Eisenberg, D., Weiss, R. M., & Terwilliger, T. C. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 140.
Evans, T. W., & Knuth, M. W. (1987) World Patent WO 8700204, January 15, 1987.
Hartman, P. A., Stodola, J. D., Harbour, G. C., & Hoogerheide, J. G. (1986) *J. Chromatogr. 360*, 385-395.
Havel, H. A., Kauffman, E. W., Plaisted, S. M., & Brems, D. N., (1986) *Biochemistry 25*, 6533-6538.
Havel, H. A., Kauffman, E. W., & Elzinga, P. A. (1988) *Biochim. Biophys. Acta 955*, 154-163.
Holladay, L. A., Hammonds, R. G., & Puett, D. (1974) *Biochemistry 13*, 1653-1661.
Horowitz, P. M., & Criscimagna, N. L. (1990) *J. Biol. Chem. 265*, 2576-2583.
Lehrman, S. R., Tuls, J. L., & Lund, M. E. (1990) in *Peptides: Proceedings of the Eleventh Peptide Symposium* (Rivier, J. E., & Marshall, G. R., Eds.) pp 571-574, ESCOM Publishers, Leiden, The Netherlands.
London, J. Skrzynia, C., & Goldberg, M. E. (1974) *Eur. J. Biochem. 47*, 409.
Nicoli & Benedek (1976) *Biopolymers 15*, 2421-2437.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266.
Provencher, S. W. (1982) *Comput. Phys. Commun. 27*, 213-227, 229-242.
Robson, B., & Pain, R. H. (1971) *J. Mol. Biol. 58*, 237.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76-85.
Stolar, M. W., Amburn, K., & Baumann, G. (1984) *J. Clin. Endocrinol. Metab. 59*, 212.
Tomich, C.-S. C., Olson, E. R., Olsen, M. K., Kaytes, P. S., Rockenbach, S. A., & Hatzenbuhler, N. T. (1989) *Nucleic Acids Res. 17*, 3179-3197.

Characterization of a Synthetic Peptide Corresponding to a Receptor Binding Domain of Mouse Interferon γ[†]

Harold I. Magazine* and Howard M. Johnson
Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611
Received December 12, 1990; Revised Manuscript Received March 1, 1991

ABSTRACT: A receptor binding region of mouse interferon γ (IFNγ) has previously been localized to the N-terminal 39 amino acids of the molecule by use of synthetic peptides and monoclonal antibodies. In this report, a detailed analysis of the synthetic peptide corresponding to this region, IFNγ(1-39), is presented. Circular dichroism (CD) spectroscopy indicated that the peptide has stable secondary structure under aqueous conditions and adopts a combination of α-helical and random structure. A peptide lacking two N-terminal amino acids, IFNγ(3-39), had similar secondary structure and equivalent ability to compete for receptor binding, while peptides lacking four or more N-terminal residues had reduced α-helical structure and did not inhibit ^{125}I-IFNγ binding. Substitution of proline, a helix-destabilizing amino acid, for leucine (residue 8) of a predicted amphipathic α-helix (residues 3-12), IFNγ(1-39)[Pro]^{8}, resulted in a substantial reduction in the helical content of the peptide, supporting the presence of helical structure in this region. However, destabilization of the helix did not reduce the competitive ability of the peptide. A peptide lacking eight C-terminal residues, IFNγ(1-31), did not block ^{125}I-IFNγ binding and had no detectable α-helical structure, suggesting a requirement of the predicted second α-helix (residues 20-34) for receptor interaction and helix stabilization. Substitution of phenylalanine for tyrosine at position 14, IFNγ(1-39)[Phe]^{14}, a central location of a predicted Ω-loop structure, did not affect the secondary structure associated with the region yet resulted in a 30-fold increase in receptor competition. Substitution with glycine at position 14 also did not affect secondary structure and resulted in a competitive ability similar to that of IFNγ(1-39). These data support the presence of a binding domain in the N-terminus of IFNγ and indicate a role for residues 3, 4, 14, and at least some of residues 32-39 in IFNγ(1-39) interaction with receptor. This study provides insight into the structural/functional basis for IFNγ interaction with receptor that should be useful for the development of potent agonists and antagonists of IFNγ action.

Interferon γ (IFNγ)[1] is a secretory glycoprotein produced primarily by antigen- and mitogen-stimulated T-lymphocytes and plays a central role in the initiation and regulation of the immune response (Johnson, 1985). IFNγ regulates class II major histocompatibility complex antigen expression, B-cell maturation, and antibody production as well as the activation of the host defense against tumor cells and microorganisms [reviewed by Vilcek et al. (1985)]. Although the mouse and human IFNγ receptor has been cloned recently (Auget et al., 1988; Gray et al., 1989; Kumar et al., 1989), the mechanism by which IFNγ exerts its many biological activities remains unclear.

[†] This work was supported by Research Grant CA 38587 from the National Institutes of Health. Florida Agricultural Experimental Station, Journal Series No. R-01566.
* To whom all correspondence should be addressed at The Albany Medical College, Department of Biochemistry A-10, 47 New Scotland Ave., Albany, NY 12208.

