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# High-Level Expression, Purification, and Characterization of Recombinant Human Tumor Necrosis Factor Synthesized in the Methylotrophic Yeast *Pichia pastoris*

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ABSTRACT: Human tumor necrosis factor (TNF) $\alpha$/cachectin was expressed in the methylotrophic yeast *Pichia pastoris* at high levels (>30% of the soluble protein) by placing the TNF cDNA under the control of regulatory sequences derived from the alcohol oxidase gene. Batch fermentor cultures at cell densities of 50 and 85 g dry cell weight/L contained approximately $6 \times 10^{10}$ and $10^{11}$ units/L TNF bioactivity (6 and 10 g/L TNF), respectively. TNF productivity of 0.108 g $L^{-1}$ $h^{-1}$ was obtained in the continuous mode on glycerol- and methanol-mixed feed at 25 g dry cell weight/L cell density. TNF contained in the yeast cell lysate was soluble, displayed full cytotoxic activity, and was recognized by antibodies prepared against TNF derived from *Escherichia coli*. TNF was purified to >95% purity with >75% recovery by using three sequential chromatographic steps with a coordinated effluent–affluent buffer scheme which allowed one eluate to also serve as the loading buffer for the succeeding column. The amino acid composition, $NH_2$-terminal amino acid sequence, isoelectric point, and minimal molecular weight determined for TNF corroborated those properties predicted from the nucleotide sequence. Sedimentation data indicated that TNF in the native form is a compact trimer held by noncovalent interactions. Circular dichroic spectra of TNF resemble those of proteins with high $\beta$ structure. TNF exhibited cachectic activity on mouse 3T3-L1 cells at about the same equivalence as the cytotoxic activity toward mouse L929 cells. In the criteria examined, TNF derived from *P. pastoris* closely resembles TNF derived from recombinant *E. coli* and human HL-60 cells.

Tumor necrosis factor (TNF)[1] is an antitumor protein found in the sera of animals that have been primed with microbial materials such as BCG (Bacillus Calmette Guerin), *Corynebacterium parvum*, or zymosan and subsequently treated with lipopolysaccharides (LPS, a major constituent of the cell wall of Gram-negative bacteria also known as endotoxin or bacterial pyrogen) (Old, 1985). The cellular origin of TNF is macrophages (monocytes) (Carswell et al., 1975; Hoffmann et al., 1978; Mannel et al., 1980; Satomi et al., 1981). TNF causes hemorrhagic necrosis and regression of certain tumors transplanted in mice (Carswell et al., 1975; Kull & Cuatrecasas, 1981). TNF shows cytotoxic activity against certain tumor cell lines (Carswell et al., 1975; Ostrove & Gifford, 1979; Haranaka & Satomi, 1981). TNF is identical with cachectin, one of the principal mediators of the lethal effect of LPS (Beutler et al., 1985). Cachectin inhibits the biosynthesis of lipoprotein lipase and other enzymes required for de novo triglyceride synthesis in adipocytes (Pekala et al., 1983). Cachectin also suppresses the expression of mRNAs produced specifically by mature adipose tissue cells (Torti et al., 1985) and prevents the morphological differentiation of adipocytes in vitro.

The human TNF gene has been cloned (Pennica et al., 1984; Shirai et al., 1985; Wang et al., 1985; Shomura et al., 1985; Maremenout et al., 1985; Yamada et al., 1985; Berent et al., 1986) and found to be present as one copy per haploid genome on chromosome 6 (Nedwin et al., 1985; Spies et al., 1986).

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[1] Abbreviations: BCG, Bacillus Calmette Guerin; CD, circular dichroism; CPG, controlled pore glass; DO, dissolved oxygen; ELISA, enzyme-linked immunosorbent assay; GdmCl, guanidinium chloride; HPLC, high-performance liquid chromatography; LPS, lipopolysaccharides; $M_r$, molecular weight; PMSF, phenylmethanesulfonyl fluoride; PTC, phenylthiocarbamyl; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TEOA, triethanolamine; TNF, tumor necrosis factor; UV, ultraviolet.

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Four exons code for a precursor molecule of 233 amino acids. The mature form is secreted as a 157 amino acid protein after an unusually long 76 amino acid prepeptide sequence has been cleaved (Shirai et al., 1985; Shomura et al., 1985). The possibility that the 76 amino acid prepeptide might serve another function has been suggested (Muller et al., 1986) and recently has been shown to be necessary for the transmembrane localization of the precursor form of TNF from which the mature TNF (secreted form) is eventually cleaved (Kriegler et al., 1988). The membrane-bound form of TNF with a cytotoxic domain exposed at the cell surface of macrophages is suggested to have physiological significance in localized cytotoxicity.

The TNF cDNA has been expressed in *Escherichia coli* and monkey Cos-1 cells (Pennica et al., 1984; Shirai et al., 1985; Wang et al., 1985; Shomura et al., 1985; Maremenout et al., 1985; Yamada et al., 1985; Berent et al., 1986). Numerous biological activities of TNF have been discovered by using the *E. coli* derived recombinant TNF, and these results have been recently reviewed (Beutler & Cerami, 1988). Caution is warranted in interpreting these results because of the possible contamination of TNF with bacterial pyrogen or some unidentified property unique to TNF produced in *E. coli*. To address this problem, we have examined the properties of TNF produced in a different host system.

Yeast is widely used as a host for production of heterologous proteins (Brunt, 1986; Kingsman et al., 1987; Bitter et al., 1987). The human proteins produced in yeast more closely resemble the natural material as compared to proteins expressed in *E. coli* in certain instances (Miyamoto et al., 1985; Hallewell et al., 1987). Expression in yeast was restricted to *Saccharomyces cerevisiae* until recently when other species of yeasts such as *Kluyveromyces lactis* and *Pichia pastoris* have been employed (Sreekrishna et al., 1984; Brunt, 1986; Thill et al., 1987). *P. pastoris* has proved to be superior to *S. cerevisiae* for the expression of certain proteins (Cregg et al., 1987; Tschopp et al., 1987). We have expressed TNF in *P. pastoris* and examined the properties of TNF purified from yeast. TNF was expressed at high levels in this yeast (>30% of the soluble protein), and unlike in *E. coli* (Davis et al., 1987), the TNF produced in yeast was completely soluble. This property enabled us to develop a fast and simple purification procedure for TNF. The variety of criteria so far examined demonstrates yeast-produced TNF is similar to TNF produced in *E. coli*.

