Sequence-specific ^1H -n.m.r. assignments and peptide backbone conformation in rat epidermal growth factor

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The solution conformation of rat epidermal growth factor (EGF) has been investigated by proton n.m.r. techniques. Two-dimensional proton n.m.r. experiments have allowed sequential resonance assignments to be made for most protons. On the basis of these assignments, two regions of anti-parallel β -sheet structure have been derived from the n.m.r. data. A β -sheet segment running from about V19 to V23 (capital letters refer to amino acids in the single-letter notation) is folded onto a β -sheet segment running from R28 to N32 and joined by a chain reversal from E24 to D27. A second region involves a β -turn from V34 to Y37, which starts a short β -sheet up to G39, followed by a chain reversal up to Q43, which leads to folding of the C-terminal β -sheet segment, i.e. H44–R45, running antiparallel to the short Y37 β -sheet segment. The N-terminal segment up to G18 exists in a multiple bend conformation and is folded on to the V29–V23/R28–N32 β -sheet such that Y10, Y13, Y22 and Y29 are proximal to each other. Structural comparison of rat, murine and human EGFs indicates a number of highly conserved structural features common to at least these species of EGF.

INTRODUCTION

Epidermal growth factor (EGF) is a protein hormone effector molecule that works directly on epidermal and epithelial cells by binding to a transmembrane receptor molecule and becoming internalized in the cell by endocytosis (Carpenter & Cohen, 1976, 1979). Physiological effects of EGF include increased uptake of salts and glucose, increased protein, RNA and DNA synthesis and, ultimately, cell division. Since rat EGF competes with murine EGF and human EGF for binding to the same cellular receptor (Schaudies & Savage, 1986), it can be postulated that a conserved structural receptor-binding domain exists on EGF. The poor reaction of rat EGF with anti-(mouse EGF) antibodies and vice versa (Schaudies & Savage, 1986), however, argues that differences in their structures do exist. Moreover, certain virally transformed tumour cells have been shown to produce an 'EGF-like' substance, called ' α -transforming growth factor' (α TGF), which competes with authentic EGF for EGF receptors (De Larco & Todaro, 1978; Sporn & Todaro, 1980; Roberts *et al.*, 1980; Todaro *et al.*, 1980; Ozanne *et al.*, 1980). These and other observations have stimulated interest in the structural elucidation of growth factors.

To date, n.m.r. structural investigations have been undertaken on murine EGF (Mayo, 1984, 1985; Mayo *et al.*, 1986a; De Marco *et al.*, 1986; Mayo & Burke, 1987; Mayo *et al.*, 1987; Montelione *et al.*, 1986, 1987) and on des-(49–53) human EGF (Carver *et al.*, 1986; Cooke *et al.*, 1987). Since a comparison of EGF structures from various species could help identify a possibly conserved receptor-binding domain, we have chosen to

study rat EGF. Fairly recently the amino acid sequence of rat EGF was reported (Simpson *et al.*, 1985). The molecule consists of a single polypeptide chain of 48 amino acid residues and is devoid of lysine, alanine, phenylalanine and tryptophan. Previously we studied the aromatic residues in rat EGF by proton n.m.r. (Mayo *et al.*, 1986b). In the present study we sequentially assign proton resonances in the rat EGF n.m.r. spectrum and investigate the peptide backbone structure and compare it with that for murine EGF and human EGF.

MATERIALS AND METHODS

Rat epidermal growth factor (EGF) was purified from rat salivary-gland extracts by using the method of Schaudies & Savage (1986). The resulting rat EGF sample gave a single band on gel electrophoresis and was further purified by h.p.l.c. to yield α -rat EGF which was devoid of any N-terminal cleavage products (Simpson *et al.*, 1985). α -rat EGF was used in these experiments.

All h.p.l.c. separations were carried out at 40 °C and a constant flow rate of 2 ml/min. Operating parameters were controlled by using a Varian LC-5500 liquid chromatograph with a Vista Series 400 data processor. Eluted protein was detected at 280 nm by using a Varian UV/VIS multi-wavelength detector. The sample was loaded on to a 15 cm $\times$ 4.6 mm (inside diameter) stainless-steel column packed with silica based C_{18} material (Waters Nova-Pak C_{18} column) equilibrated with a primary solvent of 0.2% trifluoroacetic acid/water (500:1 v/v), pH 2.3. After loading, the chromatogram was developed by running a linear 80 min gradient between 20 and 45 %

Abbreviations used: EGF, epidermal growth factor; DSS, sodium 4,4-dimethyl-4-silapentane sulphonate; n.O.e., nuclear Overhauser enhancement; f.i.d., free induction decay; 2D-n.m.r. COSY, two-dimensional n.m.r. correlated spectroscopy; 2D-n.m.r. NOESY, two-dimensional n.m.r. nuclear Overhauser enhancement spectroscopy; c.i.d.n.p., chemically induced dynamic nuclear polarization; V19 (etc.), valine-19 etc. (amino acid single-letter notation); HOHAHA, homonuclear magnetization transfer (Davis & Bax, 1985); α TGF, α -transforming growth factor.