[1] Abbreviations: IFNγ, interferon γ; CD, circular dichroism; mAb, monoclonal antibody; tBoc, *tert*-butyloxycarbonyl; PAM, (phenylacetamido)methyl; HPLC, high-performance liquid chromatography; FBS, fetal bovine serum; TFE, 2,2,2-trifluoroethanol.

0006-2960/91/0430-5784$02.50/0

An investigation of how IFNγ exerts a myriad of biological effects on a variety of cell types is perhaps best initiated at the level of receptor interaction. To gain insight into the structural basis for the interaction of IFNγ with its receptor, synthetic peptides corresponding to various regions of the IFNγ molecule were prepared and their ability to block IFNγ–receptor interaction was determined. A synthetic peptide corresponding to the N-terminal 39 amino acids of IFNγ, IFNγ(1–39), but not peptides corresponding to other regions of the molecule, blocked ^{125}I-IFNγ interaction with receptor (Magazine et al., 1988). IFNγ(1–39) competed for binding with approximately one thousandth of the affinity of native IFNγ. The mapping of the epitope specificity of a monoclonal antibody (mAb) that blocks IFNγ interaction with receptor to a region of the N-terminus encompassing a portion of a predicted amphipathic α-helix and an Ω-loop structure further supported the importance of the N-terminus of IFNγ (Magazine et al., 1988; Jarpe & Johnson, 1990). These data suggested a possible role for such structures in receptor interaction.

In the studies reported here, we employed a secondary structure prediction of the IFNγ molecule that has been described previously (Magazine et al., 1988) together with synthetic peptides corresponding to truncations and structural analogues of IFNγ(1–39) to explore the importance of various segments of the peptide in receptor interaction. The prediction, based primarily on the results of the Chou and Fasman algorithm (1974, 1977), suggests that residues 3–12 and 20–34 will adopt helical structure in the intact molecule, with residues 3–12 being an amphipathic α-helix. The helical regions are predicted to be separated by an Ω-loop structure encompassing residues 12–20. Structural and functional characterization of IFNγ(1–39) validated the structural predictions of our model and should advance our understanding of IFNγ–receptor interaction.

Materials and Methods

Peptide Synthesis. Peptides were synthesized on a Biosearch 9500/AT peptide synthesizer by the solid-phase method of Merrifield (1964), employing *tert*-butyloxycarbonyl- (tBoc-) derivatized amino acids (Advanced ChemTech). All peptides were synthesized on a (phenylacetamido)methyl (PAM) resin to produce a peptide amide after cleavage and deprotection with anhydrous hydrofluoric acid employing the low–high method of Tam et al. (1983). Peptides IFNγ(1–39), IFNγ(3–39), IFNγ(5–39), IFNγ(7–39), IFNγ(1–39)[Leu]^{6}[Glu]^{8}, IFNγ(1–39)[Pro]^{8}, IFNγ(1–39)[Phe]^{14}, and IFNγ(1–39)[Gly]^{14} were generated from a single synthesis that was interrupted after the stepwise addition of amino acids starting from the C-terminal residue to amino acid 16. A portion of the peptidyl resin was removed, with the remainder of the resin being synthesized to completion. N-Terminal truncations of IFNγ(1–39) were generated by removal of the peptidyl resin from the reaction vessel when the desired sequence length was obtained. For each of the IFNγ(1–39) analogues, a portion of the peptidyl resin was placed into the reaction vessel and synthesized to completion, containing an amino acid substitution at the desired location. IFNγ(1–31) and IFNγ(95–133) were synthesized individually as described for IFNγ(1–39) except that IFNγ(95–133) was synthesized on a Merrifield resin to obtain a peptide acid.

Crude peptides were desalted over a Sephadex G-10 column and purified by reverse-phase high-performance liquid chromatography (HPLC) (Perkin-Elmer) with use of a semipreparative C18 HPLC column (Vydak) and a linear gradient of 0.1% TFA and acetonitrile. Amino acid analysis was performed by the University of Florida Peptide Core Facility on a Beckman 6300 amino acid analyzer. The analysis of the purified peptides differed by less than 10% of that expected for a homogeneous product.

Circular Dichroism Measurement. CD spectra were determined at room temperature on a Jasco-500c spectropolarimeter calibrated with (+)-10-camphorsulfonic acid with use of a 0.1-cm path-length cuvette (Jasco). CD spectra were obtained in triplicate at pH 7.4, although similar results were obtained by measurement at pH 3.0 or 4.5. The solvent spectrum was subtracted from the spectrum obtained for each sample. Peptides at a stock concentration of 1 mM were diluted with water or phosphate buffer just prior to spectral measurement. Peptide concentration was determined by quantitative amino acid analysis. The results are expressed as mean residue ellipticity ([θ]) and calculated as described (Yang et al., 1986). Secondary structure prediction from the CD spectra was calculated by the computer program CDESTIMA as described (Cheng et al., 1978; Yang et al., 1986). Dilution of the sample over the concentration range of 50–250 μM was used to assess peptide aggregation (Marqusee et al., 1989). Since dilution of the sample had a negligible effect on the ellipticity of the peptides, aggregation was determined not to have an appreciable contribution to the CD spectra obtained.

