Vol. 157, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1988 Pages 364-37'

CHARACTERIZATION OF A RECOMBINANT MURINE INTERLEUKIN-6: ASSIGNMENT OF DISULFIDE BONDS

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Received October 6, 1988

Murine interleukin 6 (mIL-6) has been synthesized as a fusion protein using a *lac* operon inducible plasmid in *Escherichia coli* . The first 8 amino acids are from the N-terminus of bacterial β-galactosidase and the last 175 amino acids are from residue number 12 to the end of native mIL-6. This fusion protein is equipotent with the native molecule in the hybridoma growth factor assay and has comparable receptor binding characteristics. The two disulfide bridges in mIL-6 have been identified by *Staphylococcus aureus* V8 protease peptide mapping and Edman degradation of cystine-containing peptides. It has been shown that there are disulfide bonds between Cys_{46}-Cys_{52} and Cys_{75}-Cys_{85}.

Murine interleukin-6 (originally designated hybridoma/plasmacytoma growth factor) was initially identified as a T-cell derived lymphokine with growth factor activity for B-cell hybridomas and plasmacytomas (1,2). Subsequently, IL-6 was detected in the supernatants of lipopolysaccharide -stimulated macrophages (3,4) and virally-induced fibroblasts (5).

We have also identified the human hybridoma / plasmacytoma growth factor (6,7); this human counterpart has also been detected by others in different biological systems and alternatively designated 26 kDa-protein (8), interferon-β_2 (9) or B-cell stimulatory factor-2 (10). It is now apparent that IL-6 has pleiotropic biological activities which include

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0006-291X/88 $1.50

normal B-cell differentiation and T-cell growth factor activity (11), hepatocyte stimulatory activity (12-17), nerve growth factor-like activity (18) and hemopoietic activity (19-22); for review see 11). It has also been suggested that the constitutive expression of IL-6 in humans may lead to the pathogenesis of certain autoimmune diseases (11,23).

Recently, we determined the complete amino acid sequence of murine IL-6 at both the protein (25) and DNA level (24) and established that although there is virtually no similarity between the N-terminal region of mouse and human IL-6, extensive amino acid similarity exists in the middle and C-terminal regions of these molecules. As a first step in our studies to elucidate the structure-function relationship of IL-6 we have determined, and report in this communication, the assignment of the two disulfide bridges in this molecule.

MATERIALS AND METHODS

Construction of mouse IL-6 expression vector

Mouse HP1/IL-6 cDNA (24) was cloned into the EcoR1 site of pUC9 plasmid and its 5'end was partly digested with exonuclease III (25) to remove the 5'-untranslated region and the signal sequence. The most active deletion mutants were selected by a direct hybridoma growth factor (HGF) assay using whole bacteria and the IL-6-dependent hybridoma 7TD1 (see below). The most active clone, p9HP1B5B12, was found to encode a fusion protein consisting of the 8 N-terminal amino acids of bacterial β-galactosidase fused to amino acid number 12 of mature IL-6.

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                1               5                  10
mIL-6           Phe-Pro-Thr-Ser-Gln-Val-Arg-Arg-Gly-Asp-Phe-Thr-Glu-Asp-Thr-

recombinant                Thr-Met-Ile-Thr-Pro-Ser-Leu-Ala-Thr-Glu-Asp-Thr-
mIL-6
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Purification of recombinant mIL-6 (rmIL-6)

rmIL-6 was purified from bacteria grown overnight in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were washed with phosphate-buffered saline (PBS), lysed by sonication in 1% Triton X-100 and harvestered by centrifugation. The pellet, which contained most of the rmIL-6, was solubilized in 6M guanidine.HCl. After a 25-fold dilution in PBS containing 0.01% (v/v) Tween 20, the solubilized material was precipitated with ammonium sulfate first at 40% saturation to eliminate residual contaminants and then at 80% saturation to concentrate mIL-6. Final purification was achieved by affinity chromatography on a Sepharose-bound monoclonal anti-IL-6 antibody as described for native mIL-6 (26).

