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STRUCTURAL CHARACTERIZATION OF RECOMBINANT CONSENSUS INTERFERON-α

MICHAEL L. KLEIN, TIMOTHY D. BARTLEY, POR-HSIUNG LAI and HSIENG S. LU*
Amgen Inc., 1900 Oak Terrace Lane, Thousand Oaks, CA 91320 (U.S.A.)
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SUMMARY

Recombinant consensus interferon-α is derived from genetically modified *Escherichia coli* containing a synthetic gene constructed from a consensus of interferon sequences. The purified and biologically active protein has been subjected to detailed structural characterization including sequence determination and peptide isolation and identification. The homogeneous consensus interferon-α preparation contains two chromatographically indistinguishable homologous polypeptides with one containing an extra methionyl residue at the amino terminus. The delineated amino acid sequence of the protein is identical to that expected from the coding sequence of the gene. Correct oxidation of the molecule has been confirmed with two intramolecular disulfide linkages observed at Cys(1)–Cys(99) and Cys(29)–Cys(139).

INTRODUCTION

Interferons (IFNs) are a group of closely related soluble cellular proteins acting as potent biological response modifiers *in vivo* (for reviews, see refs. 1 and 2). It has been demonstrated that homogeneous interferon preparations elicit various biological activities such as antiviral effect, cell growth inhibition and immunomodulation[3]. Interferons-α are differentiated as "leucocyte type" interferons and are antigenically, physicochemically and biologically distinguishable from interferons-β (fibroblast type) and -γ (immune type)[1].

Approximately fourteen distinct human IFN-α genes coding for proteins which contain 164–166 amino acid residues have been cloned[4–6]. All the interferon-α genes yield products that are about 80% homologous at the amino acid level. At Amgen (Thousand Oaks, CA, U.S.A.), a consensus interferon-α (IFN-Con_1) gene, a hybrid of the fourteen IFN-α subtypes, was developed. The polypeptide derived from this hybrid gene corresponds to the amino acid sequence representing the most frequently observed amino acid at each position when the sequences for the fourteen IFN-α subtypes are aligned[7]. This synthetic hybrid gene was cloned into *Escherichia coli* and the expressed recombinant product was purified to homogeneity. The final product was confirmed to have a specific antiviral activity of more than $5 \cdot 10^8$. In this communication, the entire amino acid sequence of IFN-Con_1 was elucidated by direct N-

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and C-terminal sequence analyses of the intact protein and by characterization of various overlapping peptide fragments. In addition, the covalent disulfide structures in IFN-Con_1 were confirmed to be correctly oxidized and identical to the structures in authentic IFN-α[9,10].

EXPERIMENTAL

Materials

Trypsin [L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated] and iodoacetate were obtained from Sigma. Iodoacetic acid was recrystallized from light petroleum prior to use. [2-^{3}H]Iodoacetate was a product of New England Nuclear. *Staphylococcus aureus* V8 (SV8) protease was purchased from Miles, and carboxypeptidase P from the Peptide Institute (Osaka, Japan). Trifluoroacetic acid (TFA), acetonitrile and water were obtained from Burdick & Jackson. Crystalline cyanogen bromide was from EASTMAN-Kodak. All of the sequencing reagents and solvents were purchased from Applied Biosystems.

Purification of IFN-Con_1

A hybrid gene coding for IFN-Con_1 was synthesized and cloned into *E. coli*. Recombinant IFN-Con_1 produced by this organism was purified to homogeneity as follows. Intact cells of *E. coli*, harvested from fermenatation broth by centrifugation, were lysed in a Manton–Gaulin press. The insoluble pellet, containing IFN-Con_1, was collected by centrifugation at 3500 *g* and solubilized in 6 *M* guanidine–HCl, 2 m*M* dithiotreitol (DTT), 25 m*M* Tris–HCl (pH 8.5). Guanidine–HCl and DTT were removed by buffer exchange against 25 m*M* Tris–HCl (pH 8.5) to initiate refolding and oxidation. The IFN-Con_1 was purified by sequential chromatography using DEAE-Sepharose Fast Flow and Sephacryl S-200 (Pharmacia).

