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REVERSIBLE SUBUNIT DISSOCIATION OF TUMOR NECROSIS FACTOR DURING HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

Hydrophobic interaction chromatography (HIC) of recombinant tumor necrosis factor (TNF) results in reversible dissociation of the quaternary protein structure yielding separation of trimer and monomer peaks. Gel electrophoresis, size exclusion, fluorescence polarization and rechromatography were used to identify the trimeric and monomeric species. Relative amounts of these peaks varied as a function of temperature and column contact time. When TNF was chromatographed in the presence of partially proteolyzed [14 kilodalton (kD)] TNF, two additional peaks, identified as the 14-kD monomer and heterotrimer of TNF and the 14-kD fragment, appeared. Rechromatography of this heterotrimer produced TNF monomer and 14-kD peaks establishing that the multiple peak pattern in HIC was due to quaternary dissociation. Incubating TNF in denaturants prior to non-denaturing size-exclusion chromatography resulted in apparent protein unfolding. However, free, undenatured monomer was not observed. We conclude that TNF is most likely a trimer, which is tightly held together by hydrophobic forces, and that the tertiary structure of the monomer is stabilized through this subunit association. The hydrophobicity of the sorbent surface mediates reversible dissociation of the trimers to monomers through hydrophobic stabilization of the monomeric tertiary structure. After elution, the TNF monomers reassociate to form the native TNF trimer.

INTRODUCTION

In the past few years there have been several studies documenting the role of the reversed-phase and hydrophobic interaction high-performance liquid chromatography (HPLC) columns in catalyzing the denaturation of proteins during chromatography[1–5]. The general phenomenon occurs when two peaks are eluted from a column, an early-eluted peak of native conformation and a later-eluted peak of denatured conformation. Biological activity, second-derivative UV spectroscopy, fluorescence spectroscopy, and circular dichroism spectroscopy have been used to identify the native and denatured tertiary protein structures[3–7]. Typically, on-column de-

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naturation of proteins could be increased by increasing the temperature or the on-column incubation time[1–6]. Lu *et al.*[8] dramatically demonstrated the role of the column surface by using fluorescence spectroscopy to demonstrate that lysozyme undergoes tertiary structural changes while adsorbed to a reversed-phase-HPLC (RP-HPLC) column. These structural changes were reversed during the elution of the protein. Although RP-HPLC sorption often leads to irreversible tertiary protein denaturation, several examples have been studied where the later-eluted, denatured peak can later refold in solution to regenerate the native, biologically active early peak[3,4,7]. With these proteins, rechromatography of either the native or denatured peaks yields an equilibrium mixture of native and denatured species, indicating reversibility of the denaturation process.

Several examples of similar two-peak separations have also been observed in the hydrophobic-interaction mode of protein purification[5–7,9]. Since hydrophobic interaction chromatography (HIC) is much less hydrophobic in its eluent composition and column surface composition, it is inherently less denaturing than RP-HPLC. It is no surprise then that reversible tertiary protein denaturation can be mediated through sorption on the HIC column surface[7]. A different form of protein denaturation, subunit aggregation, a quaternary structural change, has been shown to be catalyzed through protein sorption on an HIC sorbent surface[10]. The on-column aggregation of α-lactalbumin was shown to be a reversible process, which was dependent upon protein concentration, and resulted in the elution of several peaks, oligomeric in composition. During the HIC of proteins, on-line spectral changes have been used to characterize conformational changes in protein structure[4]. These conformational changes (increased conformational heterogeneity) were correlated to increases in peak retention time and peak bandwidth with increasing temperature. Such HIC elution characteristics, increasing retention time and peak bandwidth with increasing temperature, are pervasive throughout the HIC literature and are an indication that proteins share a common hydrophobic retention mechanism during HIC[5,11].

Tumor necrosis factor (TNF), also known as cachectin, is capable of selectively causing necrosis of tumor cells[12]. It may also be responsible for cachexia (wasting of the body) in response to infectious or neoplastic disease. It is currently in clinical trials for the treatment of cancer. Two recent review articles describe the isolation, structure, and function of this protein[13,14]. Chromatography of TNF on a HIC column resulted in a chromatogram that contained four peaks from a sample that was expected to be homogeneous. This unexpected result gave impetus to the analytical work described in this paper. Chromatographic variables, including flow-rate, temperature, and column contact time, affected the relative amounts of the peaks observed in HIC profiles of TNF. During the course of this work, several publications have appeared, characterizing TNF as a protein of trimeric quaternary structure[15–17]. Given the multiple peak chromatograms observed for TNF in HIC, we investigated the possibility of quaternary dissociation as an explanation of the apparent chromatographic anomalies.

MATERIALS AND METHODS

TNF samples were obtained from internal sources at Cetus (Emeryville, CA,

U.S.A.). The cloning and purification of the TNF used has been described by Cetus scientists[18–21]. N-terminal sequencing, amino acid analysis, and peptide digests have been used to identify unambiguously the primary structure of TNF and the 14-kilodalton (kD) fragment, while gel electrophoresis has been used to establish the purity of the protein.

HPLC equipment

Two gradient liquid chromatographic systems were employed. System 1 consisted of two Beckman 100A pumps, controlled by a Model 421 controller (Beckman, Pleasanton, CA, U.S.A.), a Millipore/Waters WISP 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.), and a Hitachi Model 100-40 spectrophotometer (Hitachi, Tokyo, Japan), equipped with a 1-cm, 20-μl flow cell (Beckman). System 2 consisted of a Spectra-Physics SP8700 solvent delivery system (Spectra-Physics, San Jose, CA, U.S.A.), a WISP 710B automatic sample injector, a Hitachi Model 100-40 spectrophotometer, equipped with a 1-cm, 20-μl flow-cell, and a Perkin-Elmer Model 650-15 spectrofluorometer (Perkin-Elmer, Norwalk, CT, U.S.A.). A Forma Scientific bath and circulator, Model 2006 (Mallinckrodt, Paris, KY, U.S.A.), filled with 50% aqueous ethylene glycol, was used to cool the HPLC column below room temperature. Chromatographic data were processed with a Nelson 6000 chromatography data system (Nelson Analytical, Cupertino, CA, U.S.A.). The HIC column used in this study was a TSK Phenyl 5-PW (75 × 7.5 mm I.D.) (Bio-Rad Labs., Richmond, CA, U.S.A.). Electrophoresis equipment was obtained from Hoefer Scientific (San Francisco, CA, U.S.A.).