IFNγ Iodination. Purified recombinant IFNγ obtained from either Genentech (San Francisco, CA) or the American Cancer Society (New York, NY) had a specific activity of 5×10^6 or 5×10^5 antiviral units/mg, respectively. IFNγ was labeled by the chloramine-T method as described (Russell et al., 1988). The radio-specific activity of ^{125}I-IFNγ was generally 50–100 μCi/μg and had a biological activity similar to that of the unlabeled protein.

Receptor Binding. Mouse fibroblasts (L-cells) were suspended in media (Hepes/minimal essential medium, pH 7.4) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin) and allowed to grow to confluence in a 24-well plate (Falcon). Binding of ^{125}I-IFNγ alone or in the presence of peptide competitors was allowed to occur for 2.5 h at 24 °C in the presence of 25 mM sodium azide. Cells were then washed extensively with ice-cold media to remove unbound ^{125}I-IFNγ, dissolved in 1% sodium dodecyl sulfate, absorbed to a cotton-tipped applicator, and counted in a γ-scintillation counter. Enhanced binding of ^{125}I-IFNγ in the presence of peptides IFNγ(1–31) and BAR(1–33) was occasionally observed yet was not reproducible. Nonspecific binding was determined by competition of ^{125}I-IFNγ in the presence of 1000-fold excess unlabeled IFNγ and varied from 10 to 35% depending on source of the IFNγ used and the individual experiment performed.

Results

Circular Dichroism Spectra of IFNγ(1–39). The far-UV CD spectrum of IFNγ(1–39) in water or 40% 2,2,2-trifluoroethanol (TFE) is shown in Figure 1. The spectra of IFNγ(1–39) in water had minima at 222 and 209 nm and a maximum at 192 nm, suggesting the presence of α-helical structure. Dissolving IFNγ(1–39) in TFE resulted in an increase in the ellipticity of the peptide, with maximal effect occurring at a minimum concentration of 40% TFE (data not shown). Analysis of the spectra by CDESTIMA suggested an α-helical content of 17 and 48% for IFNγ(1–39) in water and TFE, respectively (Table I).

Inhibition of ^{125}I-IFNγ Receptor Binding by N-Terminal and C-Terminal Truncations of IFNγ(1–39). IFNγ(1–39) blocked the binding of ^{125}I-IFNγ to receptor on mouse L-cells, consistent with earlier work (Magazine et al., 1988). Peptides representing N-terminal and C-terminal truncations of the

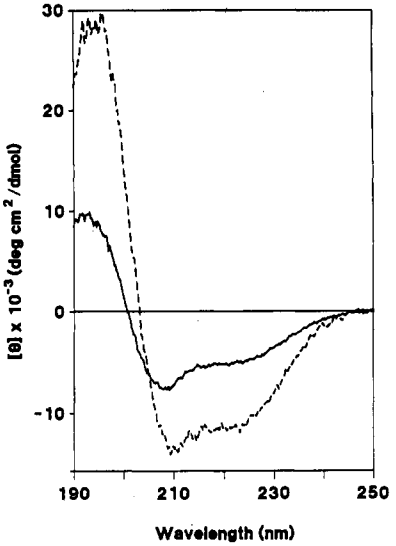

FIGURE 1: Far-UV CD spectra of IFNγ(1–39) in water or 40% TFE. IFNγ(1–39) in water, pH 7.4 (—), or 40% TFE (- - -) was used at a concentration of 50.0 and 54.1 μM, respectively.

Table I: Helical Content of Peptide Analogues[a]

peptide	$[\theta]_{222nm}$ (deg·cm²/dmol)	helical content (%)	
		calculated	predicted
IFNγ(1–39) in 40% TFE	−11917	48	69
IFNγ(1–39)	−5910	17	69
IFNγ(3–39)	−5960	17	69
IFNγ(5–39)	−3980	12	64
IFNγ(7–39)	−3676	10	59
IFNγ(1–31)	−2000	0	56
IFNγ(1–39)[Pro]⁸	−2176	4	44
IFNγ(1–39)[Leu]⁶[Glu]⁸	−7250		69
IFNγ(1–39)[Gly]¹⁴	−6180		69
IFNγ(1–39)[Phe]¹⁴	−5898		69

[a] Peptides unless otherwise noted are dissolved in water as described in Materials and Methods. The calculated helical content is derived from CDESTIMA analysis of the peptide CD spectra while the predicted content represents that estimated from secondary structure prediction for the peptide sequence as it might fold in the native IFNγ molecule.