## Materials and Methods

*Bacterial and Yeast Strains.* *P. pastoris* GS115 (*his4*)- (Cregg et al., 1985) was used for expression of TNF. *E. coli* CSH7 (*lacY rpsL thi*) was used for *E. coli* expression of TNF. *E. coli* DG75′ (*hsd1 leu6 lacY thr-1 supE44 tonA21* $\lambda^-$) was used for all plasmid constructions and propagations.

*Media Composition.* Yeast extract tryptone medium (YT) contained the following (per liter): tryptone (8 g), yeast extract (5 g), and NaCl (2.5 g). LBAp contained (per liter) yeast extract (10 g), tryptone (10 g), NaCl (10 g), and ampicillin (100 mg) adjusted to pH 7.5 with 5 M NaOH. Minimal glycerol medium (MGY) contained (per liter) yeast nitrogen base without amino acids (13.4 g), biotin (400 μg), and glycerol (10 mL). Minimal methanol medium (MM) contained (per liter) yeast nitrogen base without amino acids (13.4 g), biotin (400 μg), and methanol (5 mL). Histidine (40 mg) was added to the various minimal media when required. IM-3 medium consisted of (per liter) $KH_2PO_4$ (15 g), $K_2HPO_4$ (1 g), $MgSO_4 \cdot 7H_2O$ (500 mg), $CaSO_4 \cdot 2H_2O$ (400 mg), $(NH_4)_2SO_4$ (3 g), $FeSO_4 \cdot 7H_2O$ (16.25 mg), $CuSO_4 \cdot 5H_2O$ (1.5 mg), $ZnSO_4 \cdot 7H_2O$ (5 mg), $MnSO_4 \cdot H_2O$ (0.75 mg), and biotin (50 μg), pH adjusted to 5.4. FM21 medium (for 10% carbon source) contained the following (per liter): phosphoric acid (3.5 mL), $CaSO_4 \cdot 2H_2O$ (0.15 g), $K_2SO_4$ (2.38 g), $MgSO_4 \cdot 7H_2O$ (1.95 g), $FeSO_4 \cdot 7H_2O$ (65 mg), $CuSO_4 \cdot 5H_2O$ (6 mg), $ZnSO_4 \cdot 7H_2O$ (20 mg), $MnSO_4 \cdot H_2O$ (3 mg), KOH (0.65 g), and biotin (82 μg).

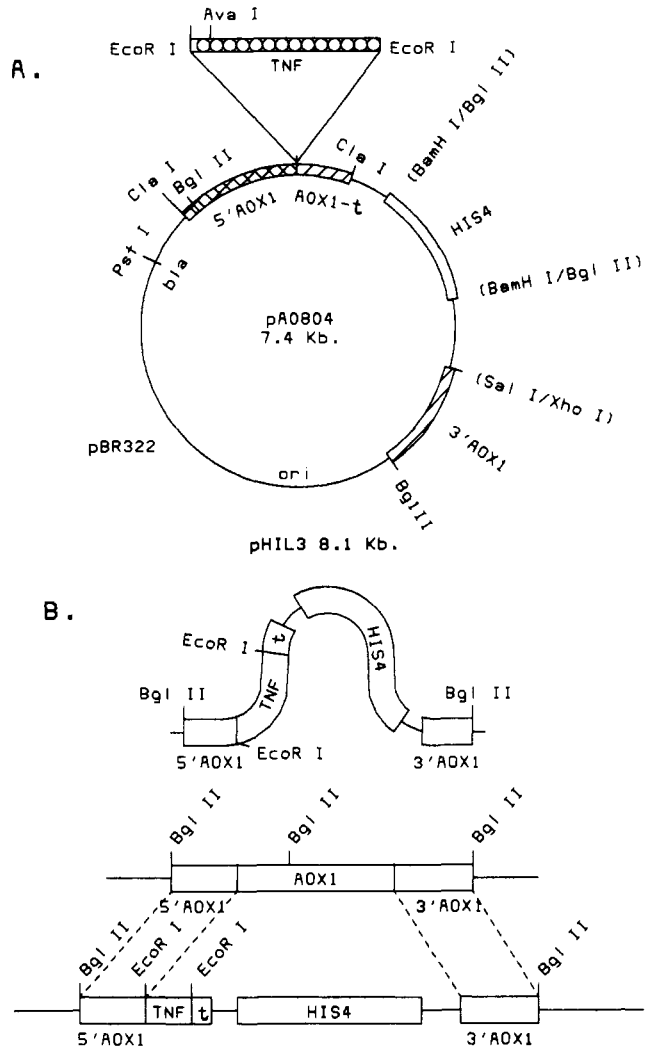


FIGURE 1: Structure of *P. pastoris* TNF expression plasmid pHIL3 (panel A). Plasmid pHIL3 contains the TNF gene inserted between the *AOX1* promoter (*5′AOX1*) and the *AOX1* terminator (*AOX1*-t) for methanol-regulated expression of TNF in *P. pastoris*, the *bla* gene and *ori* from pBR322 for propagation in *E. coli*, the *HIS4* gene from *P. pastoris* which serves as a selection marker in the transformation of GS115, and the *3′AOX1* DNA fragment which contains the 3′ noncoding portion of *AOX1*. The *3′AOX1* sequence together with the *5′AOX1* is necessary for site-directed integration of the linear DNA (derived by *Bgl*II digestion of pHIL3) containing the TNF expression cassette and *HIS4* into the *P. pastoris AOX1* locus as illustrated in panel B.

