

FOR THE RECORD

Crystallization and preliminary X-ray analysis of a 1:1 complex between a designed monomeric interferon-gamma and its soluble receptor

M. RANDAL¹ AND A.A. KOSSIAKOFF^{1,2}

¹Graduate Group in Biophysics, University of California, San Francisco, California 94000

²Genentech Inc., 1 DNA Way, South San Francisco, California 94080

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Abstract: A variant of human interferon-gamma (IFN- γ) has been created in which the two chains of the homodimeric cytokine were linked N- to C-terminus by an eight residue polypeptide linker. The sequence of this linker was derived from a loop in bira bifunctional protein, and was determined from a structural database search. This “single-chain” variant was used to create an IFN- γ molecule that binds only a single copy of the α -chain receptor, rather than the 2 α -chain receptor: 1 IFN- γ binding stoichiometry observed for the native hormone. Crystals have been grown of a 1:1 complex between this single-chain molecule and the extracellular domain of its α -chain receptor. These crystals diffract beyond 2.0 Å, significantly better than the 2.9 Å observed for the native 2:1 complex. Density calculations suggest these crystals contain two complexes in the asymmetric unit; a self-rotation function confirms this conclusion.

Keywords: interferon-gamma; receptor; structure-based design; X-ray crystallography

Interferon- γ (IFN- γ) is a homodimeric cytokine with potent antiviral, antiproliferative, and immunomodulatory activities (Wheeler, 1965; Farrar & Schreiber, 1993). It is expressed as a 143 amino acid polypeptide, with a post-translation modification resulting in a pyroglutamate amino terminus, the addition of carbohydrate, and a heterogeneous carboxyl terminus (Gray & Goeddel, 1982; Rinderknecht et al., 1984; Pan et al., 1987). IFN- γ exerts its various activities through receptor aggregation, a general mechanism common to the cytokine family (Sprang & Bazan, 1993). The first step of this process involves IFN- γ binding to its high-affinity cell surface receptor, IFN- γ R α , a member of the class-2 hematopoietic receptor superfamily (Schreiber & Celada, 1985; Langer & Pestka, 1988). While IFN- γ has been shown to induce the dimerization of IFN- γ R α (Greenlund et al., 1993), subsequent binding

of a related receptor, IFN- γ R β 1, is required for signaling via the JAK/STAT pathway. IFN- γ R β 1 is postulated to be one member of a family of accessory factors that allows for the different cellular responses of IFN- γ (Soh et al., 1994; Hemmi et al., 1994).

The ability to produce large quantities of fully active recombinant IFN- γ (Gray et al., 1982; Tanaka et al., 1983) has made it the focus of numerous mutagenesis and structural studies aimed at establishing the determinants of receptor recognition. The X-ray crystal structure has been determined at 2.9 Å resolution of IFN- γ with two copies of the extracellular domain of IFN- γ R α bound symmetrically to the homodimer (Walter et al., 1995; S. Ealick, pers. comm.). This structure has provided details of the interaction between IFN- γ and its high-affinity receptor, including the observation that the two receptors do not themselves interact. It also shows that the IFN- γ residues in contact with the receptor form a discontinuous epitope made from residues on each monomer. One segment consists of helix A, the AB loop, and helix B from monomer one; the other consists of helix F and the C-terminus from the monomer two. Unfortunately, interpretation of structural detail is affected by the relatively limited resolution of this structure. In addition, this structure does not elucidate the role of the positively charged C-terminus of IFN- γ , a segment shown by mutagenesis to be essential for receptor binding (Lundell et al., 1991). Attempts to produce a crystal form that diffracts to higher resolution have not been successful (M. Ultsch & H. Christinger, pers. comm.).

Modifications made to a protein either through enzymatic or genetic means can often have dramatic effects on crystallization. To improve the diffraction quality of the IFN- γ /IFN- γ R α crystals, as well as to construct a molecule more amenable to structure/function analysis of the hormone/receptor interface, we have produced a variant of IFN- γ engineered to bind only one copy of IFN- γ R α . This was achieved by designing a “single-chain” IFN- γ variant; the two polypeptide chains were covalently linked by a short peptide, transforming the homodimer into a twofold symmetric single-chain molecule. Incorporation of this linker removes a portion of the C-terminus shown by mutagenesis to be critical for receptor recognition (Lundell et al., 1991). A second mutation capable of eliminating receptor binding, His 111 to Asp (H111D)

Reprint requests to: Anthony Kossiakoff, Genentech Inc., 1 DNA Way, South San Francisco, California 94080; e-mail: koss@gene.com.