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acetonitrile/0.2% trifluoroacetic acid in the primary solvent.

Samples for ^1H n.m.r. measurements had been freeze-dried from $^1\text{H}_2\text{O}$ solutions and redissolved in $^2\text{H}_2\text{O}$ or 90% $^1\text{H}_2\text{O}/10\%$ $^2\text{H}_2\text{O}$ immediately before the experiment. The final protein concentration of the h.p.l.c.-purified sample was about 2 mM in approx. 50 mM-potassium phosphate buffer. The p^2H was adjusted by adding microlitre increments of NaO^2H or $^2\text{H}_3\text{PO}_4$ to a 0.4 ml sample. All measurements were done at the p^2H value indicated in the text, read directly from the pH-meter and not adjusted for isotope effects.

500 MHz ^1H n.m.r. spectra were recorded in the Fourier mode on a Bruker AM 500 MHz spectrometer. The solvent deuterium signal was used as the field-frequency lock. All chemical shifts are quoted in parts per million (p.p.m.) downfield from sodium 4,4-dimethyl-4-silapentane sulphonate (DSS).

COSY spectra were filtered for double quantum coherences (Piantini *et al.*, 1982) and NOESY spectra (Jeener *et al.*, 1979) were obtained with mixing times from 50 to 300 ms. The two-dimensional homonuclear magnetization transfer (HOHAHA) spectra, used to identify some spin systems completely, were obtained by spin locking with a MLEV17 sequence (Davis & Bax, 1985) with mixing times of 32 and 64 ms. All spectra were acquired in a phase-sensitive mode with the TPPI method (Marion & Wüthrich, 1984). In most cases suppression of the water resonance was achieved by irradiation at the water frequency during the relaxation delay between the scans. In the case of the NOESY spectra run in $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (9:1, v/v), the third detection pulse was replaced by a semi-selective 1-3-3-1 pulse sequence (Turner, 1983; Hore, 1983*a,b*) for water suppression. The 2D-n.m.r. data were transferred to a microVAX II computer and processing was done with the '2DNMR' software written in FORTRAN 77. All spectra were baseline-corrected in the Ω_1 domain and the NOESY spectra in $^1\text{H}_2\text{O}$ in the Ω_2 domain as well (Boelens *et al.*, 1985).

RESULTS

Sequential resonance assignments

The first step in sequential resonance assignments is the identification of spin systems associated with individual amino acid residues. Most of these spin systems for rat EGF were identified by using COSY and HOHAHA data recorded in $^1\text{H}_2\text{O}$ or in $^2\text{H}_2\text{O}$. Fig. 1 presents typical HOHAHA data taken at 303 K in $^1\text{H}_2\text{O}$. Many non-overlapping spin systems could be easily recognized. Some ambiguities were removed by analysing data on the same sample dissolved in $^2\text{H}_2\text{O}$, since only 17 amide protons are long-lived, which greatly simplifies the $\alpha\text{CH-NH}$ backbone fingerprint region. Other ambiguities were removed by recording data at 310 K; resonances belonging to one spin system usually shifted slightly differently with increasing temperature than those of an overlapping one. Supplementary information from COSY data sets generally resolved other spin system assignment ambiguities.

Spin systems for six glycine residues, five valine residues, two leucine residues, two isoleucine residues, and one threonine residue were thereby uniquely identified from HOHAHA and COSY spectra. Some of these, for example, are identified in Fig. 1, with continuous lines

connecting cross-peaks. The remaining identified spin systems were grouped as *AMX* spin systems for residues containing an $\alpha\text{CH-}\beta\text{CH}_2$ fragment (i.e., Asn, Ser, Cys, Tyr, Asp, His) or *long* side-chain spin systems in which further scalar coupling to more peripheral protons was observed (i.e. Gln, Glu, Met, Arg). Since there are no lysine residues present in rat EGF, three of the four arginine spin systems were uniquely identified in the HOHAHA data from their ϵNH resonance frequencies. With these combined data, all but four of the 48 possible spin systems were at least partially identified; many of the $\alpha\text{CH-NH}$ cross-peaks have been labelled in Fig. 1. Table 1 lists all known spin system assignments.

