(Porter & Kasper 1986; Haniu et al., 1986). The region from residues 144 to 158 (three threonines, four serines, and three glycines) is a candidate for the NAD(P)H- or FAD-binding region.

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SUPPLEMENTARY MATERIAL AVAILABLE

Tables of amino acid compositions and sequence analyses and figures of chromatographic analysis of the peptide fragments (11 pages). Ordering information is given on any current masthead page.

Registry No. NBSF, 349-96-2; TNBS, 2508-19-2; DT-diaphorase, 9032-20-6; DT-diaphorase (rat liver), 115756-48-4; tyrosine, 60-18-4; lysine, 56-87-1.

REFERENCES

Bayney, R. M., Rodkey, J. A., Bennett, C. D., Lu, A. Y. H., & Pickett, C. B. (1987) *J. Biol. Chem. 262*, 572–575.
Del Valle, U., & Shively, J. E. (1979) *Anal. Biochem. 96*, 77–83.
Ernster, L., Ljunggren, M., & Danielson, L. (1960) *Biochem. Biophys. Res. Commun., 2*, 88–92.
Ernster, L., Danielson, L., & Ljunggren, M. (1962) *Biochim. Biophys. Acta 58*, 171–188.
Ferrante, R. J., Kowall, N. W., Beal, M. F., Richardson, E. P., Jr., Bird, E. D., & Martin, J. B. (1985) *Science (Washington, D.C.) 230*, 561–563.
Fields, R. (1971) *Biochem. J. 124*, 581–590.
Haniu, M., Iyanagi, T., Miller, P., Lee, T. D., & Shively, J. E. (1986) *Biochemistry 25*, 7906–7911.
Hawke, D. H., Yuan, P.-M., & Shively, J. E. (1982) *Anal. Biochem. 120*, 302–311.
Hawke, D. H., Harris, D. C., & Shively, J. E. (1985) *Anal. Biochem. 147*, 315–330.
Höjeberg, B., Blomberg, K., Stenberg, S., & Lind, C. (1981) *Arch. Biochem. Biophys. 207*, 205–216.
Hollander, P. M., & Ernster, L. (1975) *Arch. Biochem. Biophys. 169*, 560–567.
Hosoda, S., Nakamura, W., & Hayashi, K. (1974) *J. Biol. Chem. 249*, 6416–6423.
Huang, M.-T., Miwa, G. T., & Lu, A. Y. H. (1979) *J. Biol. Chem. 254*, 3930–3934.
Iyanagi, T. (1987) *Chem. Scr. 27A*, 31–36.
Iyanagi, T., & Yamazaki, I. (1970) *Biochim. Biophys. Acta 216*, 282–294.
Koh, J.-Y., Peters, S., & Choi, D. W. (1986) *Science (Washington, D.C.) 234*, 73–76.
Liao, T.-H., Ting, R. S., & Yeung, J. E. (1982) *J. Biol. Chem. 257*, 5637–5644.
Lind, C., Hochstein, P., & Ernster, L. (1982) *Arch. Biochem. Biophys. 216*, 178–185.
Miller, M. G., Rodgers, A., & Cohen, G. M. (1986) *Biochem. Pharmacol. 35*, 1177–1184.
Palczewski, K., Hargrave, P. A., Folta, E. J., & Kochman, M. (1985) *Eur. J. Biochem. 146*, 309–314.
Porter, T. D., & Kasper, C. B. (1986) *Biochemistry 25*, 1682–1687.
Prochaska, H., & Talalay, P. (1986) *J. Biol. Chem. 261*, 1372–1378.
Robertson, J. A., Chen, H.-C., & Nebert, D. W. (1986) *J. Biol. Chem. 261*, 15794–15799.
Shively, J. E., Yuan, P.-M., & Hawke, D. H. (1982) *Anal. Biochem. 120*, 312–322.
Talalay, P., & Benson, A. M. (1980) *Adv. Enzyme Regul. 20*, 287–300.
Thor, H., Smith, M. T., Hartzell, P., Bellomo, G., Jewell, S. A., & Orrenius, S. (1982) *J. Biol. Chem. 257*, 12419–12425.
Wallin, R., Gerhardt, O., & Prydz, H. (1978) *Biochem. J. 169*, 95–101.
Williams, J. B., Lu, A. Y. H., Cameron, R. G., & Pickett, C. B. (1986) *J. Biol. Chem. 261*, 5524–5528.

Structure–Activity Relationships of Recombinant Human Interleukin 2

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ABSTRACT: Structure–activity relationships of recombinant human interleukin 2 were investigated by preparation, purification, and characterization of 21 missense mutants. A key role for residue Phe42 in the high-affinity interaction with receptor was indicated by (a) the reduction of 5–10-fold in binding affinity and bioactivity upon mutation of this residue to Ala and (b) the lack of evidence for conformational perturbation in Phe42 → Ala in comparison with the wild-type protein as investigated by intrinsic fluorescence, second-derivative UV spectroscopy, electrophoresis, and reversed-phase HPLC, suggesting that the drop in binding is a direct effect of removal of the aromatic ring. In contrast, the conservative mutations Phe42 → Tyr and Phe42 → Trp did not cause significant reductions in bioactivity. UV and fluorescence spectra indicated approximately 60% overall exposure to solvent of tyrosines in the wild-type molecule, the tryptophan (residue 121) being buried; fluorescence data also showed that Trp42 in Phe42 → Trp is likely to be within 1 nm of Trp121 and about 50% exposed to solvent. Phe44 → Ala, Cys105 → Ala, and Trp121 → Tyr also exhibited reduced bioactivity, but these mutants are conformationally perturbed relative to wild type. None of the remaining mutants had detectably reduced bioactivity, even though several showed signs of altered conformation. Four mutants were recovered in very low yield, probably because of defective refolding.

Interleukin 2 (IL-2)[1] is a 133-residue protein secreted by T-lymphocytes that promotes the proliferation of activated T-helper cells (Robb, 1985) and modulates growth and differentiation of other lymphocyte subsets, for example, activated

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B-cells (Pike et al., 1984), T-cytotoxic cells (Ezard et al., 1985), natural killer cells, and lymphokine-activated killer cells (Hinney et al., 1981; Mule et al., 1985). The amino acid sequence deduced from cDNA clones (Taniguchi et al., 1983; Devos et al., 1983) has been confirmed by direct amino acid sequencing of lymphoid IL-2 (Robb et al., 1984) and shows the presence of a single Trp and three Cys residues, 58, 105, and 125; Cys58 and Cys105 form a disulfide bridge (Robb et al., 1984). IL-2 receptor (IL-2R) appears to consist of two membrane-bound polypeptides, the tac (M_r 55 000) and "converter" (M_r 75 000) molecules (Robb et al., 1987).

Identification of IL-2 residues that bind to receptor would be of considerable interest; site-directed mutagenesis of recombinant IL-2 has been applied to this end by several groups (Ju et al., 1987; Liang et al., 1986; Ralph et al., 1987; Gadski et al., 1987), although mutants have generally not been characterized except in terms of activity or receptor binding. We have prepared, purified, and characterized 21 missense mutants[2] of human recombinant IL-2; characterization pertained to conformational integrity as well as bioactivity, since mutants that show alterations in folding as well as activity do not provide direct evidence of the locality of the binding site. Electrophoresis, RP-HPLC, and in selected cases UV and fluorescence spectroscopy were used to afford a preliminary guide to conformation from which to interpret any changes in activity, although such techniques may of course leave local or minor perturbations undetected.

IL-2 is expressed in *E. coli* as insoluble aggregates, which necessitated solubilization in 6 M Gdn-HCl and refolding as part of the purification procedure (Weir et al., 1987; Kato et al., 1985). In our case, the isolated pure wild-type IL-2 was found by mass spectrometry of tryptic peptides to be a 4:1 mixture of N-Met (unprocessed Met at the N-terminus) and authentic N-Ala IL-2 (W. P. Blackstock, R. J. Dennis, J. Sparks, and M. P. Weir, unpublished results); these two species have been shown to be equipotent in bioassay (Yamada et al., 1986).

Materials and Methods

Reagents. Ammonium persulfate, SDS, tetramethylenediamine, hydroxylamine, sodium acetate, and sodium dihydrogen phosphate were of AnalaR grade (BDH). Acrylamide and bis(acrylamide) were from Bio-Rad. All other chemicals were from Sigma; Gdn-HCl was of grade 1 purity.

