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^{1}H NMR Assignment and Secondary Structural Elements of Human Transforming Growth Factor α[†]

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ABSTRACT: The ^{1}H NMR spectrum of human transforming growth factor α (hTGF-α) has been completely assigned, and secondary structural elements have been identified as a preliminary step in determining the structure of this protein by distance geometry methods. Many of these structural elements closely correspond to those previously found in a truncated human EGF [Cooke et al. (1987) *Nature (London) 327*, 339-341] and murine EGF [Montelione et al. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5226-5230]. These include the presence of an antiparallel β-sheet between residues G19 and C34 with a type I β-turn at V25-D28, a type II β-turn at H35-Y38, and another short β-sheet between residues Y38-V39 and H45-A46.

Transforming growth factor α (TGF-α)[1] [see Derynk (1986) for a recent review] was originally discovered with TGF-β in the conditioned medium of virally transformed rat kidney fibroblasts (DeLarco et al., 1981). It was subsequently found in the conditioned medium of several human tumor cell lines (Todaro et al., 1980) and human tumor explants (Derynk et al., 1987) and has been detected in the urine of patients with disseminated cancers (Sherwin et al., 1983). In vitro addition of TGF-α to growth medium at nanomolar concentrations stimulates the growth of normal fibroblasts and epithelial cells (DeLarco & Todaro, 1978; Schultz et al., 1987), whereupon

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[1] Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; 2D, two dimensional; DQF-COSY, 2D double quantum filtered correlation spectroscopy; RELAY, 2D relayed coherence transfer spectroscopy; TOCSY(HOHAHA), 2D total correlation spectroscopy; 2Q-COSY, double quantum 2D correlation spectroscopy; NOESY, 2D NOE spectroscopy; hTGF-α, human transforming growth factor; th-EGF, truncated human epidermal growth factor; m-EGF, murine epidermal growth factor; EDTA, disodium ethylenediaminetetraacetate.

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they assume a transformed phenotype which is fully reversible upon removal of the growth factor from the medium (Roberts et al., 1983).

The TGF-α sequence is highly conserved, with only 4 out of 50 amino acid substitutions between the rat and human proteins (Derynk et al., 1984). Mature human TGF-α is a heat- and acid-stable polypeptide of 50 residues (M_r 5600). Complementary DNA sequence analysis has shown that the mature protein is derived from a glycosylated precursor (160 amino acids) that is processed by 2 elastase-type cleavages at Ala-Val-Val sequences to yield TGF-α and several processing intermediates (18–20 kDa) possessing similar biological activities (Bringman et al., 1987).

Although only sharing a 33% amino acid sequence identity, TGF-α and epidermal growth factor (EGF) bind to the same receptor (Massague, 1983). They share similar roles in the processes of development, proliferation, and neoplastic transformation in many cell phenotypes. However, EGF and TGF-α produce different effects on primary explants of normal and neoplastic human lung tissue (Siegfried, 1987). It can be anticipated that other differences in these mitogenic factors will be found as well. Proteins similar to TGF-α and EGF have been discovered as early gene products of the vaccinia virus (Twardzik et al., 1985) and the Shope fibroma virus (Chang et al., 1987). These proteins all share six cysteine residues similarly spaced along the sequence and are thought by analogy to have similar disulfide bond arrangements. This has been confirmed in the case of TGF-α and EGF (vide infra). Growth factor like sequences have also been found as domains in several extracellular proteins (Doolittle, 1985). The tertiary structure of the protein is required for biological activity (Hanauske et al., 1986). As of yet, no crystal structures have been determined for any of these growth factor like proteins, nor for the larger proteins containing growth factor like domains. Knowledge and comparison of the structures of TGF-α and EGF could lead to a better understanding of which portions of these proteins bind to the EGF receptor. Such knowledge is of interest for the rational design of antagonists that might control cell proliferation in several types of human tumors.

The structures in solution of several small proteins comparable in size to TGF-α have been probed by the application of modern two-dimensional NMR techniques [for a recent review, see Wüthrich (1986)]. Recently, there has been significant progress in determining the solution structures of a C-terminal truncated human EGF (thEGF) (Cooke et al., 1987) and murine EGF (mEGF) Montelione et al., 1987) by these methods. This paper reports similar progress for human TGF-α. The proton resonances of hTGF-α have been completely assigned, and quantitative distances from NOE buildup rates are being used to generate structures of the protein. The results of these studies will be reported elsewhere. Herein, we report secondary structural elements and compare them to those reported for thEGF (Carver et al., 1986) and mEGF (Montelione et al., 1986).

