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Structural Analogs of Interleukin-2: A Point Mutation that Facilitates Biological Response

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SUMMARY

Interleukin-2 (IL-2) is an immunoregulatory cytokine whose biological effects are mediated through interaction with specific receptors on the surface of target cells. Due to its presumed role in generating a normal immune response, IL-2 is being evaluated for the treatment of a variety of tumors, in addition to infectious diseases. During the study of the structure-activity relationships for IL-2 and its receptors, one analog in which threonines at positions 41 and 51 were replaced by prolines (T41/51P) was found to possess apparent signaling abnormalities. Bioassays and receptor binding assays with human peripheral blood lymphocytes revealed the EC_{50} and K_d values of this analog to be 200 pM and 5.9 nM, respectively. Although the EC_{50} is greater and the receptor affinity of T41/51P is much weaker than that of wild-type IL-2, receptor occupancy versus biological response comparisons indicated that a much lower receptor occupancy was required to generate an equivalent biological response. Competitive receptor binding analyses with both intermediate affinity (β/γ subunit complex) and low affinity (α subunit) receptors were carried out to assess the origin of this phenomenon. Similar analyses of the singly substituted T41P and T51P analogs were carried out. From these studies, it was apparent that facilitated signaling was mainly attributable to position 51, whereas mutations at position 41 primarily influenced low affinity binding. The observation that the T51P analog facilitates response, compared with wild-type IL-2, may indicate a signaling-dependent conformational change in IL-2 upon receptor binding.

IL-2 is a 15-kDa glycoprotein that belongs to a growing family of structurally related lymphokines (1, 2). It is secreted by T lymphocytes upon antigen stimulation and functions in both autocrine and paracrine manners as a growth factor. IL-2 also regulates activities of natural killer cells and B cells (3). Several clinical studies have shown that IL-2 can be effective in the treatment of melanoma, renal cell carcinoma, and hematopoietic malignancies (4). In addition, the utility of IL-2 for the treatment of infectious disease is being investigated (5).

The development of structure-activity relationships for IL-2 has proven challenging, due to the multimeric nature of the IL-2R (6–8). The IL-2R is composed of at least three cell surface subunits, the 55-kDa subunit (α subunit) (9–11), the 75-kDa subunit (β subunit) (12), and the 64-kDa subunit (γ subunit) (13, 14). These three chains cooperate to form the high affinity IL-2R complex ($K_d \approx 10^{-11}$ M), whereas the α chain alone and the β/γ complex form low affinity ($K_d \approx 10^{-8}$ M) and intermediate affinity ($K_d \approx 10^{-9}$ M) receptor sites, respectively. The α chain contains only 13 amino acid residues in its cytoplasmic domain and apparently displays no ability to transduce intracellular signals. Rather, it forms a heteromeric complex with the β subunit that serves to capture IL-2 on the surface of activated T cells (15). In contrast, the β and γ subunits associate in a ligand-dependent fashion and form the presumed signaling complex (13). The β and γ subunits (but not the α subunit) belong to the large hematopoietin family of cell surface receptors (2, 11, 16).

Thus far, most of the structure-activity relationship studies of IL-2 have focused on the search for amino acid residues that directly interact with receptor sites (17–21). Knowledge of these contact residues would provide insight into IL-2 function and could lead to the rational design of IL-2 analogs with enhanced pharmacological properties. In this study, we report a series of IL-2 analogs generated in an attempt to specifically alter the structure of the protein. Earlier studies suggested that conformational perturbation by insertion of proline residues into α-helical segments of the protein could influence the signaling characteristics of IL-2 (22). This study was designed to further test that observation by insertion of proline residues into the B and B′ helices of IL-2 based on the 3-Å X-ray structure

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ABBREVIATIONS: IL-2, interleukin-2; PBL, peripheral blood lymphocyte; HPLC, high performance liquid chromatography; IL-2R, interleukin-2 receptor.