*Plasmids.* *E. coli* TNF expression plasmid pUC9-$P_L$1-TNF1 (Berent et al., 1986) contains TNF gene under the control of the λ-phage $P_L$ promoter. This promoter is derepressed at 42 °C due to inactivation of the repressor coded by cIts857. *P. pastoris* expression vector pAO804 contains a unique *Eco*RI site flanked by the 5′ and 3′ regulatory sequences of the methanol-induced alcohol oxidase gene (*AOX1*) of *P. pastoris* (Ellis et al., 1985; Cregg et al., 1987). The TNF expression plasmid pHIL3 (Figure 1A) was constructed by excising the TNF gene out of pUC9-$P_L$-TNF1 as a *Bgl*II–*Eco*RI fragment of size 698 bp. The *Bgl*II site was converted into an *Eco*RI site by ligating with the annealed oligo-

nucleotides 5′-GTCCAGCAATCATGGAATTCCCGG and 5′-GATCCCGGGAATTCCATGATTGCTG (OCS Labs, Denton, TX). The TNF gene present on an *Eco*RI fragment was inserted into the *Eco*RI site of pAO804 to obtain pHIL3. The junction sequence between the *AOX1* promoter (*5′AOX1*) and the beginning of the TNF coding sequence is AGGA-ATTCCCGGGATCT *ATG*. There is an additional 221 bp of 3′ noncoding sequence derived from TNF cDNA in addition to the TNF coding sequence of 471 bp.

*Expression of TNF in E. coli and Preparation of Cell Extract.* CSH7(pUC-$P_L$1-TNF1) was grown to saturation in LBAp at 30 °C. TNF expression was induced by diluting the culture to an $A_{600}$ of 2 into 2 × YT medium and incubating at 42 °C for 4 h. Cell extracts were prepared by suspending cells in lysis buffer (50 mM Tris, pH 7.6, 30 mM NaCl, and 1 mM PMSF) at an $A_{600}$ of 50 and lysing either by lysozyme treatment or by two passages through a French press. The soluble fraction was recovered by centrifugation in a microfuge for 10 min or at 15000 rpm in a Sorvall SM24 or SA600 rotor for 10 min at 4 °C. The residual pellet was rinsed 2 times with lysis buffer, and the insoluble fraction was solubilized by boiling the pellet for 5 min with SDS sample buffer (1% SDS, 5% β-mercaptoethanol, 10% glycerol, 10 mM EDTA, and 0.025% bromophenol blue).

*Expression of TNF in P. pastoris Shake Flask Cultures.* Cells grown in 10 mL of MGY at 30 °C to an $A_{600}$ of 5–10 were harvested, washed once with MM, resuspended in MM at an $A_{600}$ of approximately 4.0, and incubated at 30 °C for 3–4 days to induce expression.

*Production of TNF in a High Cell Density Fermentor.* The production of TNF was examined in one continuous and two batch-type fermentation runs. Each run was performed in a modified 5-L New Brunswick Scientific fermentor equipped with monitors and controls for pH, dissolved oxygen (DO), agitator speed, temperature, air flow, and oxygen flow. Temperature was maintained at 30 °C. Cell yields were determined from washed cell dry weights. Inocula for the fermentor runs were grown in 250-mL Erlenmeyer flasks containing 100 mL of MGY. Fermentor cultures grown in the batch mode were propagated in 2% w/v glycerol–IM-3 medium until the available glycerol was exhausted. Continuous culture was established by using 10% w/v glycerol–FM21 salts feed until steady-state conditions were achieved. Methanol was added to the culture as a 5% w/v glycerol + 1% methanol–FM21 salts feed for continous run on a mixed substrate or as a discontinuous series of methanol additions for batch mode fermentation.

*Preparation of P. pastoris Cell Extract and Quantification of TNF.* Yeast cells were washed once in breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, and 5% glycerol) and resuspended at an $A_{600}$ of 50–100. Equal volumes of acid-washed glass beads (size 450–500 μm) were added. The mixture was vortexed for a total of 4 min, 30 s mixing each, followed by 30 s on ice. The sample was then centrifuged in a microfuge for 10 min or at 10000 rpm for 10 min in a Sorvall SM24 or SA600 rotor. The clear supernatant solution was transferred to a fresh tube and stored at −20 °C.

TNF as percent of total protein was determined by densitometric scanning of the Coomassie brilliant blue stained protein bands fractionated by SDS–PAGE using a Model 3 CS microdensitometer (Joyce-Loebl, Gateshead, England) or a Model GS-300 scanning densitometer (Hoefer Scientific, San Francisco, CA). The tumoricidal activity of TNF was assayed by measuring its cytotoxic activity against mouse L929 cells (Pennica et al., 1984). The cachectic activity of TNF was assayed on the basis of inhibition of 3T3-L1 cell adipose conversion (Liang et al., 1986).

*Purification of TNF.* Frozen fermentor broth (approximately 100 mL) was thawed at room temperature and adjusted to pH 8.0 with ammonium hydroxide. Equal volumes of glass beads (450–500 μm) were added, and the cells were disrupted for 5 min on a bead beater (BioSpec Products, Bartlesville, OK) equipped with an ice–water cooling jacket. Clarification was conducted by centrifugation at 30000*g* for 30 min. The resulting pellet was extracted with an equal volume of 0.01 M potassium phosphate, pH 7.5 (KP buffer). These and all subsequent steps were performed at 4–10 °C.

Combined centrifugation supernatants (approximately 120 mL) were pumped at 100 mL/h onto a 2.5 × 12 cm column of controlled-pore glass (CPG, mesh size 20/80; Electronucleonics, Fairfield, NJ) preequilibrated in KP buffer. Elution of nonabsorbed material with 0.05 M Tris-HCl (pH 8.7) was monitored at 280 nm. The flow rate was routinely lowered to approximately 50 mL/h for unattended overnight operation. TNF was eluted with 2% triethanolamine (TEOA) at 100 mL/h. When the beginning of the protein peak eluted by the TEOA was detected, the UV monitor outlet was connected to the inlet of a 2.5 × 12 cm column of Accell QMA anion exchanger (Waters Associates, Milford, MA) preequilibrated in 0.02 M Tris-HCl (pH 8.3). When the end of the peak eluted with TEOA was detected, the CPG column was removed from the system and the UV monitor connected to the outlet of the Accell column. The column was washed with 3 bed volumes (approximately 180 mL) of 0.02 M Tris-HCl (pH 8.3), and TNF was eluted from the anion exchanger with 0.2 M NaCl in the above Tris buffer at 60 mL/h. At the appearance of a peak during the 0.2 M NaCl wash, the UV monitor outlet was connected to the inlet of a 1.5 × 20 cm column of Matrex Blue agarose (Amicon Corp., Danvers, MA) preequilibrated in 0.02 M Tris-HCl (pH 8.3). When the end of the peak eluted by 0.2 M NaCl was detected, the Accell column was removed from the system and the UV monitor connected to the outlet of the Blue Dye column. The column was washed with 1 bed volume of 0.2 M NaCl and 0.02 M Tris-HCl (pH 8.3), and TNF was eluted using 1 M NaCl and 0.02 M Tris-HCl (pH 8.3).