Materials and Methods

Human transforming growth factor α was purchased as a lyophilized powder from Bachem (Torrance, CA). All deuteriated reagents were obtained from Stohler Isotopes. Ten milligrams of the protein was dissolved in purified water (Barnstead Milli-Q), the pH was adjusted to 3.4 by the addition of DCl, EDTA was added to 10 mM, and the sample was washed exhaustively in purified water (pH 3.4) using an Amicon Ultrafiltration apparatus with a 2000 molecular weight cutoff membrane (Amicon YM-2) to remove paramagnetic ions from the original sample. The final sample contained TGF-α (3.5 mM), pH 3.42, 10% D_2O, and 0.5 mM EDTA. The protein was observed to provide substantial buffering capacity at this pH. Sample prepared in 100% D_2O was titrated to a pH meter reading of 3.8 units. Sample temperature was either 10, 25, or 37 °C, as indicated in the figure legends. A small aliquot of the sample was taken periodically for purity (HPLC) and biological assays (radioreceptor binding competition and soft-agar colony stimulation). No significant degradation of the sample was observed over a period of months at 4 °C.

NMR Spectroscopy. All NMR spectra were obtained on a JEOL GX-500 instrument locked at 500.13 MHz for protons. Phase-sensitive DQF-COSY (Shaka & Freeman, 1983; Rance et al., 1984), NOESY (Macura & Ernst, 1980; States et al., 1982), and TOCSY(HOHAHA) (Braunschweiler & Ernst, 1983; Bax & Davis, 1985) experiments were obtained by using parameters given in the figure legends. TOCSY-(HOHAHA) was done by using the MLEV-17 sequence with the "trim" pulses deleted, since phase errors which these modifications correct are not significant when a strong (>10 kHz) radiofrequency field is used (Weber et al., 1987). Quadrature detection in the t_1 domain was achieved by the hypercomplex method (Mueller & Ernst, 1979; States et al., 1982). Solvent suppression was performed by 1.2-s phase-locked preirradiation. The SCUBA water suppression method was used (Brown et al., 1988) for experiments in H_2O. Briefly, the presaturation field is turned off 100 ms prior to the first preparation pulse and two composite π pulses are applied, one at the beginning and the other in the middle of the 100-ms delay period. Cycle times were 2.4 s for correlation experiments and 3.5 s for NOESY experiments as the longest nonselective T_1 of the protein was 1.3 s. All experiments used a spectral width of 5000 Hz, with 16–64 transients per t_1 point and a resolution of 2048 complex points. Generally, 400 increments were acquired in each experiment to span a t_1 domain of 80 ms. Data were transferred by magnetic tape to a microVax II for processing using the FTNMR program licensed from Hare Research, Inc. Data sets were zero-filled to 2K × 2K real matrices. Window functions were skewed 30° phase-shifted sine bells for correlation experiments and 90° phase-shifted sine bells for NOESY experiments. Spectra were t_1 noise filtered by multiplying the first point by 0.5 before the second Fourier transform. Transformed spectra were base line corrected in both dimensions by a second order polynomial.

Results and Discussion

Assignments. The first task in obtaining structural information for proteins by NMR involves the assignment of as many resonances as possible, with those of backbone protons (amide, α-, and β-protons of each amino acid residue) being most crucial for secondary structural determination. Proton resonances of hTGF-α were assigned by using the standard sequential procedure (Billeter et al., 1982; Wüthrich et al., 1982). Briefly, resonances associated within a given spin system were identified by using standard COSY, RELAY, double-quantum (2Q), and TOCSY(HOHAHA) experiments. These spin systems are assigned to specific amino acid residues within the protein sequence using the statistical properties of interresidue NOE contact distances (Billeter et al., 1982).

The first step in the assignment strategy involves grouping the amino acid side chain resonances into scalar-coupled sets and identifying these sets as specific spin systems. The DQF-COSY spectrum in D_2O, a portion of which is shown in Figure 1, is essential to this task. Each cross-peak is doubly antiphase, and information concerning passive couplings can

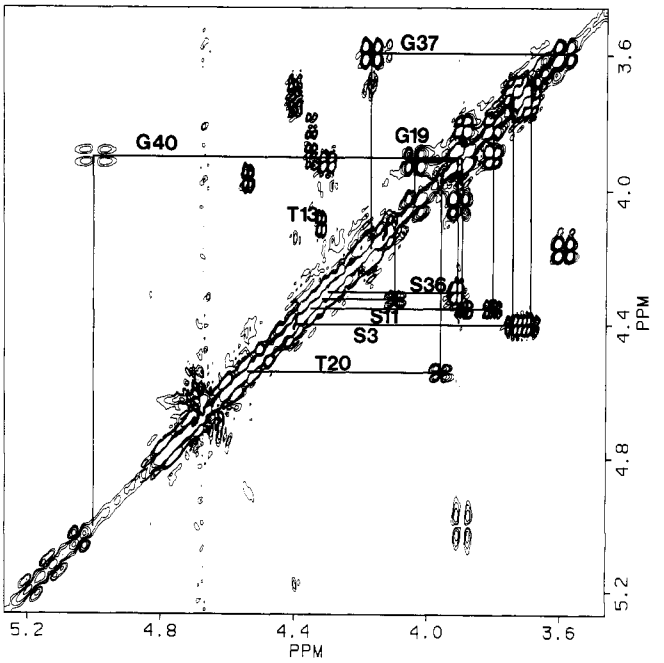

FIGURE 1: Portion of the hTGF-α DQF-COSY spectrum (D_2O, pD 3.8, 37 °C). Assignments of glycine, serine, and threonine spin systems are indicated.

