Structural Significance of the C-terminal Amphiphilic Helix of Interleukin-2*

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Interleukin-2 (IL-2, T-cell growth factor) is a lymphokine primarily responsible for mediating the proliferation of activated T-lymphocytes (1). In the brief period since the IL-2 cDNA sequence was determined (2), the sequences of this protein for two other species have been reported (3, 4), and large quantities of recombinant DNA-derived human protein have become available. Since the bacterially produced protein exhibits biologic activities identical to natural IL-2 in vitro (5), both its therapeutic exploitation (6) and function analysis (7) have been greatly facilitated.

IL-2 has been prototypic for demonstrating the utility of and problems associated with modern methods for structure-activity analysis of newly sequenced proteins. The use of antibodies as structure probes (both synthetic peptide-directed (8, 9) and epitope-mapped monoclonals (10, 11)) has indicated that the N-terminal region may be important for receptor binding. However, more precise interpretation of these results is hindered by recent findings demonstrating that large areas of the protein surface are sterically encumbered upon antibody binding (12) and that antibody binding to epitopes remote from active sites can neutralize activity via conformational perturbation (13, 14).

Site-directed mutagenesis of single and multiple residues of both human (15-17) and murine (18) IL-2 have provided considerable data. These studies have located the position of the Cys-58-Cys-105 disulfide linkage (15) and have attempted to assign significance to other residues in the sequence (16-18). However, whereas some of these investigators have carried out their biologic assays on purified recombinant proteins (15, 16), others have employed crude denatured extracts directly in biologic and receptor binding assays (17, 18). In the latter cases, quantitative interpretations of assay results are impossible since the protein is produced and stored in an inactive, reduced, and unfolded state in the bacteria (19, 20). By using crude denatured extracts, the protein is required to first refold and then elicit a response in whatever assay is being employed. Results of such studies cannot distinguish mutations which affect folding and conformation from those which are actually at or near receptor binding residues. To avoid incorrect conclusions, the use of site-directed mutagenesis in protein structure-function analysis demands purification and structural characterization of the resultant mutant proteins.

In order to avoid some of these uncertainties, we first developed a model for the core tertiary structure of IL-2 (21) to aid in a more rational approach to structural analysis. Among the features of this model were a 4-fold 4-helical (right-handed) core tertiary structure including a C-terminal amphiphilic helix spanning residues 116-132. To test this hypothesis, we developed a semi-synthetic design approach (22), which incorporated both helix stabilizing and helix destabilizing multiple simultaneous residue replacements in this region. Using only purified proteins in our assays and monitoring conformation using circular dichroism (CD), we concluded that our prediction for the C-terminal region of IL-2 was accurate.

Recently, x-ray diffraction data (3 Å) has become available which has verified this prediction (23). Although some features of the predicted model were inaccurate, such as N-terminal secondary structure assignments, IL-2 does possess 4-fold 4-helical (left-handed) core tertiary structure and a C-terminal helix spanning residues 117-133. The x-ray structure will certainly aid structure-function analysis of IL-2; however, it has not as yet provided much additional insight as to which residues may interact with the IL-2 receptor. This problem has been further complicated by the identification of a second IL-2 binding protein which participates in formation of the biologically significant high affinity receptor (1, 24).

For the potential design of IL-2 agonists and antagonists, we have expanded our design approach in the examination of
the C-terminal helical region. Using semi-synthesis as a prelude to the development of fully recombinant derivatives, we have rapidly examined several proteins having multiple residue replacements directed toward modulation of biologic activity. We have employed only purified proteins and attempted to correlate data from the biologic assays with physiochemical measurements. These results indicate that in the absence of the identification of exact receptor contact residues, it is possible to incorporate modifications designed to affect secondary and tertiary structure and thus modulate bioactivity via conformational perturbation.

