Crystallization of Trimeric Recombinant Human Tumor Necrosis Factor (Cachectin)*

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Crystals of tumor necrosis factor (TNF) have been obtained in two forms. Rhombohedral crystals grow in 1.8 to 2.0 M ammonium sulfate, pH 7.8 at 21 °C, and tetragonal crystals grow in 2.6 M magnesium sulfate, pH 5.5 at 25 °C. Analysis of TNF by isoelectric focusing under native and denaturing conditions indicates that TNF molecules exist as trimers in solution. The rhombohedral cachectin crystals belong to space group R3 and have unit cell constants a = b = c = 47.65 Å and α = β = γ = 90°. Density determinations and the space group indicate that the unit cell contains one 51,000-dalton trimer. These crystals are stable in the x-ray beam and diffract to at least 1.85 Å but are apparently twinned by merohedry. The tetragonal crystals are space group P4₁2₁2₁ or its enantiomorph P4₂2₁2 and have unit cell constants a = b = c = 117.49 Å. The asymmetric unit contains one trimer; the crystals are stable in the x-ray beam and diffract to beyond 3 Å.

Tumor necrosis factor (cachectin) is a metabolically active protein secreted by macrophages in response to certain infection-related phenomena including induction of fever, shock, and cachexia (see Ref. 1 for review). TNF is present at elevated levels in the serum of Mycobacterium bovis strain BCG-infected mice treated with endotoxin, and passive immunization of mice against TNF can prevent endotoxin-induced death (2). Further, the hormone has been demonstrated to have specific cytolytic effects against certain tumorigenic cells. TNF has been demonstrated to bind with high affinity to an as yet uncharacterized cellular receptor.

The sequence of mature TNF is 28% identical to that of human lymphotoxin, a related lymphokine that interacts with the same class of receptors (8). TNF has been isolated, purified, and characterized (3). The mature protein (157 residues, M₀ = 17,000) is derived by cleavage of the 76 amino-terminal residues from the prohormone, contains two cysteines believed to form an intrachain disulfide bond, and is not glycosylated. Under native conditions TNF has been reported to exist in oligomeric states with molecular weights ranging from 34,000 to 140,000. Recent reports, however, indicate that the hormone is active as a trimer (4).

In this report we describe the conditions for growth of crystals suitable for x-ray crystallographic structure determination and describe experiments that confirm the trimeric quaternary structure of TNF in solution.

MATERIALS AND METHODS

TNF was produced from a synthetic construct, which was expressed as an intracellular protein in yeast. The protein was purified as described previously (5) and maintained in storage buffer (0.02 M BisTris, pH 8.0, 0.15 M NaCl) at a concentration of 20 mg/ml. Analysis by SDS-PAGE and isoelectric focusing were performed on a Pharmacia PhastSystem™ unit using standard separation and development techniques. PhastGel™ gradient 10–15% gels were used for SDS-PAGE, and IEF 5–8 gels were used for the IEF separations. Two-dimensional, one dimension denatured isoelectric focusing (ODD-IEF) was run with the first dimension under native conditions on a standard IEF 5–8 gel. The sample lane (approximately 5 mm wide) was then cut out, inverted, and applied across the cathodal end of the second dimension gel, a Phastgel IEF 3–9, which had been soaked for 20 min in 5% Pharmalyte 3–10, 0.6 M urea, and 0.1% Triton X-100. The gel was then run under standard conditions for an IEF 3–9 gel and developed as above.

Chromatofocusing was performed on a Pharmacia LKB Biotechnology Inc. FPLC™ system using the Mono-P™ HR 5/20 column pre-equilibrated with 25 mM BisTris, pH 6.7. TNF was applied in the equilibration buffer at a concentration of 0.75 mg/ml and eluted at a rate of 0.5 ml/min with a solution containing 10 ml of Polybuffer 74™/100 ml of H₂O, pH 5.0. Buffer exchange and protein concentration were performed in a Centricon-10™ concentration device.