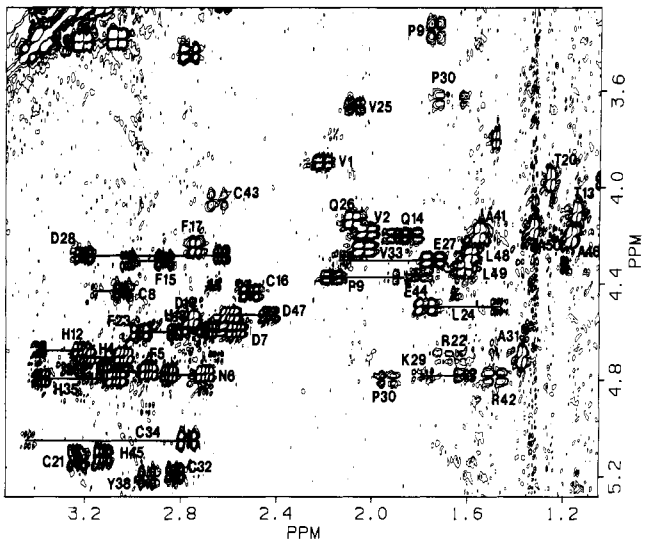

FIGURE 2: DQF-COSY spectrum (same as in Figure 1) showing H_α–H_β,$H_{\beta'}$ correlations with assignments. AMX spin systems to the left of F_2 = 2.3 ppm. Proline H_γ,$H_{\gamma'}$–H_δ,$H_{\delta'}$ and threonine H_β–methyl$_\gamma$ correlations are also labeled.

be deduced from the fine structure of the cross-peaks. For example, the three AX spin systems, corresponding to glycine H_α–H_α coherences, show a basic quartet pattern with a large geminal coupling and no passive coupling (the amide protons have been replaced with deuterons). Two of the three serine (AMX) spin systems show fine structure expressing the passive coupling between H_α and the other H_β. Figure 2 shows assignments for the H_α–H_β,$H_{\beta'}$ cross-peaks found in another portion of the same DQF-COSY spectrum. The AMX spin systems, grouped to the left half of the figure, cannot be assigned to specific types of amino acids, since cysteines (C), aspartates (D), asparagines (N), phenylalanines (F), histidines (H), tyrosines (Y), and serines (S) all exhibit AMX-type spin systems (H_α, H_β, $H_{\beta'}$). Fully half of the amino acids in hTGF-α are of this class, so specific assignment of these spin systems must be done in conjunction with sequential NOE data (vide infra).

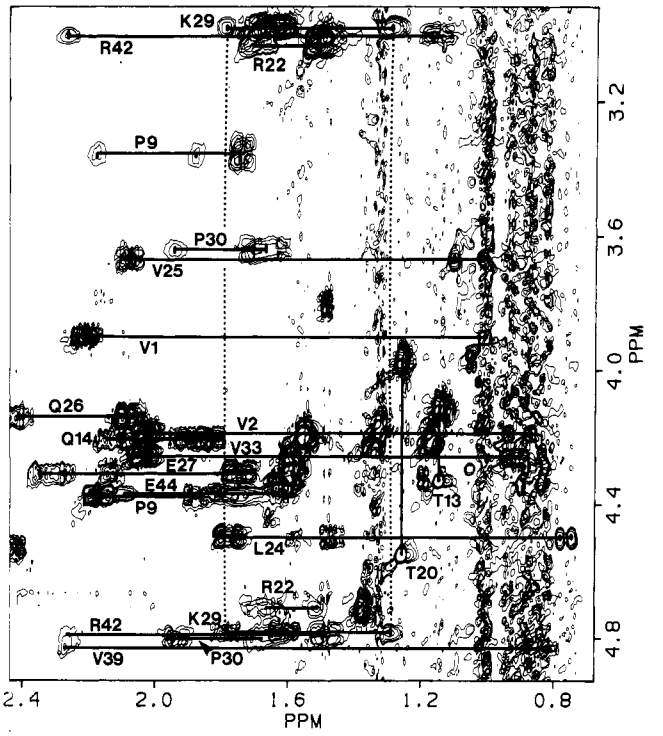

FIGURE 3: Assignment of long-chain amino acids. Superposition of DQF-COSY (antiphase peaks) and MLEV-17 (absorptive peaks, τ_m = 80 ms) experiments. Example: K29 H_α–H_β and $H_{\beta'}$ COSY peaks, relayed to H_γ,$H_{\gamma'}$ resonances in MLEV experiment. H_ϵ,$H_{\epsilon'}$–H_δ,$H_{\delta'}$ COSY peaks, relayed to H_γ,$H_{\gamma'}$ and H_β,$H_{\beta'}$ resonances.

