# Structure of Unfolded and Refolded Recombinant Derived [Ala$^{125}$]Interleukin 2

Tsutomu Arakawa, Tom Boone, Janice M. Davis, and William C. Kenney*
*Amgen, Thousand Oaks, California 91320*
*Received April 23, 1986; Revised Manuscript Received August 13, 1986*

ABSTRACT: Naturally occurring interluekin 2 (IL-2) contains an odd number (three) of cysteinyl residues and thus is susceptible to the formation of a variety of intramolecular and intermolecular disulfide bonds. The cysteine at residue 125 has been replaced with an alanine residue by site-directed mutagenesis, and hence, this analogue can form only one intrachain disulfide bond. When expressed at high levels in *Escherichia coli*, this recombinant DNA derived IL-2 analogue is insoluble, reduced, and inactive. The protein was solubilized by denaturants and, after purification, was oxidized to form an intramolecular disulfide bond. Circular dichroism (CD) has been used to investigate the effects of various denaturants on the unfolding–refolding process of the purified, oxidized protein. A similar conformation is obtained when [Ala$^{125}$]interleukin 2 [IL-2(Ala-125)] is refolded from 6 M guanidine hydrochloride, 8 M urea, or 5% acetic acid. The resultant protein, refolded from these denaturants, is monomeric and has activity comparable to or greater than that reported for naturally derived IL-2. In addition to this form, aggregates, as evidenced from gel filtration, are obtained. The specific activities of these are greatly reduced, and CD spectra indicated that they have much less helical content than the monomeric form of the protein. CD spectra also showed that the tertiary structure of IL-2(Ala-125) is entirely different in the presence of sodium dodecyl sulfate (SDS) from that of the monomeric form in the absence of SDS.

Since the discovery of human interleukin 2 (also called T-cell growth factor) by Morgan et al. (1976), several functions have been ascribed to this lymphokine. In addition to initiation and maintenance of T-cell proliferation (Morgan et al., 1976; Ruscetti & Gallo, 1981), this protein has been found to be stimulatory in the generation of cytotoxic T lymphocytes (Paetkau et al., 1980), in the activity of natural killer cells (Henney et al., 1981; Danzig et al., 1983) and of lymphokine-activated killer cells (Grimm et al., 1982), and in immune responsiveness in vitro of lymphocytes from patients with acquired immunodeficient states (Rook et al., 1983; Lifson et al., 1984).

Human interleukin 2 (IL-2)[1] purified from natural sources is heterogeneous with respect to glycosylation, isoelectric points, and molecular weights (Welte et al., 1982). The quantities of material obtainable have not been sufficient for rigorous structural characterization of the protein. By cDNA cloning and DNA sequencing of the human IL-2 gene, the protein sequence has been predicted (Taniguchi et al., 1983; Devos et al., 1983). From this information, a synthetic gene for IL-2 was constructed for optimum expression in *Escherichia coli*.[2] Analogues have been constructed with substitutions of cysteinyl residues in the protein with other amino acids and with deletions of various lengths of the amino and carboxy termini.[3] These studies have indicated that portions of the carboxy and amino termini are required for maximum cytotoxic T-lymphocyte line (CTLL) activity. Moreover, cysteinyl residues at positions 58[4] and 105 are essential for activity, and it is thought that a disulfide bond, formed between these two residues, enhances activity relative to the fully reduced form (Wang et al., 1984). Naturally occurring human IL-2 contains a third cysteine at position 125. Substitution of this residue with Asp, Asn, Ser, or Ala yields IL-2 analogues with biological activities comparable to that of the natural form; indeed, the activities of the latter two analogues seem to be slightly higher[3] (Wang et al., 1984). Moreover, these derivatives have greater stability since the natural recombinant IL-2 has the capacity to form dimers between the free cysteinyl at residue 125 as well as alternate disulfide bonds, i.e., Cys-125 to Cys-58 or to Cys-105. Such analogues have considerably reduced activity (Wang et al., 1984).