MATERIALS AND METHODS

Semi-synthetic Proteins—All of the semi-synthetic IL-2 derivatives were prepared via air-mediated disulfide formation between Cys-58 of the recombinant DNA-derived protein segment and Cys-105 of the synthetic peptide segment as described previously (22). Briefly, the appropriate synthetic peptide segment (3–6 equivalents) was added to a solution of the recombinant protein segment, and the pH was adjusted to 5.5 (NaHCO3). The mixture was stirred vigorously, and the progress of the oxidation was monitored by analytical reverse phase (C-4 Phenomenex) gradient elution (0–70% H2O/acetonitrile containing 0.1% trifluoroacetic acid, 1 min/mill; flow rate, 1 ml/min). When complete, the mixture was acidified (trifluoroacetic acid), and the desired heterodimer protein was separated from the homodimers also formed via semi-preparative reverse phase HPLC (column, C-8 Dynamax; gradient, 40–70% H2O/acetonitrile containing 0.1% trifluoroacetic acid, 1 min/mill; flow rate, 1 ml/min). When the mixtures remained as precipitates after acidification, they were resolubilized in 6 M guanidine HCl prior to HPLC purification. After removal of the acetonitrile (N2), purified fractions were stored either frozen or diluted 1:1 with protein-containing bioassay media (see below).

Analytical reverse phase HPLC was performed both on C-4 Phenomenex (as described) and C-18 Vydac columns (gradient, 0–90% H2O/acetonitrile containing 0.1% trifluoroacetic acid, 1 min/mill; flow rate, 1 ml/min).

Analytical gel filtration was performed on a Waters Protein Pak 125 column (7.8 × 300 mm) calibrated with lysozyme (14.3 kDa), β-lactalbumin (18.4 kDa), trypsin inhibitor (20.1 kDa), glycerol dehydrogenase (36.1 kDa), and bovine serum albumin (68 kDa) in 0.1% aqueous trifluoroacetic acid (flow rate, 0.5 ml/min).

Synthetic peptides were prepared as described (22) on a Biosearch 9500 solid phase peptide synthesizer. The recombinant protein IL-2(1–99) Gly was prepared as described (22). All of the segments were purified to homogeneity prior to incorporation into semi-synthetic proteins by semi-preparative reverse phase HPLC (see above).

Amino acid composition was verified by amino acid analysis (PICO-TAG®).

Circular Dichroism—Far ultraviolet CD spectra were obtained on an Instruments SA Joby Vvon circular dichrograph calibrated with (+)-10-camphorsulfonic acid and epiandrosterone. Measurements were taken at 25, 50, and 80 °C ± 0.1 in 0.1% TFA, pH 2.0. (Adjustment of solutions of these derivatives to pH 7.0 lead to excessive precipitation of the semi-synthetic proteins, however. spectra (not shown) obtained for recombinant IL-2 at pH 5.0 and 7.0 did not significantly differ from those obtained at pH 2.0.) Protein concentrations were determined by quantitative amino acid analysis (PICO-TAG®) on aliquots taken from the sample cells.

IL-2 CTL-L-2 Biosay—IL-2 bioactivity was assayed as previously described (25). Protein samples were diluted (1:1) into supplemented RPMI 1640 media containing 10% fetal calf serum and then dialyzed overnight against three changes of 25 volumes of the same media prior to the assay. These stock samples were then diluted (1:100 and 1:500) into media containing 10% fetal calf serum and titrated into 96-well microtiter plates using triplicate rows for each dilution. Protein concentrations were determined for the undiluted samples by quantitative amino acid analysis. For the bioassays of recombinant IL-2 (Takeda Chemical Co., Japan) carried out in the presence of the semi-synthetic proteins (Fig. 3B), conditions were the same as above except that IL-2 (0.1 nM) and the semi-synthetic derivative to be studied (400–500 nm) were added simultaneously in the first well of a 96-well microtiter plate and then were serially diluted (2-fold) across the plate.

