodies in *E. coli* carrying a recombinant DNA was analyzed by HPLC and was proved to be a fully reduced form with no intra- and intermolecular disulfide bonds. Refolding of reduced IL-2 in the presence of reduced and oxidized glutathione and a low concentration of guanidine hydrochloride resulted in the formation of the biologically active IL-2 quantitatively. Further purification provided a practically pure IL-2 preparation without contamination of any disulfide bond isomers.

Interleukin 2 (IL-2),[1] also referred to as T-cell growth factor, is a lymphokine produced by activated T-cells (Morgan et al., 1976; Gillis et al., 1979). The important biological activities of this protein are reported to be promotion of long-term in vitro proliferation of antigen-specific effector T-lymphocytes and induction of cytotoxic T-lymphocyte reactivity (Gillis & Smith, 1977; Watson, 1979; Gillis et al., 1980; Watson et al., 1979). Interest in IL-2 has increased recently owing to reports of a possible role for this lymphokine in the treatment of solid tumors (Rosenberg et al., 1985). Recently, mRNAs for human IL-2 from various sources, such as a leukemic T-cell line (Taniguchi et al., 1983), spenocytes (Devos et al., 1983), and tonsillar mononuclear cells (Maeda et al., 1983), were isolated and their cDNAs were cloned and sequenced. Cloned cDNA, when inserted in *Escherichia coli*, was expressed at high concentration, and the expressed protein was purified by

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[1] Abbreviations: IL-2, interleukin 2; *E. coli*, *Escherichia coli*; HPLC, high-performance liquid chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; DTT, 1,4-dithiothreitol; Cm, carboxymethylated; CTLL, cytotoxic T lymphocyte line; PAGE, polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; re-IL-2, reduced IL-2; IL-2(105–125), IL-2 with a disulfide bond between Cys-105 and Cys-125; IL-2(58–125), IL-2 with a disulfide bond between Cys-58 and Cys-125; IL-2(58–105), IL-2 with a disulfide bond between Cys-58 and Cys-105; BPTI, bovine pancreatic trypsin inhibitor.

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successive chromatography including HPLC (Rosenberg et al., 1984; Liang et al., 1985; Kato et al., 1985b). Recombinant IL-2 is produced by *E. coli* in sufficient quantity for various purposes including clinical trials, though cultured T-lymphocytes provide only a limited amount of natural IL-2 (Stern et al., 1984; Kato et al., 1985a). Additionally, no functional differences between native and recombinant IL-2 have been detected (Rosenberg et al., 1984; Naruo et al., 1985), though a little difference in the primary structure has been noted (Robb et al., 1983).

In the processing of recombinant proteins, troubles related to the structure of the protein have been often mentioned. In some cases the specific activity of the recombinant protein is lower than that of the natural counterpart, and in other cases the antigenicity differs in recombinant and natural proteins. It is believed that the origin of the problem often lies in their disulfide bonds. It was reported that a recombinant fibroblast interferon preparation which had lower specific activity than natural interferon isolated from cultured human fibroblasts contained some oligomers linked with intermolecular disulfide bonds (Freisen et al., 1981). A similar result has been reported on recombinant human leukocyte interferon (Morehead et al., 1984). In these cases it is known that the intramolecular disulfide bonds are essential for their biological activities.

Human IL-2 contains three cysteine residues located at amino acid positions 58, 105, and 125 (Taniguchi et al., 1983). Two cysteines, namely, Cys-58 and Cys-105, are involved in intramolecular disulfide bridging to form a biologically active IL-2 molecule, and the disulfide bond is essential for biological activities (Liang et al., 1985; Robb et al., 1984; Wang et al., 1984). Quite recently, structure–activity relationships of the IL-2 molecule have been partially clarified (Cohen et al., 1986; Kuo & Robb, 1986), especially concerning the region around the Cys residues (Liang et al., 1986).