reported in 1987 (23). Based on that model, threonine residues at postions 41 and 51 were selected for replacement by proline. After this analog was prepared, however, the 3-Å structure was shown to be seriously in error, with almost 90% of the peptide backbone being incorrectly traced (24, 25). The corrected structure located positions 41 and 51 outside helical segments. Nevertheless, the biological properties of an analog containing proline residues at both positions proved interesting. Analysis of high affinity receptor binding and biological response on the same population of activated human T lymphocytes revealed that, although this protein displayed weaker affinity than did wild-type IL-2, it generated significantly greater response at the same receptor occupancy. Additional analogs were prepared to further investigate the origin of this effect. This is the first observation of facilitated response for this family of cytokines and suggests that receptor binding and triggering are at least partially separable in this system.

Materials and Methods

Preparation and characterization of IL-2 analogs. IL-2 analogs were generated via cassette mutagenesis of a synthetic IL-2 gene (26). The proteins were expressed in *Escherichia coli*, refolded, and purified as described previously (22). All proteins were determined to be homogeneous by reverse phase HPLC (4.5-mm × 25-mm DuPont Protein Plus C_{18} column) and monomeric upon size exclusion HPLC (4.5-mm × 25-mm TSK-Gel G3000W_{XL} column; buffer, 25 mM sodium phosphate, pH 7.0, 100 mM KCl). The concentration of purified proteins was determined by UV absorption in 6 M guanidinium chloride ($\epsilon_{280} = 9.53 \times 10^3$ l/m-cm) (27) and, in some cases, quantitative amino acid analysis (Pico-Tag; Waters Inc.).

CD spectra. CD spectra were determined using an Instruments SA Jobin Yvon circular dichrograph calibrated with (+)-10-camphosulfonic acid and epiandosterone. Far-UV CD spectra were measured from 185 nm to 250 nm in a 0.1-cm cell at 25° in 25 mM NaH_2PO_4, pH 4.5. Near-UV CD spectra were measured from 250 nm to 310 nm in a 1.0-cm cell at 25° in 25 mM phosphate buffer, pH 6.5. Each protein sample was filtered (0.45-mm syringe filter) and the protein concentration was determined by UV absorption at 280 nm in 6 M guanidinium chloride. Spectra presented represent an average of three to five scans, after subtraction of a buffer blank.

Cell cultures. Human leukemia cell lines YT-2C2 (28) and MT-1 (29) were maintained in RPMI 1640 medium supplemented with 200 mM L-glutamine, 10% heat-inactivated calf serum (56°, 30 min), 50 μg/ml gentamycin, and 50 units/ml penicillin, in a humidified atmosphere containing 5% CO_2. Normal human PBLs were isolated from fresh blood by Ficoll/Hypaque centrifugation and were maintained in RPMI 1640 medium at a density of 1×10^6 cells/ml as described (30).

Bioassays. The bioactivity of IL-2 analogs was determined with normal human PBLs isolated as described above and stimulated with OKT3 monoclonal antibody (Ortho Pharmaceuticals) for 3 days, followed by 2 days of incubation in the absence of OKT3 (30).

The EC_{50} values for each protein were determined from the bioassay data by nonlinear least-squares fitting to eq. 1 (31). Each data point represents the average of triplicate determinations in three to 10 separate assays employing cells from different donors.

$$E = \frac{E_{max}\, C}{C + EC_{50}} \tag{1}$$

where E is the response observed at concentration C, E_{max} is the maximum response produced by the protein, and EC_{50} is the concentration of drug that produces 50% of the maximum response.