*Isoelectric Point Determination.* Isoelectric point measurements were made on cylindrical gels (0.5 × 10 cm) containing 8 M urea, 7.5% acrylamide, 0.2% bis(acrylamide), 1.6% Pharmalyte pH 5–8 (Pharmacia, Upsala), and 0.4% Ampholyte pH 3.5–9.5 (LKB, Bromma). The cathode (upper) reservoir contained 0.02 N NaOH, and 0.01 M $H_3PO_4$ served as anode solution. Gels were prefocused for 1 h at 200 V, after which approximately 20 μg of TNF in 25 μL was loaded. Samples were prepared in four configurations: (a) 60% sucrose–1% SDS; (b) 60% sucrose, 1% SDS, and 5% 2-mercaptoethanol; (c) 8 M urea; (d) 8 M urea–5% 2-mercaptoethanol. All the samples contained 0.05 M Tris-HCl (pH 8.3). Samples b and d were incubated at 37 °C for 16 h to reduce any disulfide bridges. Focusing was conducted at 400 V. Gradients of pH were measured by slicing the gel into 5-mm segments, placing them in 1 mL of deaerated water, and reading the pH. In preparation for staining, gels were fixed in 12% trichloroacetic acid and washed several times with repeated changes of 10% acetic acid and 10% methanol to remove ampholine. Protein was visualized with Coomassie blue stain.

*Circular Dichroism.* Circular dichroic spectra were measured at room temperature on an Aviv 60DS spectrophotom-

eter calibrated with *d*-10-camphorsulfonic acid (Eastman Kodak). Protein concentrations varied between 0.1 and 1.0 mg/mL in 0.05 M Tris-HCl–0.1 M NaCl, pH 8.0. Cell path lengths of 1 and 10 mm were used to obtain the optimal signal to noise ratios.

*Analytical Ultracentrifuge Measurements.* Analytical ultracentrifugation measurements were conducted in a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner (services of Dr. J. Lee of St. Louis University). Sedimentation velocity runs were conducted at 23 °C and 60000 rpm. At least eight time points were taken in each run, and the log $R$ vs time plots were linear with correlation coefficients of 1.0. Solvent densities and viscosities were either measured or taken from the literature. A partial specific volume of 0.733 mL/g was estimated for native TNF from its amino acid composition. Sedimentation equilibrium measurements were performed according to the method of Yphantis (1964). Samples were deemed to be at equilibrium when multiple scans at 1-h intervals did not show any change. All ln $C$ vs $r^2$ plots were linear for the entire solution column. The molecular weight ($M_r$) as a function of concentration in the solution column was determined by linear least-squares analysis on every five data points. Equilibrium measurements on native TNF were conducted in 0.04 M Tris-HCl while those on the subunit were conducted in 6 M GdmCl. The partial specific volume in GdmCl was estimated according to the procedure of Lee and Timasheff (1979).

*Miscellaneous Procedures.* DNA manipulations and transformations were conducted as previously described (Cregg et al., 1985; Sreekrishna et al., 1987). Protein concentrations were measured by the method of Lowry et al. (1951) or spectrophotometrically using a value of $A^{1\%}_{1cm}$ = 12.7 at 278 nm for TNF (Wingfield et al., 1987). Amino acid analysis was performed on PTC derivatives of acid hydrolysates (Bidlingmeyer et al., 1984) using the PICO-TAG system (Waters Associates, Milford, MA). The $NH_2$-terminal amino acid sequence was performed on an Applied Sciences gas-phase instrument through the services of the protein chemistry laboratory at the Washington University School of Medicine (St. Louis, MO).

## Results and Discussion

*AOX1 Disruptive Transformation of P. pastoris with the TNF Gene Expression Plasmid.* The one-step gene replacement method described for *S. cerevisiae* (Rothstein, 1983) has been successfully used by Cregg et al. (1987) for the replacement of the *P. pastoris AOX1* structural gene with the hepatitis B surface antigen gene placed under the control of *AOX1* 5′ and 3′ regulatory sequences. These strains respond to methanol induction by producing hepatitis B surface antigen. We have used a similar approach for the expression of TNF in *P. pastoris*.

Transformation of GS115 with 10 μg of *Bgl*II-digested pHIL3 gave 144 $His^+$ transformants, about 8% of which were "methanol-slow" (i.e., impaired growth on media such as MM, containing methanol as the sole carbon and energy source) presumably due to the replacement of the *AOX1* structural gene with the TNF expression cassette and *His4* as shown in Figure 1B. The reason why the *AOX1*-deleted transformants grow at all on methanol medium is due to low-level expression of alcohol oxidase activity by *AOX2* (Cregg et al., 1987; Cregg & Madden, 1987). The strains in which both the *AOX1* and *AOX2* genes have been purposely deleted are clearly "methanol-negative" (Dr. J. Cregg, personal communication).

Southern hybridization analysis of the DNA isolated from several $His^+$/"methanol-slow" transformants indicated that the *AOX1* structural gene was indeed replaced by the *Bgl*II fragment (derived from pHIL3) containing the TNF expression cassette and *His4* (data not shown). Analysis of the DNA isolated from a $His^+$/"methanol-normal" transformant indicated that the *AOX1* structural gene was intact and the entire pHIL3 DNA had integrated elsewhere (data not shown). In the *AOX1*-disruptive transformations of *P. pastoris* we have performed with various gene expression cassettes (in some instances, the DNA fragment of interest was gel purified), only 5–35% of the $His^+$ transformants are "methanol-slow", indicating the prevalence of other modes of integration.