To confirm solubility of the proteins in the bioassay media at physiologic pH, protein samples were diluted 1:1 in media containing 2% fetal calf serum, and the pH was adjusted to 7.2 by addition of Na2HPO4. The amounts of semi-synthetic proteins in solution (retention times 58–64 min) were monitored by reverse phase HPLC (C-18 Vydac column as described) both before and after incubation at 37 °C for 60 min by integration of peak area.

Human Lymphocyte Biosay—The assay was performed as described (29). Human peripheral mononuclear cells were isolated from heparinized blood by Ficoll-Hypaque centrifugation. The T-lymphocyte-containing fraction was activated using monoclonal anti-T3 (OKT3; Ortho Pharmaceutical Corp.) at a 1/10,000 final dilution in supplemented RPMI 1640 medium containing 10% fetal calf serum for 3 days. The cells were then washed free of IL-2 produced in situ and cultured for an additional 40 h in IL-2-free medium to allow for their accumulation in the early G1 phase of the cell cycle.

The cells (which still express high affinity IL-2 receptors) were then harvested and cultured for 24 h in 96-well microtiter plates (1 × 10^6 cells/well) in the presence of the appropriate semi-synthetic protein (prepared as described for the CTL-L-2 assay) or of a recombinant IL-2 standard. The extent of proliferation was monitored during the last 4 h of culture by incorporation of tritiated thymidine (2 μCi/ml). Cells were harvested on glass fiber filters with an automated harvester and were counted by liquid scintillation.

IL-2 Competitive Binding Assay—Competition for IL-2 binding to the high affinity IL-2 receptor by the semi-synthetic proteins was determined using the radiolabeled IL-2 binding assay as described (31). Protein samples were prepared as described for the CTL-L-2 bioassay.

RESULTS

Previously, we have shown (22) that an active semi-synthetic IL-2 derivative could be prepared from an inactive recombinant segment and an inactive synthetic peptide which included the C-terminal helix by formation of the Cys-58-Cys-105 disulfide. Additionally, by making 12 simultaneous residue substitutions designed to first stabilize and then destabilize this helix, semi-synthetic proteins were obtained with altered activity. These results demonstrated the critical importance of the C-terminal helix in stabilizing the biologically active tertiary conformation.

To rapidly evaluate the significance of several selected residue substitutions in this region prior to the incorporation of some of these into fully recombinant mutants, we have examined four additional semi-synthetic proteins. Fig. 1 depicts the sequences of the synthetic peptide segments used to prepare these proteins as well as the influence of residue patterns on the amphiphilicity of the C-terminal helix. As in our previous study (22), each protein has been assembled from a recombinant segment comprising the first 99 residues of IL-2 and a synthetic peptide incorporating multiple residue replacements (with respect to the authentic sequence) linked via a disulfide bond between Cys-58 and Cys-105. Variant I (Fig. 1) was prepared from a peptide which included the same multiple substitutions as our previous stabilized derivative (22) except that the Pro residues 117 and 124 were substituted with Leu residues. These changes were introduced in an attempt to help evaluate the participation of the aromatic side chains in core structural interactions. Variant II incorporates a Thr-123 to Gln replacement (instead of Thr → Ala as in Ref. 22) to increase helical amphiphilicity as well as a Thr-135 → Lys exchange to help stabilize the inherent helical dipole (26). Variants III and IV include Pro at positions 119 and 127, respectively, in order to investigate what singular contribution a proline at each of these positions might have toward destabilizing the C-terminal helix. We previously observed that the presence of Pro residues at both positions reduced helical content and inactivated the protein (22).