Chemicals

Organic solvents, reagent-grade ammonium sulfate, Tween 20, and boric acid were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). HPLC-grade water was obtained by passing deionized water through a Technic (Seattle, WA, U.S.A.) Type 1 grade water system, followed by passage through reversed-phase packing (Vydac 218TPB2030; Hesperian, CA, U.S.A.) and "ultra-filtration polishing" (Gelman, Ann Arbor, MI, U.S.A.). Mono- and dibasic phosphate were obtained from Mallinckrodt. All electrophoresis chemicals were purchased from Bio-Rad Labs. Guanidine hydrochloride, urea, and the proteins used for calibration were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium dodecyl sulfate (SDS) was obtained from Gallard Schlesinger (Carle Place, NY, U.S.A.).

Hydrophobic interaction HPLC

Mobile phase A consisted of 1 *M* ammonium sulfate, 0.1 *M* sodium phosphate (pH 8.0), 5% methanol. Solvent B consisted of 0.1 *M* sodium phosphate (pH 8.0), 5% methanol, 70% ethylene glycol. In those experiments where several pH levels were used, the mobile phases were prepared by combining appropriate amounts of mono- and dibasic sodium phosphate. The pH was adjusted with hydrochloric acid or sodium hydroxide. All mobile phases were filtered through a 0.45-μm filter (Millipore) degassed under vacuum, and purged with helium. A standard linear gradient of 0 to 100% eluent B in 120 min at 0.25 ml/min and 25°C was used in all experiments, except where noted otherwise in the text. Protein elution was monitored by absorbance at 214 nm. Blank chromatographic runs were subtracted from sample runs prior

to area calculation or display in order to compensate for excessive baseline drift due to UV absorbance and refractive index changes during gradient elution.

Size-exclusion HPLC

Non-denaturing size-exclusion chromatography (SEC) was performed with HPLC system 2 in conjunction with a 300 × 7.8 mm I.D. TSK 2000 SWXL column (Varian, Sunnyvale, CA, U.S.A.). The eluent was 0.05 *M* sodium chloride, 0.05 *M* sodium phosphate (pH 6.5) at 1.0 ml/min and ambient temperature. The non-denaturing SEC was calibrated with Bio-Rad low-molecular-weight standards. For denaturing SEC, HPLC system 2 was used in conjunction with a 250 × 9.4 mm I.D. DuPont GF-250 column (DuPont, Wilmington, DE, U.S.A.). The eluent was 6 *M* guanidine hydrochloride, 0.2 *M* sodium phosphate (pH 6.5) at 1.0 ml/min and ambient temperature. For calibration of denaturing analyses we utilized a mixture of cytochrome, lysozyme, carbonic anhydrase, ovalbumin and bovine serum albumin. Non-denaturing SEC required no sample preparation, and typically 30–100 μg were injected. Samples for denaturing analysis were denatured and reduced through the addition of a solution of 6 *M* guanidine hydrochloride, containing 5 m*M* dithiothreitol (DTT). Calibration standards were treated in a similar fashion prior to denaturing SEC. Chaotrope denaturation experiments involved the addition of 30 μl of 1 mg/ml TNF to 170 μl of the chaotropic solutions and incubating for 2–10 h at room temperature prior to non-denaturing SEC analysis. The chaotropic solutions were 1, 2 and 6 *M* guanidine hydrochloride; 1, 2 and 8 *M* urea; 0.001, 0.01 and 0.1% SDS; 10% 1-propanol; or 1% Tween 20 with each denaturant tested at pH levels of 4.0, 7.0 and 10.0 attained by the use of 0.1 *M* sodium phosphate or 0.1 *M* sodium borate.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

A non-reducing 15% polyacrylamide gel, 1.5 mm thick, was prepared and later silver-stained by the method of Laemmli[22]. Fractions were diluted 1:4 with water and then 1:1 with sample buffer.

Fluorescence depolarization

A Perkin-Elmer Model 650-10 fluorescence detector, equipped with a HPLC flow cell; slit widths, 10 nm; excitation wavelength, 280 nm; emission wavelength, 350 nm. Two polarization filters (Perkin-Elmer) were used. One was placed in the excitation beam and the other one in the emission light path. Each of these filters was rotated in the appropriate direction in order to make the polarization measurement. Calculations followed the method outlined in the Perkin-Elmer polarizer accessory manual. They are essentially identical to those described by Cantor and Schimmel[23]. Chromatographic conditions were: eluent A, 1 *M* ammonium sulfate, 0.1 *M* sodium phosphate (pH 8.0), 5% ethylene glycol; eluent B, 0.1 *M* sodium phosphate (pH 8.0), 5% ethylene glycol, 40% methanol; gradient 0–100% B in 20 min, flow-rate 1.0 ml/min; room temperature; sample, 50–100 μl of sample in eluent A, containing 0.3 mg/ml of protein.