Inspection of Figure 2 (and subsequent figures) reveals some weak, as yet unassigned, resonances in the DQF-COSY spectra of hTGF-α. These resonances must come from either degraded protein, less populated conformers, or contaminating peptides given that each resonance of the major conformer has been assigned. They all exhibit motionally averaged coupling constants, and no NOE's connecting these COSY peaks could be detected. The protein appeared >90% homogeneous by HPLC and >90% biologically active by several biological assays done periodically during the NMR experiments (M. Anzano, personal communication). All subsequent results refer to data obtained at 3.5 mM protein concentration. Self-aggregation was observed at higher protein concentrations, indicated by chemical shift changes (>0.1 ppm) of several amide and α resonances, steeper NOE buildup rates, and protection of side chain protons (K29ζ) from exchange with solvent protons.

The basic procedure used to identify the remaining spin systems is depicted in Figure 3. A combination of data from the DQF-COSY and TOCSY(HOHAHA) experiments provides a complete assignment of the larger spin systems. For example, coherence in the MLEV-17 experiment extending from H_α through H_β and $H_{\beta'}$ to Hγ and the methyl protons H_δ, $H_{\delta'}$ allows the set labeled L24 to be identified as a leucine, or an A_3B_3MPTX spin system. The single lysine, K29, could be identified by matching the extended coherence pattern from the H_α proton to that of the $H_{\epsilon,\epsilon'}$ protons in Figure 3, where a relay from H_α to $H_{\gamma,\gamma}$ is connected to a relay from $H_{\epsilon,\epsilon'}$ through $H_{\delta,\delta'}$ to $H_{\gamma,\gamma'}$ and H_β. The two threonines (A_3MX) are readily distinguished from the four alanines (A_3X) by coherence relayed from the methyl groups via H_β to H_α resonances (vertical lines). In this manner, all of the non-AMX spin systems could be assigned to amino acid types. The TOCSY(HOHAHA)-type experiments cannot by themselves provide data leading to specific assignments, since the relay of coherence is a complex function of the coupling constants

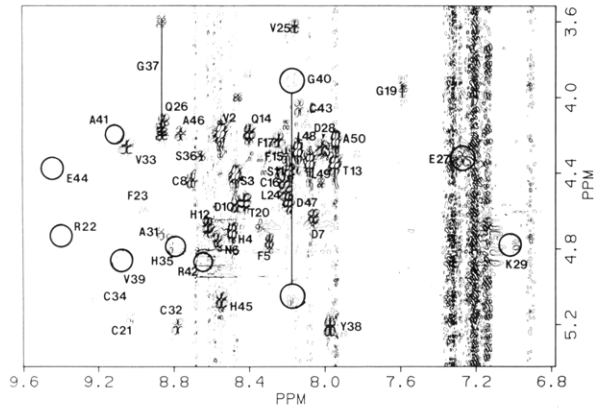

FIGURE 4: Fingerprint region of the DQF-COSY contour plot of hTGF-α (6 mM, pH 3.4, T = 25 °C). SCUBA presaturation (t_p = 100 ms) was used to suppress water. H_N–H_α cross-peaks are labeled with their assigned amino acid in the sequence.

and mixing time (Weber et al., 1987). In a protein as small as hTGF-α, inspection of the DQF-COSY cross-peak fine structures near the diagonal allowed a firm assignment of the resonances within complex spin systems such as prolines [$A_2(T_2)MPX$]. Additional information from a RELAY experiment (τ_m = 60 ms) and a 2Q-COSY experiment confirmed the assignment of $H_{\gamma,\gamma'}$ and $H_{\delta,\delta'}$ protons (data not shown) but was not absolutely necessary. Several degenerate methylene resonances could be confirmed by observation of remote peaks in the 2Q-COSY experiment.

Once the nonexchangeable resonances had been grouped into sets whose spin systems could be assigned by the aforementioned procedure, the next step in the sequential assignment method was to obtain data on amide proton chemical shifts to correlate with the H_α resonances. Only 34 of the possible 47 H_N–H_α correlations appeared in the DQF-COSY spectrum in water at 37 °C. Repeating the experiment at 25 °C revealed five other cross-peaks previously obscured by the F_2 track of the water resonance. The remaining 10 H_N–H_α correlations appeared only in MLEV-17 spectra. These resonances did not appear in COSY or 2Q spectra due to extensive line broadening. Unambiguous correlations of the H_N–H_α resonances were obtained by inspection of TOCSY-(HOHAHA) and DQF-COSY experiments done at two temperatures (25 and 37 °C). The DQF-COSY experiment was repeated at 6 mM protein concentration (Figure 4). Plots of this region at a low contour level very close to the noise clearly revealed all but two H_N–H_α correlations (H18, A41). This spectrum was acquired by using the SCUBA water suppression method (Brown et al., 1988), so that cross-peaks (e.g., V39, H35, K29) were observed that would normally be bleached out by presaturation of the water resonance.