In our preparation of recombinant IL-2, as is often the case in other recombinant proteins produced in *E. coli* with a high-level expression (Kleid et al., 1981; Simons et al., 1984; Schoner et al., 1985; Masui et al., 1984), the protein is produced as cytoplasmic inclusion bodies. It is known that such inclusion bodies are soluble only under denaturing conditions. This fact suggests that the protein in the inclusion bodies is in a denatured form or it is solubilized as a denatured form. Under such a situation, refolding of the protein and formation of correct disulfide bonds are necessary for obtaining the native protein. However, in cases of disulfide bond containing proteins the refolding yield is often poor and the final products sometimes show low specific activities. In these trials it is most important to clarify the state of disulfide bonds of the protein in the inclusion bodies and in the refolding mixture. Such characterization is expected to lead to the efficient recovery of native proteins and also to the understanding of the reason why the proteins form aggregates. However, since determination of localization of disulfide bonds in proteins is often complicated, it is desirable to establish a simplified method to analyze the proteins in terms of their disulfide bonds.

In this work, we describe the isomerization of the intramolecular disulfide bond of IL-2. The resultant isomers, including a reduced form and oligomers with intermolecular disulfide bonds, were analyzed by reversed-phase HPLC. Oxidative refolding of reduced IL-2, which was first found to be accumulated dominantly in the inclusion bodies in *E. coli*, was also studied.

Materials and Methods

Recombinant Human IL-2. cDNA coding for IL-2 of a human leukemic T-cell line, Jurkat 111, was cloned and expressed in *E. coli* (Taniguchi et al., 1983). The expressed protein, which had the characteristic biological properties of IL-2, was purified from a soluble fraction of a cell extract as described previously (Fukuhara et al., 1985). IL-2, as inclusion bodies, was produced in *E. coli* constructed and cultured as described elsewhere (Sato et al., 1987). The cells were collected by centrifugation and homogenized by lysozyme treatment and sonication at 4 °C. The resultant lysate was centrifuged at 10000 rpm for 10 min, and the precipitate was collected. The pellet thus obtained was stored at −80 °C.

Refolding and Purification of IL-2 in Inclusion Bodies. The representative procedure was as follows: The pellet was dissolved in a minimum amount of 0.1 M Tris-HCl buffer (pH 8.0) containing 6 M guanidine hydrochloride. The solution was brought to an IL-2 concentration of 0.1 mg/mL and treated with 10 mM reduced glutathione and 1 mM oxidized glutathione in the presence of 2 M guanidine hydrochloride at pH 8.0. After the solution was kept for 16 h at room temperature, the pH of the solution was brought to 6.0 by adding glacial acetic acid and the solution was concentrated with Pellicon cassettes (Millipore; mwcf 10000). The solution was centrifuged, and the supernatant was desalted by passing through a Sephadex G-25 column equilibrated with 0.05 M sodium acetate buffer (pH 6.0). The desalted solution was applied to a CM-Sepharose column (Pharmacia) equilibrated with the same buffer, and IL-2 was eluted with the same buffer containing 0.5 M NaCl. Fractions containing IL-2 were collected and applied directly to a Ultrapore RPSC column (4.6 × 75 mm; Beckman) equilibrated with 0.5 M triethylamine–acetic acid buffer (pH 4.0). IL-2 was eluted at a propanol concentration of 40% by gradient elution with 80% 1-propanol. The IL-2 fractions were collected and applied to a Sephadex G-25 column equilibrated with 0.07 M sodium acetate buffer (pH 5.0) to remove propanol. The resultant IL-2 solution was then purified by a Mono S column (5 × 50 mm; Pharmacia) equilibrated with the same buffer. IL-2 was eluted by a gradient elution with the same buffer containing 0.25 M NaCl.

Analytical HPLC. IL-2 and their derivatives were analyzed by reversed-phase HPLC with a Ultrapore RPSC column (4.6 × 75 mm) that was developed by a linear gradient of 40–64% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. Effluent was monitored by measuring absorbance at 280 or 210 nm. The quantity of IL-2 was determined by measuring a peak area at 280 nm that was previously calibrated by amino acid analysis.

Peptide Mapping. One hundred micrograms of carboxymethylated IL-2 was suspended in 200 μL of 0.1 M ammonium bicarbonate (pH 8.1) and treated with a 1/50 (w/w) amount of L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin (Sigma) for 4 h at 37 °C. The digest was directly applied on a reversed-phased column of μBondapak C_{18} (4.6 × 300 mm; Waters) that was developed with a gradient of 0–51% of acetonitrile containing 0.1% of trifluoroacetic acid at a flow rate of 1 mL/min.