RESULTS

Identity of peaks 1–4

Fig. 1A shows an HIC profile obtained by chromatographing TNF in the presence of its proteolyzed form (shown to be an N-terminal deletion resulting in a 14-kD molecular weight fragment of TNF). The peaks were labeled 1, 2, 3 and 4. (This nomenclature will be used throughout this report, regardless of the actual number of peaks present in a particular chromatogram.) Peaks 1–4 were characterized by collecting fractions from a preparative HIC run (300 μg of TNF chromatographed at 5°C and 0.1 ml/min) and analyzing the peak fractions by rechromatography in HIC mode and by SDS-PAGE. Fractions were collected from each peak and rechromatographed at 20°C and 0.5 ml/min (Fig. 1). Very little of peak 1 was recovered from the preparative run and, therefore, its subsequent chromatogram is not shown. Rechromatography of peak 2 (Fig. 1B) yielded mainly peak 2 with lower levels of peak 3 and peak 4. Rechromatography of peak 3 (Fig. 1C) gave peak 1, a trace of peak 2 and mostly peak 3. The area ratio of peak 1 to peak 3 was the same both in the parent material and in the rechromatographed peak 3 sample, indicating that the

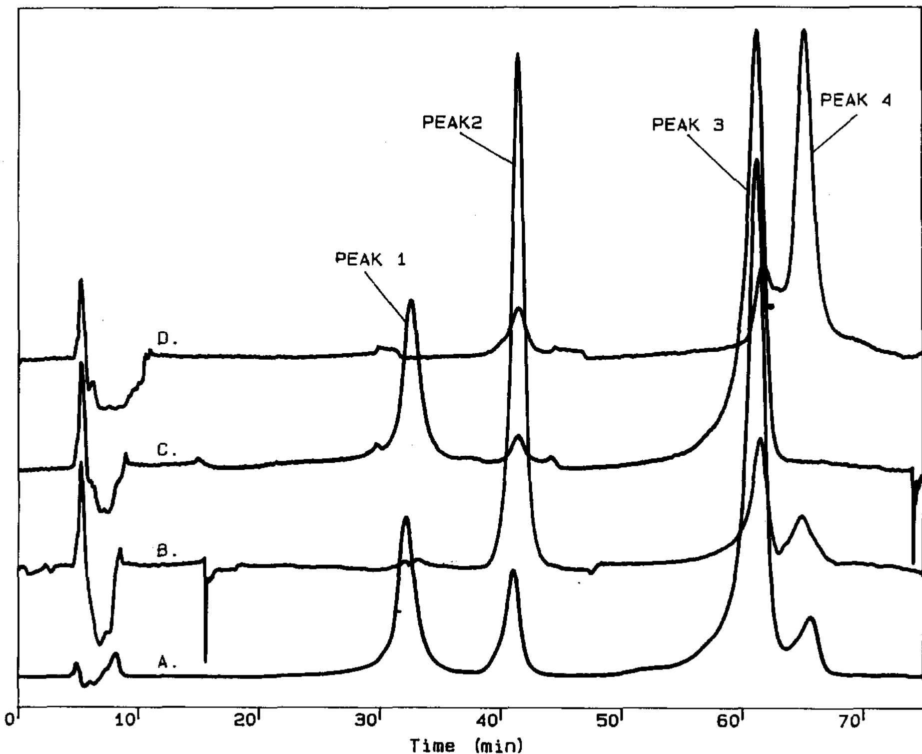

Fig. 1. HIC of peaks 2–4. (A) Parent material, containing TNF and a minor amount of 14-kD fragment. (B) Rechromatography of peak 2. (C) Rechromatography of peak 3. (D) Rechromatography of peak 4. Conditions as described in Materials and methods, except gradient 0–100% eluent B in 60 min and flow-rate 0.5 ml/min.

TNF transformation had reached equilibrium. Rechromatography of peak 4 (Fig. 1D) yielded low levels of peaks 2 and 3 and principally peak 4; the presence of peaks 2 and 3 is due to contamination of peak 4 by peak 3 during collection.

Non-reducing SDS-PAGE was performed on an isolated fraction from each peak (Fig. 2). The protein band from peak 1 migrates as TNF (17-kD). Peak 2 contains similar levels of both TNF and the 14-kD fragment. Peak 3 is TNF and peak 4 is principally 14-kD fragment but contains a small amount of TNF. Contamination of peak 4 by peak 3 was confirmed by HIC reinjection (Fig. 1).

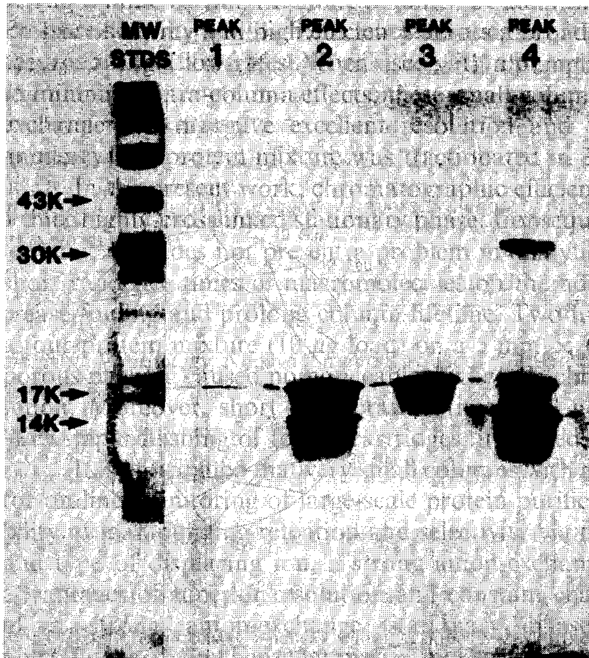

Fig. 2. SDS-PAGE of peak fractions from Fig. 1A. K = kilodalton.