Preparation of the Semi-synthetic Protein—Each of these semi-synthetic proteins was prepared from prepurified segments via air-mediated disulfide formation. The desired het-
Fig. 1. A, the amino acid sequence of the C-terminal region of IL-2 and the redesigned derivatives I-IV. The segments illustrated correspond to the final 33 residues of IL-2 and represent the synthetic peptide portion of the semi-synthetic proteins. For derivatives I-IV, only those residues which are not homologous to the authentic sequence are indicated. Residue substitutions were based on helical propensity (27). Regions within the box correspond to the C-terminal α-helix (28). B, helical wheel projections of the C-terminal helix of IL-2 (left) and derivatives I-IV (right) demonstrating amphiphilicity. Boxed residues are hydrophobic, underlined residues are hydrophilic with the remaining residues very hydrophilic (28). The helical wheel on the right represents the sequence of derivative II with substitutions indicated at the appropriate positions for derivatives I, III, and IV.

### Table I

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<th>Variant (RS)</th>
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The yields of semi-synthetic IL-2 analogs I-IV and the C-4 reverse phase HPLC retention times (see "Materials and Methods") for the recombinant (R) and synthetic (S) monomers as well as the desired heterodimeric (RS) and associated homodimeric (RR, SS) reaction products.

Since our model predicted the C-terminal helix to include residues 116 to 132 and because assignment of helix termini tends to be the least accurate, we extended the region of modification from 114 to 133. This could also contribute to the observed increase in helicity.

By monitoring changes in the CD spectrum at 50 and 80 °C, it is clear that IL-2 (Fig. 3B) is a thermally stable protein. The spectrum at 80 °C indicates only a minor loss of helical secondary structure. Similar stability was observed for each of the semi-synthetic derivatives (Fig. 3, C and D). Although the proline-destabilized proteins exhibited slightly less dichroic absorption at 222 nm than did the stabilized derivatives (e.g. β22 for II and IV = 2.5 and 2.3 × 10⁴ degree cm² dmol⁻¹, respectively), they also maintained considerable helical structure at 80 °C. Therefore, since all of these derivatives possess stable tertiary conformations, any differences observed in biologic assays cannot be attributed to gross denaturation.

To assess whether aggregation could be contributing to stabilization of secondary structure as monitored by CD, the molecular size of the hybrid proteins at similar concentrations and in the same buffer as employed for CD analysis was compared to that of recombinant IL-2 by HPLC gel filtration. Recombinant IL-2 eluted as a protein of 18-20 kDa (7.6 ml), indicating that they were predominantly monomeric under the conditions in which the CD spectra were obtained.

The CTLL-2 Bioassay—The biologic activity of the semi-synthetic proteins was first examined using the murine IL-2-dependent CTLL-2 cell line (25). As depicted in Fig. 4A, both stabilized sequences I and II exhibited an EC₅₀ (16 nm) equivalent to that previously determined for the semi-synthetic protein possessing the authentic C-terminal sequence (22). The proteins having single destabilizing proline residues in the C-terminal helix either possessed greatly reduced activity (III, Pro-119) or were completely inactive (IV, Pro-127).

When the CTLL-2 assay was carried out with recombinant IL-2 in the presence of large excesses of the semi-synthetic derivatives (Fig. 4B), relative agonist/antagonist activities...
were revealed. For example, in the presence of stabilized derivative II, the dose-response curve shifted to the left indicating partial agonist activity as would be expected from the results of the bioassay with derivative II alone. However, when destabilized derivative IV was employed, the maximal response for IL-2 was reduced by 50%, suggesting partial antagonist activity.

When the stock samples were prepared by diluting 1:1 into assay media containing 10% fetal calf serum, no precipitation was observed at physiologic pH. To confirm that the derivatives remained in solution over the period of the bioassays, their presence in solution was monitored by C-18 reverse phase HPLC. In media containing as little as 2% fetal calf serum, greater than 90% of the derivatives remained soluble after 30 h at 37 °C as determined by integration of peak area.