Preliminary studies of the IL-2(Ala-125) analogue suggested that this protein may be useful for the treatment of diseased states where IL-2 could be effective because of the characteristics of the modified protein. We have thus extended our studies to characterize this derivative of IL-2. When expressed at high levels in *E. coli*, most of the IL-2(Ala-125) is insoluble, reduced, and inactive. In order to obtain active protein, it is necessary to solubilize it in the presence of denaturants and to allow the protein to refold on removal of the denaturant. Circular dichroism has been used to investigate the effect of various denaturants on the unfolding–refolding process.

## Materials and Methods

Ultrapure guanidine hydrochloride and ultrapure urea were obtained from Heico and Schwarz/Mann, respectively. IL-2(Ala-125) was purified by a series of standard chromatographic procedures, oxidized to form an intramolecular disulfide bond, and stored in buffer A (5% glucose, 10 mM sodium acetate, pH 4). The final product was greater than 95% pure, as demonstrated by SDS–PAGE under reducing

[1] Abbreviations: IL-2, interleukin 2; IL-2(Ala-125), IL-2 analogue in which Cys at position 125 is substituted with Ala; CTLL, cytotoxic T-lymphocyte line; CD, circular dichroism; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis. IL-2(Ala-125) is being jointly developed by Amgen, Thousand Oaks, CA; Cilag, Schaffhausen, Switzerland; and Ortho Pharmaceutical Corp., Raritan, NJ.

[2] Y. Stabinski, Z. Stabinski, T. Boone, D. Murdock, L. Miller, M. Caruthers, and L. Souza, unpublished results.

[3] T. Boone, V. Chazin, W. Kenney, E. Swanson, B. Anderson, M. Carter, K. Chen, B. Bacheller, and B. Altrock, unpublished results.

[4] Amino acid residues are numbered to coincide with naturally occurring IL-2, in which the N-terminal residue is Ala. Recombinant-derived IL-2(Ala-125) contains an N-terminal Met residue (position 0) preceding the Ala.

0006-2960/86/0425-8274$01.50/0 

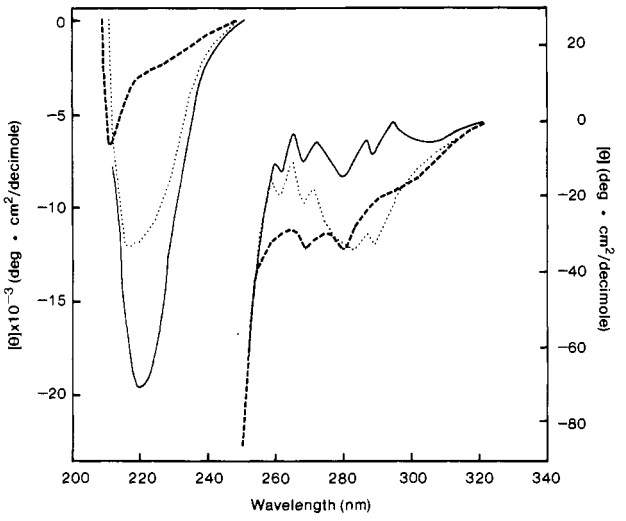


FIGURE 1: CD spectra of IL-2(Ala-125) in denaturants and 5% acetic acid: right, near-UV; left, far-UV; (—) 5% acetic acid; (– –) 6 M guanidine hydrochloride; (···) 8 M urea.

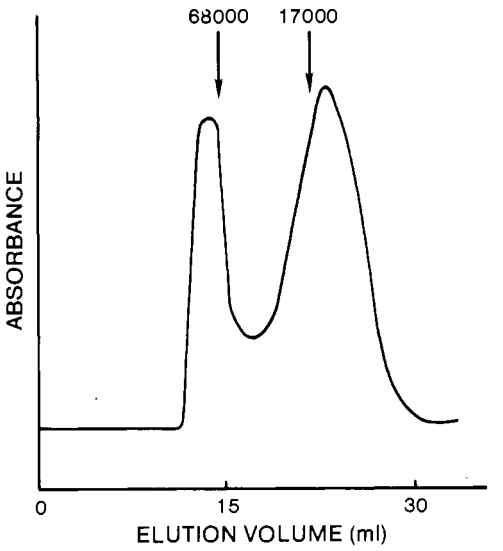


FIGURE 2: Gel filtration profile of IL-2(Ala-125) in buffer A. Myoglobin (17 000) and bovine serum albumin (68 000) were used for calibration.

