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Role of disulfide bridges in determining the biological activity of interleukin 3

(peptide synthesis/protein engineering/cysteine/lymphokine/hemopoietic growth factor)

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ABSTRACT **Total chemical synthesis of analog proteins was used to examine the requirement for specific disulfide bridges for the biological activity of interleukin 3 (IL-3), a growth factor that stimulates multiple lineages of hemopoietic cells. Four structural analogs of the mature, 140 amino acid murine IL-3 molecule were synthesized in which specific cysteine residues were replaced by alanines. In a quantitative IL-3 assay, based on [^{3}H]thymidine incorporation into factor-dependent cells, the IL-3 analog with alanines substituted for all four cysteines—i.e., [Ala^{17,79,80,140}]IL-3—had 1/500th as much activity as the molecule synthesized according to the native sequence. The two analogs [Cys^{17,79},Ala^{80,140}]IL-3 and [Cys^{17,140},Ala^{79,80}]IL-3 had similarly low activity, whereas the [Cys^{17,80},Ala^{79,140}]IL-3 analog had 2000-fold higher activity than these three analogs, and 3-fold higher than the molecule with the native sequence. This shows that in IL-3 a single disulfide bridge, between cysteines 17 and 80, is required for biological activity that approximates physiological levels. This disulfide probably stabilizes the tertiary structure of the protein to give a conformation that is optimal for function.**

The growth and differentiation of cells of the hemopoietic lineage are regulated by several glycoprotein growth factors (1). One of these, which has been best characterized in the mouse, is now widely termed interleukin 3 (IL-3) but has also been termed P-cell-stimulating factor, mast cell growth factor, burst-promoting activity, and multi-colony-stimulating factor (2–7). IL-3 stimulates multiple hemopoietic cell types, including pluripotential stem cells, committed progenitors, and some mature cells (1, 7, 8). Murine IL-3 has been purified to homogeneity (4, 9), and cDNA and genomic DNA clones that encode the protein have been isolated (10–12). The protein sequence deduced from the DNA sequences (10, 11) and N-terminal amino acid sequence analysis (9, 13) indicated that mature IL-3 contains 140 amino acid residues. However, a shorter form, lacking the first 6 amino acids, has also been identified (4). Recent experiments with antibodies to synthetic peptide fragments indicate that the IL-3 produced by activated T cells is the full-length, 140-residue form (14). Neither the location of disulfide bridges (if any) nor the three-dimensional structure of the molecule is known.

We have investigated the structural basis for IL-3 function by a total chemical synthesis approach (15). The ability to reproducibly synthesize IL-3 with high activity has allowed us to systematically vary its structure and compare the biological activities of the analogs with those of the molecule with the native sequence. In our previous study we demonstrated that the cysteine at position 17 was essential for detectable activity. A fragment corresponding to residues 18–140, and therefore lacking this cysteine, had barely detectable activity, whereas a fragment (residues 17–140) that included the cysteine at position 17 had high activity. The most likely explanation of this result was that Cys-17 is involved in a critical disulfide bridge, suggesting a role for disulfides in forming or maintaining the active structure (15).

Here we further analyze the role of disulfide bridges in determining the biological activity of IL-3. By constructing an analog with alanines substituted for all four cysteines, we have found that cysteines are not absolutely required for activity. However, an analog with a disulfide bridge between residues 17 and 80 (but not analogs with alternative disulfide bridges) had 2000-fold greater activity, suggesting that this disulfide bond stabilized a functionally favorable conformation of the molecule.