Results

Identification of Disulfide Bond Isomers. Recombinant IL-2 was isolated from the water-soluble fraction of the *E. coli* extract as described previously (Fukuhara et al., 1985). A protein with the characteristic biological activity of IL-2 was obtained as a single peak by RP-HPLC analysis. Lyophilized IL-2 thus obtained was dissolved in a buffer containing 6 M guanidine hydrochloride, and the portion was immediately analyzed by RP-HPLC as shown in Figure 1a. Besides the peak of intact IL-2, two peaks named A and B with shorter

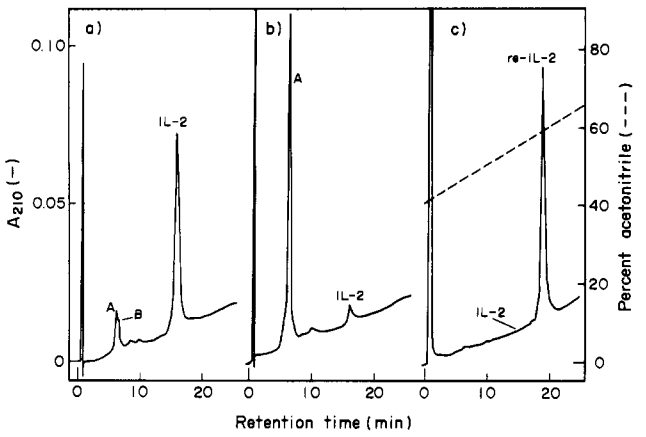

FIGURE 1: Reversed-phase HPLC of IL-2 and its disulfide bond isomers. One hundred micrograms of lyophilized IL-2 was dissolved in 100 μL of 0.35 M Tris-HCl buffer containing 6 M guanidine hydrochloride and 0.035 M EDTA (pH 8.1). (a) Immediately after dissolution; (b) 1 h at 45 °C; (c) large excess of DTT added. Five microliters of the solution was injected on a Ultrapore RPSC column and eluted at 1.0 mL/min with a linear gradient of 40–64% acetonitrile containing 0.1% trifluoroacetic acid in 24 min. See Materials and Methods. A and B indicate IL-2(105–125) and IL-2(58–125), respectively.

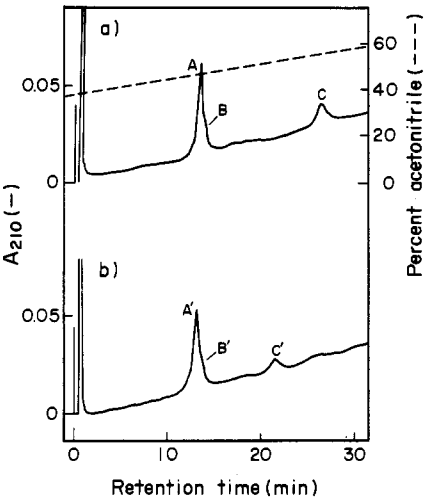

FIGURE 2: Isomerization and carboxymethylation of IL-2. Five hundred micrograms of IL-2 was dissolved in 250 μL of 0.2 M phosphate buffer containing 6 M guanidine hydrochloride and 0.02 M EDTA (pH 6.0). (a) After 16 h at 20 °C; (b) 10 M excess of iodoacetic acid was added after 16 h. Two microliters was injected. Conditions were the same as those shown in the Figure 1 legend except that a linear gradient of 36–56% in 30 min was employed. A′, B′, and C′ indicate the carboxymethylated species derived from A, B, and C, respectively, where C is native IL-2.

retention times were observed. After 1 h at 45 °C the IL-2 peak almost disappeared and peak A increased in intensity (Figure 1b). After reduction of disulfide bonds in these species with a large excess of DTT, all peaks disappeared and a new single peak of reduced IL-2 (re-IL-2) was observed as shown in Figure 1c. These results indicate that peaks A and B are probably disulfide bond isomers of intact IL-2. To certify this hypothesis, the isomerization was terminated by an addition of iodoacetic acid and the resultant carboxymethylated species were isolated by repeated runs of RP-HPLC. As shown in Figure 1, peak A is dominant when the isomerization was done at pH 8.1, but peak B is observed in a fair amount at low temperature at pH 6 as shown in Figure 2. The HPLC patterns of the carboxymethylated species are also shown in this figure. Tryptic digestion of these Cm-IL-2's was then performed, and the resultant peptide fragments were analyzed