IFNγ(1–39) sequence were synthesized and compared with intact IFNγ(1–39) to determine the relative ability of truncated peptides to inhibit ^{125}I-IFNγ binding to cell-surface receptors. A peptide lacking two amino acids from the N-terminus of IFNγ(1–39), IFNγ(3–39), had an ability equivalent to that of IFNγ(1–39) to block binding to receptor, whereas a peptide lacking the first four amino acids, IFNγ-(5–39), completely lacked the ability to inhibit ^{125}I-IFNγ binding (Table II). A peptide lacking eight amino acids from the C-terminus of IFNγ(1–39), IFNγ(1–31) also lacked blocking ability, indicating a requirement for at least some of amino acid residues 32–39 for IFNγ(1–39) interaction with receptor. An irrelevant peptide, BAR(1–33), corresponding to amino acids 1–33 of the mouse β-adrenergic receptor (Regan et al., 1988) did not block binding of ^{125}I-IFNγ. Only peptides that blocked ^{125}I-IFNγ binding also blocked IFNγ biological activity [antiviral activity, Magazine et al. (1988); induction of Class II, Ia antigen expression, unpublished observation], suggesting that binding of biologically active IFNγ was inhibited.

Circular Dichroism of N-Terminal and C-Terminal Truncations of IFNγ(1–39). CD spectra of peptides lacking N-terminal residues of IFNγ(1–39) are shown in Figure 2A. Although spectra of the peptides in TFE may reflect their secondary structure in the folded molecule, we obtained spectra

Table II: Inhibition of ^{125}I-IFNγ Receptor Binding by N-Terminal and C-Terminal Truncations of IFNγ(1–39)[a]

peptide	inhibition of ^{125}I-IFNγ binding (%)		
	56 μM competitor	167 μM competitor	500 μM competitor
IFNγ(1–39)	65	77	83
IFNγ(3–39)	70	81	89
IFNγ(5–39)	0	0	0
IFNγ(1–31)	0	0	0
BAR(1–33)	0	0	0

[a] Conditions: Synthetic peptides competed with 5 nM ^{125}I-IFNγ for binding to receptor on mouse L-cells. An irrelevant peptide, BAR(1–33), was used as a negative control. IFNγ obtained from Genentech (5 × 10⁶ antiviral units/mg) was radiolabeled to a specific activity of 112 μCi/μg. Data are normalized such that inhibition of binding in the presence and absence of 5 μM IFNγ is equivalent to 100 and 0% inhibition, respectively. Experiments were performed with duplicate samples and the standard deviation of the mean was less than 10%. Data are representative of additional competition studies where competitors were analyzed separately [IFNγ(1–39), n = 7; IFNγ(3–39), n = 5; IFNγ(5–39), n = 2; BAR(1–33), n = 2].

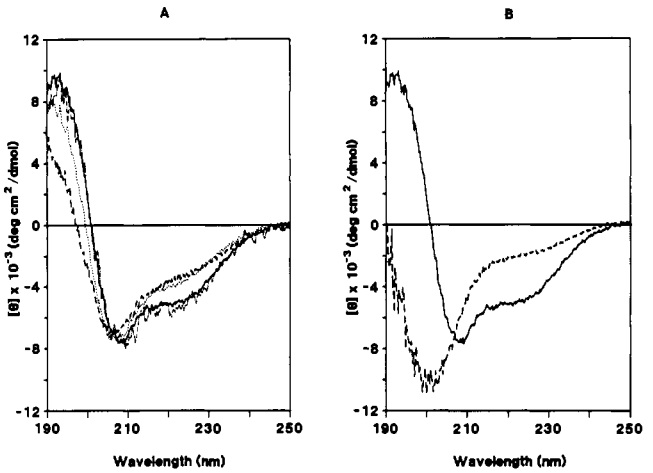

FIGURE 2: CD spectra of N-terminal and C-terminal truncations of IFNγ(1–39). CD spectra of synthetic peptides corresponding to N-terminal truncations (panel A) IFNγ(3–39) (-·-), IFNγ(5–39) (···), and IFNγ(7–39) (- - -) or C-terminal truncation (panel B) IFNγ(1–31) (- - -) of the IFNγ(1–39) sequence were obtained with use of similar peptide concentrations (46–54 μM). IFNγ(1–39) (—) is included in panel A and B to facilitate comparison of the CD spectra.

of the peptides in water to study their behavior in the assay conditions used for receptor competition studies (described above). IFNγ(3–39) had a CD spectrum superimposable with that of IFNγ(1–39), indicating that loss of two residues from the N-terminus does not affect the CD spectra. However, peptides that lack four or six amino acid residues, IFNγ(5–39) and IFNγ(7–39), respectively, demonstrate CD spectra with a reduced ellipticity at 222 nm. CDESTIMA analysis of the spectra indicated a reduction in the helical content of these peptides relative to IFNγ(1–39) (17% α-helix), with IFNγ-(5–39) and IFNγ(7–39) calculated to have 12 and 10% α-helix, respectively. Also, the observed reduction in helical content of these peptides follows a pattern similar to that predicted by the Chou and Fasman algorithm (1974, 1977) (Table I). The C-terminally truncated peptide, IFNγ(1–31), exhibited a drastic reduction in the ellipticity at 222 nm and the emergence of a strong minimum at 200 nm (Figure 2B). This spectrum suggests that the peptide had a large reduction in α-helical content relative to IFNγ(1–39), which is consistent with the calculation of CDESTIMA that failed to detect helical structure.