Competitive radio-receptor binding assays. Competitive binding of ^{125}I-IL-2 by the mutant proteins was performed with the same population of activated PBLs as used in the bioassay, after 3–5 days of OKT3 stimulation, as described (15). Competitive binding to intermediate and low affinity receptors was carried out with the YT-2C2 and MT-1 cell lines, respectively, also as described (15). The results were fitted to eq. 2.

$$\frac{B(L)}{B(0)} = \frac{1 + K^*L^*}{1 + K^*L^* + KL} = \frac{1}{1 + pL} \tag{2}$$

where L^* and L are the concentrations of labeled and unlabeled ligands, respectively, K^* and K are the equilibrium constants for the binding of the labeled and unlabeled ligands, respectively, to a receptor site, $B(L)$ is the concentration of bound, radioactively labeled ligand when the concentration of unlabeled ligand is L, and $B(0)$ is the concentration of bound, radioactively labeled ligand in the absence of unlabeled ligand (15). The parameter p is given by the expression

$$p = K/(1 + K^*L^*) = (K_d + K^*L^*/K)^{-1} \tag{3}$$

where K_d is the dissociation constant (15). The parameter p and its deviation were determined by combining data points from three to 10 assays, followed by nonlinear least-squares fitting to eq. 2. K_d values were then calculated from eq. 3.

Results

Strategy. The objective of this study was to further investigate the observation that insertion of proline residues into helical segments of IL-2 could result in minor conformational perturbation accompanied by signaling abnormalites when receptor occupancy was compared with biological response (15, 22). The initial design of the T41/51P analog was based on a 3-Å X-ray structure of IL-2 available at the onset of this study (32). In that model, threonine residues 41 and 51 were located within the B and B′ helixes, respectively. These threonine residues were replaced by two prolines in an effort to disrupt helical structure and examine the influence of these changes on biological activity and receptor binding. During the course of these experiments, the original X-ray structure was questioned, on the basis of structural homology to other cytokines of known three-dimensional structure (24), and was subsequently revised (25). In the corrected structure, both positions 41 and 51 were located outside helical regions. The biological characteristics of the T41/51P analog (see below) were sufficiently interesting to warrant examination of analogs carrying single mutations at these position, despite the unintended structural correlation.

Protein preparation. The cDNA encoding the IL-2 analogs was prepared via cassette mutagenesis of a synthetic IL-2 gene, as descibed (22). Expression, refolding, and purification of the proteins were carried out using techniques that had been optimized for wild-type IL-2 (22). The purity and aggregation state of all of the analogs were monitored by reverse phase and size exclusion HPLC. In all cases, the products were determined to be homogeneous and monomeric at the concentrations used for the bioassays and receptor binding studies (data not shown). Periodic examination of the aggregation state of stock solutions was performed to check for self-association. For all of the proteins examined, no detectable aggregation was noted upon storage.

Biological activity and receptor binding. The ability of IL-2 and the three analogs to mediate the proliferation of normal activated T cells is illustrated by the dose-response curves in Fig. 1. The EC_{50} value of T41/51P as determined from these data was 200 pM, compared with 16 pM for wild-type IL-2 (Table 1). When analogs carrying the single mutations at these positions were examined, the greatest influence on the

