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Chemical synthesis in protein engineering: total synthesis, purification and covalent structural characterization of a mitogenic protein, human transforming growth factor-alpha

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Successful approaches to protein engineering required that the desired analogs be easily and rapidly obtained in sufficient quantities and purities for unambiguous structural and functional characterizations. Chemical synthesis is the method of choice for engineering small peptides. We now demonstrate that with improved methodologies and instrumentation, total chemical synthesis can be used to produce a small protein in a form suitable for engineering studies. Active human transforming growth factor-alpha (TGF-α), a 50 amino acid long protein with three disulfide bonds, has been synthesized and purified in multiple tens of mg amounts in <7 days. The purified human TGF-α migrated as a single band on SDS–polyacrylamide gels, ran as a single sharp major band at p*I* = 6.2 on isoelectric focusing gels, displayed an MW = 5546.2 (Th.5546.3) by mass spectrometry, contained three disulfide bonds and had EGF receptor binding, mitogenic and soft agar colony formation activities. The locations of disulfide bonds were found to be analogous to those found in epidermal growth factor (EGF) and in human TGF-α expressed in bacteria.

Key words: Automated solid phase protein synthesis/protein folding/human transforming growth factor-α/protein sequencing/plasma-desorption mass spectrometry

Introduction

Human transforming growth factor-alpha (TGF-α) (Marquardt *et al.*, 1983; Derynck *et al.*, 1984) is a member of a superfamily of proteins containing characteristic domains defined by six uniquely spaced cysteines in their amino acid sequences. Identified members of this family include other growth factors such as murine and human epidermal growth factor (EGF) (Gray *et al.*, 1983; Scott *et al.*, 1983; Lee *et al.*, 1985), vaccinia virus growth factor (Stoobant *et al.*, 1985), pox virus growth factor (Porter and Archard, 1987), Shope fibroma growth factor (Chang *et al.*, 1987), amphiregulin (Shoyab *et al.*, 1989), mouse and rat TGF-αs (Lee *et al.*, 1985; Todaro *et al.*, 1985), non-growth factors such as LDL receptor (Russell *et al.*, 1984), EGF precursor protein (Gray *et al.*, 1983; Doolittle *et al.*, 1984), protein C (Foster and Davie, 1984), human proteoglycan core protein (Krusius *et al.*, 1987), human and bovine blood coagulation factors IX and X (Patthy, 1985), urokinase (Gunzler *et al.*, 1982), tissue-type plasminogen activator (Ny *et al.*, 1984), cytotactin (Jones *et al.*, 1988) and protein products of developmentally regulated genes such as *Drosophila*'s Notch gene (Wharton *et al.*, 1985) and *Caenorhabditis elegans*' lin-12 gene (Greenwald, 1985), product of the cell lineage-specific gene of sea urchin (Hursh *et al.*, 1987) and the product of the Pfs gene of *Plasmodium falciparum* (Kaslow *et al.*, 1988).

It has been shown that in murine EGF and human TGF-α the six cysteines are all identically paired into three disulfide bonds, suggesting a common folding motif that has been evolutionally conserved (Savage *et al.*, 1973; Winkler *et al.*, 1986). Whether this disulfide bonding motif extends to other members of this family remains to be seen. A systematic structure–activity study of TGF-α in relation to other growth factors in this family may reveal fundamental principles of protein folding and activities in relation to the primary amino acid sequence.

All mitogenic growth factors in this family can elicit EGF-mediated responses by binding to and activating the cell surface receptor for EGF (Todaro *et al.*, 1976; Reynolds *et al.*, 1981; Pike *et al.*, 1982; Massague, 1983a,b; Tam *et al.*, 1984; Tam, 1985; Smith *et al.*, 1985; Stoobant *et al.*, 1985). Interestingly, TGF-α has arterial blood flow enhancement (Derynck, 1986), bone resorption (Ibbotson *et al.*, 1985; Stern *et al.*, 1985), angiogenic (Schreiber *et al.*, 1986) and keratinocyte proliferation activities (Barrandon and Green, 1987), not attainable by any concentration of EGF, indicating that TGF-αs and EGFs may not be simple agonists of each other.

We report here the successful integrated use of new methodologies and instrumentation for the total chemical synthesis, folding, purification, and physical, chemical and biological characterizations of human TGF-α as a homogeneous synthetic protein. Our results demonstrated that automated chemical synthesis is now an efficient and practical alternative to biological expression in producing molecules for protein engineering studies.

Materials and methods

Materials

Protected amino acid derivatives were purchased in bulk from Protein Research Foundation, Osaka, Japan, and were weighed into bar-coded cartridges before use. Boc-L-His (DNP) was from Fluka AG (Product no. 15389). Loaded Boc-aminoacyl-OCH_2-Pam-resins (1.0 mmol/g S-DVB), and reagents for quantitative ninhydrin monitoring were from Applied Biosystems, Inc., Foster City, CA 94404. The identity and purity of all amino acids was checked by deprotection and amino acid analysis before use. Dimethylformamide (DMF), diisopropylethylamine (DIEA) and dichloromethane (DEM) were from Applied Biosystems and had been tested by use in peptide synthesis. Trifluoroacetic acid (TFA) was from Pierce, Rockford, IL, and was glass redistilled before use. Methanol was Baker HPLC grade. Dicyclohexylcarbodiimide (DCC), *N*-hydroxybenzotriazole (HOBE), dimethylsulfide, *p*-cresol and thiocresol were AR grade from Aldrich. Diethyl ether was AR grade, low peroxide content from Baker. Tissue culture media were from Gibco, Grand Island, NY. Isotopes were obtained from New England Nuclear, Boston, MA. Other chemicals were of the highest grade available.

Automated chain assembly was performed on a standard

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A. Protected peptide chain assembly

Boc-L-Ala-OCH_2-Pan-copoly(styrene-divinylbenzene)−resin

↓ stepwise solid phase sythesis

(protected) TGF-α-OCH_2-Pam-resin

B. Deprotection

↓ a. Thiolysis
b. Modified HF acidolysis
c. Thiolysis

Crude, reduced TGF-α

C. Fold/form disulfide bonds

↓ Air oxidation, pH 8.5

Crude TGF-α

D. Purify

↓ Reverse-phase HPLC

TGF-α

E. Proof of structure

(i) Homogeneity: orthogonal analytical methods.
(ii) Covalent structure: sequence; molecular mass; disulfides.

