Protein Science (1997), 6:1777–1782. Cambridge University Press. Printed in the USA.

FOR THE RECORD

Multiple conformations of a human interleukin-3 variant

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(RECEIVED March 5, 1997; ACCEPTED April 30, 1997)

Abstract: Interleukin-3 (IL-3) is a cytokine that stimulates the proliferation and differentiation of hematopoietic cells. The hyperactive hIL-3 variant SC-55494 was shown to have at least two major conformations by high-resolution NMR spectroscopy. Mutants of SC-55494 were constructed in which alanine was substituted for proline in order to test the hypothesis that proline *cis-trans* isomerization is the source of the observed conformational heterogeneity, as well as to evaluate the effect of prolyl peptide bond configuration on biological activity. NMR spectra of four single proline-to-alanine mutants (P30A, P31A, P33A, and P37A) retain doubled resonances, while spectra of the double mutant P30A/P31A and the quadruple mutant P30A/P31A/P33A/P37A are substantially free of heterogeneity. These observations suggest that the two major conformations in SC-55494 correspond to *cis* and *trans* isomers of either or both of the R29-P30 and P30-P31 peptide bonds. All six mutants had somewhat lower cell proliferative activity than SC-55494, with relative activities ranging from 40 to 80%. The P37A mutant has a binding affinity to the low-affinity IL-3 receptor α-subunit statistically equivalent to SC-55494, while P30A, P31A, and P33A each had about two-fold decreases, and P30A/P31A and P30A/P31A/P33A/P37A had four-fold decreases. These findings suggest an important role for the *cis* configuration of either or both of the R29–P30 and P30–P31 peptide bonds in IL-3 for optimal interaction with the receptor α-subunit.

Keywords: conformational heterogeneity; hematopoietic growth hormone; interleukin-3; NMR; proline *cis-trans* isomerization

Interleukin-3 (IL-3) is a hematopoietic cytokine that binds to a cell surface receptor to stimulate the proliferation of early multipotential progenitors, as well as the growth and differentiation of many lineage-restricted blood cells (see Schrader, 1986; Metcalf & Nicola, 1995). In addition, hIL-3 stimulates peripheral blood leukocytes to produce inflammatory mediators that trigger the synthesis and release of histamine. Clinical experience with recombinant hIL-3 indicates that despite its ability to relieve thrombocytopenia, several side effects may limit its therapeutic use (Biesma et al., 1992; Denzlinger et al., 1993). SC-55494 (Thomas et al., 1995) was the result of a saturation mutagenesis approach to develop human IL-3 receptor agonists with improved growth promoting activity and therapeutic index (Olins et al., 1995). It has a 10- to 20-fold increase in growth promoting activity compared to native rhIL-3 with only a modest increase in pro-inflammatory activity (Thomas et al., 1995).

The conformation of hIL-3 is of great interest for understanding its structure–function relationships. Early NMR results with several hIL-3 variants, including SC-55494, revealed two sets of chemical shifts for a limited number of nuclei (Y. Feng, unpubl. results). Eliminating the possibility of chemical heterogeneity suggested that multiple conformations cause the resonance doubling. One of these variants, SC-65369, has been extensively studied by NMR methods and the solution structure has been determined recently (Feng et al., 1995, 1996) to be a four-helical bundle with two long overhand loops, a topology observed in many other cytokine structures. Inspection of the structure of SC-65369 revealed that the majority of residues that exhibit chemical shift heterogeneity are either located in, or are spatially proximate to, the proline-rich first overhand loop, suggesting that the origin of the heterogeneity is *cis-trans* isomerization of one or more of these prolyl peptide bonds (Feng et al., 1996). ^{15}N NMR relaxation measurements (Feng et al., 1996) indicate that this loop exhibits significantly higher flexibility than the helical core of the protein.

SC-65369, which consists of 112 amino acid residues, is derived by truncating both the N- and C-termini of hIL-3 and making 14 amino acid substitutions: V14A, N18I, T25H, Q29R, L32N, F37P, G42S, Q45M, N51R, R55T, E59L, N62V, S67H, and Q69E. SC-55494 is truncated in the same way as SC-65369 and it carries an additional 13 amino acid substitutions: A73G, S76A, K79R, L82Q, L87S, T93S, H98I, D101A, N105Q, R109E, K116V, N120Q, A123E (Thomas et al., 1995). The N-terminal half of the SC-65369 and SC-55494 sequences, including the loop containing four prolines, are identical. SC-55494 contains nine prolines in the same positions as in SC-65369. We focused on SC-55494 in this study because of the continuing interest in its elevated potency, exam-

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Abbreviations: IL-3, interleukin-3; hIL-3, human IL-3; rhIL-3, recombinant human IL-3; GM-CSF, granulocyte-macrophage colony stimulating factor; IL-5, interleukin-5

ining the role of prolines 30, 31, 33, and 37 in spectral and conformational heterogeneity, and on receptor binding and biological activity.