Rhombohedral crystals were grown at 22 °C in hanging drops equilibrated by vapor diffusion. Typically, 5 μl of the protein in Tris, pH 8.0, 0.15 M NaCl at a concentration of 20 mg/ml were mounted in thin-walled glass capillary tubes, and precession photographs were taken using CuKα radiation from a Rigaku Rotaflex rotating anode generator at 50 kV and 108 mA. Data to 3 Å from the rhombohedral crystals and 4.5 Å from the tetragonal crystals were collected by 0.75 °C ω peak scans at 0.5 deg/min and 15-s background counts at room temperature with a Rigaku Rotaflex generator operating at 5.4 kilowatts on a Rigaku AFC-5R diffractometer.

RESULTS AND DISCUSSION

Characterization—Purified TNF migrates as a single sharp band with M₀ = 17,000 (Fig. 1, lane A) on SDS-polyacrylamide
gels. However, isoelectric focusing under native conditions reveals a series of four banding groups each containing from one to four sub-bands with pI values between 6.7 and 5.8 (Fig. 1, lane D). The band groups are uniformly separated, consistent with groups of species differing by integral or constant electrical charge. In the hope that a more homogeneous protein sample would improve crystal size and quality, we attempted to isolate single bands by chromatofocusing. The elution profile of the Mono-P column contained four peaks with no discernible substructure which were shown by IEF to correspond to groups I-IV. An in vitro TNF cytotoxicity assay (9) demonstrated that all fractions were equally active. Fractions containing pure band group IV TNF were pooled and concentrated to 20 mg/ml.

The source of charge heterogeneity has not been determined. SDS-PAGE analysis of band groups I-IV separated by chromatofocusing shows no evidence of degradation of the 17-kDa monomer. Amino-terminal peptide sequences obtained by automated Edman degradation (13) for both unfractionated TNF and band group IV TNF indicate the loss of the NH2-terminal valine residue (8) from roughly 50% of the molecules.2 Loss of the amino-terminal valine residue is unlikely to generate significant change in the pI of the subunit. Elman analysis (10) of the unfractionated protein indicated that no reduced thiols were present, thus ruling out the possibility of deamidation of one or more of the 16 asparagine or glutamine residues is responsible for the heterogeneity of recombinant TNF. Isoelectric focusing of TNF in the presence of 6 M urea, which would favor dissociation of oligomers, reveals three major bands (Fig. 1, lane I) along with other combinations could yield trimeric species with similar pI values.

The trimeric structure of TNF is deduced from two-dimensional ODD-IEF gels (Fig. 2) described above by identifying major bands in the native gel are shown in C.

comes markedly heterogeneous (Fig. 1, lane E) after storage in sample buffer at 4°C for 2 months (as opposed to the unfractionated sample, which was maintained at -70°C). The seven band groups visible in this lane are similar in subbanding pattern and band separation to the four band groups in the fresh TNF. Isoelectric focusing under denaturing conditions reveals additional monomeric species which are either absent or present only as minor contaminants in fresh TNF samples (data not shown).

The complex banding pattern produced by fresh purified TNF (or samples stored at -70°C) in IEF gels run under native conditions (Fig. 1, lane D) is proposed to arise from trimers containing different combinations of the three major (and several minor) monomeric species. Substructure within individual band groups is expected because two or more combinations could yield trimeric species with similar pI values.

The trimeric structure of TNF is deduced from two-dimensional ODD-IEF gels (Fig. 2) described above by identifying the monomeric species that comprise the individual band groups in the native IEF gel. The observed pattern of banding is consistent with a trimeric, but not a dimeric, molecule. The four constituents of band group IV migrate in the native dimension (Fig. 2) with a mean pI of 6.6. These dissociate in the denaturing dimension into two monomeric species, a and