Scheme I. Strategy of total chemical synthesis of human TGF-α.

Applied Biosystems 430A peptide/protein synthesizer, except that the in-line filters in the lines to the top and bottom of the reaction vessel were removed to provide a more reproducible flow. The hydrogen fluoride (HF) cleavage apparatus was from Peninsula Laboratories, Belmont, CA.

Peptide synthesis

The protected peptide chain corresponding to the human TGF-α sequence was assembled using the stepwise solid phase approach (Merrifield, 1963) and improved methods of chemical synthesis (Scheme 1). *N*-α-Boc-Amino acids with benzyl-based side chain protecting groups (D,E:OBzl; S,T:Bzl; K:ClZ; Y:BrZ; C:4MeBzl) together with His(ImDNP) and Arg(N-G-tosyl) were used in solid phase synthesis to assemble the protected resin-bound peptide by addition of each amino acid in a stepwise fashion from the C-terminal.

Target sequence. The 50 amino acid residue sequence of human TGF-α deduced from the cDNA nucleotide sequence is shown in Figure 1. From a synthetic point of view, the most significant features are the chain length, the presence of six cysteine residues and the absence of tryptophan and methionine. In addition, histidine residues can lead to artifactual chain assembly data, and it can sometimes be difficult to completely remove the His(ImDNP) side chain protecting group (see below). Finally, there are no Asp−Pro or Asp−Gly bonds, nor any other sequences expected to cause side reactions in the chain assembly, in the final removal of protecting groups or in the subsequent work-up.

Chain assembly. TGF-α was assembled from the C- to N-terminal on a 0.5 mmol scale using a cross-linked polystyrene support containing the C-terminal residue linked to the resin through a 4-(carboxyamidomethyl)benzyl ester linkage (the so-called 'Pam' resin). This peptide−resin bond is 100 times more stable to the conditions of removal of the *N*-α-Boc groups during chain

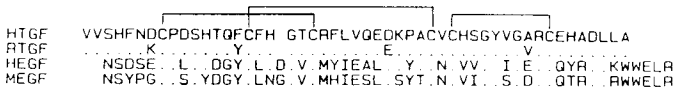

Fig. 1. Amino acid sequences of TGF-αs and EGFs.

assembly than the standard benzyl ester resin and was prepared in a form free of extraneous resin-bound functional groups (Mitchell *et al.*, 1978). The chain assembly was carried out in a fully automated fashion on an instrument specifically designed to use protocols that incorporate cumulative improvements in all major aspects of assembly of protected peptides by stepwise solid phase synthesis. The rationale for the design of the peptide/protein synthesizer has been reported (Kent *et al.*, 1985a,b) and the improved chemistry summarized (Kent and Clark-Lewis, 1985; Kent, 1988).

A highly optimized set of synthetic cycles, developed in our laboratory and used for the complete automated assembly of interleukin-3 (Clark-Lewis *et al.*, 1986) and numerous other peptides, was employed. These cycles differ in a number of important respects, both from the chemistry previously used in stepwise solid phase peptide synthesis and from standard protocols supplied with the commercially available peptide synthesizer (Clark-Lewis *et al.*, 1986). Removal of the *N*-α-Boc group at each cycle was achieved by two treatments (20 ml each) with 65% v/v trifluoroacetic acid (TFA) in dichloromethane for 1 and 14 min. Both the volume and time were critical to ensure complete deprotection. Neutralization (2 × 1 min, 10% v/v DIEA) was carried out in DMF to ensure maximal solvation of the neutral peptide−resin. Residual base was removed by thorough DMF washes (2 ml × 1 min × 2). A routine double coupling protocol was used. Both couplings used 2 mmol of Boc-amino acid. The first coupling used the preformed symmetric anhydride of the Boc-amino acid in DMF to avoid sequence-dependent coupling difficulties due to poor solvation of the peptide (Kent and Merrifield, 1980; Sarin *et al.*, 1980). The resin used had ~1.0 mmol peptide/g S-DVB (~1 phenyl ring in 10 carrying a peptide chain). Because of this, solvent volumes can be kept to a minimum while resulting in an ~0.15 M concentration of activated amino acid. This concentration is ~3- to 4-fold higher than typical protocols. In this way high yields (>99%) were routinely obtained with coupling times of only 15 min.

After a second neutralization step in dichloromethane, the second coupling employs *in situ* activation in dichloromethane in which the protected amino acid and DCC (2 mmol each) were added directly to the peptide−resin in dichloromethane to a total volume of ~10 ml. After 10 min the solution was brought to 50% v/v in DMF for the final 15 min of coupling. From previous experience this second coupling improves the yield by an average of 0.3% per residue. The activation/solvent exchange and coupling chemistry was optimized for each residue (Kent *et al.*, 1985a,b). Asn and Gln were double-coupled as the HOBt esters in DMF, formed as described (Kent and Clark-Lewis, 1985), for extended reaction times (30 min each coupling) to compensate for the slower kinetics compared with DCC activation. Arg(N-G-tosyl) was routinely double-coupled, first as the HOBt ester in DMF then by *in situ* activation in DCM (as above).

Quantitative ninhydrin assay. Resin samples (~10 mg, 2 μmol each) were automatically taken at the end of each amino acid addition cycle. The efficiency of the coupling reactions were

determined using the quantitative ninhydrin reaction for the colorimetric determination of unreacted alpha-amino groups (Sarin *et al.*, 1981). A modified resin sampling protocol was developed to avoid artifactually high ninhydrin values. We found that on prolonged standing (hours or more) His-containing peptide–resin samples gave rise to slowly increasing values for residual free amino groups. This was overcome by the addition of a small amount of DIEA (10% of final volume) to the test tube into which the peptide–resin sample was deposited in DMF. In this solvent the peptide–resin sample sank and was thus kept in basic solution. Provided resin samples were not allowed to stand for excessive amounts of time (days), this protocol minimized the problem, giving typically an order of magnitude reduction in the artifact. Background values, due to random chain scission at peptide bonds and deamidation of side chain amides under the conditions of the assay, were determined at the residues coupled to proline, which does not release a soluble chromophore from the peptide–resin during the ninhydrin reaction.

