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Tumour necrosis factor is a compact trimer

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Recombinant produced human tumour necrosis factor (TNF) has been studied to characterise the subunit structure of the protein. TNF is shown to be a trimer M_r 52000 in which the subunits are associated in a compact, triangular form. In secondary structure it belongs to the all-β class of proteins. It has high thermodynamic stability and the unfolded subunits can fold and associate spontaneously to form native, biologically active TNF.

Tumour necrosis factor; Molecular mass; Subunit structure; Secondary structure; Thermodynamic stability

1. INTRODUCTION

Tumour necrosis factor can be produced in serum by treatment with lipopolysaccharide of an animal previously primed with materials such as BCG or zymosan [1]. Its ability to bring about regression of tumours in mice makes this protein a focus of attention. Human TNF has been expressed in *E. coli* from genetic material derived from cell lines [2,3] and this has opened the door to characterisation of structure-function relationships. We have clarified the uncertainty in the literature concerning the polymeric nature of the smallest form of TNF present at concentrations as low as 50 $ng \cdot ml^{-1}$ and investigated its conformation and assembly.

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Abbreviations: TNF, tumour necrosis factor; Gdn·HCl, guanidine HCl; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of SDS; $\Delta G'_{H_2O}$, apparent free energy of stabilization in the absence of denaturant; CD, circular dichroism; UV, ultraviolet

2. MATERIALS AND METHODS

Chemicals were analar grade (BDH, Poole, England). Tris was from Sigma and Ultrapure Gdn·HCl from BRL. The buffer used in the experiments was 0.01 M Tris, 0.09 M NaCl, 1 mM disodium EDTA, adjusted to pH 8.5 with HCl unless otherwise described in the text.

Recombinant TNF was supplied by Biogen Res. Corp. (Cambridge, USA). It was assayed as described [3].

2.1. *Circular dichroism*

Circular dichroism spectra were measured using a Jobin-Yvon dichrograph IV linked to a BBC microcomputer for recording data. Spectra are averages of 4–10 scans with the baseline subtracted. Protein concentrations were estimated using a calculated value of $A^{1\%}_{1cm} = 12.7$ at 278 nm. All solutions were filtered (0.22 μm, Millipore GVWP filters) before use.

Additions of Gdn·HCl were made from a concentrated stock solution. The equilibrium unfolding data were analysed using the linear extrapolation method [4]. A plot of $\ln K_{eq}$ versus [Gdn·HCl] was extrapolated to [Gdn·HCl] = 0 to obtain a value of the apparent free energy change

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for the transition at zero concentration of denaturant, $\Delta G'_{H_2O}$. $\ln K'_{eq}$ was plotted against ln[Gdn·HCl] to obtain C_m, the midpoint of the transition where $\ln K'_{eq} = 0$ and the slope, n, which is a measure of the cooperativity of the transition.

Unfolding and refolding kinetics were measured as described in the legends.

2.2. *Absorbance*

Absorbance spectra and protein concentrations were measured using a Cary 210 spectrophotometer with thermostatted cell holders.

2.3. *Fluorescence*

Fluorescence measurements were made using a Perkin Elmer MPF3 spectrofluorimeter with thermostatted cell holder. All solutions were filtered (0.22 μm, Millipore) before use.

2.4. *Analytical ultracentrifugation*

Sedimentation equilibrium and sedimentation velocity measurements were made as described [5].

2.5. *Urea-gradient polyacrylamide gel electrophoresis*

Urea-gradient polyacrylamide gel electrophoresis was carried out in a BRL model V-16-2 vertical gel electrophoresis apparatus using 19.5 × 16.0 cm gel plates with 1.5 mm thick spacers. The electrophoresis buffer was 50 mM Tris-glycine, pH 9.2. Preparation of the urea gradient (0–8 M) and acrylamide gradient (15–11%) and conditions of polymerization were carried out according to Goldenberg and Creighton [6].

3. RESULTS AND DISCUSSION

3.1. *Molecular structure of TNF*

The relative molecular mass of human TNF as measured by SDS-PAGE in the absence or presence of reducing agent was shown to be 17 kDa in keeping with the DNA-derived sequence value of 17356 Da [3]. Molecular masses measured by sedimentation equilibrium at starting protein concentrations of 0.125 mg·ml^{-1} and 0.48 mg·ml^{-1} were 49.6 and 50.4 kDa, respectively. The linearity of the plots of protein distribution (fig.1) over a total range of 0.02–0.8 mg·ml^{-1} show that there is no dissociation or further association of TNF over this range of concentration. The