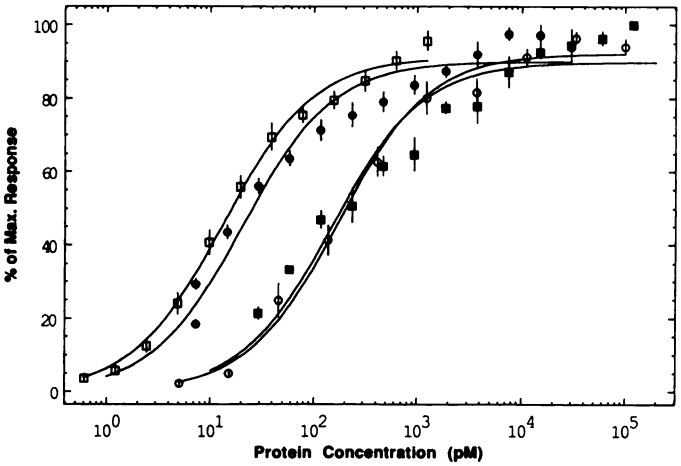

Fig. 1. Human PBL bioassays. The dose-response curves for IL-2 (□) and analogs T41/51P (○), T41P (■), and T51P (●) are shown. Each point represents the mean of three to 10 independent assays, each carried out in triplicate as described in Materials and Methods. Typical maximum response values for [^{3}H]thymidine incorporation were as follows: IL-2, 19,578 cpm; T41P, 21,960 cpm; T41/51P, 19,213 cpm; T51P, 20,254 cpm; background, 144 cpm. The respective EC_{50} values and standard deviations obtained from the data by nonlinear curve fitting are listed in Table 1.

bioactivity was attributable to position 41 (T41P). This analog displayed approximatley the same reduction in activity (EC_{50} = 190 pM) as the double mutant. In contrast, the analog carrying a proline residue at position 51 had nearly wild-type bioactivity (EC_{50} = 26 pM).

Competitive receptor binding assays were carried out on the same population of cells as used in the bioassays, to determine the relative dissociation constants (K_d) at the high affinity receptor on normal T cells. The competition curves for wild-type IL-2, compared with the three analogs, are shown in Fig. 2A. Whereas the K_d value for wild-type IL-2 determined from these data was equivalent to its EC_{50} in the bioassay (Table 1), the relationship of these parameters was quite different for the mutant analogs. For example, in the case of T41/51P the K_d was 30 times greater than EC_{50} value. When this relationship is expressed in the form of a receptor occupancy versus biological response curve over the full dose-response range (Fig. 3), it is evident that far fewer receptors need to be occupied by T41/51P, compared with wild-type IL-2, to generate equivalent bioactivity. This observation is independent of the relationship between the dissociation constants for these two proteins, because T41/51P has significantly weaker receptor affinity.

Because the receptor binding assays were performed on day 3 OKT3-activated cells due to the greater number of high affinity receptor sites and the bioassays were performed on day 5 OKT3-activated cells to eliminate background proliferation, it was possible that the timing of the two assays contributed to the observed effects. To exclude this possibility, we determined the high affinity K_d of T41/51P with day 4 and day 5 OKT3-activated cells. The K_d values for this protein obtained with day 4 and day 5 cells were 3.8 ± 0.08 nM and 7.7 ± 1.7 nM, respectively. These values compare favorably with the value obtained with day 3 activated cells (5.9 ± 0.08 nM) (Table 1), indicating that the measured K_d of the high affinity site for this analog is not dependent upon the time after activation. Likewise, the receptor occupancy versus biological response relationship determined for T41/51P with day 5 cells (Fig. 3A) is similar to that obtained with day 3 cells (Fig. 3B).

When the same occupancy-response relationships were examined for the analogs possessing only a single mutation (Fig. 3B), it was evident that the proline for threonine exchange at position 51 makes the greatest contribution to the apparent response enhancement. Although the T41P analog displayed a detectable difference in occupancy versus response, it was much less significant than that for the position 51 analog.

To further assess the origin of this phenomenon, we carried out competitive receptor binding assays on two human leukemia cell lines, YT-2C2 (28) and MT-1 (29), that express intermediate (β/γ) and low (α) affinity receptor sites, respectively. As depicted in Fig. 2B, the competition curves for binding to MT-1 cells were shifted significantly rightward for both T41/51P and T41P, whereas competition by T51P was similar to that by wild-type IL-2. Therefore, it is evident that replacement by proline at position 41 greatly reduced the affinity of IL-2 for the low affinity receptor on these cells (Table 2).

In contrast, the differences in binding to the intermediate affinity receptor were not as significant (Fig. 2C). The K_d values obtained from these competitive binding assays differed only about 2–4-fold from those for the wild-type protein (Table 2). Thus, mutations at position 41 or 51 did not have a great influence on binding to the β/γ receptor complex.