S-Carboxymethylation of IFN-Con_1

IFN-Con_1 [in 25 m*M* phosphate (pH 7), 0.1 *M* sodium chloride (phosphate buffered saline, PBS)] was desalted by passing over a Pharmcia PD-10 (Sephadex G-25) column equilibrated with HPLC-grade water. A 1-mg amount of desalted material was dried down, reconstituted with 6 *M* guanidine–HCl in 0.2 *M* ammonium bicarbonate (pH 8.2) and gently stirred at 37°C. DTT was added at a 50-fold molar excess while flushing with nitrogen gas, and stirred at 37°C for 2 h. After wrapping the reaction vessel with alumninum foil, iodoacetic acid was added at a 10-fold molar excess to DTT. Diluted sodium hydroxide (10 m*M*) was added to bring the reaction mixture back to pH 8.2. The mixtured was stirred at 37°C for 30 min, after which the reaction was quenched by the addition of 60 μl β-mercaptoethanol (5%, v/v). The mixture was immediately transferred to a dialysis bag (Spectra-Por 8000 mol.wt. cut-off), and was extensively dialyzed against 0.1 *M* ammonium bicarbonate (pH 7.8) at 4°C in the dark. The dialyzed derivative was then subjected to further analysis.

Amino acid sequence analysis

Polypeptides were sequenced from their amino termini by automated Edman degradation on an Applied Biosystems 470A gas phase sequencer. The phenylthiohydantoin (PTH) amino acids were analyzed by high-performance liquid chromato-

graphy (HPLC) according to the method of Hewick *et al.*[11]. When the entire IFN-Con_1 molecule was to be sequenced, it was spotted on a TFA-activated glass fiber (Whatman GF/C) disc; when cleavage products (peptides) were to be sequenced, they were spotted on TFA-activated GF/C discs which were treated with Polybrene containing 6.7 mg/ml sodium chloride and preconditioned with at least three sequencer cycles.

Amino acid analysis

Samples containing IFN-Con_1 were transferred to WISP vials (Waters Assoc.), dried and hydrolyzed using the vapor hydrolysis method[12]. Vials containing dried samples were placed in sealed, evacuated screw-top vacuum vials (Waters Assoc.) containing 1.5 ml of 6 *M* hydrochloric acid, 0.1% β-mercaptoethanol and 0.05% phenol. The tubes were placed in an oven at 110°C for 24 h, after which the vials were removed and dried by vacuum. The dried hydrolysates were reconstituted with 0.2 *M* sodium citrate and analyzed by high-performance cation-exchange chromatography followed by ninhydrin detection on a Beckman 6300 amino acid analyzer.

The amino acid composition of the peptide fragments was determined by a pre-column derivatization method using phenylisothiocyanate (PITC)[13,14]. The phenylthiocarbamyl (PTC) amino acids were analyzed by reversed-phase HPLC on a 3-μm Rainin C_{18} column (10 × 0.46 cm I.D.) using a Spectra-Physics 8700 HPLC system, a Waters WISP 710B autosampler, and a Waters 440 detector (254 nm detection wavelength).

Peptide mapping derived from enzymatic and chemical cleavages

Tryptic digestion. Cys(Cm)-IFN-Con_1 [Cys(Cm) is S-carboxymethylcysteine] derivative was diluted to 0.23 mg/ml with 0.1 *M* ammonium bicarbonate (pH 7.8). This solution and a solution of native IFN-Con_1 (0.23 mg/ml PBS) were treated with trypsin (trypsin:IFN-Con_1, 1:50, w/w) and incubated at 37°C for 4 h. The reactions were stopped by placing the vessels on ice. The digests were injected directly by a Waters WISP 710B onto a reversed-phase C_4 column (Vydac; 25 × 0.46 cm I.D.), and eluted with a gradient of acetonitrile in 0.1% TFA generated by a Spectra-Physics 8700 pumping system. Tryptic maps were generated by monitoring the absorbance of the effluent with a Spectraflow 757 detector (Kratos) at 220 nm. Peaks were collected in glass test tubes using a Gilson Model 202 fraction collector.