The final step in the assignment procedure involved assignment of each spin system to specific amino acids in the primary sequence of the protein. A NOESY experiment acquired with a mixing time of 200 ms contained cross-peaks corresponding to a maximum distance of 3.5 Å and was used to locate $d_{\alpha N}$, $d_{\beta N}$, and d_{NN} connectivities [see Stassinopoulou et al. (1984) for a description of this nomenclature for proton–proton distances in proteins]. Those NOE's leading to sequential assignments, with their relative intensities, are summarized in Figure 5. A sequential NOE of one type or another was found for each step along the primary sequence. Nearly all of the potentially observable sequential α-amide ($d_{\alpha N}$) NOE's, which should correspond to distances ranging from 2.2 to 3.6 Å (Billeter et al., 1982), were observed for the 200-ms data set. Pseudo-$d_{\alpha N}$ connectivities were identified

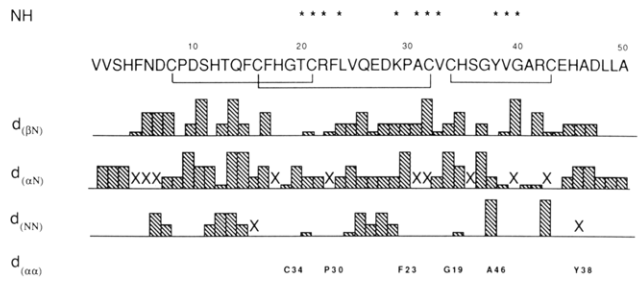

FIGURE 5: Summary of sequential NOE's found for hTGF-α. Residues with slowly exchanging amide protons are marked by asterisks. The three lines under the amino acid sequence show sequential NOE's with relative intensities indicated. The last line identifies residues whose α-proton shows an intense NOE to that of the indicated residue. NOE's not observable due to overlap or water interference are indicated by X.

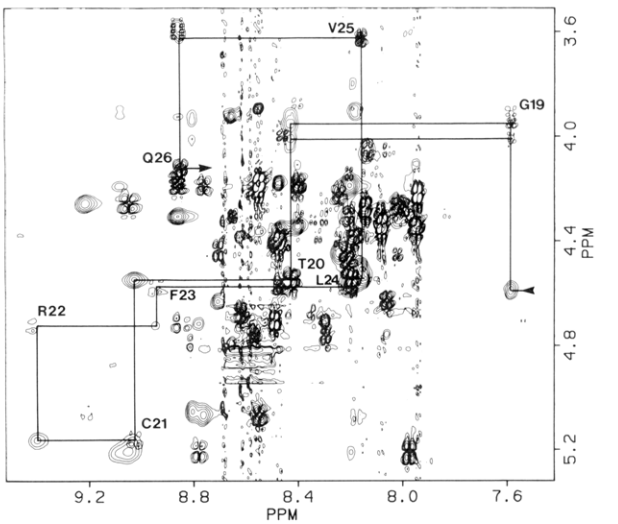

FIGURE 6: Superposition of DQF-COSY and NOESY (τ_m = 200 ms) experiments in H_2O for hTGF-α. Sequential $d_{\alpha N}$ connectivities from H18 to Q26 are traced.

for the two proline residues (see Figure 7). Figure 6 illustrates the sequential assignment process for the $d_{\alpha N}$ connectivities. The fingerprint DQF-COSY experiment and the NOESY experiment are superimposed, and the lines trace the $d_{\alpha N}$ connectivities from H_α of H18 to H_N of Q26 in the β-sheet. Only 15 of 47 amide–amide NOE's, corresponding to distances ranging from 2.0 to 4.8 Å (Billeter et al., 1982), were observed at 200-ms mixing times. Many $d_{\beta N}$ connectivities were observed as well and are indicated in Figure 5.

After the sequential assignments were completed, the aromatic resonances were assigned by NOE connectivities to H_β,$H_{\beta'}$ protons (data not shown), and the side chain amide resonances were assigned in a likewise manner. The guanidino protons of arginines were observed as very broad resonances (~6.6 ppm) and assigned by NOE contacts to their corresponding H_ϵ exchangeable protons. Hydroxyl resonances of serines, threonines, and tyrosine and the N-terminal amino group were not observed due to their rapid exchange with water protons. All of the aromatic rings are rotating rapidly enough such that their C_2-symmetry-related proton resonances are in fast exchange. A summary of the proton assignments for hTGF-α is given in Table I. All of the nonexchangeable resonances have been assigned as well as most of the exchangeable resonances.

Secondary Structure. Secondary structural elements in the protein may be located by inspection of the sequential NOE intensities given in Figure 5. Additional information was obtained by the assignment of slowly exchanging amides.