Sequential assignments were then made by combining information from HOHAHA or COSY and NOESY spectra of rat EGF dissolved in $^1\text{H}_2\text{O}$ under the same conditions. Fig. 2 shows the composite $\alpha\text{CH-NH}$ fingerprint regions from these respective data sets. Beginning with the uniquely identified spin systems mentioned above, sequence-specific connections were identified from the NOESY data for several unique peptide sequences. Two of these sequences are connected in Fig. 2; only NOESY cross-peaks have been labelled. Beginning with the valine spin system later assigned to V26, we have a pentapeptide sequence, HN-Val-*AMX*-Arg-*AMX*-Val-COOH, and beginning with the glycine spin system later identified as G17 we have the pentapeptide sequence HN-Gly-Gly-Val-*AMX*-long-COOH. By inspecting the known amino acid sequence for rat EGF (Simpson *et al.*, 1985), both these pentapeptide sequences could be unambiguously assigned to sequences V26-V30 and G17-M21 respectively. Other such sequences that were likewise identified include T4-C6, S9-G12, V34-I38, and R41-R45. From these starting points the network of $d_{\alpha\text{N}}$, d_{NN} - and $d_{\beta\text{N}}$ -type sequential n.O.e.'s was followed to supplement the sequential resonance assignments. These sequential assignments are given in Table 1.

The aromatic spin systems of four of the five tyrosine residues were connected to their associated *AMX* spin systems by strong $\beta\text{CH-NH}$ n.O.e.'s (Billeter *et al.*, 1982). This information also provided an independent check on the validity of the sequential resonance assignments in the polypeptide segments containing these aromatic residues.

Secondary and tertiary structure

Having completed most of the sequential resonance assignments, we proceeded to identify regular backbone structures in rat EGF. In particular, short backbone-proton distances were first identified by NOESY; these are summarized in Fig. 3. Several sequences, i.e. N3-P7, S9-G12, C14-N16, G18-Y22, D27-I35, Y37-G39 and H44-D46, of short $d_{\alpha\text{N}}$ distances, were recognized. In places where d_{NN} -type cross-peaks were absent or very weak, i.e. N3-G5, C14-L15, G18-Y22, R28-V34, Y37-G39 and H44-D46, extended backbone conformations are highly probable (Wüthrich, 1986). $J_{\text{HN}\alpha}$ coupling constants greater than 8 Hz for many of these amides rendered additional support for extended backbone conformations (results not shown).

The *N*-terminal segment from about C6 through G18 seems to be conformed in a multiple bend conformation, as suggested by short $d_{\alpha\text{N}}$ distances interspersed with short d_{NN} distances (Wüthrich, 1986). β -Turns, therefore, probably exist at about P8-S9, D11-G12 and N16-G17. Further chain reversals are suggested at S25-D27,

Fig. 1. Spin-lock HOHAHA 2D-n.m.r. contour plot

The amide- α CH region of a contour plot is shown for rat EGF. The sample concentration was about 2 mM in 50 mM-potassium phosphate, pH 3.1 in $^1\text{H}_2\text{O}$, whose resonance was suppressed by selective saturation for 2 s before the preparation pulse. The data set was collected as 400 f.i.d.s. containing 2K words and was processed on Digital micro VAX II computer with software written in FORTRAN 77. Labelling of resonances is as described in the text.