Quantitative Edman degradation. Quantitative Edman degradation of the protected peptide–resin was used to assess the accuracy and overall efficiency of chain assembly (Clark-Lewis and Kent, 1989). The expected amino acid phenylthiohydatoin (PTH) derivative was monitored at each cycle of the degradation to confirm that the desired target sequence of amino acids had been synthesized. In addition, the quantitation of PTH amino acids in successive cycles of the Edman degradation, after correction for background due to random chain scission during the sequencing experiment, is a quantitative measure of overall yield of the target protected peptide chain.

Deprotection and cleavage. The protected peptide–resin (600 mg) was subjected to thiolysis (10% v/v thiophenol in DMF, 2 × 1 h) to remove the ImDNP groups (Shaltiel and Fridkin, 1970). The *N*-α-Boc group was then removed to prevent side reactions in subsequent steps and the peptide–resin was neutralized, washed and air-dried. Cleavage from the resin and simultaneous deprotection of the side chains was carried out by the low–high HF method at 0°C (Tam *et al.*, 1983).

After removal of the HF the peptide was precipitated and washed with ethyl acetate, then dissolved away from the resin in dilute (10% v/v) acetic acid and lyophilized.

Folding and oxidation

The crude peptide (100 mg) was treated with 10 ml of 2 M 2-mercaptoethanol in 6 M guanidine–HCl, 50 mM Tris acetate at pH 8.5 and 37°C for 2 h to remove any residual DNP groups. The excess reagents and the DNP released were separated from the peptides by gel-filtration chromatography on Sephadex G-25 equilibrated with 1 M guanidine–HCl in 50 mM Tris acetate, pH 8.5 (standard folding buffer). The reduced and deprotected peptide was then diluted to 100 μg/ml with degassed standard folding buffer. Folding was carried out for 24 h at 25°C. The oxidation of free thiol into disulfide during folding was mediated by atmospheric oxygen introduced into the buffer by rapidly stirring the sample in a 2 l flask with a magnetic stirrer such that a smooth vortex almost reaching the bottom of the flask was formed. No visible precipitation occurred during this process. At the end of the folding period the pH of the sample was slowly adjusted to 4.0 with glacial acetic acid to prevent further thiol–disulfide exchange. About 40% of the peptide precipitated out of the solution during this procedure. This precipitate was removed by centrifugation and saved for reduction and further folding. The soluble, folded products were concentrated into 20 ml under nitrogen on YM-2 membranes using an Amicon ultrafiltration apparatus. The concentrated folded peptide was then dialyzed extensively against 1 M acetic acid at 4°C (four changes of 5 l at 8 h intervals). Another 5 mg of peptides were precipitated during guanidine–HCl removal by dialysis, and were separated by centrifugation.

Purification

The soluble, folded peptides were purified by reverse-phase HPLC on a 10 × 250 mm Vydac C-4 column. The gradient used was 0–60% acetonitrile in 0.1% TFA over 4 h at a flow rate of 3.0 ml/min. Six-milliliter fractions were collected in tared tubes. Fractions containing the product in pure form were identified by screening with analytical C-4 reverse-phase HPLC. Solvents were removed by lyophilization in a Savant Speed-Vac concentrator. Dried samples were redissolved in 0.5% acetic acid to 1 mM peptide. TGF-α activities were monitored by EGF receptor binding assay. Active fractions corresponding to a sharp early eluting peak were combined and lyophilized.

Chemical characterization

Homogeneity. The HPLC-purified product was re-analyzed by reverse-phase HPLC under conditions similar to those used for the purification. The composition of the purified product was also determined by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and by isoelectric focusing in an immobilized pH(4–10) gradient polyacrylamide gel. The synthetic protein and impurities were detected after electroblotting onto nitrocellulose.

Mass spectrometry. The mass spectrometric measurements of synthetic TGF-α were performed on the ^{252}Cf plasma desorption time-of-flight mass spectrometer constructed at the Rockefeller University and described previously (Chait *et al.*, 1981; Chait, 1987). This instrument has a mass accuracy of ±0.5 amu at *m/z* 5500 under the conditions of the experiment. One nanomole of the folded, purified synthetic material was adsorbed on a thin film of nitrocellulose from a solution of 0.2 nmol protein/μl of 0.1% TFA (Chait and Field, 1986). After adsorption the nitrocellulose film was washed thoroughly with 0.1% TFA and placed into a vacuum lock of the mass spectrometer to be dried by evacuation. The resulting layer of protein molecules bound to the nitrocellulose film was then inserted into the mass spectrometer whereupon a spectrum was accumulated to determine the molecular weight of the synthetic product. The sample film was then removed from the mass spectrometer and 10 μl of 0.08 M dithiothreitol (pH 8.5) added to the surface and allowed to react for 9 min at 25°C. The reaction was terminated by vacuum evaporation of the reagent and a spectrum of the reduced protein was then obtained.

Peptide mapping. The folded/oxidized purified product was digested with thermolysin under non-reducing conditions. Digests were analyzed by reverse-phase HPLC (Vydac, C-18; buffer A, 0.1% TFA in H_2O; buffer B, 0.1% TFA in 60% CH_3CN in H_2O; linear gradient 0–100% B over 60 min; flow rate 0.7 ml/min; detection at 214 nm). All peptide-containing peaks were collected, oxidized with performic acid and re-analyzed by C-18 reverse-phase HPLC. Peaks containing disulfide-linked fragments were identified by amino acid composition and, where necessary, sequence analysis..

Amino acid analysis. Peptide samples (~2–5 nmol) were hydrolyzed in 6 M HCl at 110°C for 24 h and the amino acid composition was determined by reverse-phase HPLC of the

Table I. Synthetic yields of human TGF-α

	Yield	
	weight (mg)	%
Boc-Ala-OCH_2-Pam–resin (0.5 mmol)	658	100
Protected peptide–resin	3280	83
Crude reduced peptide	2044	60
Crude folded TGF-α	410	19
Purified TGF-α	112	4.5[a]

[a]Subsequent folding/oxidation under more optimized conditions (10 μg/ml in 50 mM Hepes, pH 8.5, 4°C, 1 h, air oxidation) gave folding yields of 40–65%, corresponding to an overall yield of 10–12% in a single pass. Recycling the improperly folded TGF-α for repeated folding runs allowed almost complete conversion of all the HPLC-purified, reduced peptide into active TGF-α.

DABS-Cl (dimethylaminoazobenzenesulfonyl chloride) derivatized amino acid (Chang *et al.*, 1981).