Hybridoma growth factor assay

mIL-6 titrations were performed as previously described (1). Briefly, IL-6 dependent hybridoma 7TD1 (2000 cells/microwell) was incubated with serial dilutions of the test fractions in a final volume of 0.2 ml. Cell numbers were evaluated after 4 days at 37°C by measuring hexosaminidase levels (27). A titer of 1 U/ml was arbitrarily assigned to material that produced half-maximal growth of hybridoma 7TD1.

Staphylococcus aureus V8 protease mapping of recombinant mIL-6

rmIL-6 (100 µg) in 560 µl of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.01% (v/v) Tween 20 (Pierce Chemical Co.), 0.15% (w/v) (SDS) was incubated at 33°C with 4 µg *S. aureus* V8 protease (Miles Co.) for 18 h.

The *S. aureus* V8 protease digest of mIL-6, treated and untreated with 100 mM dithiothreitol, was fractionated by RP-HPLC as previously described (26,28,29). A portion of the digest was reduced in the following manner: to 60 µl of the digest (10 µg peptides) was added 10 µl of 6M guanidine. HCl 0.2 M Tris.HCl (pH 8.5), dithiothreitol (final concentration, 100 mM) and the mixture incubated at 45°C for 1 hr.

High performance liquid chromatography (HPLC)

rmIL-6 and peptide mixtures were fractionated by RP-HPLC on a Hewlett Packard model 1090A liquid chromatograph fitted with a model 1040A diode array detector as described elsewhere (28). Fractions were collected manually in 1.5-ml polypropylene tubes (Eppendorf) and stored at -20°C.

Amino acid analysis

Amino acid analysis was performed on a Beckman amino acid analyser (model 6300) equipped with a model 7000 data system. Samples were hydrolysed *in vacuo* with gaseous HCl generated from 6M HCl containing 0.1% (w/v) phenol.

Amino acid sequence analysis

Automated Edman degradation of rmIL-6 and derived peptides was performed using Applied Biosystems sequencers (models 470A and 477A) equipped with on-line phenylthiohydantion (Pth) amino acid analysers (model 120A) modified for total sample injection (30).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using the Phast System (Pharmacia). Slab gel electrophoresis was carried out on 7.5% acrylamide stacking gels and 20% polyacrylamide resolving gels using a discontinuous buffer system according to Laemmli (31). Proteins in the gel were visualised by silver staining according to the manufacturers instructions (Pharmacia, PhastGel Silver Kit Manual).

RESULTS AND DISCUSSION

The affinity purified rmIL-6 used in this study was judged homogeneous as evidenced by a simple peak on RP-HPLC (Fig. 1). As a further measure of purity, the rmIL-6 was analyzed by SDS-PAGE under non-reducing conditions Fig. 1 Inset, lane 1) or reducing conditions (5% 2-mercaptoethanol) (Fig. 1 Inset, lane 2). Purified rmIL-6 electrophoresed as a single band of apparent M_r 21-22,000 on 20% SDS-PAGE in both the unreduced and reduced state indicating it is a single subunit protein. The M_r value obtained agrees closely with that calculated from the deduced amino acid sequence of the rmIL-6; the slight difference in M_r between rmIL-6 and native mIL-6 (M_r 22-29,000) (1) is most likely due to post translational modification (O-glycosylation) of native mIL-6 reported earlier (26). The rmIL-6 migrated with a slightly lower apparent M_r when loaded and run reduced (Fig. 1 Inset, compare lanes 1 and 2).