(Lunn et al., 1992), was introduced into this binding site. This molecule has been shown to bind only a single copy of IFN- γ R α , and thus should simplify future studies directed toward quantitating the specific energetic contributions of individual residues to the binding energy. It will also make possible experiments directed toward determining the exact receptor binding stoichiometry necessary for biological activity. Surprisingly, cell based assays show that the single-chain IFN- γ induces a biological response even though it can bind only single copies of the IFN- γ R α and R β receptors (N. Randal, A.A. Kossiakoff, unpubl. results). Finally, the complex between this variant and the IFN- γ R α produced crystals that diffract to at least 2.0 Å resolution, which will enable detailed analysis of the structural features associated with this interaction.

Results and discussion: *Design of single-chain IFN- γ :* The structure of free bovine IFN- γ (M. Randal & A.A. Kossiakoff, unpubl. results) shows the N-terminal helix of one monomer to be in close proximity to the C-terminal helix of the second monomer. The C α atoms of residue 1 and residue 121' (the last residue of the second monomer that was visible in the electron density) are separated by ~12 Å (Fig. 1A). Joining these residues by a short linker would create a molecule that could be expressed as a single polypeptide. This would allow for the production of single-site mutations, something not possible in the twofold symmetric homodimer.

A method was developed to design a C' to N linker based on helix-loop-helix motifs from previously determined structures. A difference distance approach was used to screen through each structure in the Brookhaven Protein Data Bank for contiguous helices oriented similarly to the N and C-terminal helices of IFN- γ . Candidate matches were then superimposed onto the IFN- γ structure

and a C α root-mean-square deviation (RMSD) reported for the eight terminal residues of each helix. Using this approach, the entire data base of over 2,900 entries was rapidly searched.

A list of 23 helix-loop-helix motifs containing 26 residues or less and having an RMSD of less than 2.0 Å to the IFN- γ helices was produced. These 23 structures were visually evaluated in the context of the IFN- γ structure using MIDASPLUS (Ferrin et al., 1988). The helix-loop-helix motif comprising residues 10 to 29 of "bira bifunctional protein" (1BIA) was determined to be the best match, with a C α RMSD to IFN- γ of 1.8 Å for the helical regions (Fig. 1B). Features of this loop making it attractive for this application were glycines as helix terminators and a serine acting as a potential helix capping residue (Presta & Rose, 1988). To better accommodate the IFN- γ structure, three mutations were introduced into the 1BIA sequence (Fig. 1B). Glutamate 18, which was not modeled beyond the C β in 1BIA, was changed to alanine. Phenylalanine 19, buried in 1BIA but exposed in the single-chain IFN- γ model, was mutated to asparagine. Finally, histidine 20 was replaced by valine to reflect a change in solvent accessibility.

Mutagenesis, expression, and purification of single-chain IFN- γ : From a plasmid encoding the intracellular expression of IFN- γ under the control of the T7 promoter, primers were designed to create two different PCR products. One product consisted of a 5' *ndeI* site, residues 1–119 of IFN- γ , a portion of the linker, and a 3' *avaI* site. The other product contained a 5' *avaI* site, the remainder of the linker, residues 4–133 of IFN- γ , a stop codon, and a 3' *hindIII* site. These fragments were cleaved by *avaI* and ligated; the ligation product was gel purified and cleaved by *ndeI*/*hindIII*. This product was again gel purified and ligated back into