Further evidence for this peak assignment can be found in Fig. 3. The HIC profile in Fig. 3A shows that the purified 14-kD fragment was eluted as a single peak. This indicates that the 14-kD fragment did not form homomultimers (trimers) or that its multimeric form was eluted at the position of peak 4. Chromatogram B (Fig. 3) is a sample of TNF that is very low in 14-kD fragment by SDS-PAGE. It was eluted as a mixture of TNF trimer (peak 1) and monomer (peak 3). When these two samples (TNF and 14-kD fragment) were mixed and allowed to stand at room temperature for several hours, a dramatic appearance of a new peak at 75 min (peak 2) was observed (Fig. 3C). Thus, peak 4 is 14-kD fragment, peaks 1 and 3 are different forms of TNF, and peak 2 is a mixed form of TNF and the 14-kD fragment. The experiment of mixing peaks 3 and 4 in equal ratio to yield peak 2 is complicated by the possibility that peak 2 was a heterotrimer of either a 1:2 or a 2:1 TNF to 14-kD

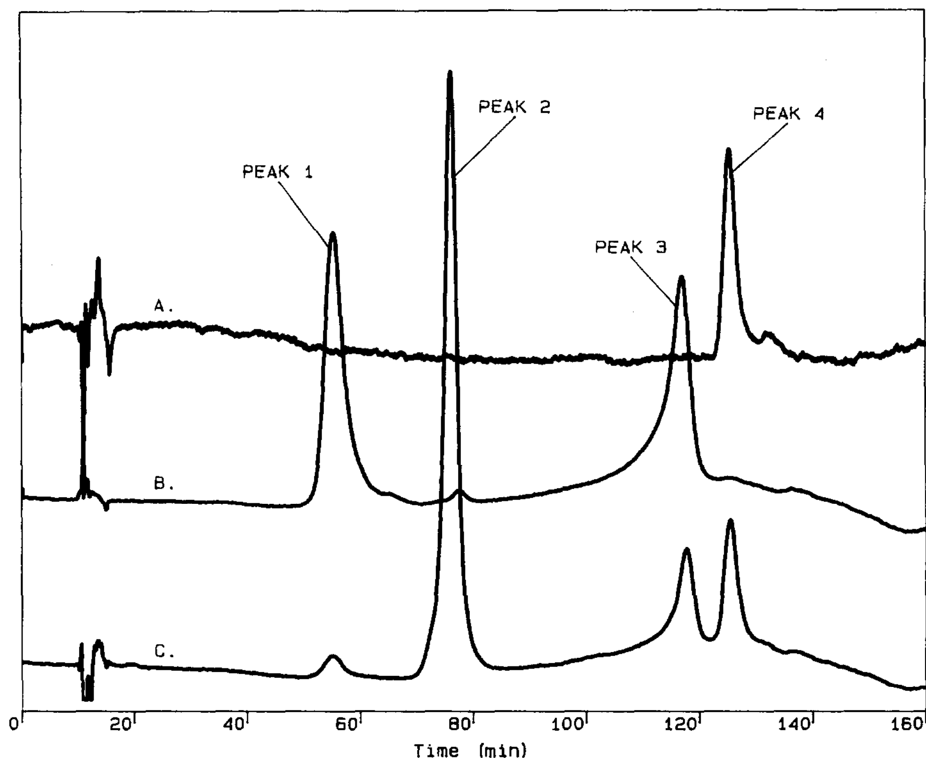

Fig. 3. Reconstitution of TNF and 14-kD fragment to yield peak 2. (A) HIC of purified 14-kD fragment. (B) HIC of purified TNF low in 14-kD contamination. (C) Equimolar quantities of TNF and 14-kD fragment (shown in chromatograms A and B), incubated at room temperature for 2 h and injected into a HIC column. Conditions as described in Materials and methods.

fragment composition. One likely possibility is that both compositions occur in equal amounts in peak 2, but that they are not easily resolved by HIC.

We suggest that the native peak 2 is a heterotrimer, composed of two 17-kD TNF molecules and one 14-kD fragment. Enzymatic proteolysis during isolation of a TNF trimer is most likely to lead to a heterotrimer of this composition. The strongest argument for this hypothesis is the integration of rechromatographed peak 2 (Fig. 1, chromatogram B). Rechromatography of peak 2 yields mostly peak 2, but some of it dissociates to give peak 3 (area count $1.55 \cdot 10^6$) and peak 4 (area count $6.40 \cdot 10^5$). If we assume that the difference in absorbance at 214 nm for TNF and the 14-kD fragment is proportional to the difference in molecular weights of peaks 3 and 4, the area ratio of peak 3 to peak 4 would be 2.0, which represents the compositional ratio of peak 2. Since peaks 3 and 4 are known to be composed of 17-kD TNF and the 14-kD fragment respectively, the composition of peak 2 is 17-kD TNF; 14-kD fragment in a 2:1 ratio. The stoichiometry of the hetero-oligomer of TNF and the 14-kD fragment comfortably fits the trimeric structure of TNF, as determined by other physico-chemical means[15–17]. Large differences in gel staining response factors between TNF and its 14-kD fragment precluded determination of composition from the silver-stained gel (Fig. 2)[24].

The SDS-PAGE analysis of peak 2, the rechromatography of peak 2, and the reconstitution experiment yielding peak 2, all indicated that TNF heterotrimer could retain its quaternary structure when chromatographed in the HIC mode. The reported in solution trimeric structure of TNF, together with our tentative heterotrimer identification, led to the speculation that quaternary structural changes of TNF were responsible for both peaks 1 and 3[15–17]. SDS-PAGE electrophoresis indicated that peaks 1 and 3 have identical molecular weights under denaturing conditions, while rechromatography experiments proved that peak 3 can yield peaks 1 and 3. These results suggest that TNF undergoes a reversible quaternary conversion.