Biological assays

EGF receptor binding assay. Murine EGF was purified and iodinated by the chloramin-T method as described (Savage and Cohen, 1972; Hollenberg and Cuatrecases, 1973). The sp. act. was $1.2-1.5 \times 10^6$ c.p.m./pmol. Human EGF receptor was purified to homogeneity from Triton X-100 extracts of human epidermoid carcinoma (A431) cells by affinity chromatography on Fractogel TSK-immobilized ricin-binding subunit, followed by EGF–Fractogel affinity chromatography (Woo, 1984). The purified receptor migrated as a single 160 kd band in SDS–PAGE, bound EGF with a K_d of 65.8 ± 3.5 nM and phosphorylated tyrosine protein kinase substrate peptide with a V_{max} of 430 nmol/mg/min in an EGF-dependent reaction.

The binding of synthetic TGF-α was assayed by competition with [^{125}I]EGF for binding to EGF receptor. Competition by unlabeled EGF was measured as a control. Two microliters of synthetic TGF-α or EGF at ×10 the test concentration were added to 18 μl of a solution containing 17 μg/ml of EGF receptor and 100 nM [^{125}I]EGF in a 50 mM Hepes (pH 8.0) buffer containing 0.1% Triton X-100 (Binding buffer) and vortexed in a 1.5 ml polyprophylene test tube at 25°C. After a 30 min incubation the assay was terminated by adding 0.3 ml bovine IgG (1 mg/ml) and 0.3 of 25% polyethylene glycol (PEG-8000), both in binding buffer, in rapid succession. Each tube was capped and mixed vigourously to precipitate macromolecules. Precipitates were collected by centrifugation in an Eppendorf microfuge at 12 000 *g* for 5 min. Supernatants were aspirated and pellets were washed once by vortex mixing with 0.5 ml of 8% PEG in 50 nM Hepes, pH 8.0, and recovered by a 1 min spin in an Eppendorf microfuge. After removal of the supernatant, pellet-associated [^{125}I]EGF was determined by gamma counting.

Mitogenic assay. NRK fibroblasts were seeded into 96-well microtiter plates in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% calf serum (Gibco) at a density of 8×10^3 cells/well. Cells were incubated at 37°C in a humidified chamber under 5% CO_2 in air. On the following day culture medium was replaced with a 50% DMEM, 50% HAM's F-12 medium containing insulin (2 μg/ml), transferrin (5 μg/ml), fatty acid-free bovine serum albumin (1 mg/ml) and linoleic acid (5 μg/ml). Cells were incubated for three more days before samples of synthetic TGF-α or EGF were added. Twenty hours after mitogen addition, cells were pulse labeled for 4 h at 37°C with 0.1 mCi of [^{3}H]thymidine (sp. act. 20 Ci/mol).

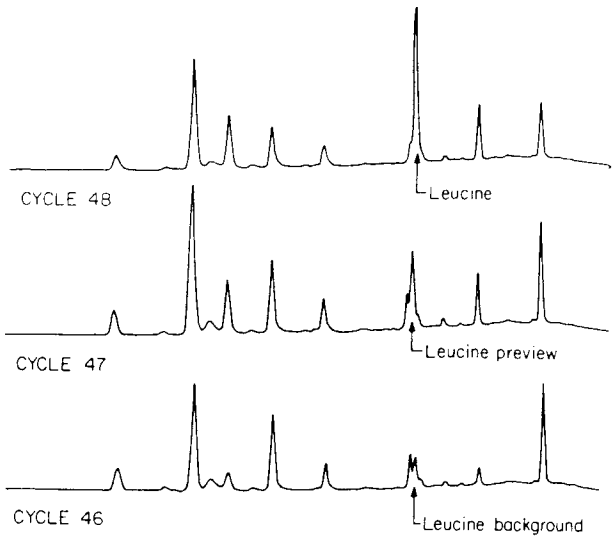

Fig. 2. Quantitative Edman degradation of synthesized human TGF-α. Synthetic human TGF-α was subjected to 49 cycles of Edman degradation starting from the N-terminal using an automatic protein/peptide sequencer. The PTH reaction products from each cycle were analyzed by HPLC. The data from cycles 46–48 are illustrated. The background signal from contaminants eluting at leucine's position is measured in cycle 46 (alanine). The accumulated amount of TGF-α synthesized as N-1 sequences ('deletion' peptides) is estimated by the percent of leucine (residue 48) observed to 'preview' at cycle 47 (aspartic acid). The average synthesis yield per cycle, *x*, over 47 residues can be calculated from the formula

$$x_{47} = 1 - \left(\frac{\text{(leucine preview} - \text{leucine background)}}{\text{(leucine} - \text{leucine background)} + \text{(leucine preview} - \text{leucine background)}} \right)$$

Uncertainties in estimating areas lead to an estimate of Leu48 preview of 16 ± 4% in cycle 47, corresponding to a synthetic yield per cycle of 99.65 ± 0.1%. A more complete discussion of quantitative Edman degradation in the evaluation of synthetic peptides can be found in Kent *et al.* (1981) and Clark-Lewis and Kent (1989).

[^{3}H]Thymidine incorporation was determined by scintillation counting of precipitated radioactivity solubilized with 0.5 M NaOH.

Soft agar colony formation assay. The soft agar colony formation activity of synthetic TGF-α, with EGF as a control, was assayed essentially as described. A single cell suspension of NRK fibroblast were obtained by trypsinization. Cells (2×10^3) were resuspended in 0.5% agar containing DMEM supplemented with 10% calf serum and varying concentrations of synthetic human TGF-α or EGF. Aliquots (0.5 ml) of each test suspension were overlaid, in duplicate, onto 6-well Costar cell culture clusters prefilled with 1.0 ml of 0.5% agar in DMEM and the exact concentration of growth factor being tested. The cells were incubated at 37°C in a moisture-saturated atmosphere of 5% CO_2 in air. Colonies formed were visually scored after 7 days of incubation, using a Nikon inverted microscope. Eight fields at ×10 magnification were scored for each concentration and averaged to obtain the data shown. Colonies containing 50 cells or more were scored as positive.

Results and Discussion

Chemical synthesis of human TGF-α

Chain assembly. Assembly of the protected TGF-α sequence was carried out by machine-assisted synthesis. The chain assembly was completed in only 75 h, without operator intervention, and yielded >3 g of protected peptide, from which >1 g of crude product was obtained (Table I).