Acetonitrile (HPLC grade S) was obtained from Rathburn and butan-1-ol from BDH; TFA (Sequanal grade), amino acid standards, and phenyl isothiocyanate were from Pierce. Sepharose CL-6B, S-Sepharose Fast Flow, and M_r and p*I* markers were purchased from Pharmacia.

[α-^{35}S]dCTPαS (1000 Ci/mmol) and [α-^{32}P]ATP (3000 Ci/mmol) were from Amersham, and dideoxyribonucleotides/deoxyribonucleotides were from Pharmacia. Isopropyl D-1-thiogalactopyranoside (IPTG) and ampicillin were purchased from Sigma, and restriction endonucleases, Klenow fragment of *E. coli* DNA polI, polynucleotide kinase, T4DNA ligase, and calf intestinal phosphatase were supplied by BCL, East Sussex. The "slot-blot" apparatus and technique were supplied by Schleicher and Schuell Inc. (Anderman Ltd., Surrey).

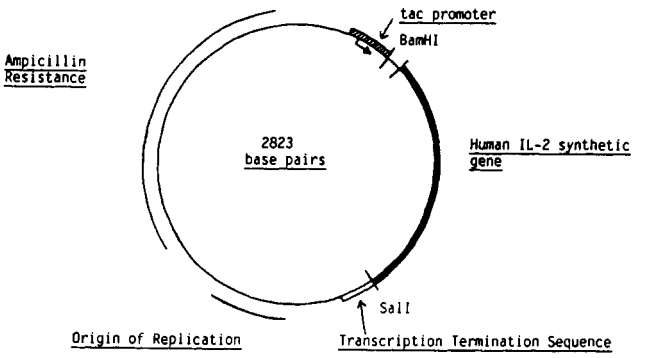

FIGURE 1: IL-2 *E. coli* expression vector pATtacIL-2C/2TT.

```
                                1
GATCCTAGGAGGTTTGGTACCATGGCTCCGACGAGCAGCTCCACCAAGAAAACCCAGCTC
BamHI                  MetAlaProThrSerSerSerThrLysLysThrGlnLeu

                                                              31
CAGCTCGAACACCTGCTGCTGGACCTGCAGATGATCCTGAACGGTATCAACAACTACAAG
GlnLeuGluHisLeuLeuLeuAspLeuGlnMetIleLeuAsnGlyIleAsnAsnTyrLys
                                                              Ala

   34       37              42    44
AACCCGAAACTGACTCGTATGCTGACCTTCAAGTTCTACATGCCGAAGAAAGCTACCGAA
AsnProLysLeuThrArgMetLeuThrPheLysPheTyrMetProLysLysAlaThrGlu
   Leu      Pro             Ala   Ala
                            Tyr
                            Trp

      55       58              64
CTGAAACACCTGCAATGGCTCGAGGAGGAGCTCAAACCGCTGGAAGAGGTTCTGAACCTG
LeuLysHisLeuGlnCysLeuGluGluGluLeuLysProLeuGluGluValLeuAsnLeu
      Asp      Ala             Gly

               78
GCTCAGTCCAAGAACTTCCACCTGCGTCCGCGCGACCTGATCTCCAACATCAACGTTATC
AlaGlnSerLysAsnPheHisLeuArgProArgAspLeuIleSerAsnIleAsnValIle
               Ala

                                103   105   107         111
GTTCTGGAACTGAAAGGCAGTGAGACTACCTTCATGTGCGAATACGCTGACGAAACCGCT
ValLeuGluLeuLysGlySerGluThrThrPheMetCysGluTyrAlaAspGluThrAla
                                Ala   Ala   Ala         Ile
                                                        Ala
                                                        Ser

            117         121      124125
ACTATCGTTGAATTCCTGAACCGTTGGATCACCTTCTGTCAGTCCATCATCTCCACCCTG
ThrIleValGluPheLeuAsnArgTrpIleThrPheCysGlnSerIleIleSerThrLeu
            Ala         Ala      AlaTyr
                        Tyr         Ala
                        Ser

ACCTAATAACTAACTAAGTCGA
ThrEnd          SalI
```

FIGURE 2: DNA and protein sequences of IL-2. The DNA sequence is of the *Bam*HI–*Sal*I fragment of pATtacIL-2C/2TT. The numbering refers to the residue position of mutations relative to the first amino acid (Ala) of mature IL-2.

Generation of Human IL-2 Structural Mutants. A synthetic DNA encoding mature human IL-2 has been expressed in *E. coli* RB791 by using the recombinant plasmid pATtacIL2C/2TT (Figure 1) as outlined previously (Weir & Sparks, 1987). Induction of the tac promoter (de Boer et al., 1982) with IPTG results in the production of insoluble IL-2 at the level of 5–10% total cell protein.

Site-directed IL-2 mutants were created essentially as described by Zoller and Smith (1982). A 438 base pair *Bam*HI–*Sal*I fragment encoding the synthetic IL-2 (Figure 2) was subcloned from the expression vector into M13mp9 to supply single-stranded template DNA. IL-2-specific oligodeoxyribonucleotides 21–28 residues in length, which carry sequence alterations appropriate for the generation of the desired mutations (see supplementary material), were used to prime the

[1] Abbreviations: IL-2, interleukin 2; IL-2R, interleukin 2 receptor; Gdn-HCl, guanidine hydrochloride; IEC, ion-exchange chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; GPC, gel permeation chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MeCN, acetonitrile; TFA, trifluoroacetic acid; R_f, fractional mobility on PAGE; λ, wavelength; NBS, *N*-bromosuccinimide; ED_{50}, dose required for 50% maximal response; HSA, human serum albumin; *E. coli*, *Escherichia coli*.

[2] Following the convention outlined by Knowles (1987), mutants will be defined by using the one-letter code for amino acids, e.g., Tyr31 → Ala becomes Y31A.

synthesis of second-strand DNA on the M13mp9–IL-2 template. Following transformation of *E. coli* JM103, IL-2 mutants were identified by plaque hybridization with the relevant ^{32}P-end-labeled primer; a predetermined critical washing temperature at which each primer was unable to anneal to nonmutant IL-2 DNA was used to differentiate between normal and mutant clones.

Most mutant IL-2 sequences were transferred into the expression vector pATtacIL2C/2TT as *Bam*HI–*Sal*I fragments, replacing the normal IL-2 gene, and mutant clones recognized by colony hybridization using the mutant primer as probe. Several mutant sequences were subcloned into the vector pATtacIL2E/2TT, which has a 10-bp rather than an 11-bp spacer between the initiator AUG and the Shine/Dalgarno sequence and gives 5-fold higher expression than pATtacIL2C/2TT. Prior to expression each mutant IL-2 sequence was subcloned into M13mp8 as a *Bam*HI–*Sal*I fragment for complete sequence verification by the method of Sanger et al. (1977).

General recombinant DNA manipulations were carried out essentially as described by Maniatis et al. (1982). M13 cloning and DNA sequencing were carried out according to the protocols supplied with the Amersham DNA sequencing kit (Amersham PLC, Bucks).

Purification of Mutant Proteins. Proteins were purified by a modification of the preparative method used for wild-type IL-2 (Weir et al., 1987). Briefly, 10-mL cultures of *E. coli* RB791 containing a plasmid bearing a mutant IL-2 gene were grown overnight and used to inoculate 1 L of medium in shake flasks. After induction and harvesting, cells (1.8–3.4-g wet weight) were disrupted by sonication and IL-2 aggregates isolated by centrifugation. Following successive 1 M Gdn-HCl and butan-1-ol washes, the pellet was dissolved in 4 mL of 8 M Gdn-HCl/10 mM DTT/50 mM Tris-HCl, pH 8.5, and chromatographed on a 40 × 2.2 cm Sepharose CL-6B GPC column equilibrated in 6 M Gdn-HCl/1 mM DTT, pH 5. IL-2 fractions were refolded/oxidized by dilution into 50 mM Tris-HCl (pH 8.5)/1.5 μM $CuSO_4$ and further purified by S-Sepharose Fast Flow cation-exchange chromatography and RP-HPLC as described below. Cl05A was purified by immunoaffinity chromatography subsequent to refolding by using "Reselute" anti-IL-2 monoclonal antibody affinity matrix (Celltech); purification was carried out according to the manufacturer's instructions and was followed by RP-HPLC as the final step.