Results and discussion: *Multiple conformations of SC-55494 characterized by NMR:* Although the NMR resonances of SC-55494 have not been fully assigned, the downfield region of its proton NMR spectrum containing aromatic side-chain resonances is remarkably similar to the previously assigned spectrum of SC-65369 (Feng et al., 1995). There is a single Trp in both SC-55494 and SC-65369, for which the side-chain spin system can be unambiguously assigned using a combination of ^{1}H-COSY and ^{1}H-TOCSY spectra. The downfield region of the proton NMR spectrum of SC-55494 is shown in Figure 1A with the Trp-104 C_4H–C_5H cross-peak indicated. All resonances of the Trp-104 side-chain are split into two chemical shifts, indicating a minimum of two distinct environments surrounding this residue, which interconvert slowly on the NMR time scale. The ratio of the two resonances is approximately 1:1, which appears to be largely independent of either the protein concentration (0.1–2.5 mM) or temperature (28–50 °C) (data not shown). In addition, the better-resolved upfield region of the proton spectrum (−1.0–0.8 ppm; Fig. 2A) was used to monitor different conformational forms, especially the existence of minor conformers. In analogy with the resonance assignments of SC-65369, this region consists of methyl resonances. The farthest upfield resonance (Peak b in Fig. 2) is one of the doublet signals arising from a methyl group of Leu-68, and the resonance at about −0.35 ppm (Peak a) arises from one of the methyl groups of Leu-111 (no splitting). Examination of the three-dimensional structure of SC-65369 reveals that both Trp-104 and Leu-68 are located in the vicinity of the proline-rich loop (Fig. 3). It was found empirically that the side-chain resonances of Trp-104 and Leu-68 could be used as reporter groups to monitor changes in the loop's conformation that result from replacing proline with alanine.

NMR characterization of the alanine mutants: As an initial test of the hypothesis that the spectral heterogeneity is caused by proline *cis-trans* isomerization within the proline-rich loop, a mutant of SC-55494 was constructed in which all four prolines (30, 31, 33, 37) were replaced by alanine. The NMR spectrum of the resultant mutant (P30A/P31A/P33A/P37A; Fig. 1E, 2G) is indeed free of resonance splitting, confirming that the heterogeneity is attributed to these prolines.

Four single proline-to-alanine mutants of SC-55494 were subsequently constructed in order to identify which specific prolyl peptide bond(s) is responsible for the heterogeneity. The aromatic side-chain resonance region of two of the single alanine mutants, i.e., P30A and P31A, are shown in Figures 1B and C. As can be