Modification of IFNγ(1–39) Predicted Secondary Structure. To determine amino acids or elements of IFNγ(1–39)

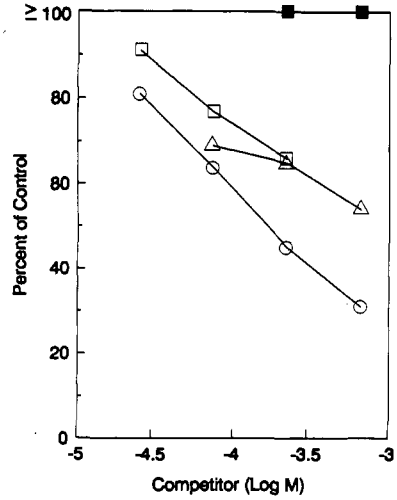

FIGURE 3: Competitive ability of IFNγ(1–39) amphipathic α-helix analogues for receptor binding. Synthetic peptides IFNγ(1–39)[Pro]^{8} (○), IFNγ(1–39)[Leu]^{6}[Glu]^{8} (□), IFNγ(1–39) (△), and IFNγ-(95–133) (■) competed with ^{125}I-IFNγ (2 nM) for binding to receptor. IFNγ used in this assay was obtained from Genentech (5×10^{6} antiviral units/mg) and radiolabeled to a specific activity of 124 μCi/μg. The assay was performed with triplicate samples, and the standard deviation of the mean was generally 10% or less.

RAT	-	-	-	C	Y	C	Q	G	T	L	I	E	S	L	E
MOUSE	-	-	-	-	-	-	H	G	T	V	I	E	S	L	E
HUMAN	-	-	-	-	-	-	Q	D	P	Y	V	K	E	A	E
BOVINE	S	G	S	Y	G	Q	G	Q	F	F	R	E	-	I	E

RAT	S	L	K	N	Y	F	N	S	S	S	M	D	A	M	E
MOUSE	S	L	N	N	Y	F	N	S	S	G	I	D	V	-	E
HUMAN	N	L	K	K	Y	F	N	A	G	H	S	D	V	A	D
BOVINE	N	L	K	E	Y	F	N	A	S	S	P	D	V	A	K

FIGURE 4: Comparison of the protein sequences of IFNγ from several animal species. Sequences from rat (Dijkema et al., 1985), mouse (Gray & Goddell, 1983), human (Gray & Goddell, 1982), and bovine (Baker, 1986) were obtained by searching the GenBank database and were aligned for maximal homology. A dash represents a gap and was inserted to facilitate comparison of the sequences. Boxed amino acid residues represent regions where the residues were identical in all four species of IFNγ.

secondary structure that facilitate (or are required for) receptor interaction, specific analogues of IFNγ(1–39) were produced. Analogue design was governed by predictive models for secondary structure of IFNγ (Magazine et al., 1988) and the epitope specificity of a neutralizing mAb mapped to a region predicted to encompass a portion of an amphipathic α-helix (Jarpe & Johnson, 1990) and Ω-loop structure (Magazine et al., 1988). Peptide IFNγ(1–39)[Leu]^{6}[Glu]^{8} was designed to disrupt the amphipathic nature of the predicted α-helix encompassing residues 3–12 but not disrupt the helical content of the region. This peptide maintained the ability to compete for receptor (Figure 3). Peptide IFNγ(1–39)[Pro]^{8}, which incorporated a proline residue at a central location in the predicted amphipathic helix, also maintained the ability to compete for receptor. Thus, either the amphipathic nature of the predicted helix or the helical structure itself may not be required for interaction of the peptide with receptor.

Comparison of the protein sequences of IFNγ in all animal species for which the sequence has been reported reveals an absolute conservation of three amino acids in the region predicted to be in Ω-loop conformation (Figure 4). The tyrosine/phenylalanine pair (residues 14 and 15 of the mouse sequence) are of particular interest since the bulkiness, hydrophobicity, and side-chain interaction of such aromatic–aromatic pairs may severely restrict the conformational flexibility of local structure (Amit et al., 1986; Murali et al., 1987) and thus may be important for stabilization of IFNγ secondary structure. Peptides having a nonconservative, IFNγ(1–39)[Gly]^{14}, or conservative, IFNγ(1–39)[Phe]^{14}, replacement of tyrosine in position 14 of this Ω-loop region were tested for competitive ability. IFNγ(1–39)[Gly]^{14} had an ability to compete for receptor similar to that of IFNγ(1–39) while IFNγ(1–39)[Phe]^{14} had a 1.5 log increase in competitive ability (Figure 5). The increase in affinity of IFNγ(1–39)[Phe]^{14} for receptor suggested that the loop region, specifically at position 14 of IFNγ(1–39), may play a role in receptor interaction.