Reversed-Phase HPLC. Semipure IL-2 mutants from IEC were chromatographed on a 25 × 0.4 cm Synchropak RP-PC18 column (Anachem) using a Varian 5020 liquid chromatograph and a UV-100 detector. Bound proteins were eluted with the following gradient, where buffer A = 0.1% TFA and buffer B = 65% MeCN/0.1% TFA: 0–77% B, 15 min; 82–91% B, 14 min; 91–100% B, 6 min; flow = 0.5 mL/min. Wild-type IL-2 eluted at about 60% MeCN.

For accurate determination of retention times of mutants relative to wild-type IL-2, 5–10-μg samples were analyzed with the Synchropak RP-P column and the following gradient: 0–69% B, 10 min; 69–100% B, 60 min; flow = 0.5 mL/min. Wild-type IL-2 retention was monitored at the beginning and end of each set of analyses and the difference in retention time of mutants from this value measured. Differences of 0.1–0.2% MeCN were not significant.

Gel Electrophoresis. SDS–PAGE was performed with 15% gels by the method of Laemmli (1970). IEF was performed with an LKB Multiphor flatbed apparatus or a Pharmacia Phast System apparatus according to the manufacturer's instructions, using respectively Pagplate pH 3.5–9.5 or Phast Gel pH 3–9 precast gels.

Electrophoresis on 15% PAGE gels at pH 4.2 was performed as described by Reisfeld et al. (1962). Samples were dissolved in 50 mM sodium acetate buffer, pH 4.6, and mixed with an equal volume of 120 mM KOH/acetic acid (pH 6.8)/20% glycerol prior to loading. Gels were Coomassie Blue stained.

Bioassay and Receptor Binding Assay. IL-2 was assayed by the murine T-cytotoxic cell (CTL-L) proliferation assay (Gillis et al., 1978). Purified IL-2 or IL-2 mutants were diluted 10-fold immediately following RP-HPLC into 20 mM phosphate–citrate buffer (pH 4)/5 mg/mL HSA and stored at −70 °C, conditions under which the wild-type and mutant proteins were stable to freeze–thawing. Samples were assayed in quadruplicate, with JURKAT lymphoid IL-2 included as a calibrant; JURKAT IL-2 was obtained from Dr. G. Thurman, NCI—Frederick Cancer Research Facility, Frederick, MD. Quadruplicates at each dose were averaged, log dose–response curves for [^{3}H]thymidine uptake were plotted, and half-maximal units were calculated by nonlinear least-squares regression analysis using the program ALLFIT (Bleackley et al., 1985). Protein concentrations were determined by amino acid analysis.

Relative receptor binding potencies of mutants were determined by measuring competitive inhibition of ^{125}I-labeled IL-2 (New England Nuclear) binding to the T-cell line HUT102B2, essentially as described by Robb et al. (1985) except that cell-bound and free IL-2 were separated by 0.22-μm membrane filtration using a Millititre 96-well plate (Millipore Ltd).

Amino Acid Analyses. Amino acid analysis was performed with a Waters PICO-TAG system as described by the manufacturer. Aminobutyric acid was an internal standard for quantitation; quantitation was based on Asp/Asn content, which was found to give the figure in consistently best agreement with the expected composition. Trp and Cys gave low and variable recoveries due to oxidation and were not included in the analyses.

Fluorescence and UV Second-Derivative Absorption Spectroscopy. Pure wild-type IL-2 and IL-2 mutants in 60% MeCN/10 mM TFA were buffered with 100 mM acetate buffer, pH 4.6 (final concentration), and most of the acetonitrile was evaporated by a gentle stream of N_2; samples were then diluted to between 25 and 155 μg/mL (1.6–10 μM IL-2) in 100 mM acetate buffer, pH 4.6; A_{285} was thus always below 0.2. Alternatively, samples were freeze-dried and dissolved in buffer, which gave the same results. Denatured IL-2 was prepared by unfolding in 6 M Gdn-HCl/100 mM acetate buffer, pH 4.6 at 37 °C, for 1 h. *N*-Acetyltyrosinamide (*N*-AcTyrNH$_2$) and *N*-acetyltryptophanamide (*N*-AcTrpNH$_2$) were dissolved in DMSO and diluted in 3:1 ratio into the appropriate buffer; Tyr and Trp are present in this proportion in wild-type IL-2.

Emission spectra (310–410 mm) were recorded at 23 ± 2 °C with base-line subtraction by using a Perkin-Elmer LS-5B fluorometer and a quartz cuvette; the excitation wavelength was 285 nm, and the excitation and emission slit widths were 15 and 2.5 nm, respectively.

Fluorescence quenching experiments were performed by addition of KI to 10 μM solutions of wild-type IL-2 or 5 μM F42W in 100 mM acetate buffer, pH 4.6; iodide was added from a stock solution of 5 M KI/100 mM acetate buffer (pH 4.6)/1 mM $Na_2S_2O_3$, the latter being included to prevent I_2 formation (Lehrer & Leavis, 1978) that caused precipitation of the protein. Fluorescence intensity (F) was estimated from

Table I: Yields and Electrophoretic and RP-HPLC Characteristics of Purified IL-2 Mutants

	yield of pure protein (μg/g of cells wet wt)	pI N-Ala IL-2 pI = 8.0 N-Met IL-2 pI = 7.7	PAGE R_f at pH 4.2	change in percent of MeCN needed for RP-HPLC elution[a]
wild type[b]	250	7.7, 8.0	0.48	0.0
Y31A	32	7.7, 8.0	ND	+0.9
F34L/T37P[b]	69	7.3, 7.4	0.48	0.0
T37P	175	7.3, 7.4	0.46	+0.2
F42A[b]	146	7.7, 8.0	0.48	−0.2
F42Y[b]	142	7.7, 8.0	0.48	0.0
F42W[b]	40	7.7, 8.0	0.48	+0.1
F44A	5.5	7.6, 7.7, 7.9, 8.0	0.48 (0.40)	0.0
H55D[b]	285	7.2, 7.5	0.47	+0.3
K64G	27	ND	ND	+0.1
F103A	23	7.6, 7.9	ND	+1.3
C105A[c]	3.9	ND	ND	−7.5
Y107A	38	7.7, 8.0	ND	−0.7
T111A	30	7.7, 8.0	ND	+0.6
T111S	9.2	ND	ND	+0.6
T111I	0.0	ND	ND	ND
F117A	0.8	ND	ND	ND
W121A	0.9	ND	ND	ND
W121Y	7.7	ND	ND	−2.3
W121S	0.0	ND	ND	ND
F124A	35	smeared	ND	−0.3
C125A	21	ND	ND	+1.2

[a] RP-HPLC retention characteristics were analyzed by reinjection of purified proteins and measured relative to wild-type IL-2 standard as the change in percent of MeCN required for elution under the same chromatographic conditions. [b] These mutants were cloned into the high-producing plasmid pATtacIL-2E/2TT; all others were in pATtacIL-2-C/2TT, which typically gave 5-fold lower expression. [c] C105A was purified by immunoaffinity chromatog. All other proteins were purified by IEC and RP-HPLC.

the peak height at λ_{max} and adjusted for the effect of KI absorption at 285 nm by using the expression $F = F_{obsd} \times 10^{\delta A/2}$, where δA is the change in absorption due to the quencher (Lehrer & Leavis, 1978); A_{285} of 5 M KI was 0.54.

Modification of tryptophan by *N*-bromosuccinimide (NBS) was performed essentially as described by Spande and Wiktop (1967): 10 μM IL-2 or 5 μM Trp42 mutant in sodium acetate buffer, pH 4.6, was reacted with NBS (1 mM stock solution in water) added to a final concentration of 40 μM. The reaction was rapid, and fluorescence spectra were recorded 10 min after addition of NBS. Higher concentrations of NBS led to precipitation of the protein.

Second-derivative UV absorption spectra were recorded with a Perkin-Elmer Lambda 5 spectrophotometer: δλ 3 nm; scan speed 30 nm/min; slit width 1 nm; response time 1 s; scan range 250–310 nm; and cuvette path length 1 cm (volume 0.5 mL). Samples were formulated as above. Tyrosine exposure was estimated from the ratio $R = a/b$, where a is the 287/283-nm peak/trough height and b is the 294/290.5-nm peak/trough height (Ragone et al., 1984).