Amino acid sequence analysis of 125 pmoles of the purified rmIL-6 yielded a single amino acid at each cycle; the first eight residues detected corresponded to bacterial β-galactosidase and the next 20 cycles yielded the sequence expected for native mIL-6 (24,26) commencing at residue 12 (see Materials and Methods). Thus, this product is different from the natural molecule in that not only is the carbohydrate moiety missing, but the N-terminal 12 residues have been replaced by an 8 residue

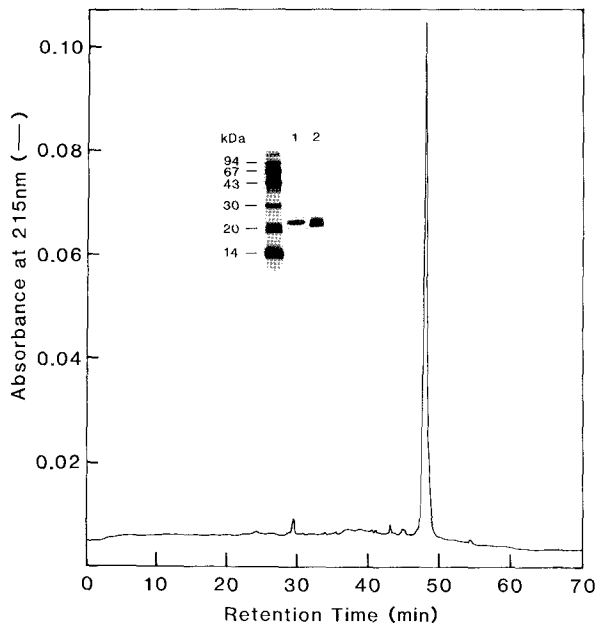


Figure 1 Analysis of recombinant mIL-6 by reversed-phase HPLC. Chromatographic conditions: column, Brownlee RP-300 (100 x 4.6 mm i.d. 7μ dp), linear 60-min gradient from 0 to 100% B, where solvent A was 0.1% (v/v) aqueous trifluoroacetic acid and solvent B was 60% acetonitrile/40% water containing 0.89% (v/v) trifluoroacetic acid. Flow rate, 1.0 ml/min. Column temperature, 45°C.

Inset. Analytical SDS-PAGE of recombinant mIL-6. Lane 1, sample prepared under non-reducing conditions. Lane 2, sample treated with 100 mM dithiothreitol before loading onto gel. Protein standards: 97 kDa, phosphorylase b; 67 kDa bovine serum albumin; 43 kDa, ovalbumin; 29 kDa, carbonic anhydrase. Proteins were visualized by silver staining.

β-galactosidase sequence. The amino acid composition of the bacterially synthesized IL-6 is in excellent agreement with the composition predicted from the nucleotide and protein sequence analyses (data not shown).

The specific activity of the recombinant protein as measured in the hybridoma growth factor assay was $0.90 \pm 0.45 \times 10^9$ U/mg which is equivalent to the value reported earlier for native mouse IL-6 (1). The recombinant protein was also as active as the native product on T cells and on IL-6-dependent plasmacytomas (data not shown). Finally, rmIL-6 has the same affinity constant ($Kd=2x10^{-11}M$) for IL-6 receptors on mouse T cells as the native protein (P. Coulie, unpublished data).

In a previous study, we established that murine IL-6 is active on both murine and human cells (7), a phenomenon seen with various other cytokines (eg. IL-1, IL-2, IL-5, macrophage colony stimulating factor and granulocyte colony stimulating factor) (32). Hence, given the lack of conservation of amino acid sequence in the N-terminal region of the human

and mouse IL-6 molecules it is not surprising that our rmIL-6, with its modified N-terminus, is equipotent with the native molecule in the hybridoma growth factor assay. This observation is in accord with that reported for a recombinant human IL-6 lacking the first 15 amino acids of the mature molecule (33). Interestingly, deletion of the sequence Arg-Gly-Asp- (residues 8-10) in the recombinant molecule, an important structural feature considered essential for cellular attachment of matrix proteins (34), does not affect its hybridoma growth factor activity *in vitro*.