MATERIALS AND METHODS

Chemical Synthesis. IL-3 and IL-3 analogs (Fig. 1) were synthesized by improved chemical methods (16–18) on a fully automated peptide synthesizer (model 430A, Applied Biosystems, Foster City, CA) (19) as described (15, 17, 18). Fully protected peptide-resins were stored in dimethylformamide in liquid nitrogen, and aliquots (750 mg) were deprotected as follows. The imidazole dinitrophenyl groups were removed from histidine residues by treatment with 2-mercaptoethanol (20%) and diisopropylethylamine (5%) in dimethylformamide; then the N^{α}-*t*-butoxycarbonyl group was removed by treatment with trifluoroacetic acid (65%) in dichloromethane (18); all remaining protecting groups were removed and the peptide was released from the resin by treatment with anhydrous hydrogen fluoride according to the "low–high" procedure (21). Peptides were precipitated and extracted with ethyl acetate, dissolved in 5% acetic acid, lyophilized, and stored at −20°C. Aliquots (100 mg) of the crude lyophilized product were dissolved in 6 M guanidine hydrochloride/0.1 M Tris acetate, pH 8.5/20% 2-mercaptoethanol (4 ml) and stirred for 2 hr at 37°C. The synthetic product was exchanged into 0.1 M acetic acid on Sephadex G-25 and added to 1000 ml of 1 M guanidine hydrochloride/Tris acetate, pH 8.5, and stirred vigorously for 16 hr at room temperature in an open beaker. After addition of 10 ml of acetic acid, the mixture was concentrated by filtration (Amicon YM5 membrane) in a stirred cell (Amicon), dialyzed against 0.1 M acetic acid, and then filtered through a 0.2-μm filter (Nalgene) and lyophilized. For bioassay a sample was dissolved in 0.1 M acetic acid (1 mg/ml) and the concentration of peptide was established by quantitative amino acid analysis.

Sulfhydryl Assay. Aliquots (200 μg) were analyzed for free thiol groups by the Ellman method (22). Dithiothreitol was used as a standard.

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Abbreviation: IL-3, interleukin 3.

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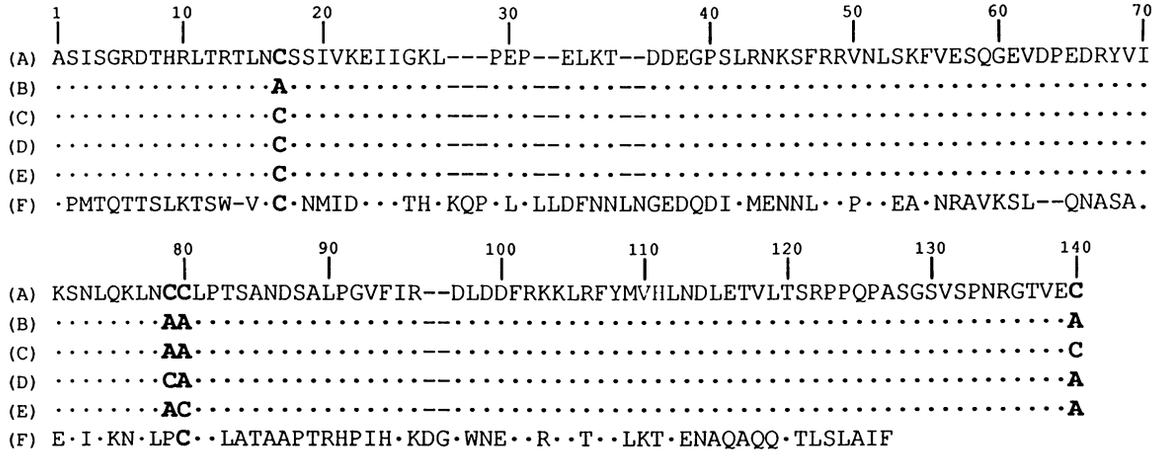

FIG. 1. Sequences of the murine IL-3 analogs synthesized and of human IL-3. The sequence of murine IL-3 (*A*) was determined by translation of the nucleotide sequences of IL-3 cDNAs and by amino acid sequence analysis of the purified native protein (9–11, 13). The analogs synthesized were [$Ala^{17,79,80,140}$]IL-3 (*B*), [$Cys^{17,140}$,$Ala^{79,80}$]IL-3 (*C*), [$Cys^{17,79}$,$Ala^{80,140}$]IL-3 (*D*), and [$Cys^{17,80}$,$Ala^{79,140}$]IL-3 (*E*). The human IL-3 sequence (*F*) was deduced by translation of a human IL-3 cDNA clone (20). Alignment of *A* and *F* was done as described (20). Amino acids are numbered according to the mouse IL-3 sequence without counting spaces necessary for alignment with the human sequence. Standard one-letter amino acid symbols are shown.