Results

Purification. Of the 21 mutant IL-2 molecules (for the wild-type sequence see Figure 2) investigated, 18 were purified successfully by sequential IEC and RP-HPLC following refolding of GPC-purified Gdn-HCl extract. Although IL-2 expression levels as judged by SDS–PAGE of cell extracts were comparable to that of wild type, yields of purified protein varied considerably between mutants (Table I) even when the 5-fold lower expression typical for the pATtacIL-2C/2TT plasmid is taken into account; in particular F117A and W121A gave very poor yields and F44A, T111S, and W121Y were lower than expected. T111I and W121S could not be isolated at all. The purification method is to some extent biased toward isolation of native-like structures due to the absorptive steps of IEC and RP-HPLC; hence, a likely explanation for low yields is that a decreased proportion of mutant protein attains a native-like structure upon refolding, due either to instability of the protein in such a conformation or to destabilization of key intermediate(s) in the folding pathway (King, 1986).

C105A can only form the wrong disulfide isomer Cys58/Cys125, which is known to have a much shorter RP-HPLC retention time (Kunitani et al., 1987), precluding purification by the usual protocol; hence, this protein was isolated by immunoaffinity chromatography. W121S could not be isolated by this method, presumably because it does not bind to the anti-IL-2 monoclonal antibody.

Reducing SDS–PAGE showed all the isolated proteins to be over 95% pure and of M_r indistinguishable from wild type (approximately 15000); nonreducing gels did not indicate the presence of dimers or oligomers. Amino acid analyses (see Table II for examples) confirmed the purity of the preparations; except for typically low values for Val and Ile due to incomplete hydrolysis, agreement with the expected compositions was generally good.

Electrophoretic Characterization. Change in p*I* from the wild-type value upon a neutral mutation can conceivably arise because of an altered tendency to deamidate, direct perturbation of the pK_a of a neighboring group or indirect perturbation via a conformational change (Righetti, 1983); thus, substantial changes in p*I* can be observed upon unfolding of proteins (Hobart, 1975). Wild-type IL-2 as isolated here was a mixture of unprocessed (N-Met) and processed (N-Ala) protein in 4:1 ratio, with p*I* values respectively of 7.7 and 8,0 (Weir et al., 1987; Kato et al., 1986); this difference can be explained by the difference in pK_a of the Met and Ala α-NH_2 group (Wingfield et al., 1987). Most mutants examined exhibited unchanged p*I* values (Table I, Figure 3). However, P34L/T37P and T37P showed a drop in p*I* of about 0.5 pH unit; pH titration analysis by IEF (Righetti, 1983; Wingfield et al., 1987) showed this change to be due to a group (or groups) of pK_a below 5 (data not shown). The most likely cause is an increased tendency to deamidate in those mutants either in vivo or during isolation, since species of this p*I* were generated by incubation of wild-type protein in 1 M TFA. F44A showed four bands, two in the wild-type position and two of 0.1 pH unit lower p*I*, that may be due to dimerization or to the presence of two sets of conformers; this protein

Table II: Amino Acid Compositions of Mutants[a]

residue	expected composition	experimentally determined compositions						
		wild type	P34L/T37P	F42A	F42Y	F42W	F44A	W121Y
D/N	12	11.8	11.5	12.4	11.8	11.8	12.4	12.2
E/Q	18	18.6	18.4	18.9	18.2	18.8	18.6	18.2
S	8	8.3	8.0	8.3	8.4	8.2	8.2	8.9
G	2	3.4	2.7	2.7	2.4	2.8	4.3	8.3
H	3	2.6	2.4	ND	ND	2.7	2.7	ND
R	4	5.1	4.9	5.1	5.2	5.2	5.9	6.5
T	12	13.2	12.8	13.3	13.7	13.4	12.1	10.1
A	5	6.0	5.4	7.1	5.9	5.7	7.8	9.8
P	5	5.6	6.7	6.2	6.4	5.8	5.6	5.4
Y	3	3.4	3.4	3.3	4.2	3.6	3.7	3.3
V	4	2.8	2.5	2.9	2.5	2.6	3.7	5.6
M	5	3.9	5.2	4.5	4.5	3.9	2.6	2.9
I	10	5.8	5.9	6.1	6.0	5.9	6.1	5.9
L	22	19.6	22.0	20.1	19	20.4	19.5	17.0
F	6	6.4	6.4	5.3	5.0	5.3	5.0	4.9
K	11	10.7	11.0	11.4	11.2	10.5	9.7	8.2

[a] Samples were acid hydrolyzed and amino acids quantitated by analysis of PTH derivatives. The expected composition given is for N-Met IL-2 excluding tryptophan and cysteine; experimental compositions are thus given as residues per 130 residues.

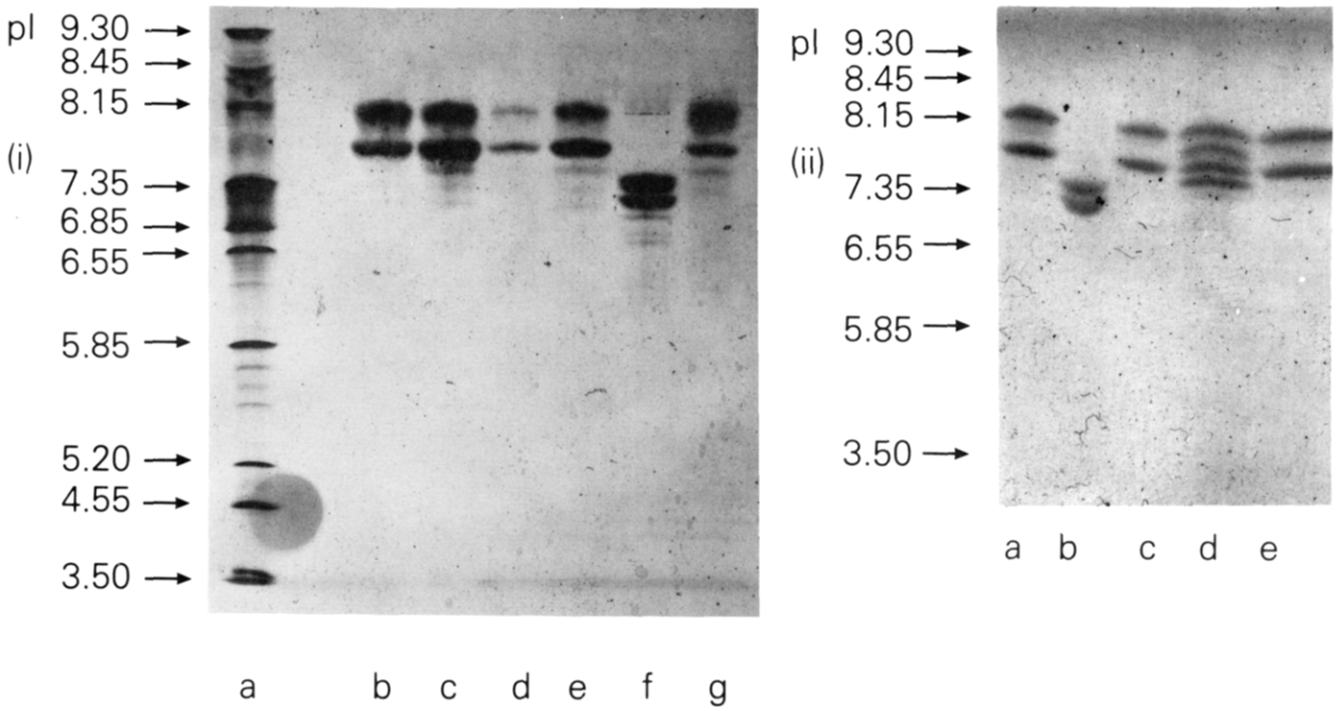

FIGURE 3: IEF was performed with freeze-dried samples dissolved in 50 mM acetic acid and loaded near the cathode end of the prefocused gels. (i) 11 × 12 cm Pagplate gel, pH 3.5–9.5: (a) p*I* markers; (b) wild-type IL-2; (c) F42Y; (d) F42A; (e) F42W; (f) T37P; (g) Y31A. (ii) Phastgel 3–9: (a) wild-type IL-2; (b) P34L/T37P; (c) wild-type IL-2; (d) F44A; (e) wild-type IL-2. Gels were Coomassie Blue stained.

showed evidence of reduced solubility and changes in fluorescence (see below). H55D exhibited a decreased p*I* as expected from the charge change upon mutation.