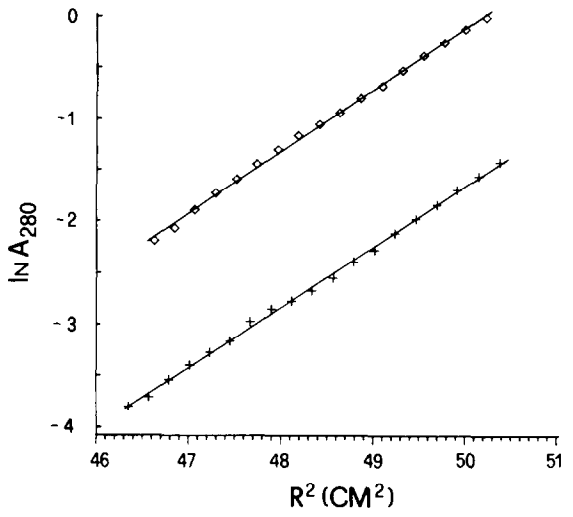
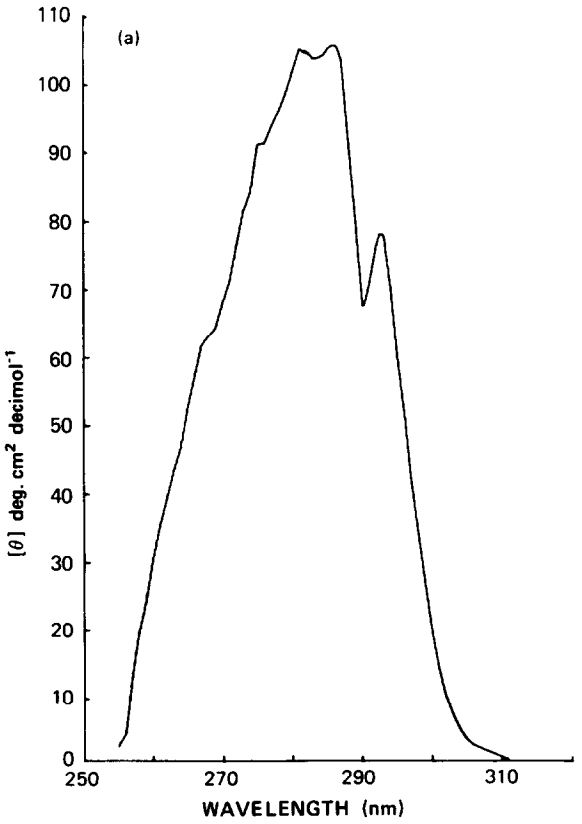


Fig.1. Sedimentation equilibrium of recombinant human TNF. The logarithm of absorbance at 280 nm ($\ln A_{280}$) is plotted as a function of the square of the distance in cm from the axis of rotation (r^2). The slopes were evaluated from the least squares fit of the data. Plots were derived from TNF in 50 mM Tris-HCl, pH 7.5, 1% (w/v) KCl at starting concentrations of 0.125 (×) and 0.48 (◇) mg·ml^{-1}, respectively. Sedimentation equilibrium was established by centrifugation at 14000 rpm for 18 h at 20°C.

molecular mass of the murine TNF was similarly shown to be 50 kDa. These results show that TNF is a trimer held together by non-covalent interactions.

The mode of association of the subunits will determine the flow properties of the trimer in solution, the two extreme configurations being a linear and a planar, triangular association, respectively. Experimental values of the geometric factor for subunit arrangement were calculated from the hydrodynamic properties of TNF (table 1) by the method of Teller et al. [7] and compared with predicted values. These results lead to the proposal that TNF is associated with two inter subunit contacts for each subunit.

The near UV CD spectrum of TNF (fig.2a) exhibits an intense positive ellipticity with a double peak at 280.5 and 285.5 nm and a minor peak at 292.5 nm. These reflect the asymmetric environment of one or both of the two tryptophan residues together with a contribution from tyrosine residues. The CD spectrum in the range