Tryptic Peptide Mapping. Tryptic digests were prepared and chromatographed by reverse-phase high-performance liquid chromatography (HPLC) on a C_{18} column (Altex) as described (15). Amino acid analysis was carried out on samples hydrolyzed in 6 M HCl vapor at 110°C for 6 hr and derivatized with dimethylaminoazobenzenesulfonyl chloride and analyzed as described (23). Peptide sequencing was carried out on a gas-phase sequenator (24) and the phenylthiohydantoins of the amino acids were analyzed by reverse-phase HPLC (25).

NaDodSO$_4$/PAGE. NaDodSO$_4$/PAGE was carried out in 12.5–20% acrylamide gels with the Laemmli buffer system (26). Standards (Bio-Rad) were phoshorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa), and α-lactalbumin (14 kDa).

Reverse-Phase HPLC. All the analogs were partially purified by reverse-phase HPLC on a Vydac C_4 column (4.6 × 250 mm) by elution with a 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid at 1 ml/min. Fractions (0.5 ml) were tested for IL-3 activity, and for purity by isoelectric focusing. In each case, fractions containing the highest activity gave a predominant band with an approximate pI of 8.5.

IL-3 Assays. IL-3 assays were performed by measuring the [^{3}H]thymidine incorporation of the IL-3-dependent mast cell line W/W.12 as described (9).

RESULTS AND DISCUSSION

Design of Analogs. Our strategy for establishing the role of disulfide bridges in determining the biological activity of IL-3 was to chemically synthesize defined structural analogs of the protein in which nonsulfhydryl-containing amino acids were substituted for cysteines and to correlate the structural changes with effects on biological activity. Two naturally occurring amino acids, serine and alanine, might be considered suitable replacements for cysteine. We chose alanine as the replacement amino acid because this represents a minimum structural change with deletion of only the sulfhydryl functionality. IL-3 contains four cysteines, at positions 17, 79, 80, and 140 (Fig. 1). We designed analogs in which alanines were substituted for one pair of cysteine residues, thus generating molecules with only two cysteine residues. With these analogs only two possible monomeric forms can exist: a polypeptide with a single disulfide bridge or a form with no disulfide bridges. An analog with alanines substituted for all four naturally occurring cysteines was used as a basis for comparison of the effects of forming the various combinations of single disulfides.

Synthesis and Characterization. The analogs were synthesized as complete 140-residue polypeptide chains by stepwise solid-phase peptide synthesis methods (27). Some analogs, for example [$Cys^{17,140}$,$Ala^{79,80}$]IL-3, were made by a divergent synthesis protocol in which about two-thirds of the peptide-resin was taken after the addition of residue 81 and stored for future assembly of analogs that share the C-terminal 60 amino acid residues. Storage in dimethylformamide in liquid nitrogen or at −80°C was found to prevent the artifactual termination of chain growth that we observed when dried peptide-resins were stored at 4°C.

The synthesizer was programmed to automatically take samples after each cycle of the chain assembly, up to the addition of residue 81 (59 cycles), for analysis of unreacted α-amino groups by the quantitative ninhydrin procedure (28). For both the [Cys^{140}]IL-3-(81–140) and [Ala^{140}]IL-3-(81–140) analogs the results showed reproducible average stepwise yields of 99.5% per residue. Samples were not taken for cycles after residue 81 because the progressive reduction in the concentration of growing peptide chains (mmol/g of peptide-resin), and the rise in background, made the results less reliable. When each 140-residue protected-peptide chain had been assembled, samples were deprotected and cleaved from the solid support by using the improved "low–high" hydrogen fluoride strategy (21) and further characterized. All the analog syntheses were performed at least twice with similar results.