and nonreducing conditions. The unfolding–refolding experiments of the purified protein were carried out at or below pH 4 to assure protein solubilization. The purified protein at ~1 mg/mL in buffer A was dialyzed overnight against 8 M urea or 6 M guanidine hydrochloride, pH 4, or against 5% acetic acid. This dialysis was sufficient to cause an equilibrium denaturation in each solvent system. The unfolded protein was refolded by diluting the protein solution (~1 mg/mL) 10-fold with buffer A and dialyzing the diluted solution overnight against the solvent. After dialysis, the protein solution was concentrated with an Amicon stirred cell.

Gel filtration was carried out at 4 °C with a column containing Sephadex G-75 (Pharmacia) in buffer A. A plastic column (1.2 × 26 cm) was used, since IL-2 (Ala-125) was found to bind to glass columns. The flow rate was adjusted to 15 mL/h. Gel filtration in the presence of guanidine hydrochloride was carried out on a Sephacryl S-200 column (1.5 × 35 cm) at the same flow rate. Protein concentration was spectrophotometrically determined with the absorptivity of $E^{0.1\%}_{280nm} = 0.90$ in buffer A. This absorptivity was determined by measuring the absorbance at 280 nm of the protein solution and quantifying the amount of the protein by amino acid composition analysis. Protein concentrations in other solvents are also calculated with the same absorptivity. The activity of IL-2(Ala-125) was estimated by measuring [$^3$H]thymidine incorporation into an IL-2-dependent murine T-cell line (CTLL) (Gillis et al., 1978). One unit of activity is the reciprocal of the dilution factor that gives 50% of the maximum incorporation of [$^3$H]thymidine.

Circular dichroic spectra were determined at room temperature on a Jasco J-500C spectropolarimeter. Spectral bandwidth was set at 1 nm. Cuvettes of 0.1 and 1 cm in light path length were used for 190–260 and 240–340 nm, respectively. The solvent spectrum was subtracted from the protein spectrum. The results were expressed as mean residue ellipticity, [$\theta$], calculated from the mean residue weight of 116 for the IL-2 analogue.

## Results and Discussion

IL-2(Ala-125) is a very hydrophobic protein. The hydrophobicity parameter of IL-2(Ala-125) was calculated, according to Bigelow (1967), to be 1230 cal/mol of residue on the basis of the amino acid composition. This value is much higher than the values for most proteins of this size ($M_r$ 15 500). Since IL-2(Ala-125) is insoluble when expressed at high level in *E. coli*, solubilization by denaturants and refolding are essential to obtain an active and stable form of the protein as described in this paper.

The CD spectra of IL-2(Ala-125) in the unfolded state are shown in Figure 1. The far-UV CD spectra show that the secondary structure of the protein is largely unfolded in 6 M guanidine hydrochloride but remains folded in 5% acetic acid or 8 M urea. It seems that the secondary structure is a mixture mainly of random structures and $\beta$-sheet, with a higher ratio of $\beta$-sheet in 5% acetic acid than in 8 M urea (Greenfield & Fasman, 1969). Near-UV CD spectra, shown in Figure 1, suggest a different tertiary structure in these three solvent systems. The unfolded protein in 6 M guanidine hydrochloride showed essentially a single peak at the elution position slightly later than that of myoglobin in the S-200 gel filtration with greater than 90% protein recovered. This indicates that the unfolded state is essentially monomeric at least in the presence of 6 M guanidine hydrochloride.

Unfolded IL-2(Ala-125) was refolded as described under Materials and Methods and loaded onto a Sephadex G-75 column in the same solvent. The gel filtration profile of the refolded protein from 5% acetic acid is shown in Figure 2. It shows two elution peaks at the excluded and included volumes. The second peak had a slightly larger elution volume than myoglobin (17 000), suggesting that the protein in the second peak corresponds to a monomeric form of IL-2(Ala-125) whereas the first peak corresponds to aggregates of the protein. The first peak was rechromatographed on the same column and resulted in the elution of essentially all the protein at the excluded volume. Thus, the first peak cannot readily convert to the second peak under these conditions. The second peak was pooled, concentrated, and loaded onto the same column. The protein eluted at the same elution position as before, indicating no aggregation. These results suggest that the protein eluted in the first peak corresponds to irreversibly formed aggregates of the protein generated in the refolding process. This does not involve intermolecular disulfide bonds, as demonstrated by the monomeric band in nonreducing SDS–PAGE. Refolding from 6 M guanidine hydrochloride or 8 M urea gave essentially the same results on gel filtration (not shown). Since the protein in 6 M guanidine hydrochloride is essentially monomeric but generates aggregates upon refolding, formation of aggregates should be ascribed to ag-