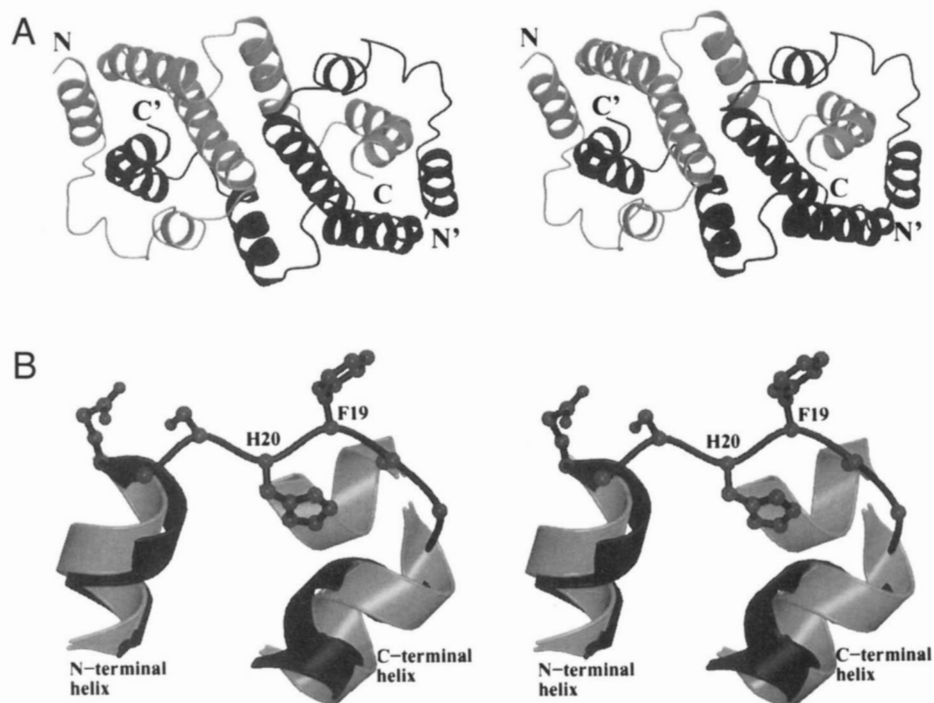


Fig. 1. A: Ribbon diagram of the 2.0 Å X-ray crystal structure of bovine IFN- γ . B: Superposition of residues 10–29 of 1BIA (black) onto the N- and C-terminal helices of IFN- γ . A portion of IFN- γ helix D is also displayed to show the environment of the 1BIA residues in the IFN- γ background.

the original vector, following excision of the wild-type gene by *ndel*/*hindIII* digestion. To preserve the original amino acid numbering scheme, the final polypeptide was numbered sequentially from -1 (the initiator methionine) to 124 for monomer 1, followed directly by residue numbers 201-333 for monomer 2. The additional mutation H311D was introduced by standard Kunkel mutagenesis (Kunkel, 1985).

Escherichia coli BL21 cells transformed with this plasmid were grown in LB until late log phase (OD_{600} 0.6-0.9) and then induced with 1 mM IPTG. Following induction, cells were allowed to grow for an additional 3-5 h and then pelleted. A rapid and efficient purification procedure was developed based on IFN- γ 's high isoelectric point ($pI = 10$) and ease of refolding. Pelleted cells were resuspended in 2 M urea, 25 mM Tris, pH 7.5, 25 mM NaCl, 5 mM EDTA, 0.1% β -mercaptoethanol and subjected to sonication. Cell debris was pelleted and the resulting supernatant loaded onto S-sepharose fast flow (Pharmacia), followed by elution with a linear NaCl gradient. Fractions containing IFN- γ were pooled and refolded by dialysis into 25 mM Tris, pH 7.5, 25 mM NaCl. This refolding step resulted in quantitative recovery of the IFN- γ as well as further purification via the precipitation of contaminant proteins. Refolded material was loaded onto a Pharmacia Mono-S FPLC column and eluted with a linear NaCl gradient. The resulting protein was pooled and concentrated using Centricon 10's (Amicon), then further purified by size-exclusion chromatography on an S-100 column (Pharmacia) equilibrated in 50 mM Tris, pH 8.0, 0.5 M NaCl.

Mutagenesis, expression, and purification of IFN- γ R α extracellular domain: Starting from a plasmid directing secretion of the extracellular domain of human IFN- γ R α (residues 1-229) under the control of an alkaline phosphatase promoter, an amino terminal epitope tag was added by standard Kunkel mutagenesis (Kunkel, 1985) to aid in purification. This epitope tag consisted of the 12 residues of herpes simplex virus glycoprotein D that are recognized by the monoclonal antibody 5B6 (Lasky & Dowbenko, 1984; Paborsky et al., 1990). This tag was followed by the peptide sequence AAHY to allow for its proteolytic removal by substrate-assisted catalysis using a variant of subtilisin in which the active site histidine has been replaced by alanine (Carter et al., 1991). As negatively charged amino acids are disfavored at position P1' for this enzyme, the amino terminal glutamate of the receptor was also mutated to alanine.