The synthetic polypeptides were each run on NaDodSO$_4$/PAGE under nonreducing and reducing conditions. As shown in Fig. 2 the reduced synthetic products each had an apparent molecular mass of 16 kDa, which was consistent with the theoretical molecular weights of between 15,636 and 15,734. Under nonreducing conditions none of the unpurified, folded peptide products contained high molecular weight material, showing that polymerization had not occurred during the synthesis or folding process. After the disulfide bridges were formed by mild oxidation, each synthetic IL-3 analog was analyzed for free sulfhydryl content by using the Ellman reagent (22). None of the folded, oxidized analogs had detectable (<0.05 mol/mol of protein) free sulfhydryl groups, although after reduction and removal of

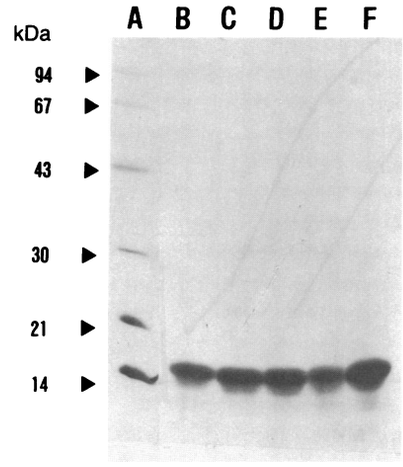

FIG. 2. NaDodSO$_4$/PAGE analysis of IL-3 analogs. Lanes: A, molecular size standards; B, [Ala^{17,79,80,140}]IL-3; C, [Cys^{17,140}, Ala^{79,80}]IL-3; D, [Cys^{17,79},Ala^{80,140}]IL-3; E, [Cys^{17,80},Ala^{79,140}]IL-3; F, [Cys^{17,79,80,140}]IL-3. Standards and electrophoresis conditions are detailed in *Materials and Methods*.

the free reducing agent, the IL-3 analogs containing cysteines had readily detectable sulfhydryl groups in the expected amounts. The [Ala^{17,79,80,140}]IL-3 analog did not give a positive reaction with the Ellman reagent under these conditions. These results, combined with the NaDodSO$_4$/PAGE data showing lack of intermolecular crosslinking, demonstrated that the appropriate intramolecular disulfide bridges were quantitatively formed.

The primary structures of the IL-3 analogs were compared by analyzing their tryptic peptide maps (Fig. 3). The reduced tryptic peptide HPLC profiles were similar, as would be expected for polypeptides with closely related primary structures. Previous peptide mapping of synthetic IL-3 (15) allowed the identification of tryptic fragments containing residues 17, 79, 80, and 140. For each analog, the tryptic peptides containing either residue 17 or 140 were collected and subjected to amino acid analysis to confirm the cysteine/alanine content. In the case of tryptic peptides covering residues 77–96 (peaks 3 in Fig. 3) and thus containing residues 79 and 80, amino acid sequence analysis was carried out. The results confirmed that the synthetic analogs had alanines located in the expected peptide fragments or, in the case of peptide 77–96, in the expected positions. Taken together these results confirmed that the synthetic products had the expected physiochemical and structural properties.

Activity of Synthetic Analogs. Before analysis for biological activity, each IL-3 analog was purified by reverse-phase HPLC. The purified analogs were analyzed for their ability to stimulate [^{3}H]thymidine incorporation in an IL-3-dependent cell line. As shown in Fig. 4 the [Cys^{17,80},Ala^{79,140}]IL-3 analog had the greatest activity in the IL-3 assay. A 50% maximal response occurred at a concentration (EC_{50}) of 4.3 $\times$ 10^{-11} M. Thus, this analog was about 2000 times more active than the [Ala^{17,79,80,140}]IL-3 analog (EC_{50} = 7.9 $\times$ 10^{-8} M). Moreover, the two analogs with alanines substituted at alternative positions—i.e., [Cys^{17,79},Ala^{80,140}]IL-3 and [Cys^{17,140},Ala^{79,80}]IL-3—had activities (EC_{50} 9.8 $\times$ 10^{-8} M and 6.3 $\times$ 10^{-8} M, respectively) that were similar to that of the analog with all four cysteines converted to alanines—i.e., [Ala^{17,79,80,140}]IL-3. In the same assay, purified, *Escherichia coli*-derived recombinant IL-3 (a gift of Biogen Research, Cambridge, MA) was about 3-fold more active (EC_{50} = 1.4 $\times$ 10^{-11} M) than [Cys^{17,80},Ala^{79,140}]IL-3. The difference could be due to residual impurities in the HPLC-purified synthetic product, since isoelectric focusing experiments indicated that, in addition to the major band that was apparent, minor silver-staining bands were also present.