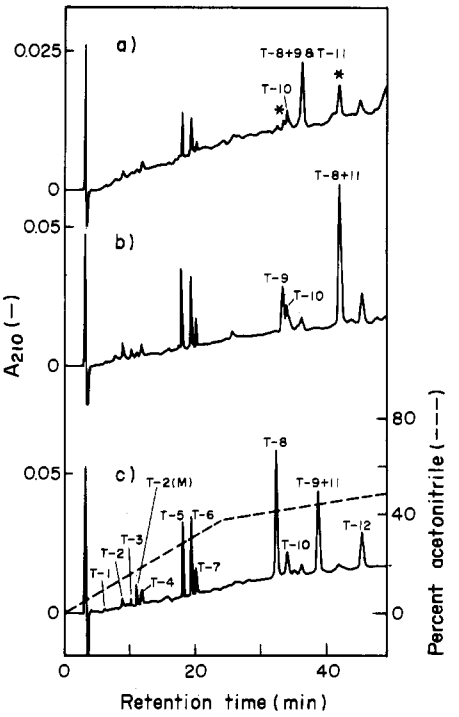

FIGURE 3: RP-HPLC tryptic maps of various disulfide forms of Cm-IL-2. About 20 μg of the trypsin digests were directly injected on a μBondapak C_{18} column and eluted by a gradient of 0–36.8% in 23 min and then 36.8–51% of acetonitrile containing 0.1% trifluoroacetic acid in 38 min. (a) Cm-IL-2(58–125) (peak B′); (b) Cm-IL-2(105–125) (peak A′); (c) Cm-IL-2(58–105) (peak C′). T-2(M) indicates a fragment with an N-terminal Met residue derived from the initiation codon, where T-2 indicates an N-terminal fragment without the Met residue. See Figure 4. T-*m*+*n* means a peak assigned to be T-*m* and T-*n* covalently linked with a disulfide bond. In (a) T-8+9 and T-11 are eluted at the same position and * denotes a peak derived from IL-2(105–125) which was not separated completely.

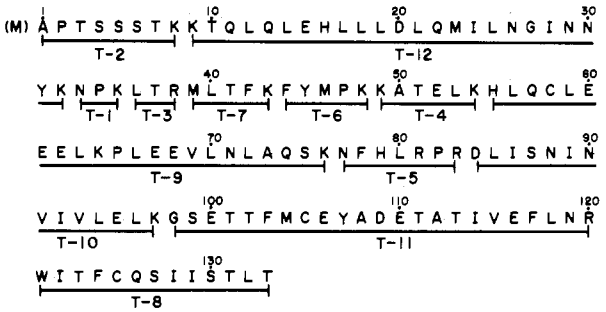

FIGURE 4: Amino acid sequence of human IL-2. T-*n* indicates trypsin peptides and corresponds to the symbols described in Figure 3. The N-terminal Met residue in parentheses is derived from the initiation codon. The single-letter notations used are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

by RP-HPLC with a C_{18} column (Figure 3). Identification of each peak was performed by both amino acid analysis and N-terminal analysis, and the assignment was shown in Figure 4. Three Cys residues in IL-2 were contained in T-8 (Cys-125), T-9 (Cys-58), and T-11 (Cys-105). In the chromatogram derived from the intact IL-2, T-8 was observed as an independent peak and T-9 and T-11 were observed as a single peak (T-9+11). Thus the location of the disulfide bond in intact IL-2 was confirmed to be between Cys-58 and Cys-105. On the other hand, the tryptic peptide map of peak A shows T-9 and a single peak of combined T-8 and T-11 (T-8+11). This pattern reveals that peak A is an isomer with a disulfide bond between Cys-105 and Cys-125. Similarly, peak B is

proved to be the other isomer, with a disulfide bond between Cys-58 and Cys-125. Thus the three possible isomers of IL-2 with an internal disulfide bond were identified.