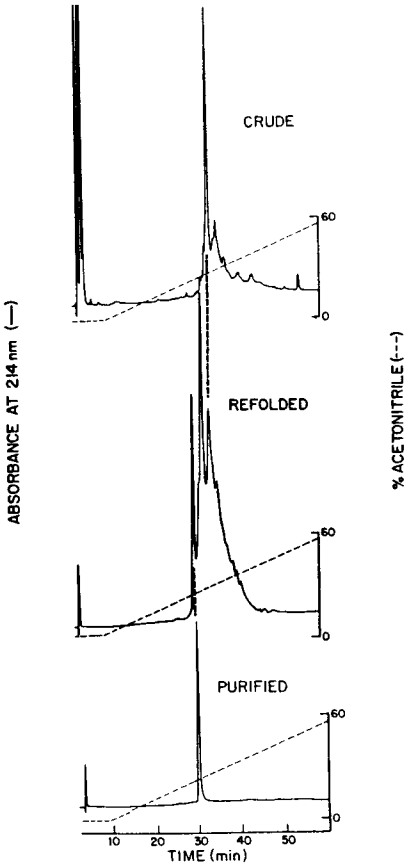

Fig. 3. Analytical HPLC of crude, folded and purified human TGF-α. 10 μg of crude cleaved human TGF-α (**a**), 10 μg of folded, oxidized TGF-α (**b**) or 10 μg of HPLC purified TGF-α (**c**) were analyzed by C-4 reverse-phase HPLC. Peptides were monitored at both 214 nm (shown) and 254 nm (not shown). The elution gradient was 0–60% acetonitrile in 0.1 TFA at a flow rate of 0.7 ml/min over 60 min.

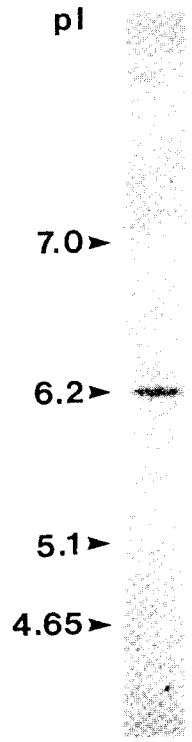

Fig. 4. Isoelectric focusing analysis of HPLC-purified human TGF-α. Samples of 10–100 μg of human TGF-α were applied to a precast, prerun immobiline gel (pH 4–10, without Gelbond™) and focused for 4 h at 3000 V. The resolved samples were blotted onto nitrocellulose filter for 1 h at 4°C and 250 V. Samples were detected by staining the filter with colloidal ink. At all sample loadings, a major band can be seen at p*I* = 6.2. A faint minor band at p*I* = 5.6 (<1% intensity of the major band) can be detected at sample loading of >25 μg/lane. Sample shown is result of loading 50 μg of purified human TGF-α in one lane.

Quantitative nihydrin analysis of resin samples taken at the end of each cycle were performed to document the efficiency of chain assembly. An average coupling yield of 99.6 ± 0.2% per residue was obtained based on background-corrected ninhydrin values. Backgrounds observed after coupling to P (30) and P (9) are 0.5% @ K 29 and 1.1% @ D 8. This calculated coupling yield corresponds to the chain assembly yield, assuming complete removal of the *N*-α-Boc group at each step, and is in good agreement with the corresponding data from quantitative sequence analysis data below.

Quantitative Edman degradation of the protected peptide–resin revealed the expected amino acid PTH derivative at each cycle of the degradation. This confirmed that the desired target sequence of amino acids had been synthesized. A total of 16 ± 4% of residue no. 48 (Leu) was seen in step no. 47 of the degradation, after correction for background due to random chain scission during the sequencing experiment (Figure 2). This represents an overall yield of the target protected peptide chain of 84 ± 4%, with respect to deletions, and corresponds to an average chain assembly yield (including both deprotection and coupling) of 99.65 ± 0.1% per residue.

In other studies of the chain assembly of the TGF-α sequence we have found that the inclusion of the second coupling involving *in situ* activation of the amino acid in the presence of the peptide–resin resulted in an average of 0.3% increased yield per amino acid, leading to an average coupling yield of 99.6 versus 99.3% typically observed with the standard protocol. Thus the routine double coupling protocol resulted in an almost 2-fold reduction in the level of deletion peptides (those missing an internal amino acid) from a calculated 27% to an observed 16% of the crude product.

Deprotection and cleavage. At the end of the chain assembly cycles, a total of 3.28 g (theoretical, 3.96 g) of protected TGF-α-resin was obtained (weight corrected for the samples taken during chain assembly), corresponding to an 83% yield (Table I). After deprotection and cleavage as described in Materials and methods, 374 mg of crude lyophilized product was obtained [81%; theoretical 462 mg, based on calculated MW of 6086 for TGF-α 9(acetate)]. Analytical HPLC of this material on a C-4 reverse-phase column revealed a sharp major peak, presumed to be reduced TGF-α, representing 45% of the total peptide products (Figure 3a).

Folding the polypeptide chain and formation of disulfide bonds. No EGF receptor binding activity was detected in the crude, reduced product as obtained from HP cleavage and deprotection. Several sets of folding/oxidation conditions were compared for their abilities to generate TGF-α activity, by monitoring the specific ability of the folded, crude product to compete with [^{125}I]EGF binding to purified human EGF receptor. The complexity of the folded, crude TGF-α was monitored by C-4 reverse-phase HPLC.

The chosen folding/oxidation conditions described in Materials and methods not only produced the maximal yield of TGF-α activity but also gave rise to a set of products from which the

active species could be rapidly and efficiently purified to homogeneity. The procedure was simple and the yield of EGF receptor-binding activity under those conditions was comparable to more complex folding systems tested. The omission of guanidium chloride from the folding buffer led to the formation of a complex mixture of folding intermediates detected by HPLC. These intermediates displayed specific EGF receptor binding activity ranging from 0 to 100% of native EGF (data not shown). Presumably, the presence of 1 M guanidium chloride helps keep incorrectly and partly folded molecules part of the productive folding pathways to native TGF-α.

An analytical HPLC profile of the crude TGF-α folded under the conditions described above is shown in Figure 3(b). Only the earliest eluting of the four major peaks detected had [^{125}I]EGF competing activity.

Purification. The early-eluting, sharp peak was purified by semi-preparative (10 mm × 25 cm) C-4 reverse-phase HPLC as described in Materials and methods. Figure 3(c) shows the reverse-phase HPLC analysis of this purified TGF-α. The overall synthetic yields are summarized in Table I and ranged from 4.5 to >10%, depending on the exact conditions used.