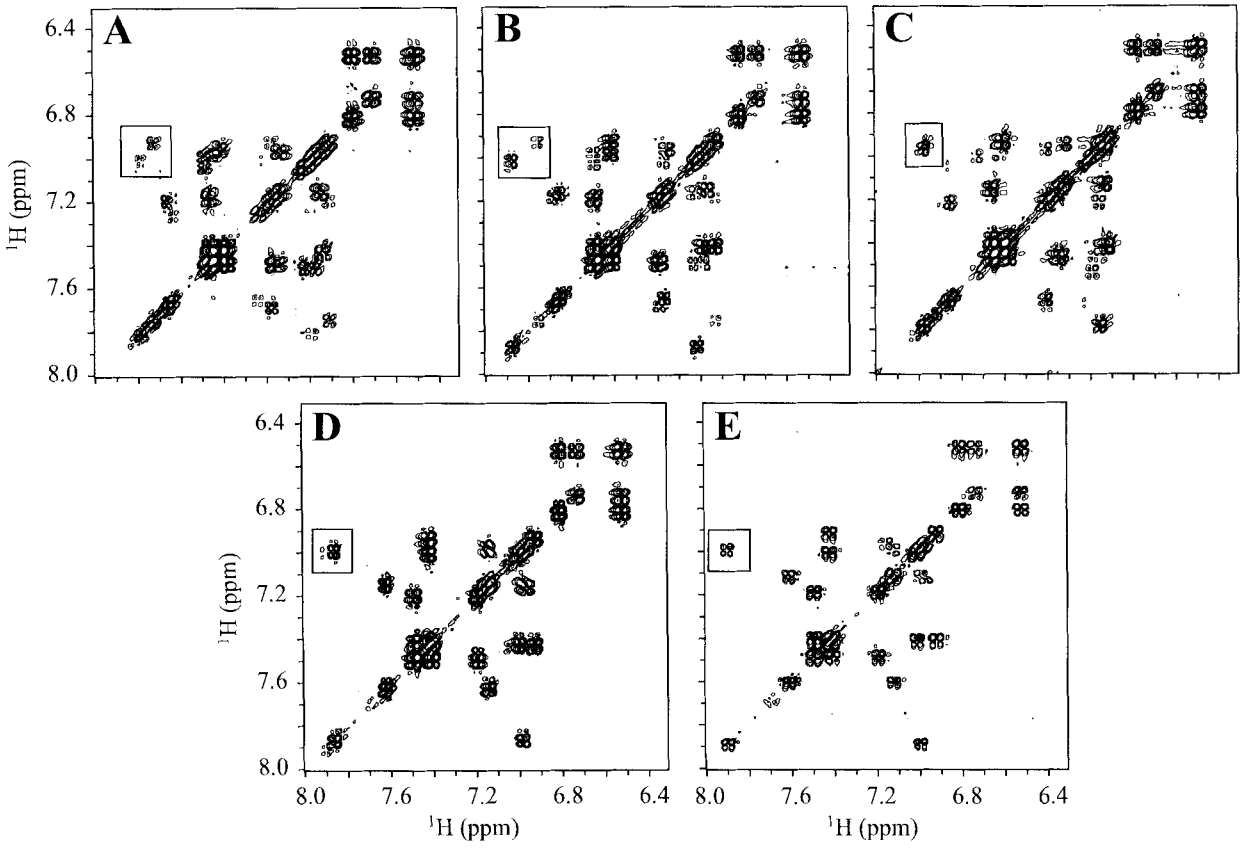

Fig. 1. Downfield regions of the 2D DQF-COSY spectra of (**A**) SC-55494; (**B**) SC-55494 (P30A); (**C**) SC-55494 (P31A); (**D**) SC-55494 (P30A/P31A); and (**E**) SC-55494 (P30A/P31A/P33A/P37A). All 2D DQF-COSY experiments were acquired with a spectral width of 6800 Hz, and acquisition times of 151 ms and 59 ms in F_2 and F_1, respectively. Residual water suppression was achieved with low-power pre-irradiation. Gaussian and sine bell multiplication were used in F_2 and F_1, respectively, and each spectrum contains 2,048 × 2,048 real points. The cross-peaks of Trp-104 C_4H–C_5H are boxed in each spectrum.