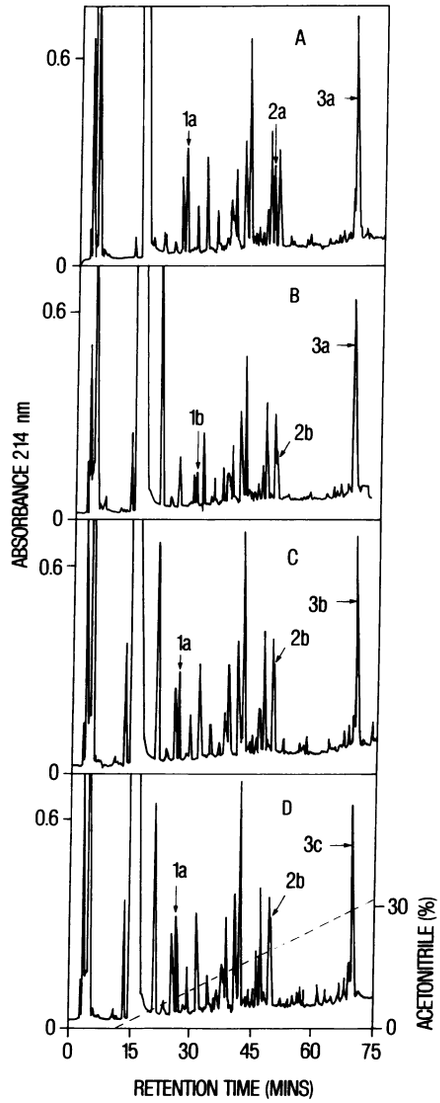

FIG. 3. Tryptic peptide maps of the IL-3 analogs. Tryptic digests of the folded unpurified IL-3 analogs were reduced with 0.1 M dithiothreitol and subjected to reverse-phase HPLC. (*A*) [Ala^{17,79,80,140}]IL-3. (*B*) [Cys^{17,140},Ala^{79,80}]IL-3. (*C*) [Cys^{17,79},-Ala^{80,140}]IL-3. (*D*) [Cys^{17,80},Ala^{79,140}]IL-3. Peaks containing cysteines or alanine replacements, labeled 1, 2, and 3, were identified by comparison with peptide maps of synthetic IL-3 (15) and the compositions determined by amino acid analysis (peaks 1 and 2) and peptide sequence analysis (peaks 3). Peaks are as follows: 1a, [Ala^{140}]IL-3-(136–140); 1b, [Cys^{140}]IL-3-(136–140); 2a, [Ala^{17}]IL-3-(14–22); 2b, [Cys^{17}]IL-3-(14–22); 3a, [Ala^{79,80}]IL-3-(77–96); 3b, [Cys^{79},Ala^{80}]IL-3-(77–96); and 3c, [Ala^{79},Cys^{80}]IL-3-(77–96).

Activity of the IL-3 analog without cysteines was somewhat surprising in view of our previous experiments showing that a fragment that lacked Cys-17 did not have activity (15). It is possible that this IL-3-(18–140) fragment, which contains three cysteines, forms disulfide bridges between cysteines 79 and 140 or 80 and 140 and that this prevents the active tertiary structure from being generated. Nevertheless, the more than 2000-fold greater activity of the [Cys^{17,80},Ala^{79,140}]IL-3 analog supports our contention (15) that a disulfide involving Cys-17 is required for full biological activity. The data further suggest that Cys-17 and Cys-80 are paired in a disulfide bridge in native murine IL-3.