E. coli strain 27C7 containing the receptor plasmid was grown at 37°C in low phosphate buffer. Following growth, cells were pelleted, resuspended in 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM benzamidine, and disrupted by sonication. Cell debris was removed via centrifugation, and the resulting supernatant loaded onto a Q-sepharose fast flow (Pharmacia) column. The column was washed with 50 mM Tris, pH 7.5, 50 mM NaCl, 12.5 mM EDTA, and eluted with a linear NaCl gradient. Fractions containing the receptor were pooled and loaded onto an affinity column consisting of the monoclonal antibody 5B6 immobilized on controlled-pore glass beads. Proteins bound nonspecifically were removed by successive washes of 50 mM Tris, pH 7.5, 0.1 M NaCl, followed by 50 mM Tris, pH 7.5, 0.5 M $(CH_3)_4NCl$, and finally 50 mM Tris, pH 7.5, 0.1 M NaCl. Bound receptor was then eluted with 0.1 M CH_3COOH , 0.15 M NaCl and immediately neutralized by 0.1 volume unbuffered Tris base. Pooled material was digested by incubation (2 h, room temperature) with a 1:10

(weight:weight) addition of the subtilisin variant described above. Digested material was adjusted to 1 M $(NH_4)_2SO_4$ by the slow addition of 3 M $(NH_4)_2SO_4$ and loaded onto a Pharmacia Phenyl-Superose FPLC column. Protein was eluted by a decreasing linear gradient of $(NH_4)_2SO_4$ in 25 mM Tris, pH 7.5.

Characterization of single-chain IFN- γ : To verify the correct folding of the single-chain molecule, both it and native IFN- γ were examined by analytical size-exclusion chromatography. Fifty microliter aliquots were injected onto a Pharmacia Superose-12 FPLC column developed at 0.35 mL/min using 50 mM Tris, pH 7.5, 0.5 M NaCl. Both proteins exhibited identical peak shape and retention time (data not shown), suggesting a native-like conformation for the single-chain variant.

According to available mutational data, the single-chain variant should bind only a single copy of IFN- γ R α . To confirm this, both mutant and wild-type IFN- γ were mixed with IFN- γ R α and analyzed by size exclusion chromatography as described above. Figure 2 shows that the retention time for the mutant and wild-type complexes are consistent with a 1:1 and a 2:1 (receptor:hormone) binding stoichiometry, respectively.

Crystallization and diffraction studies of the single-chain IFN- γ /IFN- γ R α complex: For crystallization, hormone and receptor were mixed in approximately a 1:1 ratio and fractionated via size exclusion chromatography as described above for the free hormone. The purified complex was dialyzed into 10 mM Tris, pH 7.5, 10 mM NaCl and concentrated to ~ 8 mg/mL. Initial crystallization trials were attempted using Hampton CrystalScreen I in a hanging drop format using equal volumes of protein and reservoir. Crystals grew from both 2 M $(NH_4)_2SO_4$ and 8% PEG 8000 buffered at pH 8.5 with 100 mM Tris, but these crystals were of poor diffraction quality. Further crystallization trials produced a third, better diffracting, crystal form from the above PEG conditions. As two crystal forms were observed to grow from the same condition, repeat seeding techniques were used to ensure production of the desired crystal form. These crystals diffracted to ~ 2.7 Å using a rotating anode source (Rigaku RU2000, $\lambda = 1.54$ Å) and a MAR-Research image plate system. The space group was determined to be $P2_1$ with unit cell dimensions $a = 73.1$ Å, $b = 107.8$ Å,