Comparison of molecular size using fluorescence depolarization

Since the identification of peaks 1 and 3 as TNF trimer and monomer, respectively, is based primarily upon chromatographic data, an independent method that could distinguish on a size basis between TNF monomer and trimer was sought. According to the Perrin equation, the polarization, P, of a fluorescence signal is a function of temperature, fluorescence lifetime, viscosity, and molecular volume[23]. In theory, a larger molecule rotates more slowly than a smaller one. Hence, if one observes the intrinsic fluorescence signal of a molecule that has been excited with polarized light, the degree to which the polarization is retained is a measure of its molecular size in solution. Since the polarization is directly related to solvent viscosity, methanol was substituted for ethylene glycol to minimize this factor during gradient elution. Column backpressure, a convenient measure of solvent viscosity, was virtually identical when peaks 1 and 3 were eluted. Assuming that the intrinsic fluorescence lifetime is constant for the various TNF homologues, the change in polarization must be due to changes in molecular volume (*i.e.*, size in solution). The values were measured by stopping the flow of eluent as the TNF peaks were eluted from the HIC column and taking measurements with the emission polarizer in either the parallel or perpendicular position relative to the excitation polarizer. The data in Table I indicate that the TNF peak 1 (presumed homotrimer) and peak 2 (presumed heterotrimer) are similar in size. In contrast, P values indicate that peak 3 (presumed TNF monomer) is significantly smaller in size, supporting the notion of subunit dissociation. While peaks 1 and 2 are likely to be homogeneous, the elution of peak 3 may yield a mixture of quaternary forms, created during the elution–detection process. Eluted TNF monomers may rapidly form transitory dimers, while some small fraction of monomers may even begin to undergo denaturation. Polarization measurements of purified TNF in solution showed no change ($P = 0.16 \pm 0.01$) over a pH range of 5.0 to 9.0 and over an ionic strength range of 0.1 to 2.0 M ammonium sulfate indicating in-solution stability of the quaternary structure.

TABLE I

FLUORESCENCE POLARIZATION OF TNF ELUTED IN HIC

TNF containing a minor amount of 14-kD fragment was chromatographed as described in Materials and methods.

	Peak 1	*Peak 2*	*Peak 3*
P (polarization)	0.20	0.20	0.13
R.S.D. ($n = 3$) (%)	3	6	8

TABLE II

VARIATION OF TNF PEAK AREA AS A FUNCTION OF LOAD

Purified TNF at various concentrations in a constant volume (100 μl) of eluent A. HIC at 20°C and 0.5 ml/min; other conditions as described in Materials and methods.

Load (μg)	*Peak 1**	*Peak 3**	*Peak 1/peak 3*
100	10.6	44.8	0.24
50	4.8	22.2	0.22
10	0.69	2.33	0.30

* Area counts $\times 10^{-6}$.

Variation of peak distribution with protein load

Karger *et al.*[10] have shown that aggregates of α-lactoglobulin form at the head of the HIC column, elute as a cluster of peaks, and are a function of the amount of protein injected. To test the possibility that TNF forms aggregates, purified TNF was injected over a wide range of sample loads, holding the injection volume and sample ionic strength constant. The results (Table II) show that, although there is some change in overall protein recovery, the ratio of trimer (peak 1) to monomer (peak 3) is relatively constant and independent of load. Thus, the differences in molecular size, as indicated by the fluorescence polarization, are not due to on-column aggregation but are a distinctive feature of the quaternary structure of TNF.

Variation of peak distribution with temperature

Previous studies of protein retention in HIC have clearly demonstrated that higher temperatures result in increased on-column dissociation of protein tertiary structure[5–7]. A similar experiment was performed in an attempt to vary the quaternary structure of TNF (Fig. 4). Protein recovery, assessed by summed peak area, was nearly constant, but the individual peak areas varied considerably. The area of peak 1 decreased, while the area of peak 3 increased as the temperature increased from 0 to +30°C (Fig. 4). This result clearly demonstrates the conversion of TNF trimer (peak 1) to TNF monomer (peak 3) as a function of increasing column temperature. At the highest temperature, 30°C, the area of peak 4 increased at the expense of peak 2, indicating the conversion of the heterotrimer to 14-kD monomer. Increased column temperature, up to 45°C, resulted in the complete disappearance of peaks 1 and 2 and reduced (<50%) protein recovery. Presumably, the loss of protein is due to thermal denaturation of the monomeric tertiary structure to the point of insolubility. Peaks 2, 3 and 4 were retained longer, and increased their bandwidths at lower temperatures. These unexpected results will be explored in the Discussion section.

Variation of peak distribution with flow-rate

Fig. 5 shows the effect of flow-rate changes on the elution profile of TNF. Retention times were converted to retention volumes for comparison. Summed peak areas (normalized for flow) were similar at the three flow-rates, but the area distribution of peaks 1–4 varied. As the flow-rate decreased, the area of peak 1 decreased and the area of peak 3 increased, demonstrating trimer to monomer interconversion. This effect was later explained by an experiment in which column contact time was

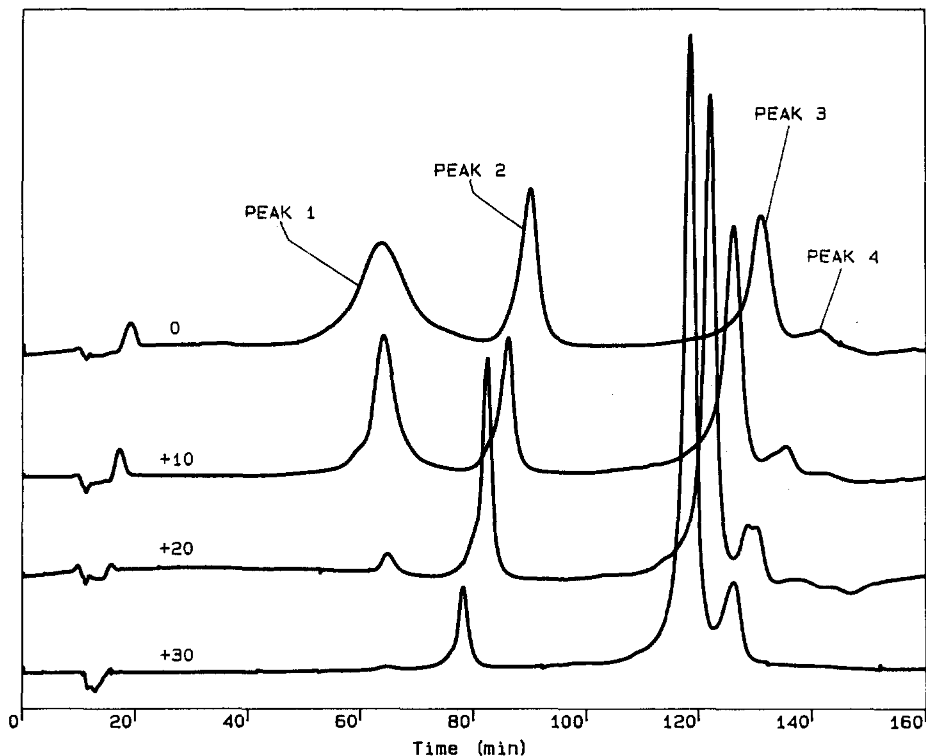

Fig. 4. HIC profile of TNF and a minor amount of 14-kD fragment at various temperatures. Other conditions as described in Materials and methods.

investigated as a variable in trimer-to-monomer conversion. At lower flow-rates, peaks 2, 3 and 4 decreased in both retention volume and bandwidth. These changes in peak retention and bandwidth are similar to the ones observed in the previous experiment with increasing temperature, but to a lesser degree.