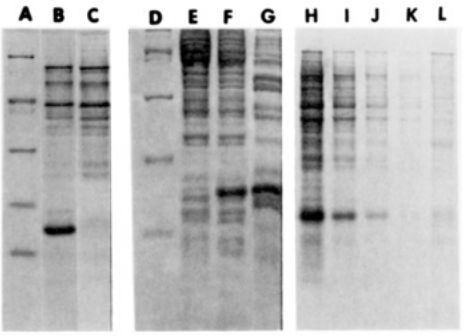


FIGURE 2: SDS–PAGE on a 15% gel of TNF in *P. pastoris* and *E. coli* cell extracts. The *N,N′*-methylenebis(acrylamide) concentrations were 0.4% (for lanes A–C and H–L) and 0.8% (for lanes D–G), respectively. Lanes A and D, low molecular weight markers detailed in Figure 5; lane B, *P. pastoris* GS115/pHIL3-2 soluble cell lysate; lane C, *P. pastoris* GS115/pAO804-1 soluble cell lysate; lane E, *E. coli* CSH7 soluble fraction (French press lysate); lane F, *E. coli* CSH7/pUC9-$P_L$1-TNF1 soluble fraction (French press lysate); lane G, *E. coli* CSH7/pUC9-$P_L$1-TNF1 insoluble fraction (French press broken cell pellet washed with lysis buffer 2 times and solubilized by boiling for 5 min with SDS sample buffer); lane H, *P. pastoris* GS115/pHIL3-5 soluble lysate from cells grown in continuous fermentor on mixed feed (see Figure 3c for details); lanes I, J, and K are the first, second, and third washes with breaking buffer of the pellet fraction from the lysed cells used in lane H; lane L, insoluble fraction from cells used in lane H (3 times washed pellet solubilized by boiling with SDS sample buffer for 5 min). The TNF contents in the cell extracts determined by quantitative densitometric analysis of the proteins separated by SDS–PAGE are 36% for lane B, 14% for lane F, 12% for lane G, and 16% for lane H.

*Methanol-Regulated Expression of TNF in AOX1-Disrupted Transformants.* Several $His^+$/"methanol-slow" transformants of GS115 with pHIL3 were examined in shake flask cultures (cell density of about 1 g dry cell weights/L) for the methanol-regulated expression of TNF. As seen in Figure 2 (lane B), GS115/pHIL3-2 cell extract contains a major protein in the expected size range of 17 kDa for TNF, and this protein is absent in the control sample (Figure 2, lane C). For GS115/pHIL3-2, the TNF cytotoxic activity of cell extracts after methanol induction was $3 \times 10^6$ units/mg of total soluble protein, as compared to $10^3$ units/mg prior to methanol induction. Similar results were obtained with another transformant, GS115/pHIL3-5 (data not shown). TNF expressed in *P. pastoris* was completely soluble (Figure 2, lanes H–L). GS115/pAO804-1 (without the TNF gene) cell extract had no detectable TNF activity. The TNF expression level in *E. coli* was considerably lower than in *P. pastoris*, and the TNF produced in *E. coli* was only partly soluble (Figure 2, lanes B, F, and G). The TNF biological activity of *E. coli* extract was $3 \times 10^5$ units/mg of total soluble protein.

The two *P. pastoris* strains which express high levels of TNF in shake flask cultures were examined for TNF production under high cell density batch type fermentor conditions (Figure 3). The basal level of TNF expression in fermentor cells on glycerol was considerably higher than that observed in shake flask cultures. Levels increased after addition of methanol,

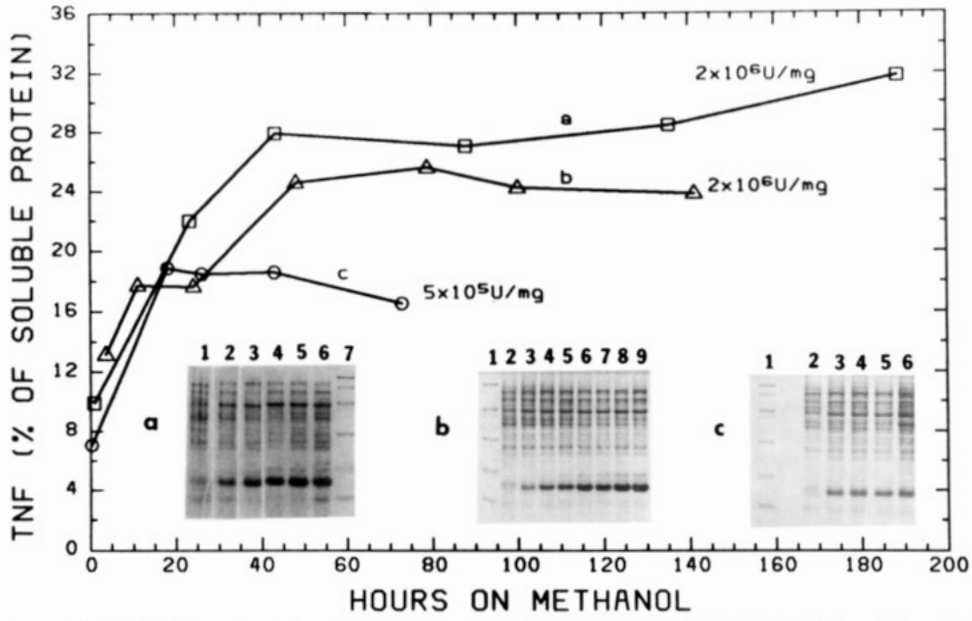


FIGURE 3: Production of TNF in the fermentor. Samples were assayed for TNF content and TNF biological activity. (a) Production of TNF by GS115/pHIL3-5 in the batch fermentor. Cells were grown on 20% w/v glycerol to a steady-state growth yield of 108 g/L dry cell weight in 2 L. To induce for TNF expression, methanol (275 mL) was added to the culture over a period of 189 h. The final cell density was 88 g dry cell weight/L. Samples were analyzed for TNF by SDS–PAGE on a 15% gel (inset a) after 0 (lane 1), 23 (lane 2), 43 (lane 3), 112 (lane 4), 160 (lane 5), and 189 h (lane 6) on methanol. (b) Production of TNF by GS115/pHIL3-2 in the batch fermentor. Cells were grown to 57 g dry cell weight/L cell density on 10% w/v glycerol medium in 2 L. Methanol (100 mL) was added to the culture over a period of 142 h to induce for TNF expression. At the end of 142 h on methanol, the cell density was 50 g dry cell weight/L. Samples were analyzed for TNF by SDS–PAGE on a 15% gel (inset b) after 0 (lane 2), 3 (lane 3), 10.5 (lane 4), 24 (lane 5), 48 (lane 6), 79 (lane 7), 100 (lane 8), and 142 h (lane 9) on methanol. (c) Production of TNF by GS115/pHIL3-5 during continuous culture. Cells were grown to 25 g dry cell weight/L cell density on glycerol (5% w/v) in a 2-L volume and shifted to methanol (1% w/v) + glycerol (5% w/v) mixed feed. Total volume (7.3 L) was collected in 73 h at a flow rate of 100 mL/h. Samples were analyzed by SDS–PAGE on a 15% gel (inset c) after 0 (lane 2), 18 (lane 3), 26 (lane 4), 43 (lane 5), and 73 h (lane 6) on methanol + glycerol mixed feed. Lanes 1 (inset b and inset c) and 7 (inset a) contain the low molecular weight markers described in Figure 5. The TNF specific activity (cytotoxicity) of the cell extracts prepared from cells at the end of the fermentor runs is also indicated in the figure.