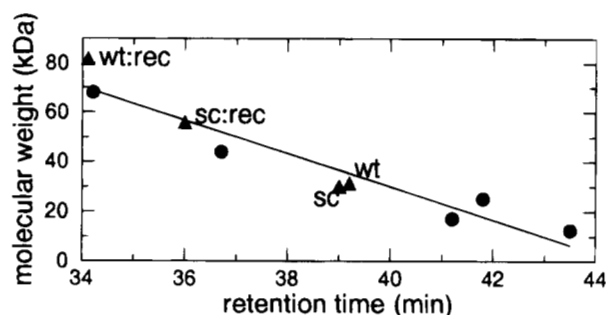


Fig. 2. Results of size-exclusion chromatography runs. Circles represent standards and are (from low to high molecular weight) cytochrome *c*, equine myoglobin, chymotrypsinogen, chicken ovalbumin, and bovine serum albumin. Triangles are: sc, singlechain IFN- γ ; wt, wild-type IFN- γ ; sc:rec, single-chain IFN- γ complexed to IFN- γ R α ; wt:rec, wild-type IFN- γ complexed to IFN- γ R α .

$c = 85.1 \text{ \AA}$, $\beta = 97.9^\circ$. Using a molecular mass of 56 kDa for the 1:1 complex, the crystal was determined to most likely contain two complexes in the asymmetric unit, corresponding to a $V_m = 2.25 \text{ \AA}^3/\text{Da}$ (Matthews, 1968), and a solvent content of $\sim 55\%$.

For data collection, crystals were briefly washed in solutions of 0.1 M Tris pH 8.0, 10% PEG 8000, 10% glycerol and 0.1 M Tris pH 8.0, 10% PEG 8000, 15% glycerol, retrieved with a rayon CryoLoop (Hampton), and plunged into liquid nitrogen. Crystals were maintained at 100 K during data collection. Data were collected at CHESS beamline A1 ($\lambda = 0.91 \text{ \AA}$) using a $2k \times 2k$ ADSC CCD detector. The crystals diffracted anisotropically, with diffraction observed to approximately 1.6 \AA in the a^*c^* plane and to approximately 2.0 \AA along the b^* axis. Two crystals were used for data collection. Starting from a random orientation, 290° of data were collected in two batches from crystal 1 using 1° oscillations at a crystal to detector distance of 100 mm; 261° of data were collected in four batches from crystal 2 using 1° oscillations at a crystal to detector distance of 85 mm. A final 90° of data were collected from crystal 2 using 1.5° oscillations at a crystal to detector distance of 100 mm. Each batch of data was reduced and scaled individually using DENZO and SCALEPACK (Minor, 1993; Otwinowski, 1993), for a total of 773,440 observations. Batches from each crystal were then scaled separately, and finally, data from the two crystals were scaled together. This final scaling gave an R_{merge} of 5.3% for 146,574 reflections of which 81,005 were unique, corresponding to 97% completeness (87% complete in the shell 2.1 to 2.0 \AA).

A self-rotation function was calculated using GLRF (Tong & Rossman, 1997) in the resolution range 10–2.8 \AA to search for possible noncrystallographic symmetry elements. Figure 3 shows the $\kappa = 180^\circ$ section from this calculation. The presence of a peak with height $\approx 25\%$ that of the origin peak suggests there exists a twofold noncrystallographic symmetry axis approximately 18° from the b axis. Structure solution by molecular replacement has been initiated using as a model the coordinates of the 2:1 complex (S. Ealick, unpubl. results) edited to the 1:1 form.

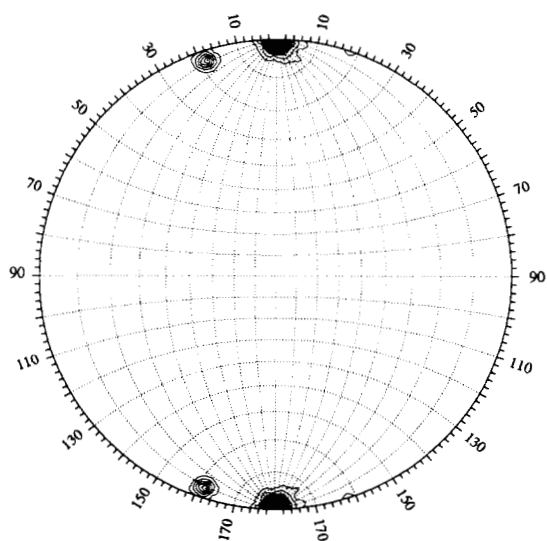


Fig. 3. $\kappa = 180^\circ$ section from a self rotation function. Contour lines are plotted beginning at 2σ and continuing at 1σ intervals. Psi angles are indicated on the circumference.

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