Variation of peak distribution with on-column contact time

As work progressed, a working hypothesis was developed which predicts that TNF is mainly a trimer in solution and can dissociate on a HIC column. If the trimer is held together by hydrophobic forces, then a hydrophobic region must be located on the surface of the monomeric protein. Dissociation of the trimer makes this hydrophobic region accessible to interaction with the sorbent surface. The monomer therefore is strongly retained by the column and is not available to reform the trimer. Hence, one may predict that allowing the TNF trimer to equilibrate with a hydrophobic column material will result in the ultimate formation of the monomer. Thus, by increasing the column contact time, more monomer will appear as trimer disappears.

A sample of TNF in eluent A was injected into the HIC column. The flow was stopped after the sample had reached the head of the column and resumed after 0,

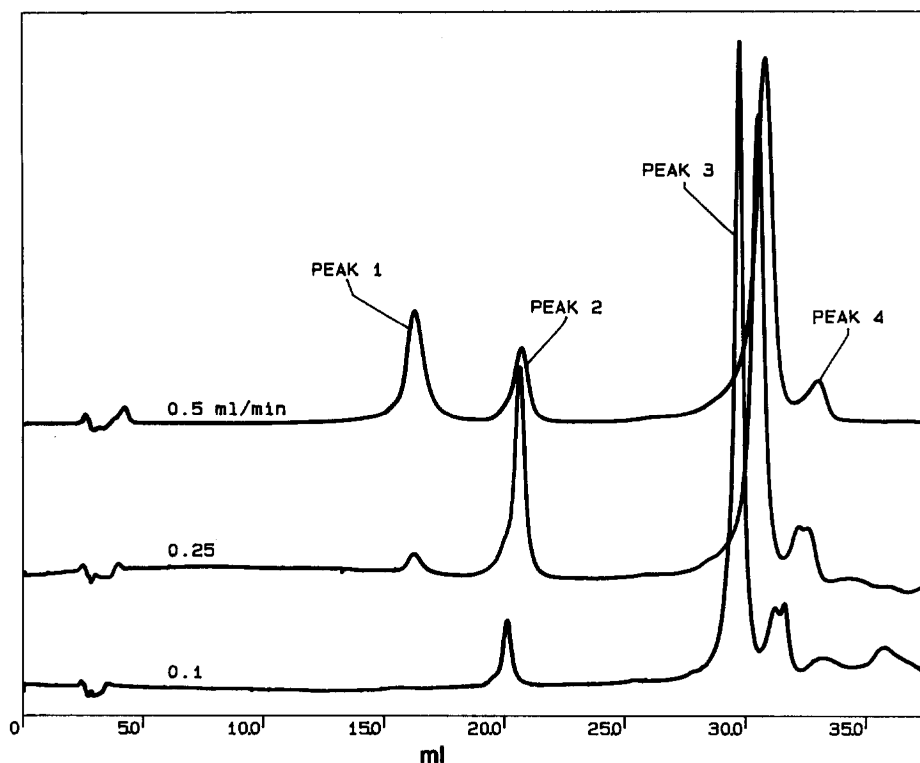

Fig. 5. HIC profile of TNF and a minor amount of 14-kD fragment at various flow-rates. For chromatogram comparison, the abcissa is plotted in volume. Other conditions as described in Materials and methods.

2 and 7 h. At that time, an identical gradient (0 to 100% eluent B in 120 min) was started. The results in Fig. 6 clearly show that peak 3 increased at the expense of peak 1. The half-life of the conversion of TNF trimer to monomer on the column at 25°C was approximately 3–4 h.

Variation of peak distribution with sample solvent and mobile-phase effects

Although the HIC column packing appears to be essential for promoting dissociation of the TNF trimer, the sample solvent and mobile phase composition also play a major role in this dissociation. When purified TNF was chromatographed in HIC mode, the ratio of peak 3 (monomer) to peak 1 (trimer) was constant (1.7 ± 0.6) over a range of eluent pH values of 7.0–8.0. However, at pH extremes (5.0 or 9.0), the ratio of peak 3 to peak 1 changed dramatically (31 ± 0.5), indicating a high degree of quaternary dissociation. When 200 μl of TNF was injected in eluent A, the addition of 30% propylene glycol to the sample solvent changed the ratio of peak 3 to peak 1 from 1.9 to 6.4. When the same mass of TNF was injected in a smaller volume (20 μl) of similar composition, addition of propylene glycol did not change the ratio of peak 3 to peak 1 (1.9).