The Human T-Cell Bioassay—Although the CTLL-2 assay is quantitative and highly reproducible, the cell line is of murine origin. Since we are investigating derivatives based on the human sequence and the precise nature of the multi-component IL-2 receptor system is still not completely understood, we have also examined the semi-synthetic proteins in an assay employing cell cycle synchronized human peripheral blood lymphocytes (HPBL) (29). The results of this assay, although qualitatively similar to the CTLL-2 assay, reveal important
A Strategy for Protein Design

**FIG. 4.** A, CTLL-2 biologic assay of derivatives I (■), II (●), and III (▲). The dotted line represents activity exhibited by a semi-synthetic protein possessing the authentic IL-2 C-terminal sequence (22). The activities are expressed as a percent of maximum [3H]thymidine ([3H]TdR) incorporation elicited by recombinant IL-2 at 0.2 nM. Each point represents the mean of triplicate determinations. Derivative IV failed to induce [3H]thymidine incorporation above background levels at concentrations 10⁶-fold higher than levels of recombinant IL-2 required to induce a detectable response. B, CTLL-2 bioassay of recombinant IL-2 (▲) in the presence of semi-synthetic derivatives I1 (●), or IV (+). Protein ratios in the mixed assays were constant starting with concentrations of: IL-2, 0.1 nM; derivative I1, 450 nM; derivative IV, 400 nM. Shifts in the dose-response curve of IL-2 by derivatives I and III were similar to those produced by derivatives II and IV, respectively.

**FIG. 5.** A, human peripheral blood leukocytes biologic assay of derivatives I (■), II (●), III (▲), and IV (+) expressed as a percent of maximum [3H]thymidine incorporation elicited by recombinant IL-2 at 0.2 nM. Each point represents the mean of triplicate determinations. B, results of the human peripheral blood leukocytes assay expressed in double-reciprocal fashion. Derivatives I (■), II (●), III (▲), and IV (+). Derivative II exhibited stronger competition than did I or III, whereas the competitive ability of IV was much weaker.

**DISCUSSION**

Interleukin-2 has played a key role in helping to elucidate the complexities associated with the T-cell-dependent immunologic response. Additionally, it has been the focus of several structure-function studies and typifies the challenges encountered when proteins are employed as pharmaceuticals. Although x-ray diffraction data is now available (23) and numerous structure activity investigations have been reported (7–11, 15–17), the precision of the results has not yet allowed the design of IL-2 agonists and antagonists based on the knowledge of which residues interact with the various components of the high affinity receptor complex.

We have employed computer-assisted methods to develop a model for IL-2 tertiary structure (22) and have investigated the validity of aspects of the prediction using a semi-synthetic design approach. In this study we have utilized the same approach to rapidly evaluate multiple modifications of the protein structure which could lead to the development of IL-2 agonists or antagonists via conformational perturbation of the binding region rather than mutagenesis of specific receptor binding residues.

The use of semi-synthesis has allowed an evaluation of multiple residue substitutions on the conformations and bioactivity of IL-2. It has afforded a rapid assessment of...
several design elements which now can be incorporated into fully recombinant analogs. The system is not ideal in that the activity of the reconstituted proteins does not equal that of natural or recombinant IL-2 (e.g. EC_{50} = 14 nM for II versus 10 pm for rIL-2 in the CTLL-2 bioassay). This may result from reduced affinity for individual receptor proteins or less efficient signal transduction due to increased conformational flexibility. However, since all of the analogs were homogeneous and of defined concentration and stability, quantitative assessments can be made when compared to a semi-synthetic protein with the authentic sequence (22).

Examination of the same series of proteins using cassette mutagenesis and fully recombinant proteins could have been utilized initially; however, the effort and problems commonly associated with customizing a gene, transformation, and expression of protein followed by purification and refolding would have certainly made the approach much more tedious.

The integrity of C-terminal α-helix of IL-2 is critical for biologic activity. Previous studies had demonstrated that modifications (11) or substitution (17) of Trp-121 as well as deletion of Phe-124, Cys-125, or Gln-126 (16) all result in the loss of bioactivity as measured in the CTLL-2 bioassay. Without structural data, these observations have led some investigators to suggest that this region contains receptor binding residues (17). Although this cannot be rigorously excluded, x-ray diffraction data has verified that this region is α-helical and has corroborated our multiple residue substitution studies (22). Therefore, the earlier observations can now be attributed to destabilization of important secondary structure.