Substantial changes in PAGE (nondenaturing) mobility are often observed to occur upon unfolding (Creighton, 1978). None of the mutants examined by PAGE at pH 4.2 showed anything but minor changes in R_f (Table I, Figure 4), and those mutants that did show changes (T37P and H55D) had altered p*I* values; such small shifts in R_f are consistent with single or partial charge differences (Picard et al., 1987). Hence, these mutants are folded to structures of comparable hydrodynamic radius to the wild-type molecule. F44A (Figure 4, track i) did exhibit a minor band of $R_f = 0.40$, which may be dimer; dimers or oligomers of F44A are presumably noncovalent since there was no evidence for them on nonreducing SDS–PAGE.

Reversed-Phase HPLC. RP-HPLC is carried out under conditions (acid/organic solvent) that might be expected to induce denaturation; indeed, conformational transitions upon chromatography frequently lead to peak splitting, a process apparently enhanced by stronger hydrophobic interactions with unfolded species (Hearn et al., 1985). How closely the conformation of a protein when bound to the hydrophobic phase resembles the native structure is therefore unclear. However, within a set of IL-2 mutants, changes in retention have been shown to be proportional to conformational changes, as indicated by the magnitude of the hydrophobic contact area parameter derived from chromatographic data (Kunitani et al., 1987). Our IL-2 mutants were thus examined for differences in retention from the wild-type molecule (Table I); the largest change was caused by formation of the wrong disulfide isomer in C105A, which presumably results in a grossly altered conformation (Kunitani et al., 1987; Browning et al., 1986). None of the other mutants showed evidence of such a large structural distortion, although W121Y eluted at a markedly lower percent of MeCN, strongly indicating a

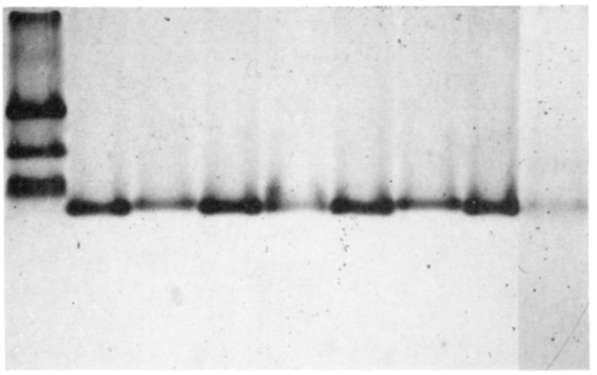

FIGURE 4: Nondenaturing polyacrylamide gel electrophoresis. PAGE was performed with a separating gel at pH 4.2. Samples were freeze-dried and loaded in 50 mM acetate buffer, pH 4.6. Gels were Coomassie Blue stained. (a) p*I* markers loaded to confirm proper running of the gel; (b, d, f, and h) wild-type IL2; (c) F42W; (e) F42A; (g) F42Y; (i) F44A. Note the faint band at reduced R_f in F44A, consistent with some dimerization of this protein.

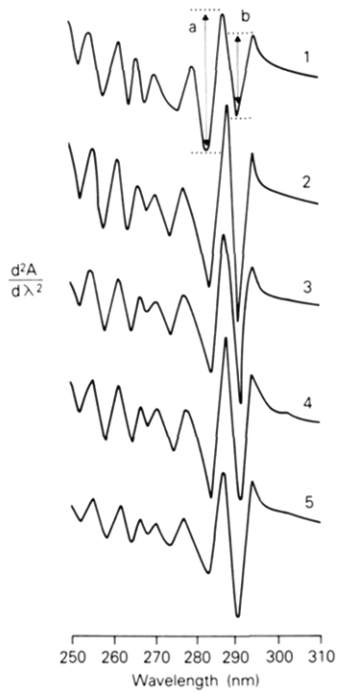

FIGURE 5: Second-derivative UV absorption spectra. Samples were formulated at 10 μM protein concentration in 100 mM sodium acetate buffer, pH 4.6, except for unfolded wild-type IL-2, which was in 6 M Gdn-HCl/100 mM sodium acetate buffer, pH 4.6. Peak/trough heights *a* and *b* were used to calculate the fractional exposure of tyrosine residues (see text). (Spectrum 1) Unfolded wild-type IL-2; (spectrum 2) native wild-type IL-2; (spectrum 3) F42A; (spectrum 4) F42Y; (spectrum 5) F42W. Note the small but significant shifts in the peaks at 266.5 and 278 nm seen in the unfolded wild-type molecule compared with the folded IL-2 species.

conformational change in this molecule. C-terminal mutants (F103A–C125A) showed greater changes in retention than N-terminal mutants, the latter group being substantially unaltered except for Y31A; it is possible that this dichotomy reflects a greater participation of C-terminal residues in binding to the hydrophobic phase.

UV Second-Derivative Spectroscopy. Protein second-derivative spectra in the region 250–310 nm show the following major features (d'Albis & Gratzer, 1974; Ragone et al., 1984; Ichikawa & Terada, 1981): a maximum at 294 nm and a minimum at 290.5 nm due to Trp; a maximum at 287 nm and a minimum at 283 nm due to Trp and Tyr; and maxima at 278 nm and 266.5 nm due to Tyr and Phe, respectively. Several differences were observed between spectra of native and Gdn-HCl-unfolded wild-type IL-2 (Figure 5, curves 1 and 2) in particular 1–2-nm shifts at λ_{max} 278 nm and 266.5 nm. In contrast, the peak/trough positions of mutants F42A, F42Y, and F42W were the same within error as native wild-type IL-2 except for a 0.6-nm blue shift at 287 nm in F42W that may be due to the additional Trp. F44A and F124A proteins were also examined but were poorly soluble in aqueous solution at the necessary concentration.

Table III: Fractional Tyrosine Exposure (α) Estimated from Second-Derivative Spectra

IL-2 protein	R_n[a]	R_u[b]	Tyr/Trp[c]	R_a[d]	α
wild type	1.04	1.71	3	0.11	0.58
F42A	1.06	1.47	3	0.11	0.69
F42W	0.80	1.04	1.5	0.39	0.63

[a] Determined in 0.1 M acetate buffer, pH 4.6. [b] Determined in 6.0 M Gdn-HCl/0.1 M acetate buffer, pH 4.6. [c] Calculated from sequence. [d] Taken from Ragone et al. (1984).

Table IV: Fluorescence Emission Spectra: λ_{max} and Relative Intensities

sample	buffer	λ_{max} (nm)	rel F[a]
wild-type IL-2	acetate, pH 4.6	324	1.0
wild-type IL-2	6 M Gdn-HCl, pH 4.6	352	1.5
P34L/T37P	acetate, pH 4.6	324	0.9
T37P	acetate, pH 4.6	324	0.9
F42A	acetate, pH 4.6	324	0.8
F42Y	acetate, pH 4.6	324	0.9
F42W	acetate, pH 4.6	344	3.5
F44A[b]	acetate, pH 4.6	333	1.0–1.6
H55D	acetate, pH 4.6	324	0.9
3:1 *N*-AcTyrNH$_2$: *N*-AcTrpNH$_2$	acetate, pH 4.6	355	1.7
3:1 *N*-AcTyrNH$_2$: *N*-AcTrpNH$_2$	6 M Gdn-HCl, pH 4.6	355	2.3

[a] Calculated from peak heights at λ_{max} and normalized to an equivalent concentration of wild-type IL-2 in acetate, pH 4.6. [b] Solutions of this protein were turbid, but the bandshape was similar to that of the wild-type molecule.

Denaturation also produced an increase in the ratio, *R*, of peak/trough heights *a* (287/283 nm) and *b* (294/290.5 nm). Such a change has been observed for several proteins and has been shown to be proportional to the extent of burial of Tyr residues, and thus Tyr fractional exposure (α) can be estimated by using the following relationship (Ragone et al., 1984): $\alpha = (R_n - R_a)/(R_u - R_a)$, where $R_n = a/b$ (native), $R_u = a/b$ (unfolded), and R_a is the value for a mixture of *N*-AcTyrNH$_2$ and *N*-AcTrpNH$_2$ of appropriate stoichiometry measured in 100% ethylene glycol, which approximates to complete residue burial. Values of R_n, R_u, and α are shown in Table III for wild type, F42W, and F42A; small increases in fractional exposure were registered for the mutants, but there was no substantial change apparent. Taken together, the second-derivative spectra do not indicate any major change in Tyr or Phe burial in the three mutants at position 42.