Table I: Proton Resonance Assignments for hTGF-α[a]

residue	NH	H_α	$H_{\beta 1}$	$H_{\beta 2}$	$H_{\gamma 1}$	$H_{\gamma 2}$	$H_{\delta 1}$	$H_{\delta 2}$	H_ϵ	other
Val-1		3.90	2.21		1.01	1.01				
Val-2	8.47	4.18	2.02		0.94	0.87				
Ser-3	8.38	4.40	3.74	3.69						
His-4	8.40	4.69	3.04	3.19			7.21		8.68	
Phe-5	8.24	4.76	2.93	3.12			7.20	7.20	7.33	7.33(ζ)
									7.33	
Asn-6	8.51	4.77	2.70	2.85			6.88	7.50		
Asp-7	7.96	4.59	2.68	2.57						
Cys-8	8.69	4.43	3.04	3.16						
Pro-9		4.37	2.17	1.87	1.73	1.74	3.35	3.37		
Asp-10	8.29	4.54	2.75	2.75						
Ser-11	8.17	4.35	3.89	3.80						
His-12	8.57	4.67	3.21	3.39			7.27		8.59	
Thr-13	7.88	4.33	4.10		1.14					
Gln-14	8.33	4.20	1.84	1.91	2.08	2.15			6.71	
									7.28	
Phe-15	8.11	4.30	2.87	3.02			7.14	7.14	7.24	7.22(ζ)
									7.24	
Cys-16	8.12	4.44	2.51	2.22						
Phe-17	8.27	4.25	2.74	2.74			7.13	7.13	7.23	7.23(ζ)
									7.23	
His-18	7.06	4.58	2.75	2.37			6.92		8.50	
Gly-19	7.51	4.05								
		3.90								
Thr-20	8.40	4.54	3.96		1.26					
Cys-21	8.91	5.13	3.23	3.13						
Arg-22	9.31	4.71	1.65	1.70	1.51	1.50	3.04	3.04	7.03	
Phe-23	8.88	4.60	2.97	2.82			6.92	6.92	7.17	7.16(ζ)
									7.17	
Leu-24	8.19	4.49	1.77	1.47	1.58		0.79	0.75		
Val-25	8.07	3.66	2.08		1.10	1.02				
Gln-26	8.72	4.13	2.08	2.08	2.42	2.42			6.82	
									7.55	
Glu-27	7.47	4.30	1.74	2.14	2.27	2.36				
Asp-28	8.01	4.28	3.21	2.63						
Lys-29	7.04	4.77	1.61	1.78	1.29	1.30	1.63	1.67	2.98	7.56[b]
									2.98	
Pro-30		4.79	1.93	1.70	1.70	1.64	3.63	3.64		
Ala-31	8.77	4.70	1.37							
Cys-32	8.68	5.18	2.82	2.71						
Val-33	8.98	4.25	2.03		0.95	0.91				
Cys-34	9.07	5.05	2.77	3.44						
His-35	8.71	4.79	3.07	3.38			7.33		8.66	
Ser-36	8.55	4.30	3.91	3.91						
Gly-37	8.80	4.17								
		3.59								
Tyr-38	7.94	5.20	2.92	2.76			6.70	6.70	6.42	
									6.42	
Val-39	9.05	4.82	2.26		0.88	0.83				
Gly-40	8.09	5.00								
		3.89								
Ala-41	9.10	4.19	1.55							
Arg-42	8.71	4.79	1.48	2.27	1.48	1.14	3.00	3.00	7.04	
Cys-43	8.13	4.05	2.65	2.94						
Glu-44	9.43	4.36	1.82	1.78	2.08	2.08				
His-45	8.63	5.11	3.12	3.24			7.20		8.58	
Ala-46	8.66	4.20	1.16							
Asp-47	8.16	4.52	2.59	2.42						
Leu-48	8.02	4.28	1.58	1.58	1.55		0.89	0.83		
Leu-49	8.05	4.34	1.61	1.63	1.58		0.90	0.84		
Ala-50	7.74	4.17	1.32							

[a] Transforming growth factor α, 3.5 mM in 90% H_2O/10% D_2O, pH 3.42, T = 37 °C. [b] Observed only at ≥6.0 mM protein.

Tertiary and secondary structural features protect amide protons from exchange with solvent (Englander & Kallenbach, 1984). Slowly exchanging amides were observed after dissolution of lyophilized protein in D_2O at 10 °C (pD 3.8) and immediate acquisition of a TOCSY(HOHAHA) experiment (MLEV-17, τ_m = 35 ms). Only 10 of the 47 amides exchanged slowly ($<\sim 0.010$ min^{-1}) under these conditions. At 37 °C, all amides are exchanged after 1 h ($>\sim 0.15$ min^{-1}). A TOCSY(HOHAHA) experiment is advantageous in that the amides can be more confidently assigned by observation of the magnetization relayed to the H_β,$H_{\beta'}$.

Figure 7 shows a portion of the NOESY spectrum (τ_m = 200 ms) of hTGF-α in D_2O. Strong NOE cross-peaks between α-protons are labeled. A series of close contacts between α-protons remote along the primary sequence are indicative of antiparallel β-sheet structure. Sequential pseudo-$d_{\alpha N}$ NOE connectivities to proline H_δ,$H_{\delta'}$ are also indicated in this figure. Remaining cross-peaks are intraresidue H_α–H_β,$H_{\beta'}$ and glycine H_α–H_α NOE contacts.