SV8 protease digestion. A solution of native IFN-Con_1 (0.23 mg/ml PBS) was teated with SV8 protease (enzyme-to-substrate ratio = 1.25) and incubated at 37°C for 18 h. The reaction was stopped by placing the vessel on ice. Mapping was performed as described above for tryptic mapping. SV8 cleavage is expected to occur at Glu-X and, to a lesser extent, Asp-X bonds.

Cyanogen bromide cleavage. A 500-μg amount of Cys(Cm)-IFN-Con_1 in 0.1 *M* ammonium bicarbonate (pH 7.8) was dried and reconstituted with 190 μl of a 10% (w/v) cyanogen bromide solution in 70% formic acid (pre-purged with nitrogen gas). The reaction was run for 16 h in the dark at room temperature. The mixture was then dried to near-completion in a hood with a stream of nitrogen, followed by the addition of 200 μl of 0.1% TFA, which gave a turbid solution. To alleviate much of the turbidity, one crystal of guanidine–HCl was added. Using this solution, cyanogen bromide mapping was performed as described above for tryptic mapping.

C-terminal analysis

Desalted IFN-Con$_1$ (in water) was brought to 0.05% (w/v) Brij-35 at pH 4.0 (final IFN-Con$_1$ concentration of 0.36 mg/ml). Carboxypeptidase P was added at a 1:400 (w/w) ratio with IFN-Con$_1$ and left to digest at room temperature. Time point aliquots were removed and quenched by adding TFA to a 10% concentration. The samples were immediately dried and subjected to amino acid analysis using pre-column PITC derivatization and narrow bore HPLC[15].

Peptide nomenclature

Peptide numbers were designated according to the elution order of each HPLC peptide map. Those peaks not numbered were recovered in low concentration and yielded no sequencing results.

RESULTS AND DISCUSSION

Amino acid analysis

Table I shows the amino acid composition of IFN-Con$_1$ derived from acid

TABLE I

AMINO ACID COMPOSITION ANALYSIS OF RECOMBINANT IFN-Con$_1$

Recombinant IFN-Con$_1$ was hydrolyzed and the hydrolysate analyzed as described in the Experimental section.

Amino acid	*Number of residues*	
	Theoretical	*Calculated*
Cys(Cm)*	–	3.85
Asx	14	14.14
Thr	8	8.29
Ser**	13	11.55
Glx	28	28.26
Pro	5	6.17
Gly	4	6.01
Ala	10	10.04
Cys	4	N/A***
Val	8	7.23
Met	4	3.65
Ile	8	7.50
Leu	20	20.59
Tyr	5	4.73
Phe	10	9.97
Lys	9	9.12
His	3	2.93
Arg	11	10.94
Trp§	2	1.82
Total	166	

* Cysteine was determined as carboxymethylated cysteine by analysis of Cys(Cm)-IFN-Con$_1$.
** Content of Ser is not corrected.
*** Not analyzed.
§ Tryptophan was recovered by 4 *M* methanesulfonic acid hydrolysis of IFN-Con$_1$.

hydrolysis. The calculated numbers of residues correspond very well with the expected values except for the low values for serine and valine, and the high values for proline and glycine. Cysteine was determined as its S-carboxymethyl derivative, and tryptophan was separately determined by hydrolysis using 4 *M* methanesulfonic acid[16]. The low value for serine is due to partial destruction during acid hydrolysis and was estimated without correction. The compositional analysis of IFN-Con_1 indicates that it is a product derived from the IFN-Con_1 gene.