and plateau values were reached within 48 h; thereafter the expression level remained fairly constant. TNF expression level (as percent soluble protein) in the batch fermentor on methanol was comparable to that observed with shake flask cultures. Batch cultures at cell densities of 50 g (with GS115/pHIL3-2) and 85 g (with GS115/pHIL3-5) dry cell weight/L contained approximately $6 \times 10^{10}$ units/L and $10^{11}$ units/L TNF cytotoxic activity, respectively, which correspond to 6 and 10 g/L TNF using a specific activity value of $10^7$ units/mg for pure TNF (see below). The production of TNF in GS115/pHIL3-5 was also examined in a continuous fermentor on glycerol + methanol mixed feed (Figure 3). TNF productivity in the continuous culture under the conditions described here was 0.108 g $L^{-1}$ $h^{-1}$.

Expression of heterologous proteins in yeast at levels similar to that observed for TNF expression in *P. pastoris* (>30% of soluble protein) has been reported for intracellular expression of hepatitis B virus core antigen (as 28-nm particles) (Kniskern et al., 1986) and human superoxide dismutase (N-acetylated protein) (Hallewell et al., 1987) in *S. cerevisiae*. High-level secretion (>2 g/L of culture medium) of *S. cerevisiae* invertase enzyme (glycosylated protein) in *P. pastoris* has also been recently reported (Tschopp et al., 1987). Certain indigenous enzymes such as alcohol oxidase of methylotrophic yeasts (Couder & Baratti, 1980; Sahm & Wagner, 1973; van Dijken et al., 1976), 3-phosphoglycerate kinase of *S. cerevisiae* (Chen et al., 1984; Chen & Hitzeman, 1987), and the alkaline extracellular protease of *Yarrowia lipolytica* (Tobe et al., 1976) are also expressed at high levels.

Perhaps one of the factors which favors high-level accumulation of a protein is proteolytic stability. The highly expressed proteins with the exception of alcohol oxidase are devoid of good PEST sequences (Figure 4). PEST sequences are found in all rapidly degraded eukaryotic proteins of known sequence; such proteins have been implicated as favored substrates for calcium-activated proteases (Rogers et al., 1986). The octameric crystalline arrangement of alcohol oxidase and its peroxisomal compartmentalization may serve to mask its PEST region as has been suggested by Rogers and Rechsteiner (1985). The proteins expressed at high levels in yeast do not contain the pentapeptide sequences XFXRQ or QRXFX (X = any amino acid), which are selective for degradation of cytoplasmic proteins by the lysosomal pathway (Dice, 1987). The nature of the $NH_2$-terminal amino acid residue is also a factor (Bachamir et al., 1986). The amino acids alanine, cysteine, glycine, methionine, serine, threonine, and valine and any blocked amino-terminal amino acid have a stabilizing effect against rapid degradation by ubiquitin-mediated pathways. The proteins expressed at high levels in yeast have a stabilizing amino-terminus amino acid residue (Figure 4). The exception is HBcAg, which is present in the particle form, and it is not clear whether the initiator methionine is cleaved. On the basis of the predictions of Huang et al. (1987), one would expect acetylation of the $NH_2$-terminal methionine of HBcAg, resulting in a stabilized amino-terminal structure.

*Purification and Physicochemical Characterization of TNF.* Rapid purification of TNF was attained through a three-step sequential chromatographic procedure in which column buffers are coordinated to minimize between column manipulations. The practical advantages of this arrangement have been described (Sofer & Britton, 1983). The entire purification can be completed in less than two 8-h days with recovery of approximately 78% of the activity present in the clarified lysate. Quantitative densitometry of a Coomassie blue stained gel