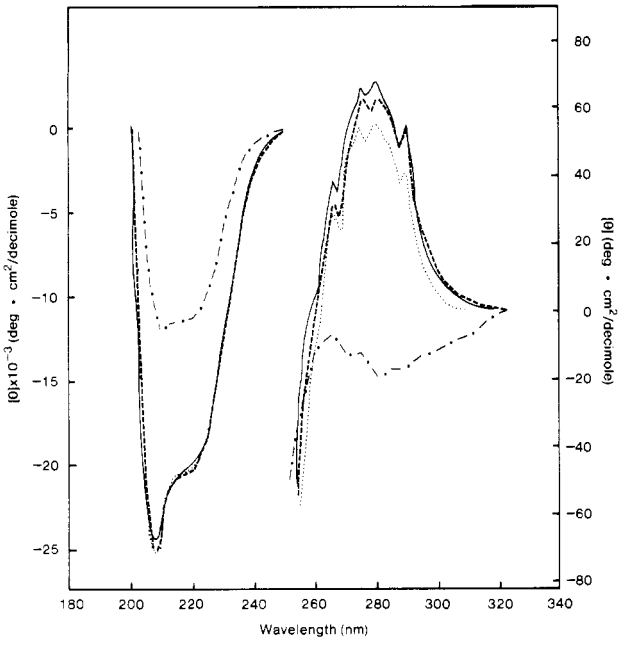


FIGURE 3: CD spectra for monomeric form of IL-2(Ala-125) in buffer A: right, near-UV; left, far-UV; (—) folded from 8 M urea, protein concentration for CD = 0.38 mg/mL; (--) folded from 5% acetic acid, 0.30 mg/mL; (···) folded from 6 M guanidine hydrochloride, 0.17 mg/mL. The spectra for the aggregated form at 0.25 mg/mL are given for comparison (-·-).

gregation during the refolding process. This suggests that manipulation of refolding would increase the yield of monomers. In fact, greater dilution resulted in a higher ratio of monomers.

CD spectra of the refolded protein are shown in Figure 3. The far-UV spectra showed essentially the same profile for refolding from 5% acetic acid, 6 M guanidine hydrochloride, and 8 M urea. The amount of $\alpha$-helix was calculated to be ~70% according to the method of Greenfield and Fasman (1969), indicating that the protein is highly helical. For comparison, the far-UV spectrum of the aggregated form refolded from urea is shown in Figure 3. This indicates much less helical content than the monomeric form of the protein. The near-UV spectra were also similar for the refolding from three different solvent systems. The refolding from guanidine hydrochloride showed slightly smaller CD intensity than when refolding was from 5% acetic acid or from 8 M urea. This is most likely due to experimental error because of low protein concentrations (0.17 mg/mL) for CD measurements and hence high noise to signal ratio. The spectra showed distinct peaks at 280 and 290 nm, which could be ascribed to the Trp transitions, although the peak at 275 nm (Figure 3), possibly corresponding to the Tyr bands, overlaps with the Trp transitions (Strickland, 1974). The bands below 270 nm could be due to Phe. These CD features indicate that the aromatic residues in the monomeric form are incorporated into the rigid tertiary structure, which provides an asymmetric environment for the aromatic residues and gives rise to the CD signals (Timasheff, 1970). On the other hand, the aggregated form, refolded from urea, exhibited a spectrum totally different from that described above (Figure 3). This spectrum is similar to that in 8 M urea, which suggests that part of the protein aggregated in an unfolded state during the refolding process. This agrees with the observation that the aggregated form had much less ordered structure than the monomeric form (Figure 3).