N-terminal sequence of IFN-Con_1

When purified Cys(Cm)-IFN-Con_1 was subjected to N-terminal sequencing, two distinct sequences were revealed. Once sequence corresponded to a species starting with the initiator methionine. The other sequence began with cysteine as the N-terminus, indicating the removal of the initiator methionine by the host expression system. The ratio of N-terminal methionine to cysteine is approximately 1.1:1, suggesting that the *E. coli* methionyl aminopeptidase[17] is able to process approximately half of the protein initiator under the conditions used to express IFN-Con_1. The N-terminal sequence analysis of the protein results in the elucidation of the first 33 amino acid residues from the amino terminus as shown in Fig. 1. Cys-1 and Cys-29 were positively identified by sequencing the Cys(Cm)-IFN-Con_1 derivative. However, due to the interference of the two N-terminal sequences of IFN-Con_1, the extended sequencing run yielded inconclusive results, as carryover and lag made further assignment unclear after cycle 33.

C-terminal sequence analysis

The intactness of the C-terminal sequence of IFN-Con_1 was confirmed by two separate analyses. The C-terminal sequence was identified by isolation of an SV8 protease-cleaved peptide, peptide S-1 (Figs. 1 and 2). The peptide contains six amino acids and has a sequence of Arg-Leu-Arg-Arg-Lys-Glu-COOH as revealed by compositional and sequence analyses. This C-terminal sequence is supported by carboxypeptidase P digestion of the intact protein (Table II). Kinetic analysis of the data is consistent with the fact that the C-terminal sequence of the protein is · · · (R)-L-R-R-K-E, indicating that the recombinant IFN-Con_1 indeed contains the intact C-terminal amino acid sequence as predicted from gene sequence. The release of arginine at higher value is expected as two arginine groups are located at the third and fourth positions near the C-terminal of the molecule. No degradation or further C-terminal processing generated by *E. coli* proteases was observed.

Isolation and characterization of peptides

To elucidate the complete amino acid sequence of IFN-Con_1, the native protein or S-carboxymethylated derivative was subjected to a series of enzymatic and chemical cleavages and the resulting peptide mixtures subjected to separation by reversed-phase HPLC. Fig. 2–5 represent HPLC peptide maps obtained from various cleavages of IFN-Con_1. All peaks were collected and analyzed by PTC-amino acid analysis (data not shown). All of the numbered peptides were characterized and actually found in the IFN-Con_1 sequence (Fig. 1). Those fractions which showed low recovery in amino acid analysis were not further analyzed.