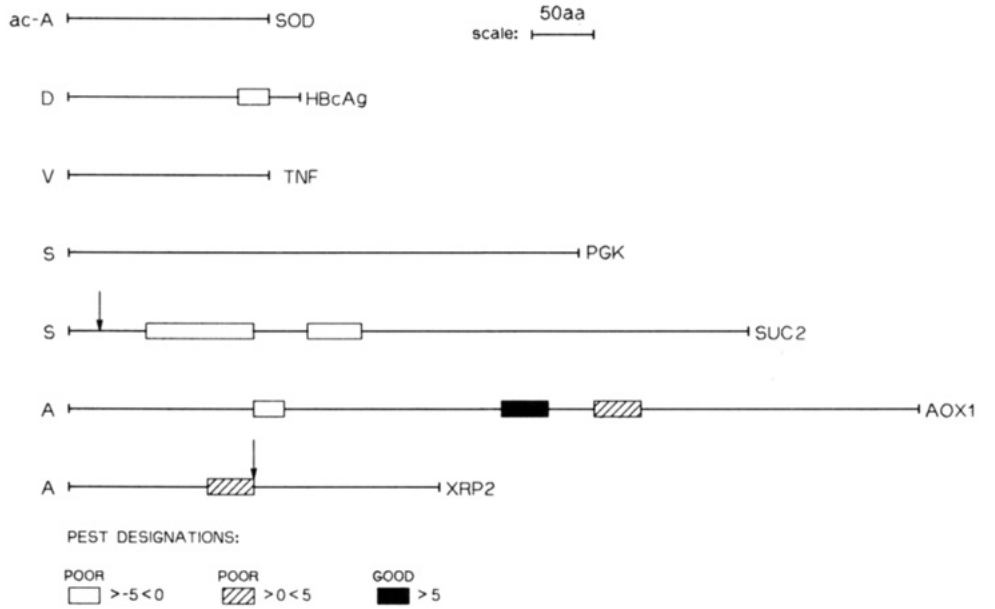


FIGURE 4: Diagrammatic representation of the PEST regions and the $NH_2$-terminal amino acid of the mature proteins expressed at high levels in yeast. Each protein is represented by a thin horizontal line proportional to the length of its amino acid (aa) sequence. At the beginning of each protein, the predicted $NH_2$-terminal amino acid residue is indicated (ac-A, aminoacetylalanine; D, aspartic acid; V, valine; S, serine; A, alanine). The algorithm developed by Rogers et al. (1986) was used for identifying PEST regions of proteins. This algorithm identifies and scores 10 or more amino acids enriched for P (proline), E (glutamic acid) S (serine), and T (threonine) and flanked by basic residues R (arginine), K (lysine), or H (histidine). These characteristics are combined with a hydrophilicity prediction to yield possible scores of approximately −45 to +50. PEST regions with scores greater than 5 are shown as black boxes, those scores between 0 to 5 are shown as striped boxes, and those between −5 and 0 are shown as white boxes. Arrows indicate the position where mature protein starts for the proteins with a cleavable presequence. Abbreviations: SOD, human Cu/Zn superoxide dismutase; HBcAg, hepatitis B viral core antigen; TNF, human tumor necrosis factor; PGK, phosphoglycerate kinase from *S. cerevisiae*; SUC2, invertase from *S. cerevisiae*; *AOX1*, alcohol oxidase from *P. pastoris*; XRP2, alkaline extracellular protease from *Y. lipolytica*.

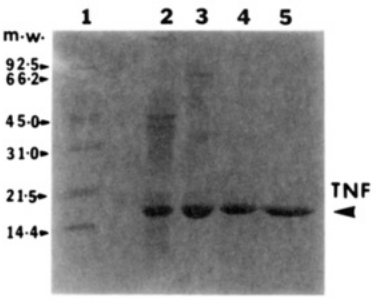


FIGURE 5: Purification of TNF from yeast cell lysate as followed by SDS–PAGE on a 15% gel. Lane 1, low molecular weight markers (Bio-Rad, Richmond, CA); lane 2, clarified cell lysate; lane 3, CPG eluate; lane 4, Accell QMA eluate; lane 5, Matrex Blue A eluate.

(Figure 5) indicated a purity of >95%.

$NH_2$-terminal amino acid sequence analysis of the purified TNF indicated the presence of two species. Approximately 80% of the material had the expected $NH_2$-terminal sequence Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-His-Val-Val-Ala-Asn-Pro, and the remainder began with the second residue Arg and had the expected sequence thereafter. The major sequence is identical with that reported for natural human TNF (Aggarwal et al., 1985). Occasionally, we have noticed larger deletions into the $NH_2$-terminal region. The $NH_2$-terminal initiator methionine was not detected in any of the TNF preparations analyzed, suggesting that the methionine residue was efficiently cleaved from the $NH_2$ terminus. Huang et al. (1987) have observed that in *S. cerevisiae* that initiator methionine is cleaved from recombinant thaumatin, if the second residue is proline, valine, or cysteine. The second residue in TNF is valine, and the $NH_2$-terminal methionine is efficiently cleaved from TNF, suggesting a similarity in the amino-terminal processing of proteins in these two yeasts. TNF purified from *P. pastoris* consistently had a specific activity of (1–2) × $10^7$ units/mg in the cytotoxicity assay, which suggests that the observed $NH_2$-terminal heterogeneity does not have a significant effect on cytotoxic activity. Studies with TNF modified by site-specific mutagenesis have suggested that the first 10 $NH_2$-terminal amino acid residues of TNF can be deleted or modified without significantly affecting the ability of TNF to kill its target cell (Mark et al., 1987).

TNFs expressed in *P. pastoris* and *E. coli* were equivalently recognized by polyclonal antibodies prepared in rabbit for TNF purified from *E. coli* as measured in quantitative ELISA (data not shown). TNFs present in *P. pastoris* and *E. coli* cell lysates were purified to >95% purity in a single step by using an immunoaffinity column containing anti-TNF antibodies prepared against TNF purified from *E. coli*. The TNF purified from *P. pastoris* extract had the expected $NH_2$-terminal amino acid sequence, and there was no evidence for the presence of $NH_2$-terminal methionine. With the material purified from *E. coli*, $NH_2$-terminal methionine was detected in about 25% of the material, suggesting that *E. coli* is less efficient than *P. pastoris* in removing $NH_2$-terminal methionine from TNF. Several other recombinant proteins expressed in *E. coli* also seem to retain $NH_2$-terminal methionine (Nakagawa et al., 1987). It remains to be seen whether *P. pastoris* is in general more efficient than *E. coli* in cleaving the $NH_2$-terminal methionine from other proteins.

The amino acid composition of purified TNF compares favorably to the composition predicted by the nucleotide sequence (Berent et al., 1986). Of the 17 amino acids quantitated, 16 differed from the expected values by an average of 10.6%; recovery of cysteine was only 35% of the expected (0.7 residue vs 2 residues expected). The subunit molecular weight of our recombinant TNF as estimated by SDS–PAGE was 17 250 with a standard deviation of 800 (data from eight gels). Similarly, the subunit molecular weight as measured

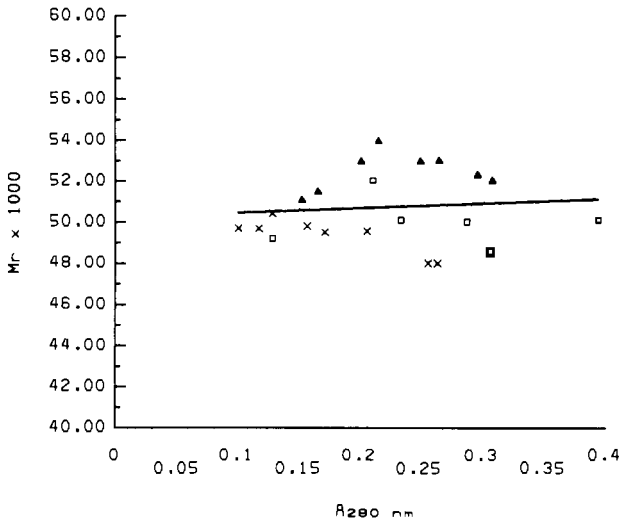


FIGURE 6: Sedimentation equilibrium analysis of native recombinant TNF purified from *P. pastoris*. Measurements were performed using 40 mM Tris-HCl, pH 8.5, at 23 °C. Initial protein concentrations were between 0.08 and 0.2 mg/mL. All ln $C$ vs $r^2$ plots were linear (correlation coefficient 0.992–0.997) for the entire solution column. Each point in this figure is the linear least-squares fit to five data points about the concentration ($A_{280nm}$) indicated for the point. The designations are (□) centrifugation at 24000 rpm and (▲ and ×) centrifugation at 30000 rpm.