CD spectra. When the CD spectra of all three analogs in the far-UV region were compared with the spectrum of wild-type IL-2, no significant changes were observed (data not shown), indicating that the secondary structure of the protein was unaltered. This is not surprising, considering that the locations of the mutations are outside the helical segments of the protein. In contrast, when the near-UV CD spectra were examined important differences were revealed (Fig. 4). Although the T41P analog provided a CD spectrum similar to that of wild-type IL-2, both T51P and T41/51P displayed significantly different near-UV CD spectra. These results indicate that proline at position 51 of these mutants sufficiently alters the tertiary conformation of the protein to perturb the asymmetric environment surrounding tryptophan at position 121, the major aromatic chromophore contributing to the CD signal in this region.

Discussion

At the onset of this study, our goal was to examine the influence on tertiary conformation and bioactivity of placement of proline residues in the B and B′ helices of IL-2. Earlier

TABLE 1

Comparison of K_d and EC_{50} values

The EC_{50} and K_d values were determined by bioassays and receptor competitive binding assays, as described in Materials and Methods. The values are expressed as mean ± standard error (three to 10 independent assays). The standard errors for K_d values were determined from the standard deviation for p.

	IL-2	T41/51P	T41P	T51P
EC_{50} value (M)	$1.6 \pm 0.05 \times 10^{-11}$	$2.0 \pm 0.07 \times 10^{-10}$	$1.9 \pm 0.05 \times 10^{-10}$	$2.6 \pm 0.06 \times 10^{-11}$
K_d value (M)	$1.6 \pm 0.02 \times 10^{-11}$	$5.9 \pm 0.08 \times 10^{-9}$	$2.9 \pm 0.04 \times 10^{-10}$	$1.3 \pm 0.02 \times 10^{-10}$