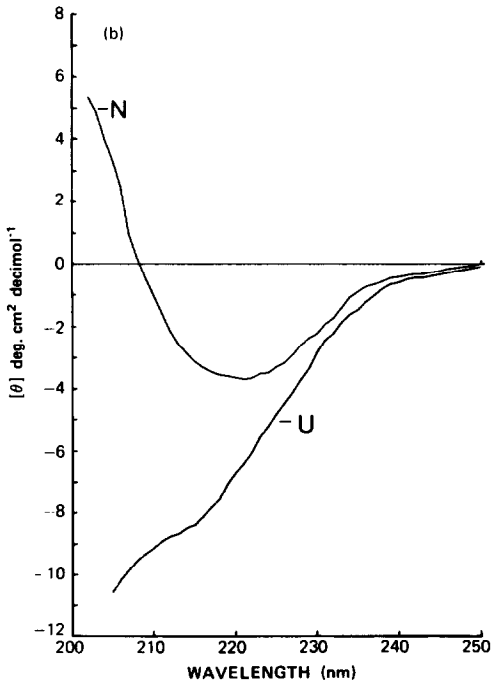


Table 1

Hydrodynamic properties of recombinant human TNF

Parameter	Value
v (ml·g^{-1})	0.729
s^0	4.13 S
r_s cm × 10^8 (a)	3.01
r_s cm × 10^8 (b)	2.70
r_s cm × 10^8 (c)	2.47 (2.81)
f/f_0 (d)	1.22 (1.07)
F_n (e)	0.967 (0.956, 0.864)

(a) Stokes' radius estimated from M_r of 52000, $s_{20,w}$ of 4.13 S and v of 0.729 ml·g^{-1} according to the Svedberg equation [18]. (b) Stokes' radius estimated from gel permeation chromatography on LKB Ultrogel AcA54. (c) Stokes' radius predicted for an anhydrous sphere or a hydrated sphere (value in parentheses) of M_r of 52000 and v = 0.729 ml·g^{-1}. A hydration value of 0.350 g H_2O/g protein was calculated from the amino acid composition [19]. (d) Frictional coefficient calculated using f_0 values calculated for an anhydrous sphere or a hydrated sphere (value in parentheses). (e) Geometric factor for subunit arrangement calculated according to the method of Teller et al. [7]. The values in parentheses are the predicted values for a planar triangular trimer and a linear trimer, respectively

200–250 nm (fig.2b) is of relatively low intensity with a trough centred at 221 nm. Analysis of the spectrum in terms of secondary structure [8] gives 3% helix and 45% β-structure placing TNF, like interleukin 1 [9], in the all-β class of proteins [10].

Human TNF contains two cysteine residues which form a disulphide bond as shown by reaction with 2-nitro-5-thiobenzoic acid [11]. This bond can readily be cleaved under non-denaturing conditions and the resultant sulphydryl groups alkylated with iodoacetamide. The product has M_r = 50000, $s_{20,w}$ = 4.0 S and biological activity similar to that of native TNF. The disulphide does

Fig.2. Circular dichroism spectra of TNF. The spectra are an average of 4 (near UV) and 8 (far UV) scans with the baseline subtracted. Protein concentration 0.87 mg/ml in 0.01 M Tris, 0.09 M NaCl, 1 mM disodium EDTA, pH 8.5. Spectra were recorded at 20°C using 1 cm and 0.01 cm pathlength cells at 1 nm and 2 nm bandwidths for near and far UV regions, respectively. The far UV spectrum is shown for native TNF (N) and for TNF unfolded (U) in 4 M Gdm·HCl.

not therefore appear to play an important role in stability of subunit association or activity.

3.2. *Subunit interactions*

On urea-gradient electrophoresis, TNF shows a single transition between 0 and 8 M urea which occurs at ~3.8 M urea at pH 9 and 8°C. The transition is continuous and independent of whether TNF is applied in zero or 8 M urea (fig.3). Under these conditions therefore TNF unfolds reversibly and rapidly relative to the time of electrophoresis. The thermodynamic stability was estimated [12] under these conditions as being 26 kJ·mol^{-1}.

Initial attempts to study the unfolding equilibria in free solution showed that at 25°C unfolded TNF in 5 M Gdn·HCl aggregates significantly so that refolding after 60 s in that solvent results in only 50–60% yield of native protein. Yields of 80–90% can be obtained however when TNF is unfolded in high concentrations of Gdn·HCl at 6°C over the pH range 5.9–9.5. Samples which refolded at pH 8.5 were active on bioassay. The reversible unfolding transitions at pH 8.5 (fig.4) measured by near and far UV ellipticity at 11°C are superimposable. This fact and the high cooperativity (n = dln K'/dln[Gdn·HCl] = 17.6) support the assumption of a 2-state transition and strong interaction of the subunits. The thermodynamic stability of the native protein relative to the fully unfolded state is 40.6 ± 3.6 kJ·mol^{-1} based on results from both near and far UV CD. The midpoint of the transition under these conditions occurs at C_m = 1.14 M Gdn·HCl.