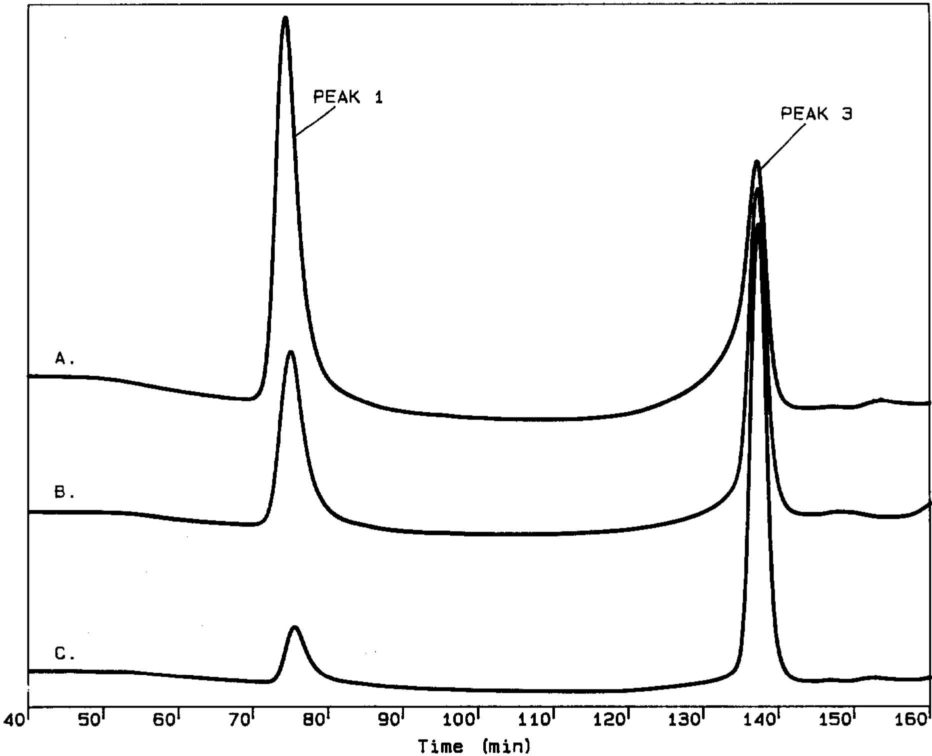

Fig. 6. HIC profile of TNF after various on-column incubation periods. (A) No on-column incubation. (B) 2-h on-column incubation. (C) 7-h on-column incubation. After 5 min of eluent A, the flow was stopped and restarted after the delay indicated. For clarity, chromatograms have been adjusted to align the peaks. Other chromatographic conditions as described in Materials and methods.

Comparison of molecular size using SEC

Previous studies have shown that, under non-denaturing conditions, TNF has a molecular weight of 34–140 kD[17]. Under the non-denaturing SEC conditions described under Materials and methods, purified TNF exhibited an apparent molecular weight of 35 kD. Under the denaturing sizing conditions described, TNF sizes with an apparent molecular weight of 17 kD, in agreement with SDS-PAGE. One could conclude from these apparent-molecular weight determinations and the amino acid sequence that TNF is a dimer. However, previous physico-chemical characterization of TNF argues very strongly for a quaternary structure that is a compact trimer under non-denaturing conditions[15–17]. The conclusion of previous studies that the TNF quaternary structure is quite compact and has an apparent smaller molecular weight due to cooperative hydrophobic forces between the subunits, is consistent with the HIC and SEC data presented.

In an attempt to identify a monomeric TNF species under non-denaturing conditions, a HIC peak presumed to be monomeric TNF, peak 3, was collected from HIC and immediately injected into the non-denaturing SEC system. The result was always a TNF peak with an apparent molecular weight of 35 kD. From these studies

it was presumed that TNF monomers eluted from the HIC column rapidly equilibrate (during SEC) to reform the compact trimeric structure. Transient intermediate forms, such as the dimer, are likely to be of very similar size to the stabilized (compact) trimer and not resolvable.

In an attempt to simulate the denaturing forces of the HIC sorbent surface, TNF was incubated with various chaotropes at different pH levels and analyzed by non-denaturing SEC. The chaotropes included ionic surfactants (SDS), non-ionic surfactants (Tween 20), denaturing reagents (guanidine, urea) and 2-propanol, at pH levels of 4, 7 and 10. Many of the chromatograms showed only a single peak with an apparent molecular weight of untreated TNF, 35 kD. At the higher levels of ionic or non-ionic surfactants a badly distorted peak of higher molecular weight was seen, in addition to the 35-kD peak. This larger molecular weight was interpreted as representing gross secondary, tertiary, and quaternary denaturation. Many of these incubations resulted in poor protein recovery or no recovery at all, indicating that denaturation had occurred to the point of protein insolubility. In none of the incubation mixtures was a smaller 17-kD species ever detected. These results suggest that in-solution quaternary denaturation leads to extensive tertiary denaturation, followed by precipitation or detergent solubilization of the unfolded monomer.

DISCUSSION

The experiments described in this report conclusively establish that the multiple peak equilibria seen in HIC are due to quaternary dissociation. First, reconstitution of TNF with the 14-kD fragment (Fig. 3), combined with the results of SDS-PAGE of peak 2 (Fig. 2) indicate that peak 2 is a hetero-oligomer. The results of peak 2 rechromatography (Fig. 1B) suggest that this hetero-oligomer is composed of TNF and 14-kD fragment in a 2:1 ratio, and conclusively demonstrates on-column dissociation of the TNF quaternary structure during HIC. Peak 2 is well separated from its nearest neighbors, precluding possible contamination by other peaks. Secondly, SDS-PAGE (Fig. 2) identifies both peaks 1 and 3 as TNF. The on-column incubations (Fig. 6) show that peak 1 can be converted to peak 3 during extended column contact, while rechromatography of peak 3 (Fig. 1C) shows that the transformation of peak 1 to peak 3 is reversible in solution. Since previous physico-chemical characterization established TNF as a trimer, the fluorescence polarization experiments (Table I) suggest that peaks 1 and 2 are the trimer and heterotrimer, respectively, and that peak 3 is due to monomeric TNF[15,16]. The extreme difference in hydrophobicity between the trimeric species (peaks 1 and 2) *versus* the monomeric species (peaks 3 and 4) argue for a qualitatively different hydrophobic binding surface, consistent with the on-column dissociation of the trimer to monomers.