To characterize the isomerization reaction, native IL-2 was heated to 60 °C in a nondenaturing buffer, but no isomers were detected. Further, to investigate the location of Cys-125 and the disulfide bond in the native state, native IL-2 was treated with an excess amount of iodoacetate acid or DTT under nondenaturing conditions. Though native IL-2 was not carboxymethylated in its native state, reduction of IL-2 occurred to form precipitate of reduced IL-2.

The biological activity measured by [^{3}H]thymidine incorporation of IL-2-dependent CTLL cells (Gillis et al., 1979; Watson et al., 1979) of carboxymethylated IL-2 and its isomers was examined, since the isomers are unstable and easily isomerize under the condition to measure the biological activity. Though Cm-IL-2 with a disulfide bond between Cys-58 and Cys-105 showed a specific activity almost identical with that of intact IL-2, the other isomers without a disulfide bond between Cys-58 and Cys-105 had no activity. This result indicates that the disulfide bond between Cys-58 and Cys-105 is essential for demonstration of the biological activity. The present result is also compatible with the results reported previously (Liang et al., 1986).

Additionally, isomers with intermolecular disulfide bonds, namely, oligomers, were prepared by air oxidation of reduced IL-2. The resultant oligomers, which were characterized by a nonreductive SDS–PAGE analysis, were also provided for RP-HPLC analysis to show multiple peaks between peak B and the peak of intact IL-2 under the condition shown in the Figure 1 legend. Especially a dimeric IL-2 with a disulfide bond between Cys-125 and Cys-125′ was prepared by air oxidation of intact IL-2 in the presence of a trace amount of Cu(II) ion in 40% 1-propanol, and the dimer gave a single peak between peak B and the peak of intact IL-2 (data not shown).

Analysis of IL-2 in the Inclusion Body. As described above, all disulfide bond isomers of IL-2 could be identified by RP-HPLC analysis. Then the IL-2 protein in the inclusion bodies was analyzed in terms of the state of Cys residues. After cultivation of *E. coli*, lumps, namely, inclusion bodies, were observed by an optical microscope in the elongate cells. Since the free sulfhydryl group of a Cys residue is very unstable, the inclusion bodies were carefully collected as described under Materials and Methods. After separation from the cell debris by centrifugation, the collected inclusion bodies showed an IL-2 content of at least about 80% as determined by reducing SDS–PAGE analysis. The inclusion bodies were quite insoluble in neutral buffers, and they were only soluble in the presence of a denaturant such as 6 M guanidine hydrochloride and 8 M urea or in the presence of a strong detergent such as 0.5% SDS. Then, the inclusion bodies were dissolved in an acidic buffer containing 6 M guanidine hydrochloride and EDTA, which was designed to minimize the air oxidation of –SH groups and isomerization of a disulfide bond, and immediately analyzed by RP-HPLC. As shown in Figure 5, only a peak of reduced IL-2 was observed and the intact IL-2, and the isomers with disulfide bond(s) were practically not detected. Thus the IL-2 protein accumulated in the inclusion bodies was revealed to be a reduced form without any disulfide bond.

Refolding of Reduced IL-2. Since reduced IL-2 is not biologically active, the disulfide bond formation is necessary to obtain the biologically active IL-2 from the inclusion bodies. Since it is well-known that a protein forms a correct disulfide bond spontaneously under physiological conditions, air oxidation was first tested after lowering the concentration of guanidine hydrochloride by mere dilution or dialysis. But the conversion of the reduced IL-2 to the native form was poor because of the formation of insoluble materials. Combination of an oxidized and reduced form of glutathione was utilized for regeneration of a native protein from a reduced protein (Ahmed et al., 1975). Application of this system to the oxidative refolding of reduced IL-2 resulted in success. Since the presence of a high concentration of guanidine hydrochloride, which was required for solubilization, disturbs the correct folding, it must be removed prior to the oxidation. However, complete removal of the denaturant resulted in precipitation of reduced IL-2, and the precipitate was not solubilized even under oxidative conditions. Therefore, the oxidation was carried out in the presence of a lower concentration of guanidine hydrochloride. The optimum concentration to solubilize reduced IL-2 and to bring about a correct folding proved to be in a range of 1.5–3.0 M. The upper limit is in good agreement with the denaturing point of native IL-2 monitored by circular dichroism spectra (data not shown). The concentration of reduced IL-2 is also important. For the quantitative recovery it must be lower than 150 μg/mL to avoid an intermolecular disulfide bond formation. Under these conditions reduced IL-2 was treated with 5–100 mM reduced glutathione and a $^1/_{10}$ amount of oxidized glutathione at pH 8, room temperature for several hours. The representative chromatograms of the time course of refolding process were shown in Figure 6. Reduced IL-2 was quantitatively converted to the correctly folded IL-2 with a disulfide bond between Cys-58 and Cys-105, and no isomers were observed. It is noticeable that isomers formed during storage were also converted to intact IL-2 as shown in Figure 6. Further purification of refolded IL-2 as described under Materials and Methods provided a practically pure IL-2 preparation (>99% pure) without contamination of any disulfide bond isomers.