The strategy adopted for identifying the disulfide bridges in rmIL-6 involved peptide mapping a reduced (100 mM dithiothreitol) and unreduced *S. aureus* V8 protease digest of the molecule and locating the disulfide-containing peptides by their differential chromatographic behaviour upon reduction.

A comparison of Figs. 1A and 1B reveals that upon reduction, peptide fractions II and III (Fig. 1A) disappear and peptide fractions IV and V appear (Fig. 1B). Also, there is a significant, and reproducible, shift in retention time (0.47 min) of fraction I upon reduction. These data suggested that peptide fractions I, II and III contain disulfide bridges.

Sequence analysis of peptide fraction II (Table 1) identified two peptides; one 15 residue peptide S1 IQRNDGXYQTGYNQE (residues 69-83) and a peptide S2 IXLLKISS.... (residues 84-). These data, together with amino acid analysis (data not shown) indicate these peptides resulted from cleavage at Glu-68 and Glu-83 and are joined by a disulfide bond between Cys-75 and Cys-85 (Table 2).

Upon reduction, peptides S1 and S2 chromatograph at 12.20 min (fraction IV, Fig. 2b) and 41.46 min (fraction V, Fig. 2b), respectively; the identity of these peptides was revealed by amino acid sequence analysis (data not shown). Sequence analysis of peptide fraction III, containing peptide S3, confirmed the assignment of a disulfide bond between Cys-75 and Cys-85 (Tables 1 and 2). Peptide S3 resulted from lack of *S. aureus* V8 protease cleavage at Glu-83. Upon reduction, the non disulfide bond form of S3 co-chromatographed with S2 at 41.46 min (sequence data not shown). The second disulfide bond, between Cys-46 and Cys-52, was found in peptide Fraction I (Fig. 1A) which comprised peptide S4 LCNGNSDCMNNDDALAE (residues 45-61) (Table 1 and 2). A slight, but significant, shift in retention time (0.47 min) of this peptide occurred upon reduction.

Further evidence for identification of disulfide bonds in peptide fractions I,II and III was provided by the release of the di-Pth derivative of cystine during Edman degradation after cleavage of the second half of the cystine residue (35). The major product chromatographed

TABLE 1

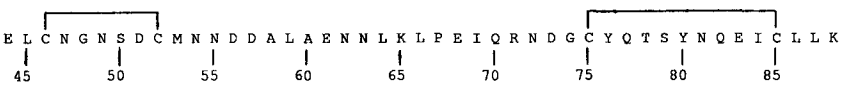
Sequence analysis of *Staphylococcus aureus* V8 peptide fractions of recombinant mIL-6 from Fig.2a

Cycle number	Pth-Xaa (pmol)		
	Fraction I	Fraction II	Fraction III
1	Leu (280)	Ile (133)	Ile (46)
2	X	Gln (36)	Gln (26)
3	Asn (187)	Arg (36), Leu (30)	Arg (16)
4	Gly (115)	Asn (61), Leu (54)	Asn (31)
5	Asn (104)	Asp (80), Lys (39)	Asp (27)
6	Ser (10)	Gly (50), Ile (26)	Gly (41)
7	Asp (42)	Ser (10), X[a]	X
8	X[a]	Ser (10), Tyr (30)	Tyr (20)
9	Met (76)	Gln (24), Gly (14)	Gln (19)
10	Asn (63)	Leu (13), Thr (2)	Thr (7)
11	Asn (105)	Leu (20), Gly (10)	Gly (28)
12	Asp (38)	Tyr (15), Glu (8)	Tyr (18)
13	Asp (53)	Asn (15), Tyr (7)	Asn (16)
14	Ala (32)	Gln (7), His (5)	Gln (12)
15	Leu (38)	Glu (4), Ser (1)	Glu (11)
16	Ala (24)	Tyr (3)	Ile (11)
17	Glu (5)	Leu (5)	X
18			Leu (11)
19			Leu (13)
20			Lys (5)
21			Ile (6)
22			Ser (1)
23			Ser (1)
24			Gly (2)
25			Leu (2)

X, unidentified Pth-amino acid derivative. [a]The di-Pth derivitive of cystine (eluted in tyrosine position) was identified in cycle 8 of fraction I and cycle 7 of fraction II.