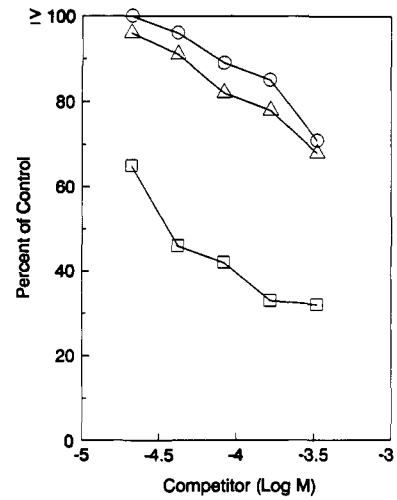

FIGURE 5: Competitive ability of IFNγ(1–39) Ω-loop analogues for receptor binding. Synthetic peptides IFNγ(1–39)[Gly]^{14} (○), IFNγ(1–39)[Phe]^{14} (□), and IFNγ(1–39) (△) competed with ^{125}I-IFNγ (5 nM) for binding to receptor. IFNγ used in this assay was obtained from the American Cancer Society (5×10^{5} antiviral units/mg) and radiolabeled to a specific activity of 117 μCi/μg. The assay was performed with duplicate samples, and the standard deviation of the mean was generally 10% or less.

Circular Dichroism of Peptide Analogues. The extent of helical formation can be monitored by following the minimum in CD spectra occurring at 222 nm (Johnson, 1988; Marqusee et al., 1989). The ellipticity of the peptide analogues at 222 nm, with the exception of IFNγ(1–39)[Pro]^{8}, does not indicate a loss of helical content relative to IFNγ(1–39) (Table I). IFNγ(1–39)[Pro]^{8} exhibits a profound reduction in ellipticity at 222 nm, suggesting a large loss in helical content. Alterations in the CD spectra obtained for the peptide analogues are consistent with that expected on the basis of predictive models.

Discussion

We have reported previously that the amino terminus of IFNγ may encompass a functional site required for binding of the molecule to receptor as evidenced by the ability of a synthetic peptide corresponding to the N-terminus, but not internal regions or the C-terminus of IFNγ molecule, to block ^{125}I-IFNγ binding to cell-surface receptors (Magazine et al., 1988). Also, a mAb produced against recombinant IFNγ that abrogates binding of IFNγ to receptor was mapped to N-terminal residues 3–14 of IFNγ, further suggesting the importance of the region in receptor binding (Magazine et al., 1988; Jarpe & Johnson, 1990).

IFNγ has been shown to exist as a monomer (Yip et al., 1981) and homodimer (Yip et al., 1981; Pestka, 1983) in solution. Although the functional form of IFNγ remains

unknown, isolated monomeric IFNγ has been reported to possess biological activity (Rinderknecht et al., 1984), suggesting that dimerization may not be required for function. CD spectroscopy of IFNγ(1–39) in water failed to indicate aggregation of the peptide, suggesting that monomeric peptide may be involved in the blockage of IFNγ receptor binding. IFNγ(1–39) has one thousandth of the affinity of intact IFNγ, which may suggest a requirement for other regions of the molecule or dimerization to achieve the affinity of the intact molecule.

Circular dichroism studies on IFNγ(1–39) revealed that IFNγ(1–39) may have appreciable secondary structure in solution. Comparison of the CD spectra of IFNγ(1–39) in water and TFE suggests an increase in the α-helical content of the peptide in TFE. Helix-stabilizing agents, such as TFE, are commonly used for CD spectroscopy of peptides since they may enhance and stabilize existing structure, allowing an assessment of the helical potential of a peptide (Martenson et al., 1985). The increased secondary structure of the peptide in TFE, however, does not attain the level of secondary structure predicted [by the algorithm of Chou and Fasman (1974, 1977)] for the region in the native IFNγ molecule (Table I) and may reflect a lack of stabilizing forces present in intact IFNγ or inaccuracies associated with secondary structure prediction.

IFNγ(3–39) maintained the ability to compete for receptor and had a CD spectrum similar to that of IFNγ(1–39), suggesting that residues 1 and 2 of IFNγ(1–39) may not be required for receptor interaction or maintenance of secondary structure. Residues 1 and 2 are not predicted to attain ordered structure in the intact molecule, which is consistent with our data that suggest that IFNγ(1–39) and IFNγ(3–39) have similar α-helical content. The recent observation that IFNγ produced by recombinant methods that lacks the first two N-terminal amino acid residues maintains biological activity (Zavodny et al., 1988) is also in agreement with our observations.

Peptides lacking four or more N-terminal residues have a reduction in α-helical content and lack competitive ability, suggesting that helical structure may play a role in receptor interaction. Disruption of the amphipathic nature of the helix predicted for residues 3–12 by transversion of Glu and Leu at position 6 and position 8, respectively, did not affect the competitive ability of the peptide, indicating that the amphipathic nature of the helix is not required for receptor interaction. Furthermore, IFNγ(1–39)[Pro]^{8}, which has a replacement of Pro for Leu at position 8 of IFNγ(1–39), had a reduced α-helical content as confirmed by its CD spectrum. Nevertheless, this peptide maintained the ability to compete for receptor, suggesting that helix 3–12 may not be required for receptor interaction. However, although the peptide has reduced helical content in solution, receptor-induced stabilization of helix 3–12 cannot be discounted.