The mobile phase or the sample solvent can destabilize TNF quaternary structure during HIC but does not cause observable dissociation in solution. HIC of TNF at mobile phase pH extremes or injection of large sample volumes (containing low salt or high ethylene glycol concentration) can dramatically increase dissociation. However, in-solution fluorescence depolarization values and sedimentation coefficients of TNF do not change over the pH range of 5 to 10, suggesting that the size distribution of the in-solution species is independent of pH[15]. In addition, denaturing incubations at pH 4–10 prior to non-denaturing SEC has not resulted in measurable

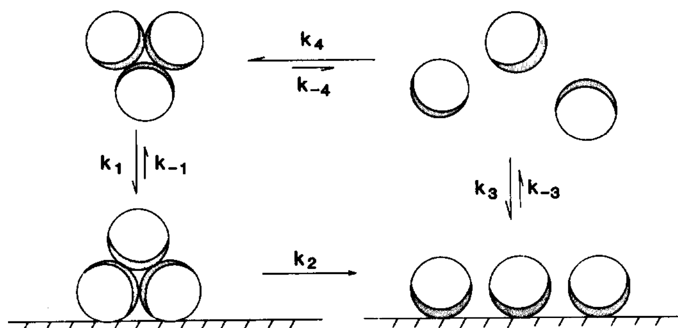

Fig. 7. Model of TNF on-column subunit dissociation and in-solution reassociation. The shaded areas represent a hydrophobic surface on the TNF monomer structure. For explanation, see Discussion section.

monomeric TNF. Thus, the HIC column surface is necessary to promote the dissociation of TNF trimer to monomer.

A possible explanation consistent with the results of our HIC experiments is presented in Fig. 7. The TNF monomer contains a strongly hydrophobic region on its surface (shaded in the figure) which is strongly attracted to a similar surface on other subunits, forming a trimer in solution. The trimer is bound to the HIC sorbent surface at high salt concentrations (k_1) through a second, less hydrophobic, surface on the TNF trimer. The hydrophobic attraction of the trimer to the sorbent surface is rather weak, and the intact TNF trimer is eluted early by the decreasing salt gradient (k_{-1}). However, while on the sorbent surface, the trimer may dissociate into monomers (k_2). This is facilitated by stabilization of the monomeric tertiary structure by association with the hydrophobic sorbent surface. Because the surface hydrophobicity of the monomer is greater than that of the trimer, the monomers are eluted very late in the decreasing salt gradient (k_3). The monomers rapidly reform to trimers when in solution (k_4). The effects of higher temperature and longer column contact time both increase the effect of on-column dissociation (k_2) and thus increase trimer dissociation to monomers prior to elution.

The model of TNF subunit equilibria shown in Fig. 7 can be extended to explain the relationship of TNF structure to apparent anomalies in HIC behavior. We propose that TNF is normally a multimeric protein with a high concentration of hydrophobic residues in one region of its monomeric surface, not in its interior. This hydrophobic surface keeps the quaternary structure (trimer) intact and stabilizes the protein tertiary structure through hydrophobic subunit association. Thus, the trimer is similar to a conventional monomeric protein, with its hydrophobic surfaces hidden from the aqueous environment,. whereas the TNF monomer has its hydrophobic residues oriented on its surface. Supporting this view, Wingfield *et al.*[15] have characterized TNF as a "compact trimer" with a Stokes radius that is unusually small for a trimer of that molecular weight. Through sedimentation experiments and circular dichroism measurements in guanidine hydrochloride titrations, these workers have concluded that the TNF trimer becomes denatured to the unfolded monomer without intermediate species[15]. Our inability to isolate non-denatured TNF monomers by SEC is consistent with this view. It appears that the in-solution tertiary structure of monomeric TNF is unstable relative to the trimeric form and that the

hydrophobic ligands of the HIC sorbent surface mimic the hydrophobic surface of other monomeric subunits and thus stabilize the tertiary structure of the sorbed TNF monomer.

The anomalous temperature behavior of TNF during HIC supports this hypothesis of stabilization of monomeric tertiary structure through hydrophobic attraction. Wu *et al.*[6] have correlated increases in temperature to changes in tertiary protein structure (denaturation and longer retention on HIC). This increase in retention with increasing temperature has been observed consistently for monomeric proteins in HIC[5,6,11]. TNF behaves quite differently, as peaks 3 and 4 decrease their retention with increasing temperature (Fig. 4) or with lower flow-rates (Fig. 5). The lower flow-rates of Fig. 5 represent essentially longer on-column incubations prior to peak elution. Higher temperatures or longer on-column incubations allow the TNF monomer to maximize its hydrophobic attraction to the HIC sorbent surface, similar to other proteins in HIC[5,6]. However unlike normally monomeric proteins, with TNF monomers (peaks 3 and 4) maximal hydrophobic contact produces shorter retention. This unusual result suggests conformational change which, we propose, represents enhanced tertiary stabilization.

Wu *et al.*[6] also correlated the broadening of peaks with rising temperature to increased destabilization of tertiary protein conformation during HIC. Fig. 4 shows that, as the temperature is increased, TNF peak widths decrease, in contrast to the typical behavior of proteins in HIC[5,6]. In Fig. 6, TNF trimer (peak 1) tails and TNF monomer (peak 3) fronts, typical of protein separations involving conformational interconversion[1,3,5]. Upon extended on-column incubation, peak 3 dramatically sharpens to become symmetrical (Fig. 6). We suggest that increased temperature or extended on-column incubation maximizes the tertiary stability of monomeric TNF resulting in a minimum of structural heterogeneity and in sharper peaks.

TNF is an unusual protein in that during HIC it is reversibly dissociated to monomeric subunits, which appear to be stabilized by the mildly hydrophobic sorbent. Conclusively establishing this transformation has been elusive due to the rapid reversibility of the reaction in solution, the probability of transient forms, such as the dimer, and the behavior of the trimer as a compact molecule. We have presented a hypothesis of quaternary on-column denaturation which is consistent with the HIC of TNF as well as the published characterization of the TNF protein. However, other hypotheses of multimeric on-column dissociation such as trimer–dimer, are possible. The one presented provides the simplest explanation of the data.

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