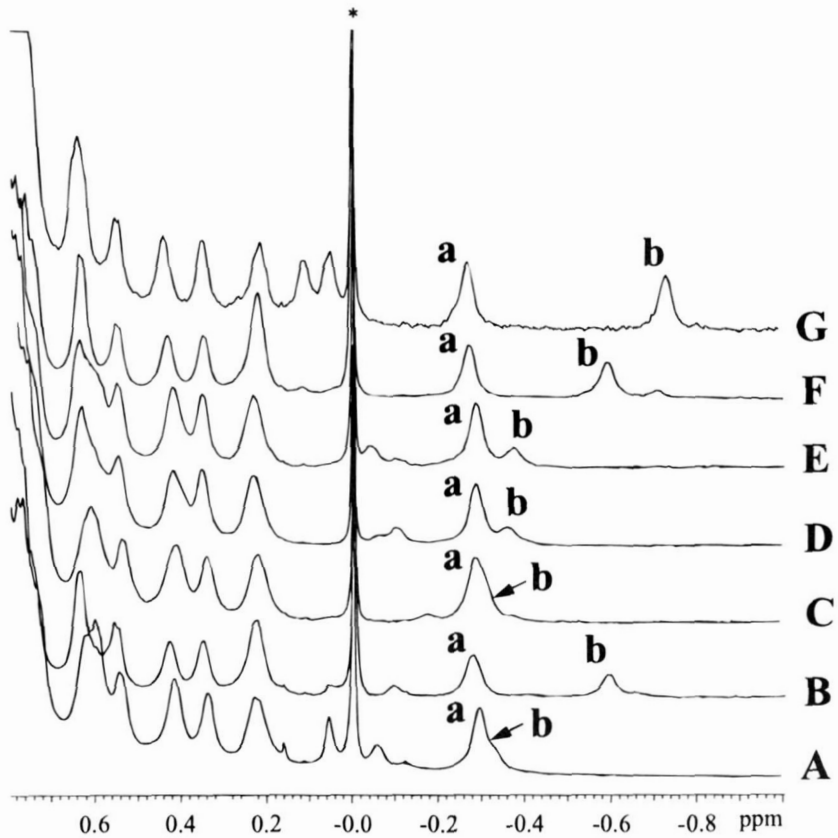

Fig. 2. Upfield regions of the 1D proton spectra of (**A**) SC-55494; (**B**) SC-55494 (P30A); (**C**) SC-55494 (P31A); (**D**) SC-55494 (P33A); (**E**) SC-55494 (P37A); (**F**) SC-55494 (P30A/P31A); and (**G**) SC-55494 (P30A/P31A/P33A/P37A). The internal reference TSP signal is marked with *, the methyl resonance of Leu-111 is marked with a, and that of Leu-68 is labeled with b (both assigned by analogy to SC-65369). The minor conformation present in the spectrum of SC-55494 (P30A/P31A) is ≤10%.

seen from this representative data, resonance splitting was observed for these single mutants. Similar data were obtained for P33A and P37A (data not shown). Thus, eliminating the prolines individually failed to remove the conformational heterogeneity, or to dramatically alter the ratio of the split signals. However, when a double alanine mutant (P30A/P31A) was tested, the spectrum was largely free of heterogeneity (Fig. 1D), with only a small percentage of a minor conformer present (Fig. 2F).

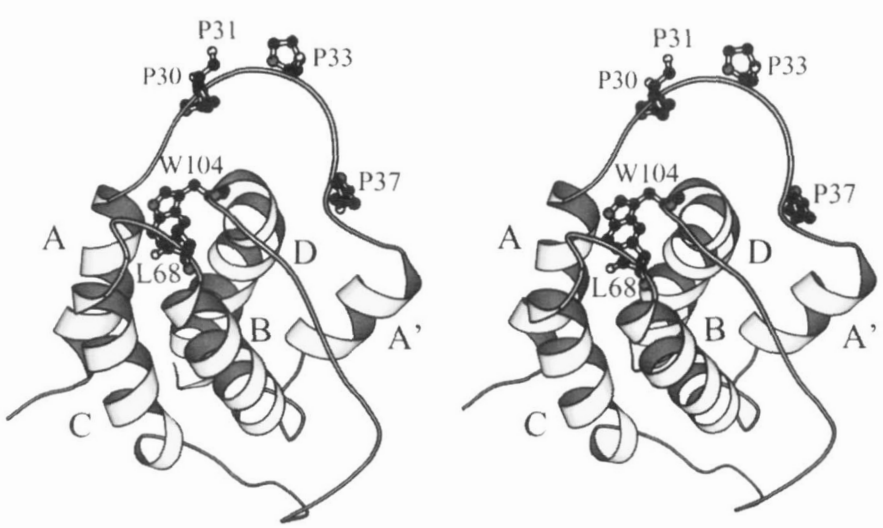

Fig. 3. Ribbon diagram of SC-65369 (PDB entry 1JLI) drawn in stereo with MOLSCRIPT (Kraulis, 1991). The five helices are labelled as A, A′, B, C, and D. Pro-30, Pro-31, Pro-33, Pro-37, Leu-68, and Trp-104 are displayed with a ball-and-stick representation.

Functional studies of the alanine mutants: IL-3 promotes multilineage hematopoietic cell growth by binding to a heterodimeric cell surface receptor (Kitamura et al., 1991). The receptor consists of an α-subunit that is specific to IL-3 and a β-subunit that is common to the GM-CSF and IL-5 receptors. IL-3 binds to the α-subunit with low affinity, while no binding can be detected to the isolated β-subunit. A high-affinity complex is formed in the presence of both α- and β-subunits. Signaling occurs only after high-affinity complex formation.