Fig. 3 shows the HPLC peptide map obtained after chromatography of a tryp-

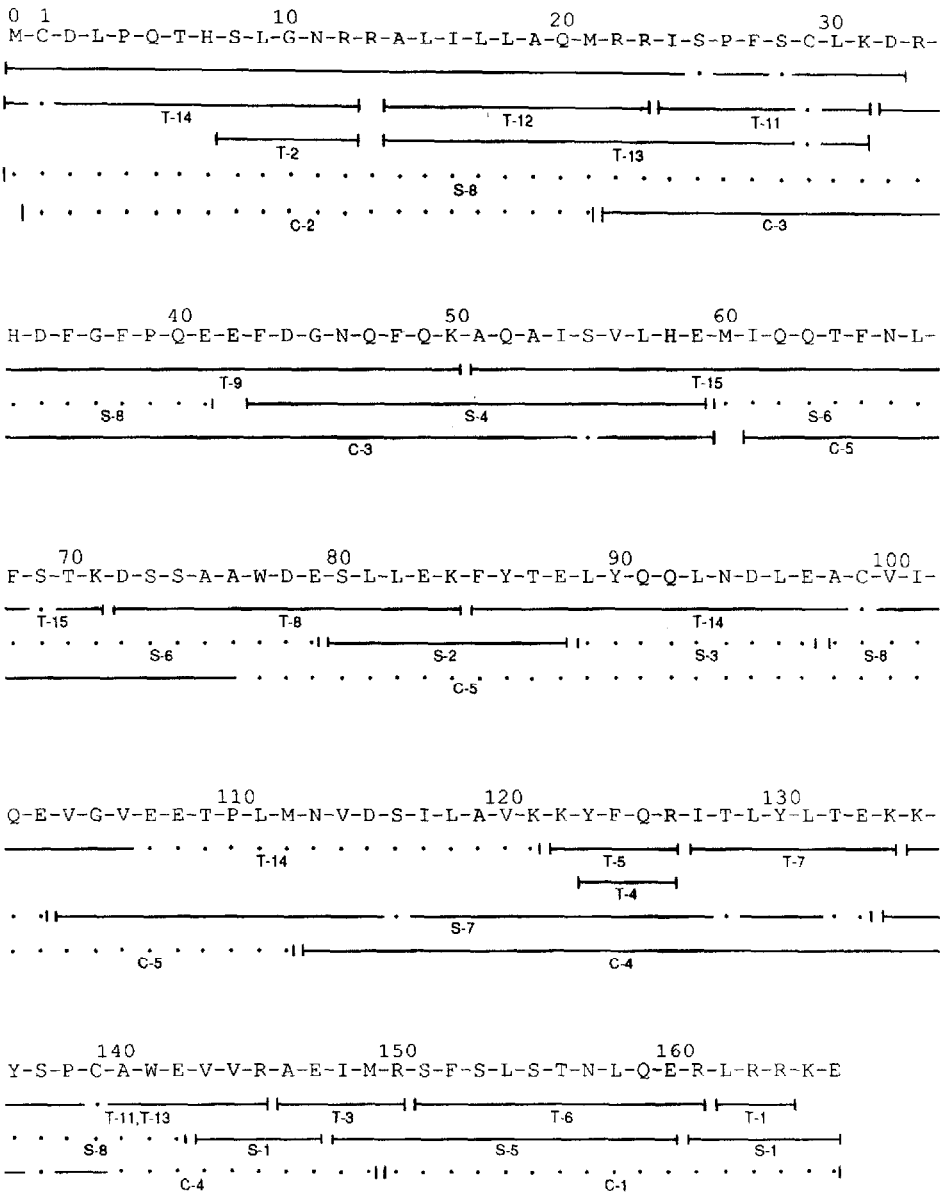

Fig. 1. Amino acid sequence of recombinant IFN-Con_1. Sequences analyzed with intact Cys(Cm)-IFN-Con_1, and peptides derived from native and S-carboxymethylated IFN-Con_1 are indicated by solid lines under the residues comprising the protein or the peptide. The letter codes below these solid lines correspond to the peptides generated by enzymatic or chemical cleavage of native and S-carboxymethylated IFN-Con_1 which were separated and isolated by HPLC (refer to Figs. 2–5): T = tryptic peptides; S = SV8 peptides; C = cyanogen bromide peptides. The solid line without a letter code corresponds to the sequence of intact Cys(Cm)-IFN-Con_1. Solid lines indicate the results of automated Edman degradation. Dots indicate residues deduced from amino acid analysis but not identified by automated Edman degradation.

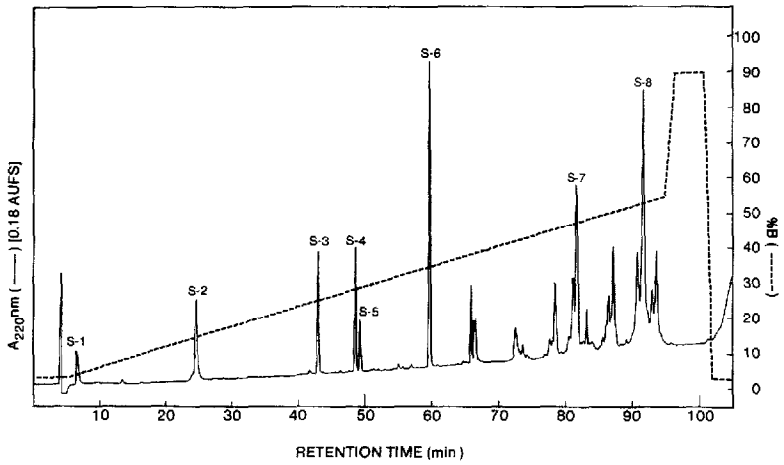

Fig. 2. HPLC peptide map of SV8 protease derived peptide from native IFN-Con_1. Native IFN-Con_1 was incubated with *S. aureus* V8 protease, and 46 μg of the resulting peptide mixture was separated by reversed-phase HPLC (see Experimental section). Peaks which were analyzed after collection are numbered according to order of elution. Solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile–water (90:10).