2 C. Slaughter, M. J. Eck, and S. R. Sprang, unpublished data.
Crystallization of TNF

Crystallization—We have obtained crystals of cachectin in two space groups. Rhombohedral crystals (Fig. 3B) are produced when ammonium sulfite is used as a precipitant while tetragonal crystals have been grown from magnesium sulfate. Rhombohedral crystals were obtained with the unfraccionated protein in ammonium sulfite at concentrations ranging from 1.5 to 2.0 M at a pH of approximately 7.8 at 21 °C. The crystals are highly birefringent and rhombohedral in shape. Crystals first appear after 36 h and grow to 0.1 mm on edge in a few days. Crystallization of ammonium sulfite have been entirely unsuccessful, even in trials containing 10 mM ammonium sulfite. Also, attempts with ammonium sulfite and either β-mercaptoethanol or dithiothreitol as a reducing agent have been unproductive. SDS-PAGE (Fig. 1, lane B) and IEF (Fig. 1, lane H) of washed, redissolved rhombohedral crystals indicate that the lattice contains primarily band group I protein, which is present in barely detectable quantities in the original crystallization solution. This may explain our lack of success with the purified band group IV TNF; crystallization attempts with this fraction resulted in showers of microcrystals.

Analysis of precession photographs and diffractometer data indicate a rhombohedral unit cell with \( a = b = c = 47.65 \text{Å} \), and \( \alpha = \beta = \gamma = 88.1^\circ \), space group R3. By using measured density (1.22 g/cc) and calculated partial specific volume (0.74 cc/g), the number of molecules per unit cell was calculated to be 2.55, not inconsistent with a trimer in the unit cell, considering possible experimental error in density measurements. For a trimer, the computed Matthew’s coefficient, \( V_m = 2.12 \text{Å/dalton} \), well within the range of reported values (12). The reciprocal lattice shows approximate R3M symmetry (consistent with space group R32); however, the extent of 2-fold symmetry about the \( a^* \) and \( b^* \) axes varies from crystal to crystal, suggesting that the pseudo-symmetry is due to twinning by merohedry (14). R32 symmetry implies six molecules per unit cell, resulting in an unreasonable value for the packing density (\( V_m \) of 1.08 Å³/dalton). Twinning fractions (15) estimated from data sets for selected crystals range from 35 to 45%. Despite the observed twinning, the crystals are well formed and highly birefringent. Measurements from 15-min still photographs and preliminary data collection trials indicate that the crystals diffract to at least 1.86 Å, are stable to a monochromatized x-ray beam, and show a loss of only 10% in intensity of three low order reflections after 70 h of irradiation at room temperature. All attempts to grow un-twinned crystals in this space group, including alteration of pH, precipitant, and crystal growth rate, have been unsuccessful.

Tetragonal crystals of TNF were obtained with the unfraccionated protein in 2.6 M magnesium sulfate at pH 5.5. Crystal growth is quite temperature-dependent; the largest single crystals are grown when the crystallization plate is kept at approximately 20 °C for the first 48 h and then shifted to 25 °C. Crystals typically appear after 72 h and grow to 0.2 × 0.2 × 0.8 mm in several days. Analysis of precession photographs and diffractometer data indicate that the crystals belong to space group P4_2_2 or its enantiomorph P4_1_2_1 and have unit cell dimensions \( a = b = 95.08 \text{Å} \) and \( c = 117.5 \text{Å} \). The crystals diffract to at least 3.0 Å and are stable in the x-ray beam. A search for heavy atom derivatives is in progress. The computed packing density (\( V_m = 2.6 \text{Å}^3/\text{dalton} \)) is consistent with the presence of three monomers in the asymmetric unit or 24 monomers per unit cell. A self-rotation function (16) computed with a native 4.5 Å resolution data set reveals a local 3-fold axis of symmetry parallel to the \( a^* \) and \( b^* \) axes, indicating the presence of trimers of TNF in the asymmetric unit. The two crystal forms of TNF reported here and those reported by other groups (11,17) all contain multiples of three TNF molecules in the unit cell, suggesting that TNF is a trimer in the crystalline state as well as in solution.

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