Peptide IFNγ(1–31), which lacks eight C-terminal amino acids, had no detectable α-helical structure and failed to compete for receptor, suggesting that at least some of the α-helical structure may be required for receptor interaction. Interestingly, destabilization of helix 3–12 or loss of a section of helix 20–34 [in IFNγ(1–39)[Pro]^{8} and IFNγ(1–31), respectively] results in a greater reduction in helical content than can be explained by complete loss of either helix, suggesting that the helices may interact and stabilize each other. Insertion of a proline residue within helix 3–12 reduces the helical potential of the region (due to incorporation of inappropriate bond angles within the helix), possibly weakening the interaction of helix 3–12 and 20–34, which may explain the greater than expected reduction in helical content of the peptide. In addition, at least some of residues 32–39 may be critical for helix stabilization of IFNγ(1–39), since the lack of these residues results in a complete loss of helical content. Peptide IFNγ(7–39), which maintains appreciable helical content, may retain sufficient helical structure in region 7–12 for interaction with helix 20–34, resulting in packing and stabilization of the helices.

Predicted helices 3–12 and 20–34 are separated by a predicted Ω-loop structure. Conservation of several amino acids within this putative loop structure among the protein sequences of IFNγ for different animal species (Figure 4) and the mapping of a mAb that inhibits receptor binding of IFNγ to a portion of this structure suggest a functional role for the region. Peptide IFNγ(1–39)[Phe]^{14}, which has a modified Ω-loop structure due to a conservative substitution of phenylalanine for tyrosine at position 14 of IFNγ(1–39), had a 1.5 log increase in competitive ability relative to IFNγ(1–39) for binding to receptor while a nonconservative substitution with glycine did not significantly affect the competitive ability of the peptide. Although further investigation is necessary to explain the enhanced competitive ability of IFNγ(1–39)-[Phe]^{14}, substitution with phenylalanine may enhance binding by shifting the conformation of the peptide to a form that better mimics receptor-bound IFNγ(1–39).

Our model for the secondary structure of IFNγ (Magazine et al., 1988) is consistent with the data obtained. Receptor competition studies and CD spectroscopy of truncations and analogues of IFNγ(1–39) suggested a role for residues 3, 4, 14, and at least some of residues 32–39 in the interaction of IFNγ with its receptor. The large reduction in α-helical content of peptides IFNγ(1–39)[Pro]^{8} and IFNγ(1–31) supports the presence of predicted helical segments 3–12 and 20–34 of our model. Since loss of two N-terminal residues or exchange of residues 6 and 8 can be accomplished without loss of competitive ability, amino acid residues 1, 2, 6, and 8 appear not to be required for interaction of IFNγ(1–39) with receptor. These data have provided information concerning the secondary structure of an IFNγ binding domain. Such knowledge provides insight concerning the mechanism of IFNγ–receptor interaction that may aid in the production of agonists and antagonists of IFNγ action.

Acknowledgments

We thank Dr. J. Gander and Dr. S. Bonetti for technical assistance with the CD measurements, Dr. R. J. Cohen for advice concerning this study, Dr. J. T. Yang and Dr. C. C. Wu for generously providing computer programs for analysis of the CD spectra, and Dr. T. T. Andersen and Dr. G. D. Wilner for assisting in the editing of the manuscript.

References

Amit, A. G., Mariuzza, R. A., Phillips, S. E. V., & Poljak, R. J. (1896) *Science 233*, 743–753.

Auget, M., Dembic, Z., & Merlin, G. (1988) *Cell 55*, 273–280.

Baker, P. E. (1986) *J. Immunol. 136*, 4561–4564.

Cheng, C. T., Wu, C. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13–31.

Chou, P. Y., & Fasman, G. D. (1974) *Biochemistry 13*, 222–245.

Chou, P. Y., & Fasman, G. D. (1977) *J. Mol. Biol. 115*, 135–175.

Dijkema, R., Meide, P. H., Pouwels, P. H., Caspers, M., Dubbeld, M., & Schellekens, H. (1985) *EMBO J. 4*, 761–767.