sin digest of the native protein. At least 15 peptides were sequenced and the results are summarized in Fig. 1. Both peptides T-11 and T-14 were found to contain two distinct sequences, corresponding to the two disulfide-containing peptides [Cys(29)–Cys (139), and Cys(1)–Cys(99), respectively]. Further characterization of disulfide assignment is described below. A broader peak which eluted at 95 min. in the tryptic map corresponds to uncleaved or incompletely cleaved IFN-Con_1, indicating that the present cleavage conditions result in incomplete digestion of the protein substrate.

The alignment of tryptic peptides was obtained by isolation and characterization of overlapping peptides derived from SV8 protease digestion (Fig. 2) and cyanogen bromide cleavage (Fig. 4) of IFN-Con_1. Eight SV8 protease-derived peptides and five cyanogen bromide-cleaved fragments are actually analyzed to construct the entire sequence as shown in Fig. 1. In summary, proteolytic and chemical cleavages of both native and S-carboxymethylated IFN-Con_1, along with sequencing of the

TABLE II

RELEASED AMINO ACIDS FROM CARBOXYPEPTIDASE P DIGESTION OF RECOMBINANT IFN-Con_1

Data were obtained and calculated as number of residues released per mol of protein (see Experimental).

Amino acid	*Time (min)*				
	0	*5*	*10*	*20*	*40*
Glu	0	0.18	0.48	0.75	0.86
Arg	0	0.17	0.57	1.14	1.80
Leu	0	0.10	0.12	0.19	0.22
Lys	0	0.14	0.38	0.53	0.78

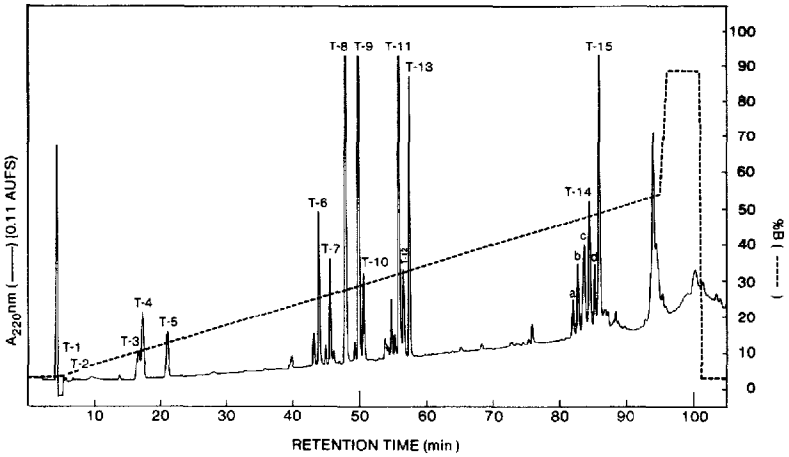

Fig. 3. HPLC peptide map of tryptic peptides derived from native IFN-Con$_1$. Native IFN-Con$_1$ was incubated with trypsin, and 46 μg of the resulting peptide mixture was separated by reversed-phase HPLC (see Experimental section). For details see Fig. 2.

N- and C-termini of the intact protein, provide for a complete overlapping sequence of this molecule. This analysis confirms that the sequence of recombinant IFN-Con$_1$ protein corresponds exactly to the coding sequence of the synthetic gene.