It is clear that the task of purifying TGF-α from the crude product after folding/oxidation was simplified because the correctly folded protein was the earliest-eluting component and eluted as a single, very sharp peak compared with other components of the mixture. Presumably this is because the correctly folded globular protein has many of the more hydrophobic side chains buried and inaccessible to the surrounding aqueous medium, is stabilized by disulfide bonds and does not undergo large-scale (slow) conformational exchanges typical of loosely folded, random-coil, long polypeptide chains. This represents a potential advantage over the corresponding situation for the purification of synthetic peptides.

Characterization of synthetic TGF-α

Because of the size of the product molecule and because of the very similar physical and chemical properties of closely related polypeptide by-products expected to be found, it was essential to first evaluate the homogeneity of the purified product and to then prove the covalent structure of the synthetic protein. In particular, it was necessary to verify the correct sequence of amino acids, the presence of three disulfide bonds and their locations, and the absence of unexpected chemical modifications and/or residual protecting groups.

Homogeneity. The synthetic protein, assembled, deprotected, folded/oxidized and purified as described above, was re-analyzed by C-4 reverse-phase HPLC under conditions comparable to those used for the preparative HPLC purification. As expected, a single homogeneous peak was observed (Figure 3c).

Immobilized pH gradient isoelectric focusing, which separates proteins based on their intrinsic isoelectric point, was used to establish the homogeneity of the product isolated by reverse-phase HPLC. This technique was high resolving power because the isoelectric point of a protein is affected not only by the number and type of charged functional groups but also by their microscopic environments within the folded polypeptide chain. Purified synthetic TGF-α was focused in an immobilized pH gradient, followed by electrophoretic transfer to nitrocellulose and staining with amido black. This revealed a single major component (>95%) with p*I* = 6.2 (Figure 4) and with barely detectable minor components.

The detection of a major component as a sharply focused band with only trace impurities was good evidence of the high degree

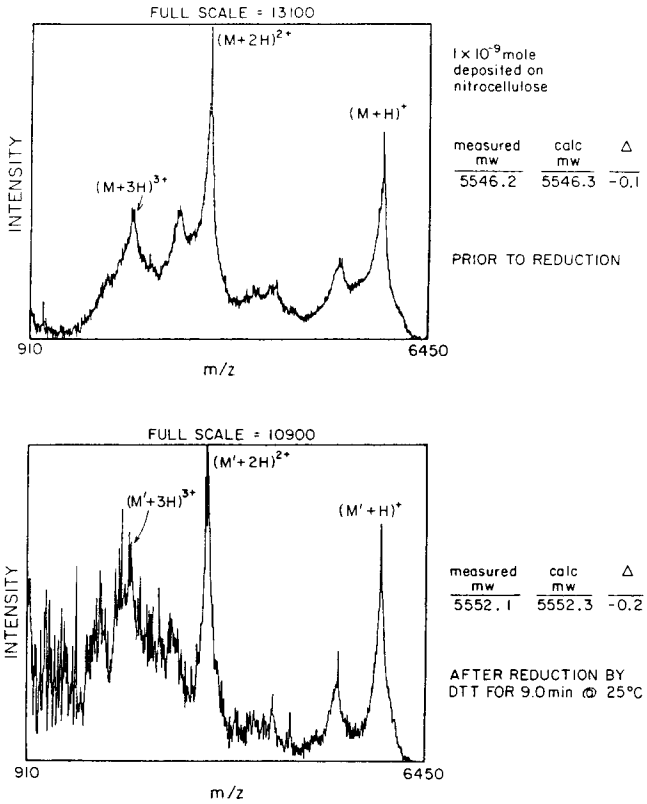

Fig. 5. Mass spectrum of human TGF-α. One nanomole of human TGF-α was deposited onto a nitrocellulose filter and used for mass spectrometry. The mass spectrum of purified, folded human TGF-α is shown in (**A**) and the mass spectrum of the same sample after reduction with dithiothreitol is shown in (**B**).

of purity of the synthetic protein, particularly since the separation mechanism of the analytical method is based on completely different principles than the reverse-phase HPLC method used for the isolation.

Amino acid composition and sequence. The amino acid composition of the synthetic TGF-α was determined after hydrolysis in 6 M HCl. Within an experimental uncertainty of ±5 mol%, the composition agreed with the values predicted from the target sequence (data not shown). The purified synthetic protein was also subjected to amino acid sequence analysis by automated gas-phase Edman degradation. This confirmed the expected amino acid sequence. No anomalous PTH derivatives were observed, and quantitative analysis of the data indicated on overall purity of >95% with respect to deletion peptides (data not shown).

Mass spectrometry. Synthetic TGF-α was analyzed by ^{252}Cf plasma desorption time-of-flight mass spectrometry (Chait and Field, 1986). Figure 5(A) shows the resulting mass spectrum between *m/z* 910 and 6456. Three peaks corresponding to singly, doubly and triply protonated TGF-α were observed. The presence of a well-defined $(M + 3H)^{3+}$ peak and the absence of a $(2M + 3H)^{3+}$ peak confirmed that the molecule was present exclusively as a monomeric species and not as a dimer. The measured molecular weight was 5546.2 [average of the values deduced from the $(M + H)^{+}$ and $(M + H)^{2+}$ ion peaks] versus the calculated molecular weight of 5546.3, consistent with the presence of three disulfides. The combined results of mass spectrometry, homogeneity data and the amino acid sequence analysis above demonstrated that the target protein has been

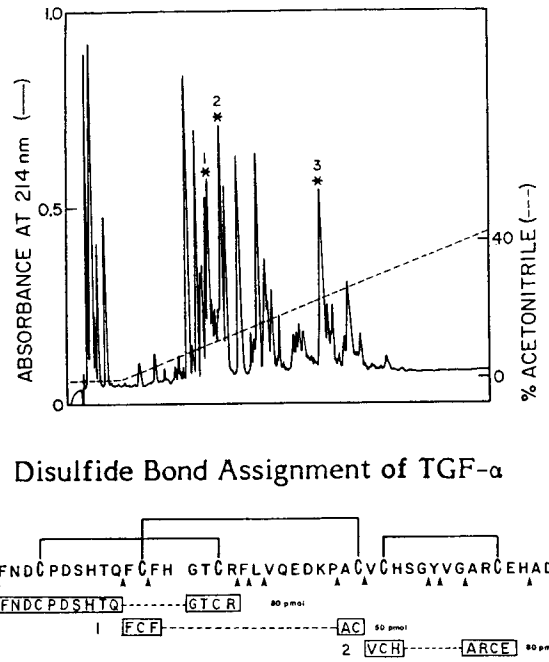

Fig. 6. Peptide map and disulfide bond assignment of human TGF-α. One nanomole of human TGF-α was digested with thermolysin at an enzyme to substrate ratio of 1:100. The resultant mixture of peptides was resolved and isolated by C-18 reverse-phase HPLC (**a**). Peptides containing disulfide bonds were identified by the appearance of new peptide peaks upon rechromatography after performic acid oxidation of each individually collected peak. Amino acid composition analysis was then performed on aliquots of the peptide peaks. Predicted thermolysin cleavage sites along human TGF-α are indicated by ▲. Amino acid composition of the three peptides (marked by *) that led to the disulfide bond assignment are listed.