TABLE 2

Identification of disulfide bridges in recombinant mIL-6



Peptide fraction	Structural data	Disulfide bond Cys-Cys
I	S4 LXNGNSDCMNDDALAE	46-52
II	S1 IQRNDGCYQTGYNQE	75-85
	S2 IXLLKISSGLLEY	
III	S3 IQRNDGXYQTSYNQEIXLLKISSGLL	75-85

Sequences indicate the results of Edman degradation of unreduced peptide fractions. X denotes a Cys residue expected from the known sequence but not observed; C denotes the di-Pth derivative of cystine.

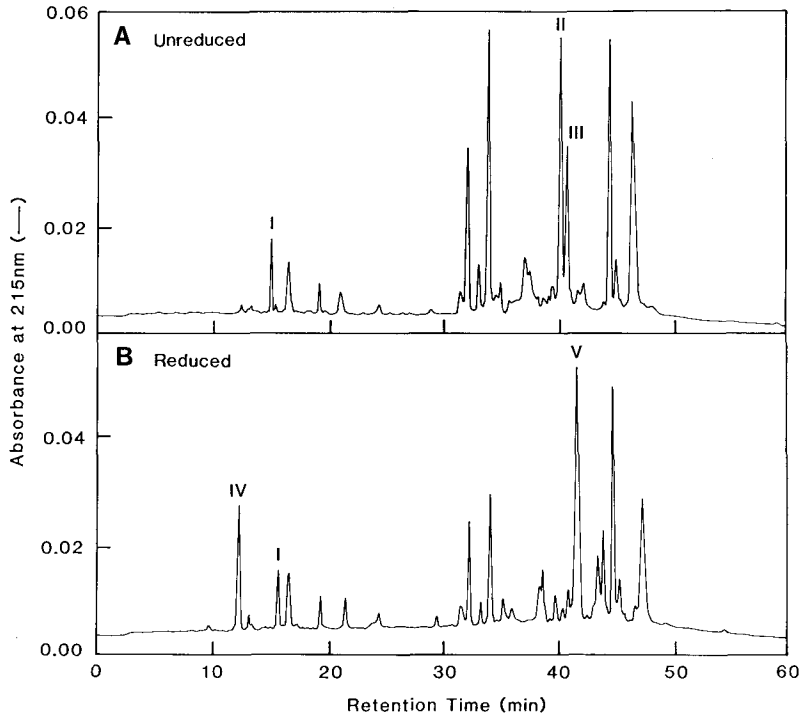


Figure 2 *Staphlococcus aureus* V8 protease mapping of recombinant m IL-6. The peptides (10 µg) were loaded onto a Brownlee RP-300 column (100 x 4.6 mm i.d., 7µ dp). Chromatographic conditions were the same as those described in Fig.1 digest rmIL-6.

A, unreduced *S. aureus* digest rmIL-6 (10 µg peptide) loaded directly.

B, prior to loading. *S. aureus* digest of rmIL-6 (10µg peptide) was treated with 100 mM dithiothreitol (see Materials and Methods). Peaks (400-600 µl) were collected manually in Eppendorf tubes and stored at -20°C. For sequence analysis, peptides were concentrated (to 40-60 µl) by microbore reversed-phase HPLC (36).

in the tyrosine position; the yield was 5-10% of the signal found for leucine/isoleucine.

It is interesting to note that the disulfide pattern for mIL-6 (Cys 1-2, Cys 3-4) is identical to that reported for granulocyte-colony stimulating factor (G-CSF) (37). Taken together with previously reported similarities between IL-6 and G-CSF at both the amino acid sequence (9,26) and gene organization (37) levels, the results of this present study provide further evidence that these molecules may have evolved from a common gene.

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