The above results indicated that the monomeric form of IL-2(Ala-125) can be obtained whether the protein was refolded from 5% acetic acid, 6 M guanidine hydrochloride, or 8 M urea. Since the protein structure is different in these denaturing solvents (cf. Figure 1), the refolding course might be different for the three solvents. Nevertheless, the same structure is obtained in the final stage, which indicates that the structure obtained by the method described is thermodynamically stable. Natural IL-2 has not been purified in sufficient quantities for CD measurements and, therefore, cannot be compared with the recombinant protein with regard to conformation.

Table I: Specific Activity of High and Low Molecular Weight Forms of IL-2(Ala-125) after Refolding from Various Denaturants

| refolded from | sp act. ($\times 10^{-6}$ units/mg) high $M_r$ form | low $M_r$ form |
|---|---|---|
| 8 M urea | 0.52 | 15.7[a] |
| 6 M guanidine hydrochloride | | 9.1[a] |
| 5% acetic acid | 0.53 | 9.2[a] |

[a] From ±40% variations in the standard assay conditions, differences in these values are not deemed significant.

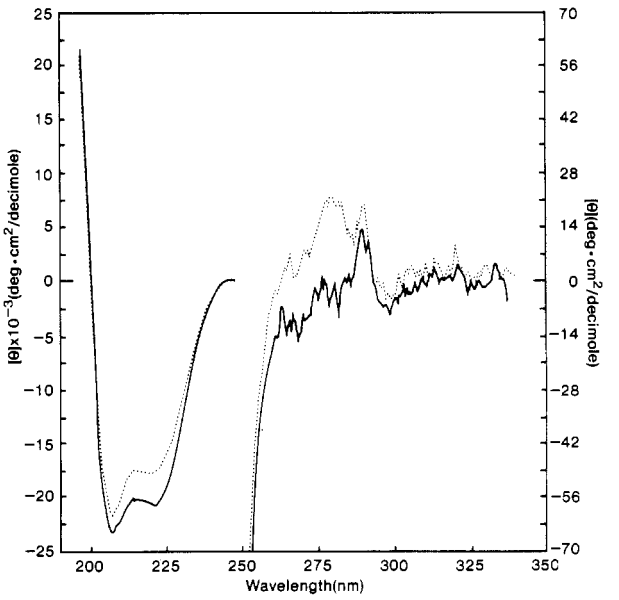


FIGURE 4: CD spectra for IL-2(Ala-125) in buffer A containing 3.3% SDS (—) or 0.33% SDS (···). All spectra are the average of five scans.

In addition to differences in the circular dichroism spectra of the aggregated forms vs. the monomeric forms of IL-2-(Ala-125) obtained by refolding from various denaturants, it was found that the biological activity of the aggregated forms in the CTLL assay were greatly diminished relative to that of the monomeric form (Table I). The specific activity of the low molecular weight form was comparable to or greater than reported values of natural sequence recombinant IL-2 (Liang et al., 1985) or of IL-2 derived from human cells (Robb et al., 1984).

Clinical trials have been performed with recombinant human IL-2 formulated in SDS, which shows a specific activity of (2–6) × $10^6$ units/mg (Lotze et al., 1985). Therefore, we examined the conformation of IL-2(Ala-125) in buffer A in the presence of 3.3% and 0.33% SDS at a protein concentration of 0.27 mg/mL. These SDS and protein concentrations were comparable to those used by Matory et al. (1985) for toxicity testing in rats. Far- and near-UV CD spectra are shown in Figure 4. The far-UV CD spectrum of IL-2(Ala-125) in the presence of 0.33% SDS differed only slightly from that without SDS, indicating similar secondary structure. This is not surprising since IL-2(Ala-125) is highly helical in buffer A

(Figure 3) and SDS is known to be a stabilizer or inducer of $\alpha$-helix (Jirgensons, 1976). The near-UV spectrum with 0.33% SDS showed CD signals that are different and greatly reduced in magnitude compared to those of the sample without SDS, indicating that the environment of the aromatic residues has changed (Strickland, 1974). SDS at 3.3% causes similar, but slightly greater, changes in the spectrum. These results suggest that the tertiary structure of IL-2(Ala-125) is altered in the presence of both 0.33% and 3.3% SDS relative to the absence of SDS, consistent with observations for other proteins (Jirgensons, 1976).