The analog with the "correct" cysteines paired (17 and 80) was actually 3-fold more active than the synthetic molecule

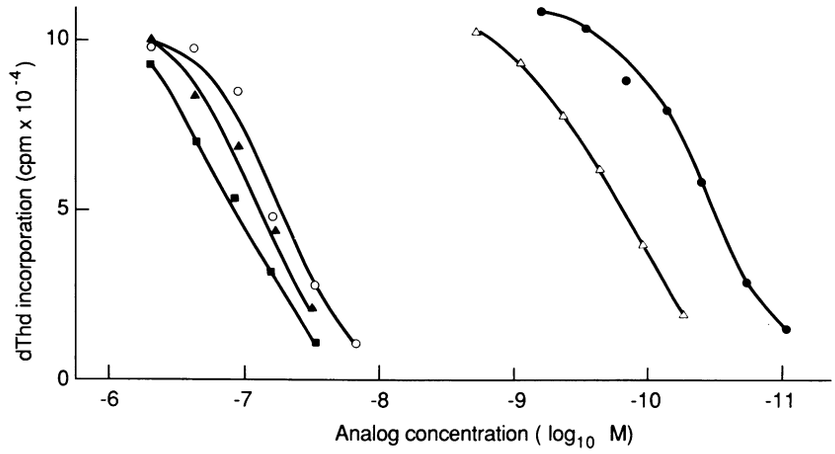

FIG. 4. Biological activity of HPLC-purified synthetic IL-3 analogs. Shown are titrations of [$Cys^{17,79}$,$Ala^{80,140}$]IL-3 (■), [$Cys^{17,140}$,$Ala^{79,80}$]-IL-3 (○), [$Cys^{17,80}$,$Ala^{79,140}$]IL-3 (●), [$Ala^{17,79,80,140}$]IL-3 (▲), and [$Cys^{17,79,80,140}$]IL-3 (△). Protein concentrations were established by measuring the absorbance at 214 nm (1 mg/ml = 15 absorbance units).

corresponding to the natural sequence (EC_{50} 4.3×10^{-11} M and 1.5×10^{-10} M, respectively). This probably reflects the fact that the IL-3 molecule contains four cysteines and it would be expected that some "incorrectly" folded molecules, stabilized by mispaired disulfides, would form during the folding/cysteine-oxidation process. These incorrectly folded products were probably not completely resolved during the HPLC purification and from the data presented (Fig. 4) would be predicted to have substantially lower activity.

In our earlier study (15) we showed that the disulfide-bridged form of the synthetic fragment IL-3-(1–79) had low but detectable activity ($EC_{50} = 3 \times 10^{-5}$ M). In view of the high activity of the full-length analog in which Cys-17 is paired with Cys-80, it seemed possible that a fragment containing Cys-17 and Cys-80 might have greater activity. However, this was not the case, in that both IL-3-(1–79) and [Ala^{79}]IL-3-(1–80) had similar EC_{50} values of about 3.3×10^{-5} M. The fact that Cys-79 is replaced by a proline in the human sequence suggested that proline may be a more optimal replacement in the human fragment. This was indeed found to be the case, as [Pro^{79}]IL-3-(1–80) had a significantly lower EC_{50} (7.6×10^{-6} M) than [Ala^{79}]IL-3-(1–80).

Conclusions. The results described here represent strong evidence that Cys-17 and Cys-80 in murine IL-3 form a disulfide bridge and are crucial for the biological activity of this lymphokine, whereas Cys-79 and Cys-140 are not required for activity. However, the results do not rule out the possible existence of a second disulfide, between the cysteines at positions 79 and 140, in native mouse IL-3. Interestingly, there are only two cysteine residues present in the human IL-3 molecule, and these coincided with cysteines 17 and 80 in the mouse IL-3 molecule when the two sequences were computer-aligned (20). This suggests that during evolution there was no selective pressure to retain the cysteines at positions 79 and 140. That a disulfide bridge between Cys-17 and Cys-79 does not yield a fully active murine molecule and that Cys-79 is replaced by a proline in the human molecule suggest the spatial orientation of the 17–80 disulfide is critical for correct folding. Our findings support the contention that, despite the relatively weak homology (29% amino acid identity), the gene for human IL-3 molecule isolated by Yang *et al.* (20) is indeed a homolog of the murine molecule, not only at the level of primary sequence but also at the level of the three-dimensional structure as determined by the disulfide bridge between residues 17 and 80.

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