All the alanine mutants had modest decreases (40 to 80% of SC-55494) in their proliferative activity relative to the parental molecule SC-55494 (Table 1). A more direct assessment of the effect of alanine substitution is provided by receptor binding assays. The alanine mutants were examined for their ability to compete with an ^{125}I-labeled hIL-3 variant in binding to the receptor α-subunit in transfected BHK cells (Thomas et al., 1995). As summarized in Table 1, the single alanine mutant P37A has a binding affinity that cannot be statistically distinguished from that of SC-55494. The other three single alanine mutants, P30A, P31A, and P33A, exhibited marginally significant differences in affinity from SC-55494 (about two-fold). The binding affinities of the multiple alanine mutants P30A/P31A and P30A/P31A/P33A/P37A are decreased to a greater extent, nearly four-fold lower than SC-55494 ($P < 0.005$). Thus, the binding data suggest that simultaneous mutation at Pro-30 and Pro-31 has a significant impact on molecular recognition.

Conformational heterogeneity caused by proline cis-trans isomerization: The ability of an X-Pro peptide bond to adopt both *cis* and *trans* configurations has been observed to cause chemical shift heterogeneity in several other proteins (e.g., Evans et al., 1987; Alexandrescu et al., 1989; Chazin et al., 1989; Chao et al., 1993; Amodeo et al., 1994; Scanlon & Norton, 1994). Both conformers often occur when the proline is in a flexible region of the structure while only a single conformer (either *cis* or *trans*) is observed in more rigid regions. In other proteins, the heterogeneity has often disappeared upon substituting a specific proline residue with a non-proline amino acid. In SC-55494, however, simultaneous substitution of the dipeptide unit at Pro-30–Pro-31 was required to reduce the heterogeneity to less than 10%, while only simultaneous replacement of four prolines completely eliminated the heterogeneity. The above observations point directly to isomerism of the peptide bonds at Arg-29–Pro-30 and/or Pro-30–Pro-31 as being the predominant source of heterogeneity. The minor remaining heterogeneity in the spectrum of the P30A/P31A variant is likely due to a small percentage of *cis* configuration at Pro-33 or Pro-37. Pro-37 in SC-55494 replaces Phe in native hIL-3 sequence. A variant related to SC-55494 with Phe at position 37 exhibited heterogeneity similar to that of SC-55494 (Y. Feng, unpubl. results). Although wild-type hIL-3 is not sufficiently soluble to make high resolution NMR studies possible, it is likely to display similar conformational heterogeneity because it has a nearly identical primary sequence in the proline-rich loop, including both Pro-30 and Pro-31.

Table 1. *Receptor-binding affinity and cell proliferation potency of SC-55494 alanine mutants*

Proteins	Affinity[a] (nM)	Relative potency[b]
SC-55494	4.7 ± 1.0	1.00
SC-55494 (P30A)	8.2 ± 2.2	0.54
SC-55494 (P31A)	9.3 ± 2.3	0.48
SC-55494 (P33A)	9.9 ± 3.7	0.58
SC-55494 (P37A)	5.2 ± 1.9 (ns)	0.83
SC-55494 (P30A/P31A)	18.4 ± 4.8 ($P < 0.005$)	0.40
SC-55494 (P30A/P31A/P33A/P37A)	16.8 ± 4.4 ($P < 0.005$)	0.61

[a]Affinity is defined as the concentration that gives half maximal binding of the ^{125}I-labeled ligand to the IL-3 receptor α-subunit in the competition assay. Significant differences are indicated with P values from the student t-test and insignificant difference indicated with ns.

[b]Potencies are listed relative to SC-55494 in the TF-1 cell proliferation assay.

The microscopic nature of the apparent (macroscopic) two states is difficult to delineate. All four prolines investigated in this study are located in a flexible long loop without any obvious conformational constraint. The observation of an essentially homogeneous spectrum for the P30A/P31A variant allows us to simplify the discussion by focusing on the four possible configurations of the Arg-29–Pro-30 and Pro-30–Pro-31 peptide bonds, rather than on the 16 possible configurations associated with all four prolines. One scenario is that only two of the four possible isomers are energetically favored (e.g., *trans-trans* and *cis-trans*; see below) and thus significantly populated to yield the two signals observed. Support for this scenario comes from the studies of small peptides containing $(Pro)_n$ sequences (Poznanski et al., 1993). It was reported that for the sequences Trp–$(Pro)_2$–Tyr, Trp–$(Pro)_2$–Met, and Tyr–$(Pro)_2$–Met, 80–100% of the Pro–Pro peptide bond is in a *trans* configuration. These results suggest that the *trans* configuration of the Pro-Pro peptide bond is strongly favored. The hypothesis that SC-55494 exists as a mixture of *trans-trans* and *cis-trans* conformers about the Arg-29–Pro-30 and Pro-30–Pro-31 peptide bonds is consistent with the spectroscopic data.