Table 1. ^1H chemical shifts for rat EGF at 303 K and pH 3.1

Residue	Chemical shift (p.p.m.)			
	NH	αCH	βCH	Others
1. Asn	—	—	—	—
2. Ser	—	—	—	—
3. Asn	8.92	5.04	2.96, 2.85	—
4. Thr	8.42	4.60	4.52	—
5. Gly	8.39	4.00, 4.30	—	—
6. Cys	8.65	4.52	3.08, 2.75	—
7. Pro	—	4.77	2.42, 2.07	γCH_2 1.93, 1.85 δCH_2 3.32, 2.72
8. Pro	—	4.29	2.41, 1.94	γCH_2 2.12, 2.41 δCH_2 3.84, 3.77
9. Ser	7.88	4.07	3.81, 3.81	—
10. Tyr	8.22	4.16	3.29, 2.67	2,6H 6.48; 3,5H 6.02
11. Asp	7.98	4.48	2.89	—
12. Gly	8.80	4.00, 3.92	—	—
13. Tyr	7.95	4.16	3.13, 3.06	2,6H 7.11; 3,5H 6.83
14. Cys	8.80	4.38	2.62, 2.28	—
15. Leu	8.13	4.32	1.60, 1.30	γCH 1.60 δCH_3 0.73
16. Asn	8.98	4.05	1.98, 1.36	—
17. Gly	8.72	4.03, 3.62	—	—
18. Gly	7.46	4.34, 3.42	—	—
19. Val	8.08	4.38	2.05	γCH_3 1.04
20. Cys	8.90	5.07	3.37, 3.18	—
21. Met	9.64	4.93	1.99	γCH_3 2.48
22. Tyr	8.77	4.75	2.75	2,6H 6.28; 3,5H 6.38
23. Val	6.57	3.94	1.83	γCH_3 0.88, 0.80
24. Glu	8.28	3.45	2.04	γCH_2 2.55, 2.47
25. Ser	—	—	—	—
26. Val	6.59	4.48	2.30	γCH_3 0.85, 0.68
27. Asp	9.52	3.96	3.25, 2.82	—
28. Arg	7.01	4.65	1.42, 1.72	γCH_2 1.57 δCH_2 3.15 ϵNH 7.18
29. Tyr	8.22	5.11	2.36, 2.43	2,6H 6.97; 3,5H 6.47
30. Val	9.29	4.83	2.12	γCH_3 0.88, 0.78
31. Cys	8.87	4.75	2.81, 2.60	—
32. Asn	9.48	5.02	2.98, 2.80	—
33. Cys	8.90	4.75	2.69	—
34. Val	8.41	4.00	3.87	γCH_3 1.13, 1.04
35. Ile	8.05	3.95	1.85	γCH_2 1.66, 1.32 γCH_3 0.96 δCH_3 0.95
36. Gly	8.58	4.28, 3.53	—	—
37. Tyr	8.31	5.29	3.03, 2.88	2,6H 6.88; 3,5H 6.66
38. Ile	9.13	4.82	1.98	γCH_2 1.36, 0.96 γCH_3 0.86 δCH_3 0.84
39. Gly	8.08	4.88, 3.90	—	—
40. Glu	9.40	4.20	2.25	γCH_2 2.59
41. Arg	8.31	4.70	2.32, 1.24	γCH_2 1.93, 1.41 δCH_2 2.92, 2.74 ϵNH 7.72
42. Cys	7.84	3.89	3.59, 3.15	—
43. Gln	9.51	3.95	2.01, 1.66	γCH_2 2.35, 1.62
44. His	8.72	5.25	3.21, 3.05	—
45. Arg	8.83	4.33	1.60, 1.72	γCH_2 1.39 δCH_2 2.96 ϵNH 7.03
46. Asp	8.42	4.61	2.61, 2.40	—
47. Leu	7.99	4.33	1.56	γCH 1.56 δCH_3 0.89, 0.83
48. Arg	7.99	4.21	1.73, 1.87	γCH_2 1.39 δCH_2 2.96 ϵNH 7.28

Fig. 5. Secondary and tertiary structures in rat EGF

Anti-parallel β -sheet V19–V23/R28–N32 segment, and double-hairpin region of V34–I38 with the C-terminus are shown. Some of the slowly exchanging amide protons are shown here, and postulated interstrand hydrogen bonds are indicated by wavy lines ($\sim$). Double-headed lines between pairs of hydrogen atoms give some of the inter-proton n.O.e.'s observed in NOESY experiments.

and M21 and between G5 and M21 were also observed, suggesting an *N*-terminal interaction, i.e., at least residues T4 and G5, with the V19–V23 β -sheet. This folding pattern is depicted in Fig. 5 with the phrase '*N*-Terminus interaction domain'.

Further aspects of backbone chain folding are suggested by cross-peaks in the NOESY spectrum (Fig. 4) regarding tyrosine–tyrosine inter-residue n.O.e.'s. From these data it can be deduced that Y10 is proximal to Y13, which supports the previously mentioned β -bend structure at D11–G12. An n.O.e. between Y29 (2,6) and Y10 (3,5) proton resonances suggests proximity of the *N*-terminal multiple bend segment with β -sheet segment R28–N32; this is not too surprising, since cystine disulphide bonds, i.e., C6–C20 and C14–C31, limit the number of possible accessibly allowed conformations between these two domains. Furthermore n.O.e.'s

among Y22 and Y29 ring proton resonances render confirming evidence for the β -sheet structure shown in Fig. 5 and indicate that both Y22 and Y29 are positioned on the same side of this β -sheet domain.