## CONCLUSIONS

It was shown that IL-2(Ala-125), when unfolded by acid or denaturant and refolded by dilution, formed both an unfolded aggregate and a monomeric form. The monomeric form has a distinct secondary and tertiary structure and as high or higher in vitro activity as the natural protein. This raises a possibility of using the recombinant material for extensive biochemical and biophysical investigations of IL-2.

## ACKNOWLEDGMENTS

The excellent technical assistance of Vicki Chazin, Yeuh-Rong Hsu, and Steven Chan is gratefully acknowledged.

**Registry No.** SDS, 151-21-3; L-Ala, 56-41-7; AcOH, 64-19-7; $NH_2CONH_2$, 57-13-6; $NH_2C(=NH)NH_2$·HCl, 50-01-1.

## REFERENCES

Bigelow, C. C. (1967) *J. Theor. Biol. 16*, 187–211.

Danzig, W., Stadler, B. M., & Herberman, R. B. (1983) *J. Immunol. 130*, 1970–1973.

Devos, R., Plaetinck, G., Cheroutre, H., Simons, G., Degrave, W., Tavernier, J., Remaut, E., & Fiers, W. (1983) *Nucleic Acids Res. 11*, 4307–4323.

Gillis, S., Ferm, M. M., Ou, W., & Smith, K. A. (1978) *J. Immunol. 120*, 2027–2032.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.

Grimm, E. A., Mazumder, A., Zhang, H. Z., & Rosenberg, S. A. (1982) *J. Exp. Med. 155*, 1823–1841.

Henney, C. S., Kuribayashi, K., Kern, D. E., & Gillis, S. (1981) *Nature (London) 291*, 335–338.

Jirgensons, B. (1976) *Biochim. Biophys. Acta 434*, 58–68.

Liang, S.-M., Allet, B., Rose, K., Hirschi, M., Liang, C.-M., & Thatcher, D. R. (1985) *Biochem. J. 229*, 429–439.

Lifson, J. D., Benike, C. J., Mark, D., Koths, K., & Engleman, E. G. (1984) *Lancet 1984-1*, 698–702.

Lotze, M. T., Matory, Y. L., Ettinghausen, S. E., Rayner, A. A., Sharrow, S. O., Seipp, C. A. Y., Custer, M. C., & Rosenberg, S. A. (1985) *J. Immunol. 135*, 2865–2875.

Matory, Y. L., Chang, A. E., Lipford, E. H., III, Braziel, R., Hyatt, C. L., McDonald, H. D., & Rosenberg, S. A. (1985) *J. Biol. Response Modif. 4*, 377–390.

Morgan, D. A., Ruscetti, F. W., & Gallo, R. C. (1976) *Science (Washington, D.C.) 193*, 1007–1008.

Paetkau, V., Shaw, J., Caplan, B., Mills, G. B., & Clee, K. (1980) *J. Supramol. Struct. 14*, 276.

Robb, R. J., Kufny, R. M., Panico, M., Morris, H. R., & Chowdhry, V. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6486–6490.

Rook, A. H., Masur, H., Lane, H. C., Frederick, W., Kasahara, T., Macher, A. M., Djeu, J. Y., Manischewitz, J. F., Jackson, L., Fauci, A. S., & Quinnan, V., Jr. (1983) *J. Clin. Invest. 72*, 398–403.

Ruscetti, F. W., & Gallo, R. C. (1981) *Blood 57*, 379–394.

Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113–175.

Taniguchi, T., Matsui, T., Takaoka, C., Kashima, N., Yoshimoto, R., & Hamuro, J. (1983) *Nature (London) 302*, 305–310.

Timasheff, S. N. (1970) *Enzymes (3rd Ed.) 2*, 371–443.

Wang, A., Lu, S., & Mark, D. F. (1984) *Science (Washington, D.C.) 224*, 1431–1433.

Welte, K., Wang, C. Y., Mertelsmann, R., Venuta, S., Feldman, S. P., & Moore, M. A. S. (1982) *J. Exp. Med. 156*, 454–464.