It is well known that both the kinetics and the equilibrium ratio of the isomerization can be markedly affected by variation of the sequence either in or outside the immediate environment of the proline residue (Brandts et al., 1975; Grathwohl & Wüthrich, 1981; Dyson et al., 1988). Thus, the inability of the single alanine mutants to remove the heterogeneity could be simply explained by the fact that upon substitution of one of the successive prolines, the equilibrium of the remaining X-Pro bond can adjust to manifest two new isomers at nearly equal population.

Another possibility is that all four isomers at Arg-29–Pro-30 and Pro-30–Pro-31 coexist in SC-55494 but, due to either chemical shift degeneracy or relatively fast interconversion rates, only two apparent states are observed experimentally. A new apparent two-site or a true two-site situation may be generated by the remaining X-Pro peptide bond when either one of the prolines is replaced with alanine. Although the current data cannot distinguish these microscopic models, the latter situation is considered less likely because not more than two sets of resonances were identified for any of the over 100 heterogeneous ^{1}H, ^{13}C, or ^{15}N nuclei in spectra of SC-65369.

The role of prolines in hIL-3: Due to proline's unique chemical structure, the energy difference between *cis* and *trans* configurations is much less for proline than for other amino acid residues (Schultz & Schirmer, 1978). As a result, the occurrence of a *cis* peptide bond preceding proline residues has been found to be

much more frequent than for all other amino acid residues (~6% for proline and 0.05% for other residues; Stewart et al., 1990; MacArthur & Thornton, 1991). The occurrence of a *cis* bond is even more frequent for an X-Pro-Pro sequence (14%; MacArthur & Thornton, 1991). *Cis-trans* isomerization is a relatively slow event and has been shown to be important in protein folding kinetics (e.g., Brandts et al., 1975). It has been long recognized that because of the special properties of proline, it is often conserved and plays a special role in protein structure, function, as well as biological processing (Brandl & Deber, 1986; Barlow & Thornton, 1988; Vanhoof et al., 1995). Thus, the special role of proline may be relevant to the observation that the Pro-30–Pro-31 dipeptide sequence is conserved in all six available primate IL-3 sequences (human, chimpanzee, gibbon, rhesus, tamarin, and marmoset).

This study has established that the conformational heterogeneity observed in SC-55494 is due to proline *cis-trans* isomerization in the proline-rich first overhand loop of IL-3. More specifically, the requirement for simultaneous mutation of Pro-30 and Pro-31 to remove the heterogeneity points to this dipeptide segment as being responsible for the heterogeneity. It further establishes that either one of these prolines is sufficient to cause multiple conformations. The significant diminution of the binding affinity for the IL-3 receptor α-subunit exhibited by the multiple alanine mutants strongly implicates the importance of the dipeptide segment Pro-30–Pro-31 to the initial binding event. These two residues can be important either because of an optimal binding interaction achievable only by having one or both of these peptide bonds in the *cis* configuration, or because of the direct involvement of the pyrolidine rings in receptor binding.

Although the data do not allow for a definitive assignment of the configuration of these peptide bonds, the model peptide studies suggest that the spectral and conformational heterogeneity observed in SC-55494 results from a true two-state equilibrium in which the Arg-29–Pro-30 peptide bond is similarly distributed between *cis* and *trans* configurations and the Pro-30–Pro-31 peptide bond adopts the *trans* configuration. One explanation for the importance of the Pro-Pro sequence could be that binding to the receptor requires both prolines in hIL-3 to adopt a *cis* configuration in order to allow for optimal interaction,[1] which is difficult although not impossible to achieve for the multiple alanine mutants due to the considerable energy cost. Such tandem *cis* peptide bonds have been observed in the crystal structures of molecules such as carbonic anhydrase II (PDB entry 1CNX or 1CNY) and GMP synthetase (PDB entry 1GPM).