Gray, P. W., & Goeddell, D. V. (1982) *Nature 298*, 859–863.
Gray, P. W., & Goeddell, D. V. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5842–5846.
Gray, P. W., Leong, S., Fennie, E. H., Farrar, M. A., Pingel, J. T., Fernandez, L., & Schreiber, R. D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8497–8501.
Jarpe, M. A., & Johnson, H. M. (1990) *J. Interferon Res. 10*, 243–252.
Johnson, C. W. (1988) *Annu. Rev. Biophys. Biophys. Chem. 17*, 145–166.
Johnson, H. M. (1985) *Lymphokines 11*, 33–46.
Kumar, C. S., Muthukumaran, G., Frost, L. J., Noe, M., Ahn, Y. H., Mariano, T. M., & Pestka, S. (1989) *J. Biol. Chem. 264*, 17939–17946.
Magazine, H. I., Carter, M. J., Russell, J. K., Torres, B. A., Dunn, B. M., & Johnson, H. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1237–1241.
Marqusee, S., Robbins, V. H., & Baldwin, R. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5286–5290.
Martenson, R. E., Park, J. Y., & Stone, A. L. (1985) *Biochemistry 24*, 7689–7695.
Merrifield, R. B. (1964) *Biochemistry 3*, 1385–1390.
Murali, R., & Subramanian, E. (1987) *Int. J. Pept. Protein Res. 29*, 187–192.
Pestka, S., Kelder, B., Familletti, P. C., Moschera, J. A., Crow, R., & Kempner, E. S. (1983) *J. Biol. Chem. 258*, 9706.
Rinderknecht, E. B., O'Connor, B. H., & Rodriguez, H. (1984) *J. Biol. Chem. 259*, 6790–6799.
Regan, J. W., Kobilka, T. S., Yang-Feng, T. L., Caron, M. G., Lefkowitz, R. J., & Kobilka, B. K. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6301–6307.
Russell, J. K., Hayes, M. P., Carter, J. M., Torres, B. A., Dunn, B. M., Russell, S. W., & Johnson, H. M. (1986) *J. Immunol. 136*, 3324–3328.
Tam, J. P., Heath, W. F., & Merrifield, R. B. (1983) *J. Am. Chem. Soc. 105*, 6442–6445.
Vilcek, J., Gray, R. W., Rinderknecht, E., & Sevastopoulos, C. G. (1985) *Lymphokines 11*, 1–32.
Yang, J. T., Wu, C. S. C., & Martinez, H. M. (1986) *Methods Enzymol. 130*, 208–269.
Yip, Y. K., Pang, R. H. L., Urban, C., & Vilcek, J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1601–1605.
Zavodny, P. J., Petro, M. E., Chiang, T. R., Narula, S. K., & Liebowitz, P. J. (1988) *J. Interferon Res. 8*, 483–494.

Biosynthesis of the Immunosuppressant Immunomycin: The Enzymology of Pipecolate Incorporation†

Jennifer B. Nielsen,* Ming-Jo Hsu, Kevin M. Byrne, and Louis Kaplan
Merck and Company, P.O. Box 2000, Rahway, New Jersey 07065
Received December 13, 1990; Revised Manuscript Received March 18, 1991

ABSTRACT: Immunomycin, an immunosuppressant closely related to FK 506, contains a pipecolate residue in amide linkage with an acyl group in its polyketide backbone. An enzyme activating L-pipecolic acid has been isolated from *Streptomyces hygroscopicus var. ascomyceticus*, which produces immunomycin. Purification results in a monomer of 170 kDa exhibiting N-terminal heterogeneity, apparently arising from proteolysis of a single species. It is a dimer under native conditions. The reaction appears to use an aminoacyl adenylate as an intermediate in the activating reaction, as do most activating enzymes involved in nonribosomal peptide synthesis. A range of pipecolate and proline analogues act as substrates in the pyrophosphate–ATP exchange resulting from the adenylation reaction. Several analogues are inhibitors of the subsequent thioesterification of the enzyme. Antibody raised to the purified enzyme was used to follow antigen during the course of fermentation. Maximal levels of antigen are found when synthesis of immunomycin is maximal. Ten of twelve immunomycin nonproducing mutants lack detectable pipecolate-activating enzyme in Western blots. From the enzymatic characteristics, substrate specificity, and immunological properties, we propose that we have isolated the enzyme responsible for activating pipecolic acid for immunomycin biosynthesis.

Immunomycin, first isolated under the name ascomycin (Arai et al., 1962; Hatanaka et al., 1988b) and subsequently named FR-900520 or FK520, is a peptidolactone produced by *Streptomyces hygroscopicus* strains. It is structurally related to FK 506, the potent immunosuppressant produced by *Streptomyces tsukabiensis* (Kino et al., 1987; Hatanaka et al., 1988b) differing only at one position of the polyketide chain (Figure 1). A four-carbon unit of the polyketide region of immunomycin originates from the incorporation of a butyrate residue while the corresponding five-carbon chain arises in FK 506 from the condensation of a propionate with an acetate residue (Byrne et al., 1991). Each product consists of a lengthy polyketide region bridging a substituted cyclic seven-carbon unit and an amino acid, pipecolic acid, or proline, via a lactone bond involving the carboxyl of the amino acid (Figure 1). In *S. tsukabiensis* the pipecolate-containing component is the principal product, with proline incorporation occurring only to a minor degree. The proline analogue (Hatanaka et al., 1988b) is considerably less potent in immunosuppression, implying that the amino acid portion of the structure is important for bioactivity. Since the amino acid is in amide linkage with an acyl group of the polyketide, it is reasonable to speculate that it is activated in the manner described for the nonribosomally synthesized peptide antibiotics (Kleinkauf & von

† Aspects of this work were presented at the Society for Industrial Microbiology Conference on Novel Microbial Metabolites in Florida, August 1990.

* Address correspondence to this author at Merck and Co., RY 80Y-210, P.O. Box 2000, Rahway, NJ 07065.

0006-2960/91/0430-5789$02.50/0