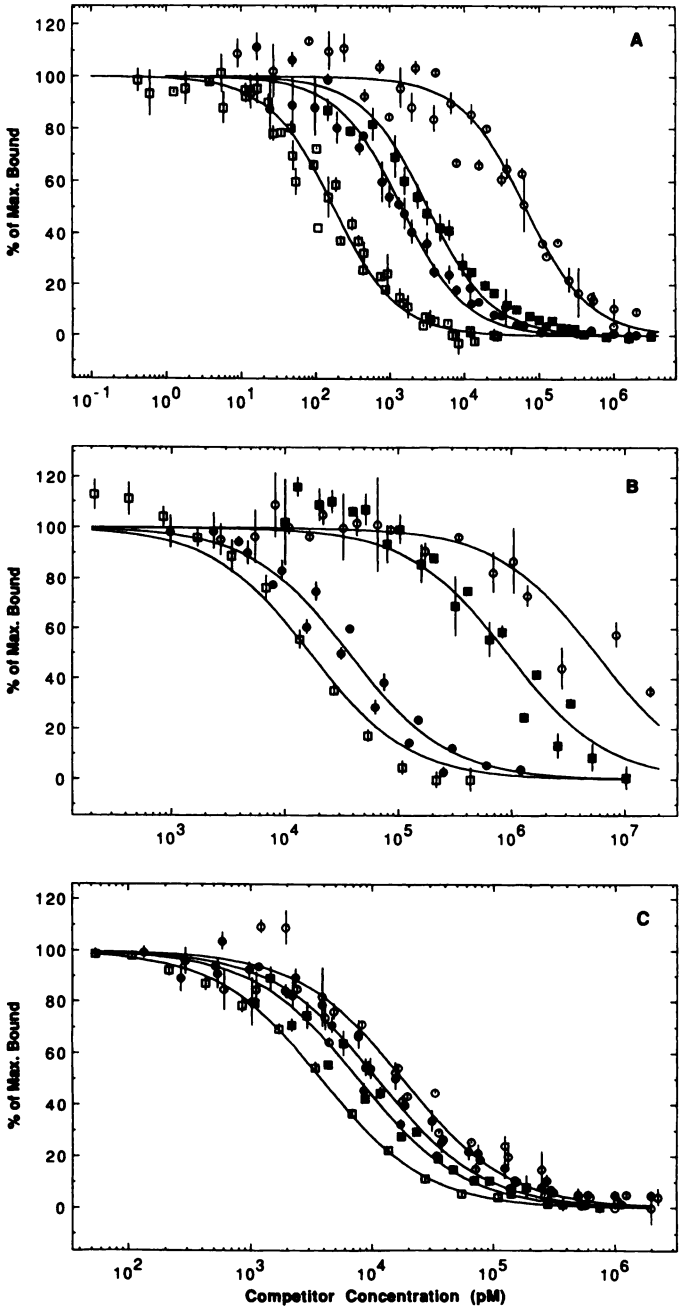

Fig. 2. Competitive radioligand receptor binding. The competitive binding curves for IL-2 (□) and analogs T41/51P (○), T41P (▨), and T51P (●) at the high affinity receptor on activated human T lymphocytes (A), at the low affinity (α) receptor sites on MT-1 cells (B), and at the intermediate affinity (β/γ) receptor sites on YT-2C2 cells (C) are shown. Each *point* represents the mean of three to 10 independent assays, each carried out in triplicate as described in Materials and Methods. The respective dissociation constants and standard deviations obtained from the data by nonlinear curve fitting are listed in Table 2.

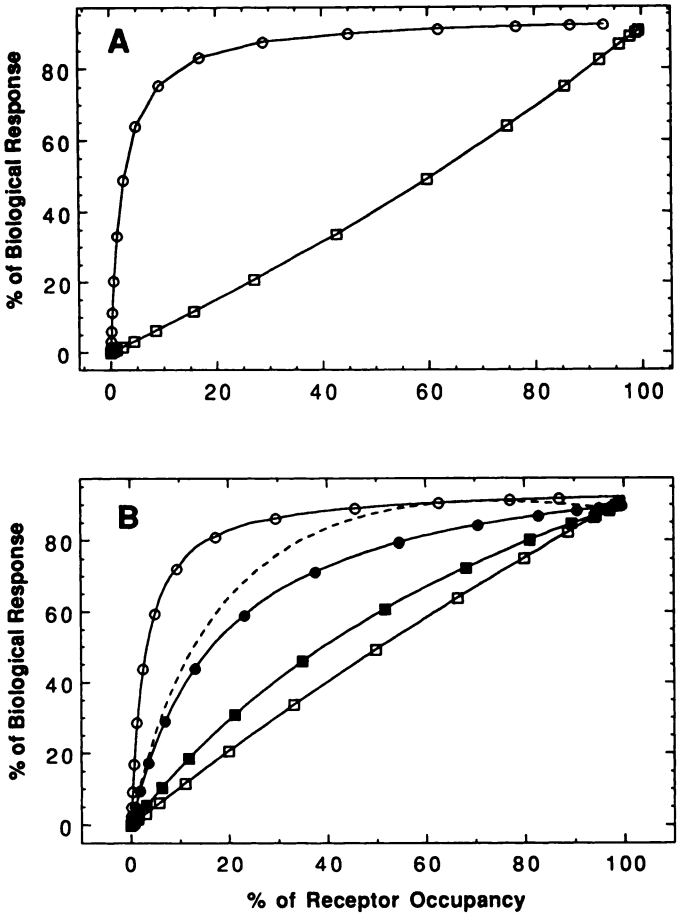

Fig. 3. Receptor occupancy versus biological response curves. The receptor occupancy versus response curves for IL-2 (□) and analog T41/51P (○) where both high affinity K_d and EC_{50} values were determined on day 5 activated cells (A) and for IL-2 (□) and analogs T41/51P (○), T41P (▨), and T51P (●) where the high affinity K_d values were determined on day 3 activated cells (B) are shown. *Dotted line*, sum of T41P and T51P. The receptor occupancy was calculated from the following equation: occupancy = [ligand]/(K_d + [ligand]).