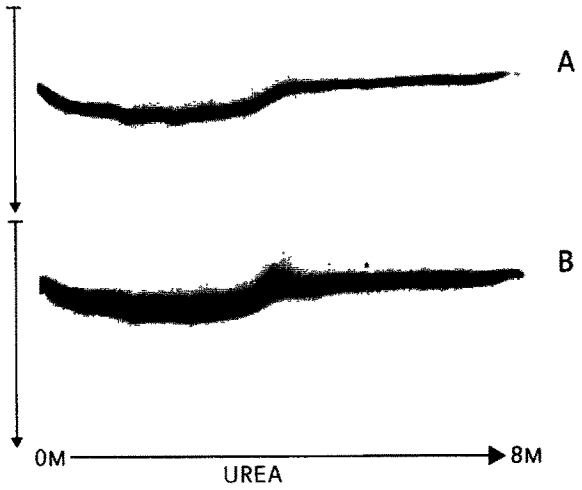


Fig.3. Urea-gradient electrophoresis of recombinant human TNF. The buffer for electrophoresis and sample application was 50 mM Tris-glycine, pH 9.2. Electrophoresis was towards the anode at 6.5 V·cm^{-1} for 15 h at 8°C. (A) Native TNF (150 μg) and (B) native TNF (150 μg) migrated into the gel before application of unfolded TNF (150 μg) dissolved in 8 M urea

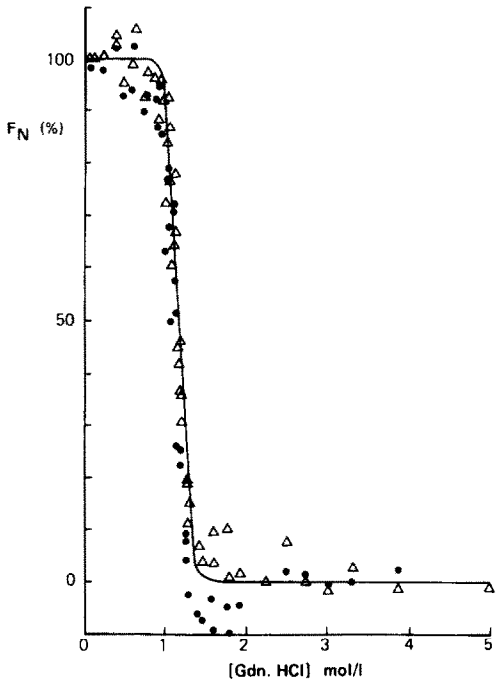


Fig.4. Reversible unfolding transition for TNF. The unfolding and refolding were monitored by ellipticity at 285.5 nm (●), 292.5 nm (△) and 208 nm (solid line). Protein solutions in 0.01 M Tris, 0.09 M NaCl, 1 mM disodium EDTA, pH 8.5, were titrated with small weighed additions of a concentrated stock solution of Gdn·HCl made up in the same buffer to give final protein concentrations of 0.34–0.82 mg/ml.

This transition was further characterised by sedimentation experiments carried out under the same conditions. At zero and 0.5 M Gdn·HCl sedimentation coefficients were 4.0 S and 3.8 S, respectively, and at 4 M Gdn·HCl, $s_{20,w}$ = 1.0 S. At intermediate concentrations within the transition high s values were obtained indicating aggregation. Similar experiments carried out in urea solutions showed similar phenomena, with a transition occurring between 4 and 5 M urea. Sedimentation equilibrium in 6 M urea led to a convex plot of ln$c$ vs r^2 indicating non-ideality. The estimated

value of M_r = 16000 was obtained using a partial specific volume v = 0.712 ml·g^{-1} [13].

These results show that the single denaturation transition is between folded trimer and unfolded monomer with no intermediate species other than artifactual aggregates. The trimer is stable over a wide pH range, there being no change in sedimentation coefficient between pH 4.8 and 10.5. Measurements of fluorescence show that TNF is denatured on lowering the pH with a pK of approx. 4.3 leading to a product shown by sedimentation experiments to be moderately aggregated. This is consistent with the irreversible loss of TNF found on dialysis for 24 h at pH values below 5.5 [14].

The strength of the interaction between the monomers is supported by a gel filtration experiment in which TNF was radiolabelled during biosynthesis. At an initial concentration of 200 ng·ml^{-1} this eluted at a volume identical to that for unlabelled TNF at higher concentrations (7 mg·ml^{-1}) showing that no dissociation of the trimer could be detected at the lower concentration.