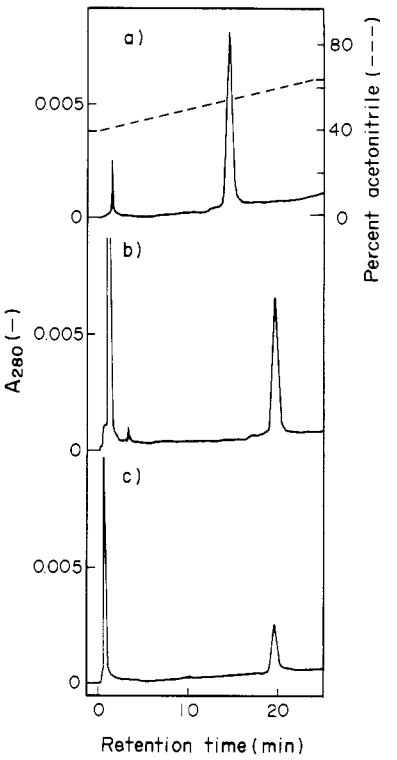

FIGURE 5: HPLC of IL-2 in inclusion bodies: (a) native IL-2; (b) IL-2 treated with DTT; (c) inclusion bodies dissolved with 0.2 M phosphate buffer containing 6 M guanidine hydrochloride and 0.02 M EDTA (pH 6.0). HPLC analysis was performed as described in the Figure 1 legend.

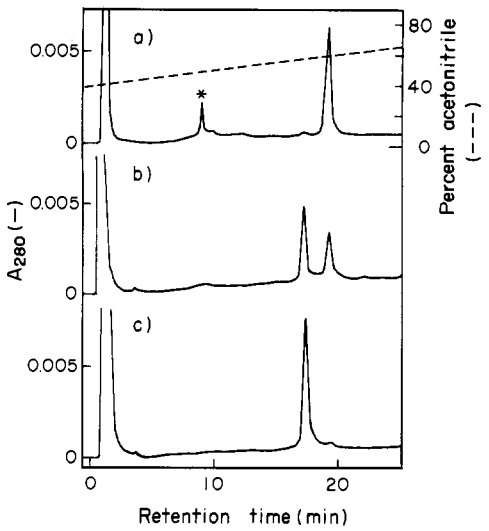

FIGURE 6: Oxidative refolding of reduced IL-2 in inclusion bodies. Inclusion bodies collected by centrifugation were dissolved in 0.1 M Tris-HCl buffer containing 6 M guanidine hydrochloride and treated with reduced and oxidized glutathione as described in the text. (a) Immediately after the initiation of refolding; (b) 0.5 h at room temperature; (c) 6 h at room temperature. About 5 μg of IL-2 was injected, and the chromatographic runs were performed as described in Figure 1. * denotes a disulfide bond isomer formed during the storage of the solution of inclusion bodies.

Discussion

As described above, the biologically active IL-2 has an intramolecular disulfide bond between Cys-58 and Cys-105 and a free sulfhydryl group in Cys-125. The free sulfhydryl group in Cys-125 is reactive and nucleophilically attacks the disulfide bond, and as a result, IL-2 isomerizes to its disulfide bond isomers under denaturing conditions. Additionally, IL-2 dimerizes by an oxidative coupling between Cys-125 and Cys-125′ under an oxidative condition in the presence of organic solvents such as 40% 1-propanol. Such an oxidative coupling does not occur by oxidation under nondenaturing conditions. The sulfhydryl group of Cys-125 is easily carboxymethylated under denaturing conditions, but the carboxymethylation does not occur under native conditions. These results indicate that the sulfhydryl group of Cys-125 is buried in the molecule and located in the position neighboring the disulfide bond between Cys-58 and Cys-105. The fact that the carboxymethylation of Cys-125 does not affect the specific activity also indicates that the Cys-125 is buried in the molecule and does not concern the IL-2–receptor interaction. Since reduction of the disulfide bond under native conditions with DTT gave reduced IL-2, the disulfide bond may possibly hinder the attack of electrophiles to Cys-125.