The residue substitution patterns shown in Fig. 4A were chosen to investigate the influence of specific residues on helical stability and how these changes could affect biologic activity. Conversion of phenylalanines 117 and 124 to Leu (I) would preserve amphiphility and helical tendency but would be expected to influence core steric interactions if the aromatic side chains are involved. Improvement of amphiphility (Thr-123 to Gln) and accommodation of the helical dipole (Thr-133 to Lys) in derivative II could be expected to result in more stable secondary structure. Singular substitutions of Pro at positions 119 or 127 would help determine which of the residues has the greatest destabilizing influence and suggest targets for the design of potential antagonists.

Examination of the resulting semi-synthetic proteins by far UV circular dichroism (Fig. 3) indicated a greater helical content than recombinant IL-2 (see "Results") for all of the derivatives. The Pro-destabilized derivatives display smaller but detectable decreases in helical content with respect to the stabilized sequences. Thermal denaturation studies failed to reveal any significant influence of residue substitution patterns on stability of the semi-synthetic derivatives. However, this is most likely a reflection of the fact the IL-2 possesses an extremely stable tertiary conformation even when assembled from two inactive segments. Minor modifications may perturb local conformation yet do not greatly influence overall stability. This is fortunate since effects of these modifications on biologic activity cannot be attributed to gross denaturation or the loss of the ability to correctly fold. Furthermore, it demonstrates that it is possible to make multiple residue replacements which greatly influence biologic activity but still result in a stable protein.

When the derivatives were examined in the CTLL-2 bioassay (Fig. 4A), both proteins having stabilizing sequences (I and II) exhibited activity identical to that previously determined for a semi-synthetic protein assembled from components having the authentic IL-2 primary sequence (22). Destabilizing the C-terminal helix by substitution of Pro residues at positions 119 (III) or 127 (IV) significantly lowered or completely abrogated activity. The influence of these derivatives on the dose response of recombinant IL-2 (Fig. 4B) suggests that at high concentrations all of the derivatives interact with the IL-2 receptor on CTLL cells resulting in shifts in the dose-response curve typical for partial agonists (e.g. II) and antagonists (e.g. IV). Evaluations of these results with respect to the design of IL-2 agonists/antagonists is complicated by the fact the CTLL-2 cell line is of murine origin. Since murine IL-2 has little activity in human T-cell assay systems (18) and the human IL-2 high affinity receptor is composed of at least two IL-2 binding proteins, only one of which has been sequenced (1, 24), the cross-species CTLL-2 bioassay may not be the most appropriate for quantitative structure-activity studies. In fact, when the modified proteins were examined in a cell cycle synchronized human T-cell assay (28), important differences were noted (Fig. 5A). Derivatives I and II were no longer equal in activity. The substitution of leucines for Phe-
117 and -124 in I resulted in a less active protein indicating potential induction of unfavorable core steric interactions. The influence of the Pro substitutions also differed. Substitution of Pro at position 119 resulted in a derivative with much greater activity than did substitution at position 127. Therefore, substitutions at position 127 are more likely to influence conformation at the binding site(s) and thus bioactivity. The results of the HPBL assay displayed in a double-reciprocal plot generates a series of lines having a spectrum of slopes (Fig. 5B). This clearly demonstrates how the proliferative activity can be modulated over a wide range by manipulating a single region of secondary structure. These differences are a result of apparent changes in the $K_d$ for these derivatives (different x intercepts). The differing affinities of IL-2 receptor complex were demonstrated in a competitive binding assay (Fig. 6). The ability of each derivative to compete with radiolabeled IL-2 for its high affinity receptor corresponded to their ranking in the biologic assay. The conclusions arrived at using these methods represent a step toward the rational design of totally recombinant IL-2 derivatives having agonist and antagonist characteristics induced by conformational perturbation.

REFERENCES