DISCUSSION

Nearly complete proton resonance assignments for rat EGF in solution at pH 3.1 and 303 K have been reported here. Furthermore, the sequence-specific ^1H -n.m.r. assignments are in agreement with the chemically determined amino acid sequence (Simpson *et al.*, 1985). These resonance assignments provide the basis for the structural analysis presented. From this analysis, two structural domains have been derived: (1) an *N*-terminal domain formed by folding multiple bend *N*-terminal

residues 1–18 on to a section of anti-parallel β -sheet, i.e. residues 19–23 and 28–32, and (2) a C-terminal domain formed by folding V34 via a β -bend at I35–G36 on to a short β -sheet stretch, Y37–I38, which is in turn folded on to another short β -sheet segment from H44 to D46 via a chain reversal from G39 to Q43.

Since mouse, human and rat EGF can compete for binding to the same cell receptors in at least some cell lines (Schaudies & Savage, 1986), similarly spatially oriented surface amino acid residues are probably present in these three EGF species. This, of course, leads one to seek similar structural features. As mentioned in the Introduction, solution structure information is currently available on the mouse and human species. At the level of present structural resolution of these EGF species, only the gross backbone conformations can be compared. However, even this initial comparison shows considerable secondary and tertiary structural similarity among these EGFs. Given primary structural similarities and the conserved positions of the three disulphide bonds, this observation might have been expected. Comparatively speaking, continuous segments of short $d_{\alpha N}$ distances suggest extended backbone conformations for residues 14–15, 18 (or 19)–23, 28–34, 37–39 and 44– (at least) 47. The N-terminus up to about residue 18 probably exists in a multiple bend structure, as evidenced by alternating short $d_{\alpha N}$ and d_{NN} distances. Chain reversals are indicated between residues 23–28, 34–37 and 39–43, where segments show continuous short d_{NN} distances and $d_{\alpha N}$ distances are large.

Totally conserved residues among rat, mouse and human EGF included G18, V19, M21, E24, D27, Y29, C31, N32, C33, V34, G36, Y37, G39, R41, C42, Q43, R45, D46 and L47. Position 35 is always an isoleucine or a valine residue, and position 40 is always a glutamic acid or an aspartic acid residue; these residues might, therefore, also be added to this group. Interestingly, most of these residues do form a conserved structural domain which is constrained by the C33–C42 disulphide bridge. The short anti-parallel β -sheet segment, V19–M21 and C31–N32, is, moreover, also conserved, and is structurally proximal to the C33–C42 double hairpin segment.

The N-terminus interacts weakly with the β -sheet segment containing residues 19–23 and is folded such that the Tyr-10/Tyr-13 segment is proximal to the Tyr-29 β -sheet segment. In at least the mouse, rat and human species of EGF, the N- and C-termini seem to be relatively highly mobile, whereas the central portion of the peptide, i.e., from residue 15 to about 44, seems to be rigidly constrained by the presence of three disulphide bonds and hydrogen-bonded anti-parallel β -sheet structure. This concept is supported by the presence of 12 (human) (Carver *et al.*, 1986), 13 (mouse) (Mayo, 1985), and 17 (rat) long-lived hydrogen-bonded amide protons which are found within the central portion of any of these EGFs.

EGFs belong to a family of mitogenic peptides which include the α TGFs (Derynck *et al.*, 1984). Primary structural similarities have been noted among EGFs and α TGFs from different species, especially in the C-terminal domain, i.e. residues 33–47. Synthetic fragments of the 'C-loop' from these peptides, i.e. C33–C42, exhibit weak binding affinity for EGF receptors (Nestor *et al.*, 1985). Interestingly, interaction studies of EGF with amphiphatic micelles show that the C-terminal domain interacts preferentially at the micellar surface (Mayo

et al., 1987). Although micelles are far removed from real receptor membranes, and synthetic fragment activity studies could reflect either coincidental or incomplete receptor interactions, these observations may act to guide future experimentation in EGF/ α TGF protein–receptor interaction studies.

This work was supported by a grant from the National Institutes of Health (U.S.A.) (no. GM-34662) and from a generous gift from the Glenmede Trust Fund to Temple University, and benefitted from n.m.r. facilities made available to Yale University through grant no. CH3-7916210 from the National Science Foundation. This work was also supported by a grant from the North Atlantic Treaty Organization (NATO) Scientific Affairs Division (RG.85/0424). R.C.C. received a Swern Graduate Research Fellowship in memory of the late Professor Swern; this work is in partial fulfilment of the Ph.D. requirements in Chemistry for R.C.C.

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Received 13 June 1988; accepted 27 July 1988