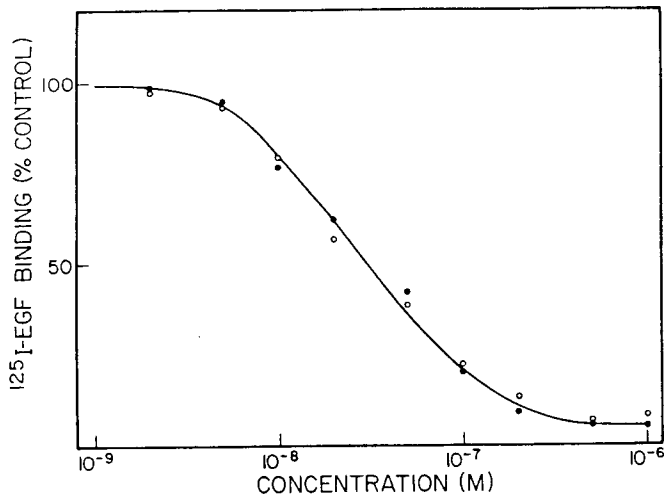

Fig. 7. Competition of human TGF-α and murine EGF with [^{125}I]EGF for binding to purified human EGF receptor (●, TGF-α; ○, EGF). Values shown are averages of two triplicate experiments. Experimental errors are less than ± 2% of total.

synthesized, that it contains three disulfides bonds and that there were no chemical modifications of the purified synthetic TGF-α.

In a second mass spectrometry experiment the TGF-α sample used for the above-described essentially non-destructive analysis was removed from the mass spectrometer and treated with an alkaline solution of dithiothreitol as described in Materials and methods. This reduced sample was then reintroduced into the mass spectrometer and a spectrum obtained (Figure 5B). The

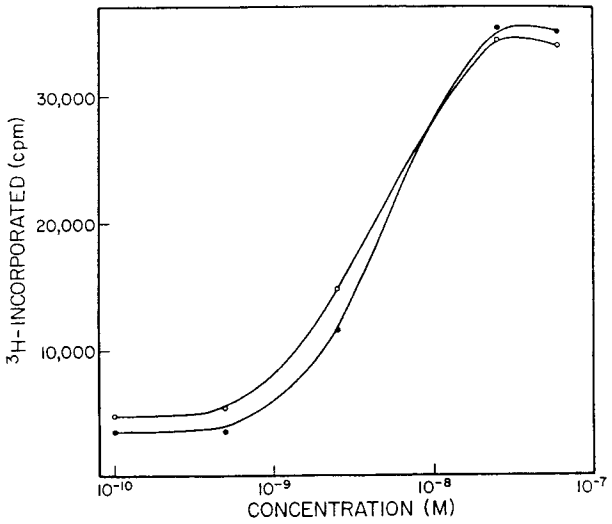

Fig. 8. Mitogenic response of NRK fibroblasts to human TGF-α and murine EGF (●, TGF-α; ○, EGF). Values shown are averages of three triplicate experiments. Observed error ranges are ± 5%.

measured molecular weight was 5552.1 (calculated: 5552.3 amu). This increase of 5.9 mass units on reduction confirmed the presence of three intact disulfide bonds in the purified synthetic TGF-α.

Disulfide bonds in human TGF-α. The location of the three disulfide bonds in purified synthetic TGF-α was determined as described in Materials and methods and in the legend to Figure 6. Disulfide-containing peptides were identified by their amino acid compositions. All peptides had compositions consistent with the target sequence and the known specificity of the protease used. Amino acid composition of components derived from the three peaks indicated with an asterisk (*) unambiguously define the disulfide bonds between Cys8 and Cys21, between Cys16 and Cys32, and between Cys34 and Cys43 respectively (Figure 6B). This pattern of cysteine pairing is identical with the disulfide bonds found in native murine EGF (Savage *et al.*, 1973), and is also identical with the disulfide paring of human TGF-α produced by overexpression in genetically engineered microorganisms (Winkler *et al.*, 1986).

Taken together, the above analytical and structural data demonstrate that a protein with the structure proposed for human TGF-α has been synthesized and purified to homogeneity.

Biological activities of synthetic TGF-α

The biological and biochemical properties of the homogeneous active synthetic human TGF-α were characterized and its specific activity compared to purified native mouse EGF in three assays.

Binding to EGF receptor. The ability of synthetic human TGF-α to compete with EGF in binding to EGF receptor purified to homogeneity from human A431 cells is illustrated in Figure 7. The interaction of EGF with solubilized EGF receptor has an intrinsic dissociation constant of 65.8 ± 3.5 nM at 25°C (Woo, 1984). Equal molar amounts of TGF-α and EGF displace identical amounts of [^{125}I]EGF bound to EGF receptor throughout the concentrations tested. These results indicated that, in this assay, the synthetic human TGF-α is equipotent to EGF in its ability to bind to EGF receptor. Synthetic human TGF-α also stimulated the tyrosine kinase activity of purified human EGF receptor (data not shown).