Fluorescence Spectroscopy. Wild-type IL-2 contains one tryptophan at position 121 that serves as an intrinsic fluorescence probe. Contributions from tyrosine to the fluorescence are expected to be small relative to those of tryptophan (Lin & Dowben, 1983). Fluorescence emission spectra are shown in Figure 6i, and data on λ_{max} and relative intensity at this wavelength are shown in Table IV; values for a 3:1 mixture of *N*-AcTyrNH$_2$:*N*-AcTrpNH$_2$ are also given.

The λ_{max} value of 324 nm seen for the wild-type molecule is in agreement with the report of Cohen et al. (1986) and is indicative of a hydrophobic environment for Trp121 (Campbell

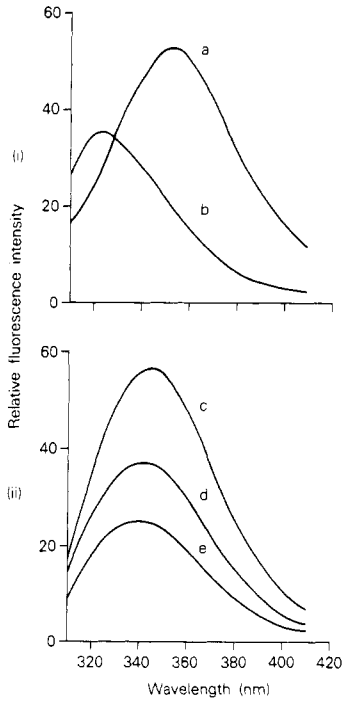

FIGURE 6: Fluorescence spectroscopy. Emission spectra (excitation wavelength 285 nm) of 10 μM wild-type IL-2 or 5 μM F42W were measured at pH 4.6 in 100 mM sodium acetate buffer. (i) (Spectrum a) Wild-type IL-2 in 6 M Gdn-HCl. Unfolding caused a 28-nm red shift from native wild-type in acetate buffer (spectrum b). (ii) (Spectrum c) F42W in 450 mM KCl. Addition of KCl did not significantly perturb the spectrum. (Spectrum d) F42W in 300 mM KI/150 mM KCl, showing quenching and a blue shift of λ_{max} compared to spectrum c. (Spectrum e) F42W after reaction with 40 μM *N*-bromosuccinimide. Oxidation of Trp42 caused a reduction in intensity and blue shift.

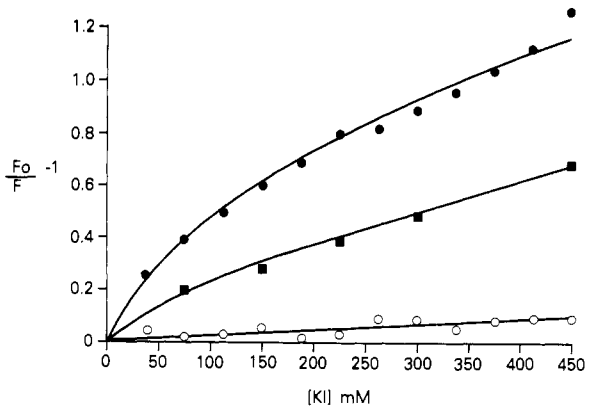

FIGURE 7: Concentration dependence of fluorescence quenching. Stern-Volmer plot of iodide quenching of wild-type IL-2 at variable ionic strength (○), F42W at variable ionic strength (●), and F42W with ionic strength held at 450 mM by addition of KCl (■). F = fluorescence intensity; F_0 = fluorescence intensity at 0 mM KI. The data show that Trp121 is largely inaccessible to KI in the wild-type molecule, but Trp42 is exposed in F42W (see text).

& Dwek, 1984); i.e., the chromophore is isolated from solvent by burial within the folded structure of the protein. This assignment is confirmed by the large increase in λ_{max} observed upon unfolding. Trp121 fluorescence also appears to be quenched within the folded protein; there was a 50% increase in intensity upon unfolding, whereas chromophores normally fluoresce less strongly in polar environments than in a hydrophobic medium (Betton et al., 1984; Campbell & Dwek, 1984).

All the mutants examined with the exceptions of F42W, F44A, and F124A showed spectra virtually identical with that of wild type, indicating that the folding around Trp121 was unperturbed in these molecules. Both F124A and F44A were poorly soluble at pH 4.6, and the former gave a broad, featureless fluorescence spectrum; F44A solutions, although somewhat turbid, showed an increase in λ_{max}, which indicates partial unfolding. Whether this is a consequence of aggregation is unclear.

F42W showed a 20-nm red shift in λ_{max} and a 3.5-fold increase in intensity compared to that of wild-type (Figure 6ii). Since two chromophores were present, splitting or increased asymmetry of the emission band might be expected, but the width and shape were very similar to those of unfolded wild-type IL-2 (Figure 6i) and *N*-AcTrpNH$_2$, except for a slight shoulder in the 320–330-nm region. It is proposed that radiationless resonance transfer of energy (Eisinger et al., 1969) has occurred from Trp121 to Trp42 and the latter has refluoresced at a longer wavelength because it is more exposed, that the intensity increase was more than 2-fold because Trp42, unlike Trp121, is unquenched, and that the slight shoulder at lower wavelengths was due to residual Trp121 direct fluorescence. A λ_{max} of 344 nm is indicative of approximately 50% Trp exposure as shown by UV solvent perturbation measurements (Craig et al., 1987).

The extent of resonance transfer is among other things proportional to the distance between, and relative orientation of, the donor and acceptor chromophores (Campbell & Dwek, 1984). Since the relative orientation of the two Trp chromophores is unknown, their distance cannot be calculated with any accuracy, but since transfer of energy appears to be over 90%, they are likely to be within 1 nm of each other (Eisinger et al., 1969).

It follows from the above interpretation that F42W should be more accessible to quenching by iodide (Eftink & Ghiron, 1981) and that a blue shift should be observed upon quenching; both of these effects were observed in F42W (Figure 6ii), which showed a 4-nm blue shift and a 30% drop in intensity with 300 mM KI. Fluorescence quenching curves at constant ionic strength and at variable ionic strength are shown in Figure 7, using the Stern-Volmer representation (Eftink & Ghiron, 1981). In contrast to F42W, the wild-type molecule was largely refractory to KI, consistent with burial of Trp121 and in agreement with reported data (Cohen et al., 1986). F42W fluorescence quenching was biphasic and was also of reduced magnitude when the ionic strength was kept constant at 450 mM; since iodide is a charged quencher, this effect may well be due to the close proximity of a positive charge from the Lys43 side chain (Eftink & Ghiron, 1981).

If Trp42 is exposed and Trp121 buried, then the former residue should show a greater susceptibility to oxidation by *N*-bromosuccinimide to the nonfluorescent oxindole derivative (Spande & Wiktop, 1967; Imoto et al., 1971); thus, NBS modification of F42W is expected to effect a sharp decrease in fluorescence and a blue shift, since resonance transfer in modified molecules would be abolished and Trp121 would directly fluoresce at 324 nm. Such an effect was observed (Figure 6ii); treatment of F42W with a 4-fold molar excess of NBS over tryptophan brought about a 60% decrease in fluorescence intensity and a 5-nm blue shift. A greater degree of modification was not possible due to precipitation of the protein at high NBS concentrations. The wild-type molecule showed only a 7% decrease in intensity upon NBS treatment.

In summary, fluorescence studies indicate that mutants P34L/T37P, T37P, F42A, Y42A and H55D are conformationally unperturbed relative to wild type in the region of their buried tryptophan residue 121; F44A fluorescence suggests some unfolding in this molecule. F42W spectra are consistent

Table V: Bioactivities and Relative Receptor Binding Affinities for IL-2 Mutants

	bioactivity[a] (units/mg × 10^{-6})	rel receptor binding affinity[b] (%)
wild type	16 ± 4.5	100
Y31A	19	103
P34L/T37P	18	ND
T37P	16	ND
F42A	2.7	8.0
F42Y	7.8	30
F42W	12	39
F44A	1.8	ND
H55D	11	ND
K64G	19	ND
F103A	8.6	ND
C105A	0.01	<1
Y107A	9.0	ND
T111A	10	ND
T111S	16	ND
T111I	ND	ND
F117A	ND	ND
W121A	ND	ND
W121Y	4.3	ND
W121S	ND	ND
F124A	19	ND
C125A	18	ND

[a] Measured on mouse CTL-L cells relative to JURKAT IL-2 standard (10^7 units/mg). [b] Measured with ^{125}I-labeled IL-2 and HUT102 human leukemia cells.

with an unperturbed molecule in which the second tryptophan, residue 42, is considerably exposed to solvent and is within 1 nm of Trp121 in the folded structure.