Assignment of disulfide bonds

Recombinant IFN-Con$_1$ when produced by genetically modified *E. coli* cells is recovered using processes including the denaturation–renaturation step. This step also allows formation of two disulfide bonds. The fully active, recombinant protein was analyzed to identify and characterize the disulfide structures.

When the protein was reacted with a 20-fold molar excess of [^{3}H]iodoacetate in

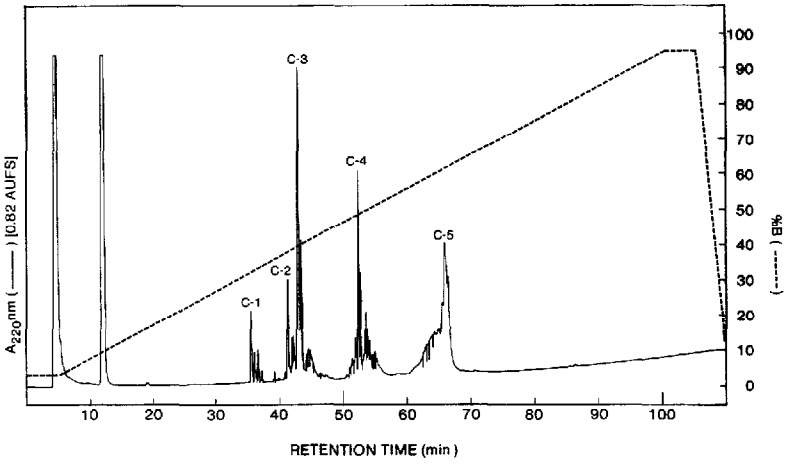

Fig. 4. HPLC peptide map of cyanogen bromide cleaved peptides from Cys(Cm)-IFN-Con$_1$. Cys(Cm)-IFN-Con$_1$ was incubated with cyanogen bromide, and 83 μg of the resulting peptide mixture was separated by reversed-phase HPLC. For details, see Fig. 2.

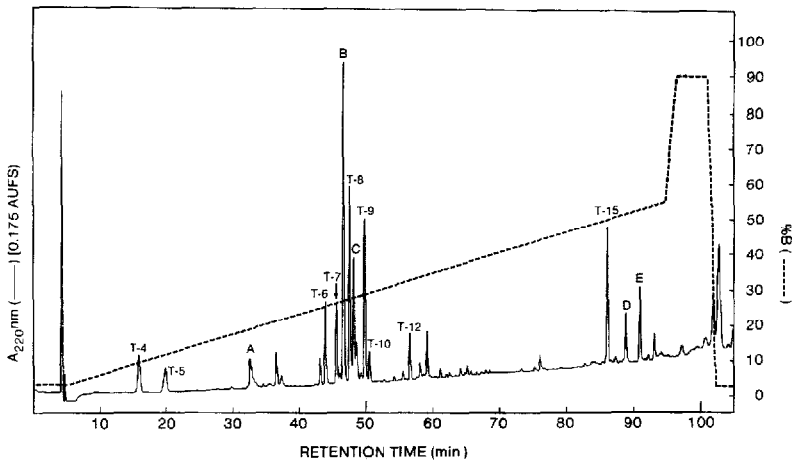

Fig. 5. HPLC peptide map of tryptic peptides from Cys(CM)-IFN-Con$_1$. Cys(Cm)-IFN-Con$_1$ was incubated with trypsin, and 46 μg of the resulting peptide mixture was separated by reversed-phase HPLC (see Experimental section). Peaks labeled A, B, C, D and E correspond to peptides containing SA-carboxymethylcysteine (see Table III).

0.2 *M* ammonium bicarbonate (pH 8.2) containing 6 *M* guanidine–HCl, 0.01 mol of radioactive label was incorporated per mol of protein. This result confirmed that all the cysteinyl residues in the molecule are involved in disulfide bond formation, and no residual unpaired cysteine was observed. This observation was further corroborated by Ellman's reaction[18], since no free sulfhydryl could be detected. When the protein was first reduced with DTT in 0.2 *M* ammonium bicarbonate (pH 8.2) containing 6 *M* guanidine–HCl and followed by alkylation with [2-^{3}H]iodoacetate (see Experimental section), 3.9 mol of radioactive label was incorporated per mol of protein. This result further indicated that the four cysteinyl residues in recombinant IFN-Con$_1$ form two pairs of disulfide bonds.