studies had indicated that this approach, when applied to the carboxyl-terminal helix of the protein, resulted in analogs with altered signaling properties (15, 22). We chose threonine residues at positions 41 and 51 for replacement by proline based on the reported 3-Å X-ray structure (23). Subsequent revision of this structure revealed that both of these positions are located outside helical segments. Threonines 41 and 51 are located in a loop connecting the A and B helices. Thus, the peculiar observation that replacement of these residues with proline had little influence on the overall helical content, as judged by far-UV CD analysis, was resolved by their true placement.

Comparison of the biologcal activity and receptor binding properties of the T41/51P analog (Table 1) suggested that insertion of proline residues outside helical regions in the protein also resulted in altered signaling properties. Unlike the earlier carboxyl-terminal analogs that suffered reduced signaling capabilities, this protein possessed an apparent enhancement in signaling characteristics, in that a response equivalent to that to wild-type IL-2 was observed at much lower receptor occupancy (Fig. 3). For example, only 15% receptor occupancy was required to generate >80% of the biological response, compared with the linear relationship observed for wild-type IL-2. This observation was highly reproducible with samples from multiple protein preparations in several independent assays using T cells from a variety of different donors.

To determine whether the origin of the facilitated response resided in either position 41 or 51 alone, we prepared each of the singly substituted analogs (T41P and T51P). A similar comparison of receptor occupancy at the high affinity site versus biological response, using the same population of T cells

TABLE 2
Dissociation constants

Cells	K_d			
	IL-2	T41/51P	T41P	T51P
		M		
Human PBL	$1.57 \pm 0.02 \times 10^{-11}$	$5.94 \pm 0.08 \times 10^{-9}$	$2.91 \pm 0.04 \times 10^{-10}$	$1.30 \pm 0.02 \times 10^{-10}$
YT-2C2	$2.55 \pm 0.05 \times 10^{-9}$	$1.20 \pm 0.01 \times 10^{-8}$	$5.17 \pm 0.08 \times 10^{-9}$	$7.39 \pm 0.09 \times 10^{-9}$
MT-1	$1.35 \pm 0.02 \times 10^{-8}$	$5.28 \pm 0.12 \times 10^{-6}$	$8.8 \pm 0.133 \times 10^{-7}$	$3.50 \pm 0.05 \times 10^{-8}$

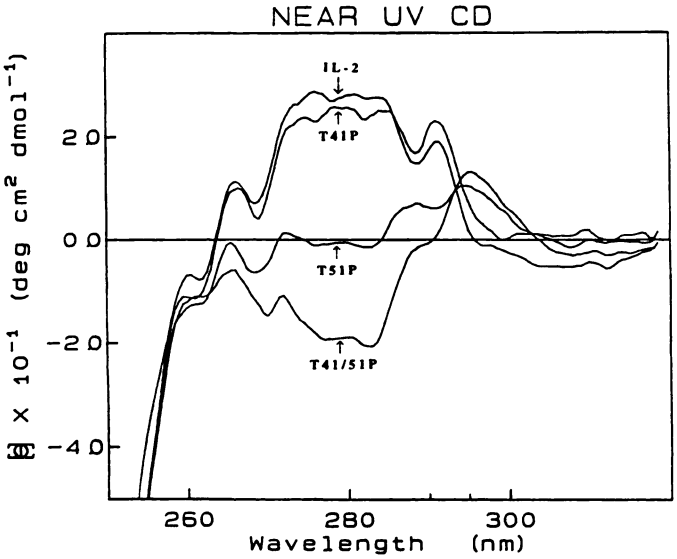

Fig. 4. Near-UV CD spectra. The near-UV CD spectra of the four proteins examined in this study were determined as described in Materials and Methods, over the range of 250–320 nm.