Bioassays and Receptor Binding Assays. The best quantitative functional assay available for IL-2 is based on its T-cell growth factor activity (Gillis et al., 1978). The specific activity for lymphoid IL-2 purified from JURKAT cell culture supernatants is defined as 10^7 ED_{50} units/mg (Thurman et al., 1986), and the bioactivity values given in Table V for purified recombinant IL-2 species were calibrated against JURKAT IL-2. The amplitude of the response for the various mutants was always near 100% wild type; hence, no partial agonists were discovered. Good replication of data points within a given assay was found (Figure 8), but values for wild-type IL-2 varied in the range 10–20 × 10^6 units/mg between assays with a mean plus or minus standard deviation of 15.6 ± 4.5 (n = 4); hence, only F42A, F44A, W121Y and C105A were significantly reduced in bioactivity. These mutants were consistently low in bioactivity upon reassay of the same samples and upon assay of fresh preparations.

Receptor binding data (Table V) were obtained on the same samples used for bioactivity measurements by competitive binding assay with ^{125}I-labeled IL-2 and the adult T-cell leukemia human cell line HUT102B2, which expresses high levels of high-affinity IL-2R (Robb et al., 1985). For those mutants analyzed, it is clear that within the limits of the assays the binding affinities of mutants broadly parallel their bioactivities, which was in most cases also observed in a set of IL-2 analogues by Nakagawa and Tager (1986). Thus, F42A is sharply reduced in both binding and bioactivity.

Discussion

In these experiments we have attempted to define IL-2 residues that are directly involved in association with IL-2R, which we will term "binding residues"; such residues might form part of the contact region in the equilibrium IL-2/IL-2R complex. Our principal assay for altered association in mutants has been the murine CTL-L cell proliferation bioassay; how valid a measure of association is this? The murine IL-2R Tac molecule is highly homologous with human IL-2R

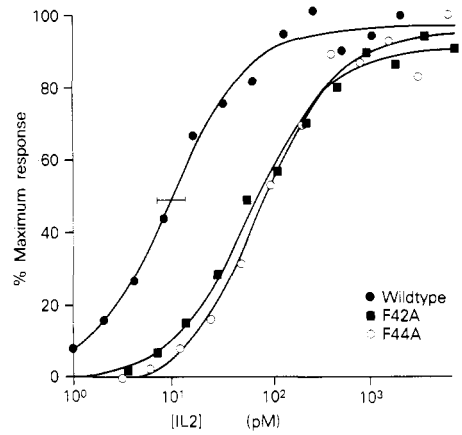

FIGURE 8: Bioassay of wild-type and mutant IL-2 species. IL-2 samples were diluted to below 10 μg/mL in 5 mg/mL HSA, pH 4.0, and stored at −70 °C prior to bioassay on mouse CTL-L cells. Data shown are for quadruplicate determinations within single assays of wild-type IL-2 (●), F42A (■), and F44A (○), normalized to JURKAT native IL-2 included within each assay. The standard deviation on ED_{50} values determined from such data was 30% and is indicated by the error bar on the wild-type curve. Clearly, F42A and F44A are of significantly reduced bioactivity.

(Shimuzu et al., 1985), and human IL-2 has been shown to be equipotent with murine IL-2 and CTL-L cells (Bleackley et al., 1985), so the human IL-2/murine IL-2R interaction is probably of a nature similar to the high-affinity human IL-2/human IL-2R interaction probed directly here for selected mutants by the HUT102 binding assay. It follows that CTL-L bioactivity is a fair measure of agonistic binding to receptor, but the possibilities of cross-species differences and of activation events that do not directly correlate with binding should be borne in mind.

If the binding of a mutant changes, then the mutated side chain can properly be considered as being in direct contact with receptor if the remainder of the protein structure shows no evidence of alteration. We have used the relatively rapid techniques of native gel electrophoresis, RP-HPLC, and electronic spectroscopy to probe mutant conformation to a first approximation and to help us interpret bioactivity data. Mutant IL-2 proteins fall into five categories: those having (1) no evidence of altered binding or conformation (F42Y, F42W, H55D and K64G); (2) no evidence of altered binding but some evidence of changed conformation (Y31A, P34L/T37P, T37P, F103A, Y107A, T111A, T111S, F124A, and C125A); (3) reduced binding and changed conformation-(F44A, C105A and W121Y); (4) reduced binding but no evidence of a conformational change (F42A); and (5) no or very little protein isolated, probably because of poor refolding (T111I, F117A, W121A and W121S).

The most striking facet of the mutants in the first two categories is the number of nonconservative mutations that result in apparently unaltered binding, in spite of in some cases causing structural perturbations, for example, F124A. This argues strongly that these residue side chains are not involved in binding to any large degree and confirms our impression gained from random mutagenesis that IL-2 is a relatively robust molecule with respect to mutations that alter activity (unpublished observations). To some extent the data may also reflect the insensitivity of the bioassay to the changes that may be occurring; protein–protein interactions tend to involve a large number of residue contacts, 10–20 per protein in the cases of lysozyme/antibody and trypsin/trypsin inhibitor complexes (Amit et al., 1986; Janin & Chothia, 1976), and individual residue contributions can sometimes be correspondingly small.

Results for mutations at Tyr31, His55, and Cys125 have been previously reported (Ju et al., 1987; Wang et al., 1984; Liang et al., 1986) and are consistent with our results. The conformation of C125A was examined by circular dichroism (Arakawa et al., 1986), which indicated a high helix content, similar to the wild-type molecule (Cohen et al., 1986).

In the third category, C105A had drastically reduced bioactivity, consistent with the distortion resulting from formation of the wrong disulfide, Cys58/Cys125 (Browning et al., 1986; Liang et al., 1986); receptor binding was also very low (Table V), in contrast with the report of Ju et al. (1987), although in the latter case there was the difference that assays were performed on crude extracts.

The fluorescence data presented here and by Cohen et al. (1986) show Trp121 to be buried; hence, it would not be surprising if W121A and W121S were to fold incorrectly, as appears to be the case. A previous report of W121S activity (Ju et al., 1987) showed it to have very low bioactivity. The conservative mutant W121Y, however, was reported to have 20–30% wild-type bioactivity (Ju et al., 1987); we have confirmed this result and also report a substantial shift on RP-HPLC (Table I), indicative of preturbed folding. It is likely that Phe117, Phe124, and Phe44 are also buried since mutants are either not recovered on purification (F117A) or show strong evidence of structural perturbation (F124A, F44A). Also, the fluorescence data presented here for F42W indicate a close proximity of Phe42 and Trp121, with the side chains probably within 1 nm. A recently published X-ray structure of IL-2 (Brandhuber et al., 1987) at 0.3-nm resolution traces the C_α backbone and shows the molecule to be largely helical, with helix B (residues 33–47) apparently packed against helix F (residues 117–133), which would indeed place Phe42 relatively close to Trp121. Furthermore, it is possible that Phe117, Trp121, Phe124, and Phe44 are buried at the helix B/helix F interface, which might place Phe42 on the solvent side of helix B, available for interaction with IL-2R (see below). Finally, the Cys125 thiol may be the quencher of Trp121 fluorescence, since it would be close to this residue in helix F.

The mutant F42A is most interesting of all from the viewpoint of structure–activity since there is a substantial loss of binding without any sign of a conformational change, pointing to Phe42 as a likely binding residue. It follows that Trp42 and Tyr42 also interact with IL-2R in the appropriate mutants. Alternatively, mutation of Phe42 to Ala may induce a local structural perturbation that leads in turn to reduced binding. Mutations at residues Leu36, Leu40, Tyr45, and Lys54 (Ju et al., 1987) and double mutants at Phe42 and Phe44 to Tyr and Trp (Gadski et al., 1987) all produced reductions in activity, although no data regarding the conformation of these mutants were presented. A neutralizing monoclonal antibody has also been shown to bind to this region (residues 33–54) and to block association with the β subunit of IL-2R (Robb et al., 1987).