Determination of the location of the correct disulfide bonds was performed by

TABLE III

IDENTIFICATION OF PEPTIDES CONTAINING DISULFIDE OR S-CARBOXYMETHYLCYSTEINE

Conditions used for isolation of these peptides are described in Figs. 3 and 5. These peptides were identified by sequence and/or amino acid analysis as described in the Experimental section.

Peptides containing disulfide	*Sequence positions*	*Peptides containing S-carboxymethylcysteine*	*Sequence positions*
11	24–31/135–145	A	0–12
13	14–31/135–145	B	135–145
14	0–12/85–121	C	24–31
a	0–13/85–122	D	85–122
b	0–12/85–122	E	85–122
c	0–12/85–121		
d	1–13/85–122		

isolation, characterization and sequencing of the cysteine-containing peptides. As shown in Fig. 3, two tryptic peptides, peptides T-11 and T-14, were confirmed to be disulfide-containing peptides by amino acid analysis and direct sequence determination. Peptide T-11 contains residues 24–31 and 135–145; and peptide T-14 contains residues 0–12 and 85–121 (Table III). Both are held together by disulfide bonds at Cys(29)–Cys(139) and Cys(1)–Cys(99), respectively. Peptide T-13, which was confirmed to contain residues 14–31 and 135–145, also contains the Cys(29)–Cys(139) disulfide. It is interesting to note that other fractions, designated as a, b, c and d shown in Fig. 3, are also Cys(1)–Cys(99) disulfide-containing peptides (Table III). This heterogeneity is explained by the fact that the C-termini of the peptides on each side of the disulfide bond contain two possible cleavage sites [Arg(11)–Arg(12) and Lys(121)–Lys(122)], and that the amino terminus of the final product contains both methionine and cysteine, giving several disulfide-containing peptides.

To further assign the disulfide bonds, a tryptic map of Cys(Cm)-IFN-Con_1 was developed in comparison to the map of native protein (Fig. 5). Peptides T-11, T-13 and T-14 together with a, b, c and d shown in Fig. 3 disappear in the map of the reduced and alkylated protein, further supporting that these fractions correspond to the disulfide-paired peptides. Peaks corresponding to the S-carboxymethylcysteine-containing peptides appear in place of the disulfide-paired peptides, and are labeled A–E shown in Fig. 5. Amino acid composition and sequence analyses allowed the assignment of these half-cysteine peptides (Table III and Fig. 1). These results unambiguously corroborate previous evidence determining that the two disulfide bonds of IFN-Con_1 are Cys(1)–Cys(99) and Cys(29)–Cys(139), and are identical to the structures of IFN-α isolated from natural sources[9,10]. HPLC of SV8 protease-digested protein also allowed recovery of a disulfide-containing peptide, peptide S-8 (see Fig. 2). Peptide S-8 is composed of three distinct peptide sequences, residues 0–41, 98–103, 134–142, and these sequences are held together by two disulfide bonds (Fig. 1). Characterization of peptide S-8 was not pursued.

In summary, detailed structural characterization indicates that the primary structure of recombinant IFN-Con_1 expressed in *E. coli* completely matches that deduced from the IFN-Con_1 gene. The incomplete N-terminal processing of the protein by the host enzyme, methionyl aminopeptidase, results in the generation of two homologous species, *i.e.*, methionyl and desmethionyl IFN-Con_1. These two forms behave similarly so that they tend to co-elute in various chromatographic procedures. IFN-Con_1 purified through denaturation–renaturation–reoxidation steps was found to properly oxidize to form a biologically active molecule that is similar to natural IFN-α[8].

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