The NOE data summarized in Figure 5 were examined for patterns indicative of regular protein secondary structure. Helical structure is characterized by a series of d_{NN} close

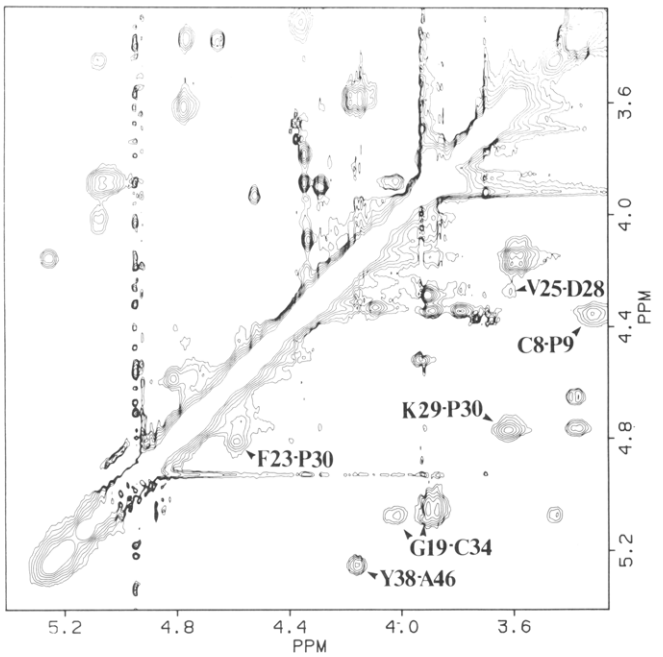

FIGURE 7: Region of hTGF-α (3.5 mM, pD 3.8, T = 10 °C) NOESY spectrum (τ_m = 200 ms) in D_2O showing NOE's between H_α remote in the primary sequence. Pseudo-$d_{\alpha N}$ NOE's to proline H_δ,$H_{\delta'}$ resonances are also labeled.

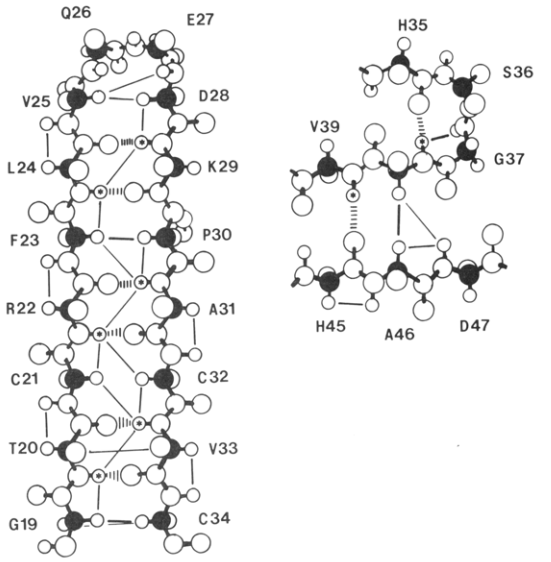

FIGURE 8: Representation of secondary structures identified in hTGF-α. Only those atoms connected to backbone atoms are shown. C_α atoms are labeled by filled circles, slowly exchanging amide protons are labeled with stars, and observed NOE contacts between protons are indicated by lines. Putative hydrogen bonds are indicated by dashed lines to the most likely acceptor consistent with NOE distance constraints.

contacts, corroborated by $d_{\alpha N}(i, i + 3)$ (for α-helices) or $d_{\alpha N}(i, i + 2)$ (for 3_{10} helices) NOE's, or $d_{\alpha\beta}(i, i + 3)$ NOE's (Wüthrich et al., 1984). The only stretches of strong d_{NN} NOE's are found at V25–D28 and S11–F15, both just long enough for a complete helical turn, but none of the corroborating NOE's were observed. Therefore, no evidence for the presence of helices was found.

Both the N-terminal (V1–D7) and C-terminal (D47–A50) residues at pH 3.2 and 3–6 mM protein concentrations appear to be uninvolved in a regular secondary or tertiary structure. Only sequential NOE's are observed for these residues, their resonance line widths are narrow, and intraresidue NOE's are weak. We see no evidence for the formation of a short strand of β-sheet in the region V1–D7 prior to the first disulfide bond, as reported in the case of mEGF S2–G5 (Montelione et al., 1986) and thEGF S2–E6 (Cooke et al., 1987). Since N-terminal deletions of rat TGF-α have been shown not to greatly affect the biological activity (Simpson et al., 1985), it appears unlikely that this structural feature contributes to such activity for hTGF-α.

The region between residues S11 and F15 of the first disulfide loop appears to form a multiple bend region similar to that described for mEGF (Montelione et al., 1986). As shown in Figure 5, strong d_{NN} NOE's indicate a general type I bend for the region S11–Q14, but no weak $d_{\alpha N}(i, i + 2)$ or d_{NN} $(i, i + 2)$ NOE's were identified to suggest a distorted helix, as found in mEGF (Montelione et al., 1987).