Nucleophilic attack of the sulfhydryl group of Cys-125 to the disulfide bond gave two isomers. Between them an isomer with a disulfide bond between Cys-105 and Cys-125 [IL-2(105–125)] is predominantly formed. However, in the early stage of isomerization or under controlled conditions (low temperature and low pH), another isomer with a disulfide bond between Cys-58 and Cys-125 [IL-2(58–125)] is formed in a fair amount. These results indicate that IL-2(105–125) is thermodynamically more stable than IL-2(58–125) under denaturing conditions. Formation of IL-2(58–125) is kinetically favored, and IL-2(58–125) may reisomerize to IL-2(105–125); as a result, IL-2(105–125) is accumulated.

Creighton reported that such disulfide bond isomers were formed during the oxidative refolding of BPTI (Creighton, 1978). However, identification and separation of such isomers are generally difficult because isomers resemble one another except in their molecular shapes. In this sense, reversed-phase HPLC is a novel and useful tool for the analysis of such disulfide bond isomers.

RP-HPLC is also useful for the analysis of the state of Cys residues in IL-2 accumulated in *E. coli* cells. Though it is reported that in several cases recombinant proteins produced in *E. coli* are accumulated as insoluble inclusion bodies, the oxidation state of these aggregates has rarely been reported. Schoemaker and his co-workers examined the inclusion bodies of calf prochymosin, and they concluded that the inclusions were mainly composed of multimers of prochymosin molecules that were interlinked partly by disulfide bonds (Schoemaker et al., 1985). In the present case, IL-2 is accumulated as a reduced form with an open disulfide bond. The reduced IL-2 forms precipitates without oxidation, because solubility of reduced IL-2 is low. Since the cytoplasm of *E. coli* is reducing and most intracellular proteins are rich in thiols (Freedmann & Hillson, 1980), such a situation may often be the case with other recombinant proteins. Formation of inclusion bodies is advantageous for mass production of foreign proteins. However, some proteins do not form inclusion bodies. Since the foreign protein as a solubilized form in cytoplasm of *E. coli* may be toxic against the host, the accumulation cannot be so high in these cases. Such a difference may depend on the solubility of the reduced protein in addition to the rate of protein synthesis.

In the present case, oxidative refolding is required to obtain biologically active IL-2. Since reduced IL-2 is insoluble in neutral buffers, care must be taken for the quantitative recovery of oxidized IL-2. The redox buffer system utilizing oxidized and reduced glutathione is quite effective for the refolding of IL-2. Determination of the conditions under which reduced IL-2 is solubilized and refolded correctly is most important. Protein concentration must be kept low, which affects the solubility of reduced IL-2 and, more importantly, the formation of oligomers. As has been clarified by prominent works performed by Konishi et al. (1981, 1982a–c), reduced glutathione catalyzes isomerization of incorrectly folded species. Since solubilities of these species are lower than that of the correctly folded one, accumulation of these species results in formation of insoluble materials. The redox buffer system may be applicable to the refolding of other recombinant protein aggregates in *E. coli* (Winkler et al., 1985).

In general, contamination of incorrectly folded species is not desired. To avoid this problem, both a careful choice of refolding conditions and an establishment of a method to analyze disulfide bond isomers are required as shown in this study. An alternative way to avoid this problem is to replace an unnecessary Cys residue with other amino acids by site-directed mutagenesis (Robb et al., 1984; Mark et al., 1984). However, this is applicable only in limited cases. The necessity of techniques described here increases in relation to the number of Cys residues in a protein molecule. In the case of IL-2, which has only three Cys residues in a molecule, the number of isomers was limited; however, refolding of proteins with many Cys residues may be a further problem.

Acknowledgments

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