(Fig. 3B), revealed that the origin of this phenomenon is mainly attributable to position 51. Although some nonlinearity is also observable for T41P, the effect is minor, compared with the results for the analogs possessing proline at position 51. In addition, the effects of the individual mutations are not simply additive (see Fig. 3B). Of note is the linear occupancy-response relationship exhibited by the wild-type protein (this is also indicated by the close correspondence of the EC_{50} and K_d values in Table 1), suggesting that during T cell activation no spare high affinity IL-2Rs exist.

To further characterize the receptor binding properties of these analogs, interactions with the low and intermediate affinity IL-2Rs were examined. This analysis revealed that the large decrease in affinity for the high affinity site observed for the T41/51P analog (Fig. 2A) was principally a result of the position 41 mutation. Furthermore, this substitution affected binding primarily to the low affinity receptor (Fig. 2C). The observation that a mutation at threonine 41 should selectively influence low affinity binding is consistent with previous reports locating several residues in the position 35–43 region as potential α subunit contact sites (20, 33). Unlike those earlier studies, in which mutations in this region had little effect on bioactivity, the T41P analog suffered a 10-fold reduction in potency. The fact that the T41P analog showed an 80-fold reduction low affinity binding, whereas binding to the high affinity receptor was reduced only 18-fold, provides additional evidence that at least the α and β subunits of the IL-2R are preassociated on the cell surface before ligand binding. A similar reduction in binding affinity for both sites would be expected if interaction with the α subunit were a prerequisite for high affinity binding, as suggested by others (34).

The approximately 10-fold reduction in high affinity binding observed for the T51P analog was a result of lesser reductions at both the low and intermediate affinity sites (Fig. 2, B and C). Residue 51 is located in a seven-residue loop between a short β-strand and the beginning of helix B (25). This region has not been implicated as a contact area for any of the receptor subunits.

For T51P, high affinity receptor binding was reduced almost 10-fold, whereas the EC_{50} for this analog decreased <2-fold. Thus, the ability of the protein to mediate T cell proliferation does not correspond to its dissociation constant at either the high affinity site or the β/γ subunit intermediate affinty site (the presumed signaling complex). Therefore, this analog possesses an apparent facilitated response. The origin of this effect is unclear. One explanation is that receptor signaling in this system requires a conformational change in the ligand that is partially mimicked by the mutation to proline at position 51. If this is the case, then such a conformational change may facilitate β/γ subunit cross-linking. Consistent with this model is the finding that, unlike T41P, the T51P analog does possess altered tertiary structure, as revealed in the near-UV CD spectrum (Fig. 4). An alternative explanation is that the proline 51 mutation slows the internalization rate for the ligand-bound receptor complex, allowing a greater signaling interval before endosomal dissociation. For the IL-2R system, internalization may be the rate-limiting step in signal transmission across the membrane (7, 35). Whether the facilitated response of T51P is also observable as enhanced receptor signaling is not addressed by the data in this study. It should be emphasized that receptor occupancy-response relationships are defined by experimentally determined K_d and EC_{50} values. In this study, although the same cells were used for both determinations, receptor binding was carried out over 1–2 hr, whereas bioactivity was measured in a 48-hr assay. Thus, any direct receptor signaling enhancement may be amplified or attenuated before the response measurement. Studies are ongoing to resolve the mechanism of this effect.

Regardless of molecular origin, enhancement of biological response may prove useful in the development of more potent IL-2 analogs. An improvement in potency has been achieved by increasing the receptor binding affinity of IL-2, although the increase was minor (17). The ability to increase response in a fashion independent of receptor binding affinity has yet to be reported for any cytokine. Although T51P remains less potent, its activity does approach wild-type levels. An understanding of the mechanism of response enhancement may lead to analogs with potencies greater than that of wild-type IL-2. Finally, combinations of mutations that increase both receptor

affinity and response could lead to analogs that possess activites greater than those achievable by either route alone.

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