Identifiable secondary structure has been located in regions along the sequence corresponding to those found for the two EGF structures. Short distances derived from the NOE data and the identification of slowly exchanging amides provide most of the data supporting these structures. Additional evidence in some cases was provided by the qualitative measurement of $^3J_{HN\alpha}$ coupling constants. Coupling constants from 6 to 12 Hz were measured with errors of ±1.5 Hz, so these data were used only to locate possible inconsistencies with the NOE data. No coupling constants less than 9 Hz were measured for residues involved in β-sheet strctures.

As is seen in Figure 8, an almost complete set of short- and medium-range NOE distances defines the antiparallel β-sheet from residues [G19 to C34]. Most of the expected cross-strand $d_{\alpha\alpha}$, $d_{\alpha N}$, and d_{NN} NOE contacts are observed. The expected strong $d_{\alpha\alpha}$ NOE contact between C21 and C32 is not observed due to the near superposition of these resonances. Several cross-strand side chain–side chain contacts have been found as well. This structure is further characterized by slowly exchanging amides found at residues flanking those showing short cross-strand $d_{\alpha\alpha}$ contacts, indicative of antiparallel structure (Wüthrich et al., 1984). Additionally, strong $d_{\alpha N}$ NOE contacts are observed, consistent with an extended backbone. One notable feature of this β-sheet is the presence of proline (P30) in the middle of one strand. Proline is considered a strong breaker of β-sheet structure, yet its frequency in this type of secondary structure is roughly 10% (Chou & Fasman, 1978). NOE patterns within the turn region V25–D28 are consistent with a type I rather than type II hairpin turn, which is also observed in the EGF structures. The extent of this β-sheet for residues G19–C34 in hTGF-α corresponds closely to that found in both EGF structures, involving residues G18–C33 in mEGF (Montelione et al., 1987) and thEGF (Cooke et al., 1987). This β-sheet therefore appears to be a common feature for the three growth factor like proteins examined to date.

The configuration of the final disulfide loop C34–C43 is also similar to the structures reported for thEGF and mEGF. A type II β-turn is indicated between residues H35 and Y38 by the strong $d_{\alpha N}$ NOE between S36 and G37 (position 2) and the strong d_{NN} NOE between G37 and Y38 (at position 3). A slowly exchanging amide for residue Y38 and small $^3J_{HN\alpha}$ for S36 support the determination of this local structure (Figure 8). All growth factor like proteins sequenced to date contain a glycine at this relative position within the disulfide loop. Since large steric clashes exist for amino acids other than glycine at position 3 in type II turns (Chou & Fasman, 1977), it seems likely that such strict conservation is required to maintain the structure of this loop and not to preserve a receptor interaction per se (Gly-37–receptor contact). The subsequent necessary turn within residues G40–C43 seems not well described by "classical" bend structures. The sequential

NOE distances do not fit any of these categories (Wüthrich et al., 1984), so clarification of the local structure, if possible, must await the results of the distance geometry analysis. The corresponding region of mEGF was found to have an unusual left-handed bend (Montelione et al., 1987). It should be noted that all of the H_N resonances in this region (G40–E44) are broaded by an as yet unidentified mechanism that probably reflects some dynamic effects in this bend. One of the G40 H_α resonances is substantially broadened as well. Broadening of the corresponding resonances in mEGF was reported (Montelione et al., 1987). Comparison of the data for these two proteins indicates a possible slight difference in their structures within this region. No G40 H_α to nonsequential H_N NOE's were observed, as reported for mEGF (G39 H_α to C42, Q43, and T44 H_N). The G40 and C43 H_N NOE's were found to be slowly exchanging, whereas in mEGF the C42, Q43, and T44 amide protons were identified as such. A detailed analysis of this structural difference requires comparison of the structures generated from the NMR data.

Finally, a short antiparallel β-sheet was identified between residues Y38–V39 and H45–A46 as shown in Figure 8. The most slowly exchanging amides were found for this region (Y38 and V39), indicating a dynamic stability for this structure close to the C-terminus of the protein. Again, this structure is observed in the thEGF and mEGF structures, but seems to be more extensive than that found for hTGF-α. Since the highest density of conserved amino acids among the growth factor like proteins occurs in this region (G37–R42), it is thought to be the region interacting with the receptor. A complete comparison of the structures of these proteins may provide insight into which amino acids are conserved for structural reasons alone and which are involved in receptor recognition.

In conclusion, many of the secondary structural elements reported for the EGF family have also been found in hTGF-α. Identification of NOE contacts between disulfide pairs in hTGF-α (unpublished data) confirms the disulfide linkages postulated by analogy with EGF. It can be expected that the tertiary structures among this family of growth factors are similar as well. Preliminary analysis of distance geometry generated structures of hTGF-α indicates that slight differences exist when compared to the structures published for thEGF and mEGF.

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