Further support for the importance of the 30–31 dipeptide segment comes from molecular modeling studies of the IL-3/IL-3 receptor complex (Klein et al., 1997). A molecular model built using mutagenesis data of hIL-3 and the crystal structure of human growth hormone with its receptor (de Vos et al., 1992) indicates that Pro-30 and Pro-31 may be in contact with the low-affinity receptor α-subunit. Unfortunately, the flexibility of the loop in which the Pro–Pro sequence resides hampers a detailed assessment of their impact on the ligand–receptor interaction. The hIL-3 variant with substitution at all four prolines in the loop is free of chemical shift heterogeneity, and is thus a good candidate for deriving a higher resolution solution structure in order to better define the interaction of IL-3 with its receptor.

[1] Although non-proline residues have been observed to adopt the *cis* configuration (e.g., *cis*-Ser in PDB entry 1CNX and 1CNY, *cis*-Ala in 1CLC, and *cis*-Gly in 1DYR), this normally occurs under conditions of considerable structural constraints such as being in a tight turn. Because the Pro-30–Pro-31 sequence of IL-3 is located in a long flexible loop, it is unlikely that any of the substituted alanines will be constrained to a *cis* configuration.

Materials and methods: *Mutagenesis and purification of SC-55494 variants:* The gene encoding SC-55494 was subcloned into pAlter-1 (Promega, Madison, Wisconsin). Site directed mutagenesis was performed using the Transformer Mutagenesis Kit (Clontech, Palo Alto, California). Mutagenesis was confirmed by DNA sequence analysis. Variants were subsequently subcloned into an *Escherichia coli* expression vector under the control of the *E. coli recA* promoter. All proteins were cytoplasmically expressed in *E. coli* strain JM101 and purified as described in Thomas et al. (1995).

NMR experiments: All NMR spectra were recorded at 30 °C on a Varian UNITY-500 spectrometer. For the alanine mutants, the sample concentrations were between 0.4 and 0.8 mM and for SC-55494 between 0.1 mM and 2.5 mM. Samples of the alanine mutants were prepared in 0.6 mL of D_2O containing 5 mM ammonium bicarbonate at pH 9.5 (uncorrected reading). The high pH condition was necessary because some of the mutants had lower solubility at neutral pH. An additional sample of 0.1 mM SC-55494 was dissolved in 90% H_2O/10% D_2O at pH 7.0 to take advantage of the well-resolved Trp-104 indole proton resonances (ca. 10.3 ppm) in the 1D spectrum. For SC-55494 and SC-55494 (P31A), samples were prepared at both pH 9.5 and 7.0, and 2D DQF-COSY spectra (Rance et al., 1983) were recorded to ensure the lack of significant pH-related differences. All data were processed on a SUN workstation using Varian VNMR software.

Receptor binding and cell proliferation assays: Specific binding assays used BHK cells transfected to express the IL-3 receptor α-subunit and an IL-3 variant, which was labeled with ^{125}I (Thomas et al., 1995). The binding assays were initiated by adding ice cold BHK cells to incubations containing [^{125}I]-SC-65355 (an hIL-3 variant with seven amino acid changes in SC-55494 and a 14-amino acid N-terminal extension; see Thomas et al., 1995) and competing compound at 0–4 °C and continued until steady state was achieved in approximately 3 h, then halted by centrifugation. Nonspecific binding was defined as the residual binding which occurred in the presence of excess SC-55494 (1 μM).

TF-1 cells (C. Sanderson, Searle) were seeded at 1.25×10^4 cells per well in Costar microwell plates with or without cytokines in serum-free Iscove's modified Dulbecco's medium (IMDM) containing bovine serum albumin (500 μg/mL), human transferrin (100 μg/mL), a lipid substitute of 2.5 mg phosphatidyl choline per mL BSA (500 μg/mL) and 50 μM 2-mercaptoethanol. After 72 h, cells were incubated with [methyl-^3H] thymidine at 0.5 μCi (18.5 kBq) per well for 4 h and then harvested onto a glass fiber filter mat for measurement of radioactivity with a beta counter. All the alanine variants were compared in activity to SC-55494 and relative potencies reported.

Acknowledgments: We thank Mark Yocum and Dr. Kevin Duffin for assistance with protein purification and characterization, Drs. B. Klein and N. Summers for helpful discussions. We also gratefully acknowledge the encouragement of Dr. John McKearn and the Synthokine Project Team.

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