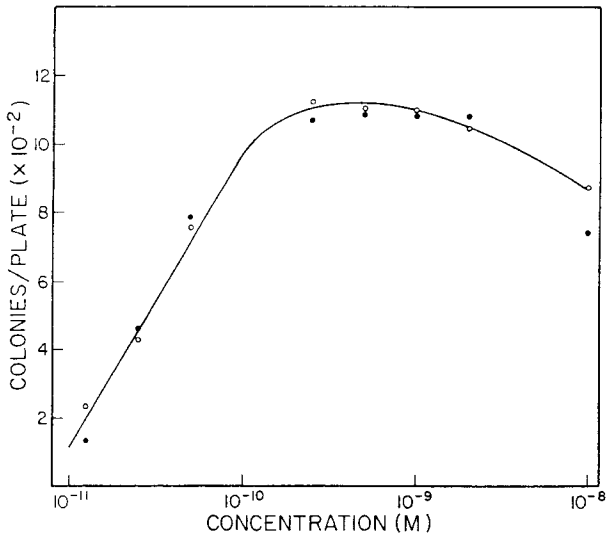

Fig. 9. Dose response of colony formation in soft agar by NRK fibroblasts (●, TGF-α; ○, EGF). Value shown for each concentration represent the average number of colonies observed for either randomly selected fields at that concentration of TGF-α.

Mitogenic activity. The mitogenic properties of purified synthetic human TGF-α on quiescent NRK cells was compared with that of native EGF in Figure 8. Half-maximal mitogenic response to TGF-α by NRK cells in a defined medium containing TGF-α, insulin, transferrin and linoleic acid, as measured by [^{3}H]thymidine incorporation, occurs at 4 nM TGF-α. Half-maximal mitogenic response of these cells to EGF under identical conditions was obtained at 3.3 nM.

Colony formation in soft agar. The dose response of NRK cells in soft agar colony formation to varying concentrations of purified synthetic human TGF-α in the presence of a fixed concentration of TGF-β, supplied in the form of 10% calf serum, is illustrated in Figure 9. After 7 days of incubation in soft agar medium containing only 10% calf serum and no TGF-α, cells did not grow and remained as a suspension of viable single cell suspension. A large number of aggressively growing colonies (Figure 10) were detected in all samples containing TGF-α at concentrations in excess of 100 pM. The half-maximal soft agar colony formation response to synthetic human TGF-α was estimated to be 30 pM (Figure 9). EGF is as potent as our synthetic human TGF-α in this assay. We also found that there was a toxicity or inhibitor effect towards colony formation at TGF-α or EGF concentrations above 1×10^{-9} M. Both the number and the size of the colonies were reduced, the effect being more pronounced for TGF-α than for EGF (Figure 9).

Conclusions

The results reported in this work show that machine-assisted 'automated' chemical synthesis is a practical tool for the preparation of small proteins as defined molecular species, rapidly and in good yield with high purity. This represents an important advance over the manual syntheses of human TGF-α (Tam *et al.*, 1986) and of similar molecules previously reported (Tam *et al.*, 1984; Heath and Merrifield, 1986). Doubts expressed concerning the ability of chemical synthesis to produce defined products for structural studies can now be put to rest (Wetzel, 1986; Offord, 1987). The approach reported may be a prototype for future work. We feel strongly that if valid structure–function correlates are to be drawn, the covalent structure of an engineered protein

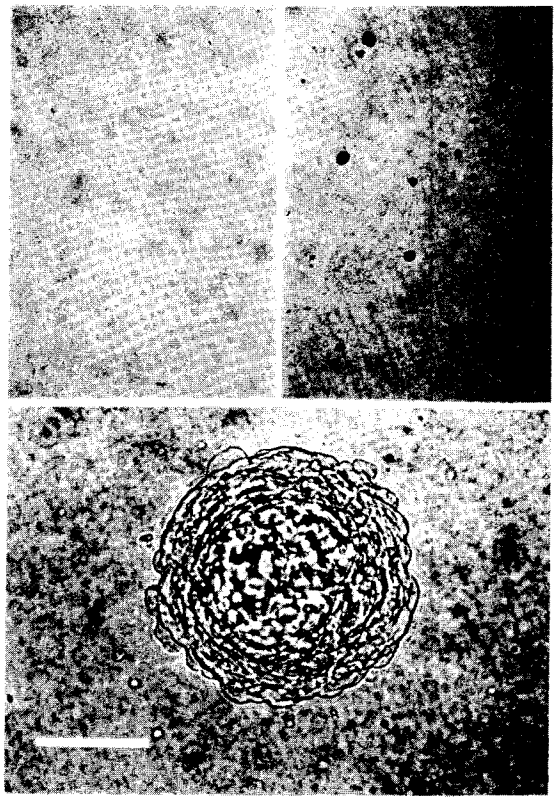

Fig. 10. Appearance of NRK colonies in the absence and presence of synthetic human TGF-α.

should first be directly established, and only then should the conformation of the molecule and its biological activities be studied. While meaningful results can be obtained under less rigorous conditions (Clark-Lewis *et al.*, 1988), caution should be exercised in interpreting results, especially if the results are negative.

Application of these techniques to the family of EGF–receptor ligands, including TGF-α, will be a powerful approach to systematic structure–function studies of this interesting group of biologically active proteins. TGF-α itself is among the smallest proteins: molecules which owe their activities to the precise three-dimensional folding of a linear polypeptide chain. With the optimized synthetic procedures described in this paper, the amino acid sequence of TGF-α can be systematically varied to explore the mechanism of folding of the protein and the relationship of primary structure to the equilibrium-folded three-dimensional structure of the molecule. This can be undertaken with far greater flexibility than currently available genetic engineering techniques allow. For example, the role of disulfide bonds in the stability of this protein can be examined by incorporation of unnatural isosteric analogs of cysteine, such as L-α-amino-*n*-butyric acid, by the forcing of unnatural cysteine pairings, or by the creation of non-reducible disulfide analogs.

Chemical synthesis offers increased versatility in the investigation of the structures and activities of proteins. In this regard, the ability to incorporate isotopic probe nuclei at specifically designated single atom locations in the molecule, in general only possible by chemical means, will be of great assistance to the high-resolution NMR studies necessary to determine the solution structures of novel 'designed' proteins, and for mechanistic studies of the same proteins. Our approach with TGF-α is to use the solved structure of the parent molecule

(Carver *et al.*, 1986; Cooke *et al.*, 1987; Montelione *et al.*, 1987; Brown *et al.*, 1989; Kohda *et al.*, 1989; Campbell *et al.*, 1989) to compare the 'NMR fingerprints' of synthetic analogs to rapidly establish similarities and differences in folded structures.

In the future it will be possible to refine the synthetic purification and analytical techniques described here and extend their application to the preparation of larger proteins in chemically purified form. These studies are currently underway in our laboratory and those of our collaborators (Wlodawer *et al.*, 1989).

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