3.3. *Conclusions*

There is a species variation in the extent of TNF glycosylation, for example, murine TNF appears in nature to be a glycoprotein [14] whereas there is no evidence for the glycosylation of human TNF. Both human and murine recombinant TNF have been shown to be trimers so that glycosylation does not appear to affect the degree of polymerisation. The recombinant protein studied here has identical chemical composition to authentic human TNF and therefore reflects properties of the authentic molecules. Existing uncertainties concerning the subunit structure, e.g. [15], have been resolved by the determination of an absolute molecular mass using equilibrium sedimentation which shows beyond doubt that TNF is a trimer of 17 kDa polypeptides. The significance of higher molecular mass species with TNF activity, e.g. [16], has yet to be clarified. The subunits are associated strongly in a compact form, showing no sign of dissociation at low concentrations. The bioassay however is carried out in the picogram concentration range, probably similar to levels in serum, so it has yet to be established conclusively whether the monomer or the trimer is active. However, the local concentration of the protein at the region of the cell surface receptor in vivo may be significantly higher than the normal serum levels. The demonstrated strong tendency to associate would be important if the TNF receptor is activated by being cross linked by the ligand, as proposed for the IgE stimulation of mast cells [17] and for stimulation of the primary immune response [18].

The protein, which is of the all-β type, can be dissociated into fully unfolded subunits and will then refold spontaneously into the active, native conformation. The 17 kDa polypeptide thus contains the information required for folding to the species which associates specifically to give biologically active TNF. This shows that the unusually long 76 residue presequence is not required for folding as in proinsulin [19] or pepsinogen [20] but is conformationally independent and presumably therefore has a separate biochemical function in secretion or targetting.

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REFERENCES

[1] Old, L.J. (1985) Science 230, 630–632.
[2] Pennica, D., Nedwin, G.E., Hayflick, J.S., Seeburg, P.H., Derynck, R., Palladino, M.A., Kohr, W.J., Aggarwal, B.B. and Goeddel, D.V. (1984) Nature 312, 724–729.
[3] Marnenout, A., Fransen, L., Tavernier, J., Van der Heyden, J., Tizard, R., Kawashima, E., Shaw, A., Johnson, M.-J., Seman, D., Mueller, R., Ruysschaert, M.-R., Van Vliet, A. and Fiers, W. (1985) Eur. J. Biochem. 152, 515–522.
[4] Pace, N.C. (1975) CRC Crit. Rev. Biochem. 3, 1–43.
[5] Wingfield, P., Payton, M., Tavernier, J., Barnes, M., Shaw, A., Rose, K., Simona, M.G., Demczuk, S., Williamson, K. and Dayer, J.-M. (1986) Eur. J. Biochem., in press.
[6] Goldenberg, D.P. and Creighton, T.E. (1984) Anal. Biochem. 138, 1–18.

[7] Teller, D.C., Swanson, E. and De Haen, C. (1979) Methods Enzymol. 61, 103–124.
[8] Provencher, S.W. (1982) Comput. Phys. Commun. 27, 229–242.
[9] Craig, S., Schmeissner, U., Wingfield, P. and Pain, R.H., submitted.
[10] Levitt, M. and Chothia, C. (1976) Nature 261, 552–558.
[11] Thannhauser, T.W., Konishi, Y. and Scheraga, H.A. (1984) Anal. Biochem. 138, 181–188.
[12] Hollecker, M. and Creighton, T.E. (1982) Biochim. Biophys. Acta 701, 395–404.
[13] Prakash, V. and Timasheff, S.N. (1981) Anal. Biochem. 117, 330–335.
[14] Haranaka, K., Carswell, E.A., Williamson, B.D., Prendergast, J.S., Satomi, N. and Old, L. (1986) Proc. Natl. Acad. Sci. USA 83, 3949–3953.
[15] Aggarwal, B.B., Kohr, W.J., Hass, P.E., Moffat, B., Spencer, S.A., Henzel, W.J., Bringman, T.S., Nedwin, G.E., Goeddel, D.V. and Harkins, R.N. (1985) J. Biol. Chem. 260, 2345–2354.
[16] Kull, F.C. and Cuatrecasas, P. (1981) J. Immunol. 126, 1279–1283.
[17] Maeyama, K., Hohman, R.J., Metzger, H. and Beaven, M.A. (1986) J. Biol. Chem. 261, 2583–2592.
[18] Vogelstein, B., Dintzis, R.Z. and Dintzis, H.M. (1982) Proc. Natl. Acad. Sci. USA 79, 395–399.
[19] Anfinsen, C.B. (1966) Harvey Lect. 61, 95–116.
[20] Ahmad, F. and McPhie, P. (1978) Int. J. Pept. Protein Res. 12, 155–163.
[21] Van Holde, K.E. (1975) The Proteins (3rd Edn) 1, 226–287.
[22] Kuntz, I.D. (1971) J. Am. Chem. Soc. 93, 514–516.