In conclusion, we have obtained evidence that residue Phe42 of IL-2, or a region adjacent to it in the tertiary structure, is directly involved in or associated with high-affinity IL-2 receptor; furthermore, this residue can be successfully replaced with a Trp, which could act as a spectroscopic probe of such an interaction. Because of the widely recognized difficulties in demonstrating conclusively the absence of a conformational change upon mutation (Knowles, 1987), further mutations will be necessary at residues adjacent in the tertiary structure; a consistent pattern should then emerge if the effects of mutations are indeed direct, as has been observed elsewhere for carefully constructed sets of mutants (Fersht et al., 1986).

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Supplementary Material Available

Table of oligonucleotides used for site-directed mutagenesis of IL-2; oligonucleotides can be matched to the DNA sequence given in Figure 2 of the text (1 page). Ordering information is given on any current masthead page.

References

Amit, A. G., Mariuzza, R. A., Phillips, S. E. V., & Poljak, R. J. (1986) *Science (Washington, D.C.) 233*, 747–753.

Arakawa, T., Boone, T., Davis, J. M., & Kenney, W. C. (1986) *Biochemistry 25*, 8274–8277.

Betton, J. M., Desmadril, M., Mitraki, A., & Yon, J. M. (1984) *Biochemistry 23*, 6654–6661.

Bleackley, R. C., Barr, P. J., Havele, C., Hooton, J., Ng, J., Meier, M., Merryweather, J. P., Gribbs, C., & Paetkau, V. (1985) *Lymphokine Res. 4*, 117–1331.

Brandhuber, B. J., Boone, T., Kenney, W. C., & McKay, D. B. (1987) *Science (Washington, D.C.) 238*, 1707–1709.

Browning, J. L.,m Mattaliano, R. J., Pingchang Chow, E., Liang, S.-M., Allet, B., Rosa, J., & Smart, J. E. (1986) *Anal. Biochem. 155*, 123–128.

Campbell, I. D., & Dwek, R. D. (1984) *Biological Spectroscopy*, pp 91–126, Benjamin/Cummings, Menlo Park, CA.

Cohen, F. E., Kosen, P. A., Kuntz, I. D., Epstein, L. B., Ciardelli, T. L., & Smith, K. A. (1986) *Science (Washington, D.C.) 234*, 349–352.

Craig, S., Schmeissner, U., Wingfield, P., & Pain, R. H. (1987) *Biochemistry 26*, 3570–3576.

Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231–297.

d'Albis, A., & Gratzer, W. B. (1974) *Companion to Biochemistry*, pp 163–196, Longman, Harlow, U.K.

de Boer, H. A., Camstock, L. J., Yansura, D. G., & Heynecker, H. L. (1982) in *Promotors, Structure and Function* (Rodriguez, R. L., & Chamberlin, M. J., Eds.) pp 462–481, Praeger, New York.

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

Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.

Eisinger, J., Fuer, B., & Lamola, A. A. (1969) *Biochemistry 8*, 3908–3915.

Ezard, R., Corthesy, P., Nabholz, M., Lowenthal, J., Zaech, P., Plaetinck, G., & Macdonald, H. (1985) *J. Immunol. 134*, 1644–1652.

Fersht, A. R., Leatherbarrow, R. J., & Wells, T. N. C. (1986) *Trends Biochem. Sci. (Pers. Ed.) 11*, 321–325.

Gadski, R., Strand, J., Deriso, P., Belagaje, R., Van Frank, R., Smith, K., & Butler, L. (1987) in *Immune Regulation by Characterised Polypeptides*, pp 363–372, Alan R. Liss, New York.

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

Hearn, M. T. W., Hodder, A. N., & Aguilar, M.-I. (1985) *J. Chromatogr. 327*, 47.

Hinney, C. S., Kuribayashi, K., Kern, D. E., & Gillis, S. (1981) *Nature (London) 291*, 335–338.
Hobart, M. J. (1975) in *Isoelectric Focusing* (Arbuthnott, J. P., & Beeley, J. A., Eds.) pp 275–280, Butterworths, London.
Ichikawa, T., & Terada, H. (1981) *Biochim. Biophys. Acta. 671*, 33–37.
Imoto, T., Forster, L. S., Rupley, J. A., & Tanaka, F. (1971) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1151–1155.
Janin, J., & Chothia, C. (1976) *J. Mol. Biol. 100*, 197–211.
Ju, G., Collins, L., Kaffka, K. L., Tsien, W.-H., Chizzonite, R., Crowl, R., Bhatt, R., & Kilian, P. L. (1987) *J. Biol. Chem. 262*, 5723–5731.
Kato, K., Yamada, T., Kawahara, K., Onda, H., Asano, T., Sugino, H., & Kakinuma, A. (1985) *Biochem. Biophys. Res. Commun. 130*, 692–699.
King, J. (1986) *Bio/Technology 4*, 297–303.
Knowles, J. (1987) *Science (Washington, D.C.) 236*, 1252–1258.
Kunitani, M., Johnson, D., & Snyder, L. R. (1986) *J. Chromatogr. 371*, 313–333.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lehrer, S. S., & Leavis, P. C. (1978) *Methods Enzymol. 49*, 222–237.
Liang, S.-M., Thatcher, D. R., Liang, C.-M., & Allet, B. (1986) *J. Biol. Chem. 261*, 334–337.
Lin, T. S., & Dowben, R. M. (1983) in *Excited States of Biopolymers* (Steiner, R. F., Ed.) pp 59–113, Plenum, New York.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mule, J. J., Shu, S., & Rosenberg, S. A. (1985) *J. Immunol. 135*, 646–652.
Nakagawa, S. H., & Tager, H. S. (1986) *J. Biol. Chem. 261*, 7332–7341.
Picard, B., Goullet, P., & Krishnamoorthy, R. (1987) *Biochem. J. 241*, 877–881.
Pike, B. L., Raubitschek, A., & Nossal, G. J. V. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7918–7921.
Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., & Irace, G. (1984) *Biochemistry 23*, 1871–1875.
Ralph, P., Nakoinz, I., Doule, M., Lee, M.-T., Jeong, G., Halenback, R., Mark, D. F., & Koths, K. (1987) in *Immune Regulation by Characterised Polypeptides*, pp 453–462, Alan R. Liss, New York.
Reisfeld, R. A., Lewis, U. J., & Williams, D. E. (1962) *Nature (London) 195*, 281–283.
Righetti, P. G. (1983) *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier, Amsterdam.
Robb, R. J. (1985) *Behring Inst. Mitt. 77*, 56–67.
Robb, R. J., Kutny, R. M., Panico, M., Morris, H. R., & Chowdhry, V. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6486–6490.
Robb, R. J., Mayer, P. C., & Garlick, R. (1985) *J. Immunol. Methods 81*, 31–42.
Robb, R. J., Rusk, C. M., Yudoi, J., & Greene, W. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2002–2006.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Shimuzu, A., Kondo, S., Takeda, S., Yodoi, J., Ishida, N., Sabe, H., Osawa, H., Diamantstein, T., Nikaido, T., & Honjo, T. (1985) *Nucleic Acids Res. 13*, 1505–1516.
Spane, T. F., & Witkop, B. (1967) *Methods Enzymol. 11*, 498–506.
Taniguchi, T., Matsui, H., Fujita, T., Takaoka, C., Kashima, N., Yoshimoto, R., & Hamuro, J. (1983) *Nature (London) 302*, 305–310.
Thurman, G. B., Maluish, A. E., Rossio, J. L., Schlick, E., Onozaki, K., Talmadge, J. E., Procopio, A. D. G., Ortaldo, J. R., Ruscetti, R. W., Stevenson, H. C., Cannon, G. B., Iyar, S., & Herberman, R. B. (1986) *J. Biol. Response Modif. 5*, 85–107.
Wang, A., Lu, S.-D., & Mark, D. F. (1984) *Science (Washington, D.C.) 224*, 1431–1433.
Weir, M. P., Sparks, J., & Chaplin, A. M. (1987) *J. Chromatogr. 396*, 209–215.
Wingfield, P. T., Graber, P., Buell, G., Rose, K., Simona, M. G., & Burleigh, B. D. (1987) *Biochem. J. 243*, 829–839.
Yamada, T., Kato, K., Kawahara, K., & Nishimura, O. (1986) *Biochem. Biophys. Res. Commun. 135*, 837–843.
Zoller, M. J., & Smith, M. (1982) *Anal. Biochem. 10*, 6487–6500.