by sedimentation equilibrium in 6 M GdmCl was 17000 ± 500. Sedimentation equilibrium measurements on our native recombinant TNF yielded a molecular weight of 51000 ± 3000 with no indication of size heterogeneity (Figure 6). Our estimate for the native molecular weight is in agreement with recently reported values of 52000 (Arakawa & Yphantis, 1987) and 50400 (Wingfield et al., 1987) by sedimentation equilibrium measurements on recombinant TNF synthesized in *E. coli*. Sedimentation velocity measurements on our native recombinant TNF yielded an $s^0_{20,w}$ of 4.2 S. This, together with the molecular weight, yields an $f/f_{min}$ of 1.15 for native TNF. This value agrees well with the value of 1.22 reported by Wingfield et al. (1987) and suggests that recombinant TNF exists in solution as a compact trimer. Both X-ray scattering in solution and single-crystal X-ray diffraction studies are consistent with a trimeric structure for native TNF (Lewit-Bentley et al., 1988). Our native TNF eluted from an HPLC size-exclusion column (7.5 × 600 mm TSK SW 2000 column; Phenomenox, Palo Alto, CA) as though it were significantly smaller ($M_r$ 40000) than a globular protein of $M_r$ 51000. This aberrant behavior by TNF during gel filtration has been observed by a number of others (Aggarwal et al., 1985; Maremenount et al., 1985; Shirai et al., 1985; Yamada et al., 1985; Davis et al., 1987). In the one instance where the expected elution behavior for a globular protein of $M_r$ 51000 was observed for TNF from both natural and recombinant sources, the gel filtration was performed in the presence of bovine serum albumin (Smith & Baglioni, 1987); such results suggest that TNF may interact with the chromatographic media.

TNF produced in this yeast expression system exhibits a CD spectrum in the UV quantitatively and qualitatively like those reported for other recombinant TNFs (Davis et al., 1987; Wingfield et al., 1987). The positive ellipticity in the near-UV manifests the asymmetric environments of one or both of the tryptophans (291-nm band and 286- and 281-nm double peak), one or more of the seven tyrosines (281- and 286-nm double peak), and one or more of the four phenylalanines (267- and 262-nm shoulders). As pointed out by others (Davis et al., 1987; Wingfield et al., 1987), the CD spectrum in the far-UV is qualitatively like those of proteins whose predominant periodic secondary structure is β. The fraction of β-structure estimated by different procedures (Provencher, 1982; Siegel et al., 1980) was around 45%.

Table I: Cachectic Activity of TNF Produced in *P. pastoris*

| insulin addition | TNF amount (ng/mL) | TNF act. (units/well)[a] | TNF concn (nM) | % cell conversion[b] |
|---|---|---|---|---|
| − | 0 | 0 | 0 | <10 |
| + | 0 | 0 | 0 | >80 |
| + | 0.6 | 100 | 0.35 | <10 |
| + | 60 | 10000 | 35 | cell death |

[a] Tumoricidal activity of TNF measured in the L929 cell assay. [b] Inhibition of adipocyte conversion is a measure of TNF cachectic activity.

Isoelectric focusing experiments were carried out for 5.5 and 7 h. The pH gradient obtained from both experiments was linear in the range from pH 4 to 8. The current in the gel system reached a constant minimum value after 3 h of focusing at 400 V, indicating that system had attained equilibrium. Stained gels revealed a similar pattern of distribution of protein bands irrespective of the composition of sample solution (see Materials and Methods). In all cases, a closely spaced doublet corresponding to values of 6.7 and 6.8 on the pH gradient was observed. These values compare well with the theoretical p$I$ value of 6.6 calculated from the gene sequence. Additional faint and diffuse bands which appeared in the more acidic portion of the gel were not further characterized. However, in separate experiments, clarified *E. coli* and *P. pastoris* cell lysates containing TNF were subjected to two-dimensional polyacrylamide gel electrophoresis. A series of five spots attributable to TNF were observed in both the extracts (data not shown). These species corresponded to different isoelectric points in the range 5–7, but all had a molecular weight of about 17500 (Dr. B. Dunbar, personal communication). We have no explanation for the apparently diminished levels of three of the five isoelectric species in our purified TNF.

In the denaturing system employed by us for p$I$ measurement, a random-coil configuration is assumed, resulting in complete exposure and noninteraction of charged groups. Although systemic artifacts (e.g., protein–ampholyte interaction) are possible explanations for focusing heterogeneity even in the presence of urea, it is more likely that inherent chemical modifications (e.g., $NH_2$-terminal acetylation or formylation, deamidation of glutamine/aspargine) contribute to charge heterogeneity. It has been suggested that most proteins contain some degree of heterogeneity caused by deamidation (Robinson & Rudd, 1974).

*Biological Activity of TNF.* TNF purified from *P. pastoris* consistently had a specific activity of (1–2) × $10^7$ units/mg of protein in its cytotoxic activity toward L929 cells. TNF purified from *E. coli* had a similar specific activity. The bioactivity of purified TNF (lyophilized or frozen with or without protein stabilizers) as well as that of the TNF in the frozen yeast cell extract containing 1 mM PMSF remained stable for at least 6 months when stored at −20 °C. Purified TNF and the TNFs contained in *P. pastoris* and *E. coli* cell lysates (with 1 mM PMSF) remained stable at temperatures up to 70 °C for 90 min.

TNF purified from *P. pastoris* exhibited cachectic activity (see Table I). Complete inhibition of adipocyte conversion was observed at a TNF concentration of 3.5 × $10^{-10}$ M in the cachectic assay. The same amount of TNF in the tumoricidal assay will cause complete killing of the target cells (L929), indicating that TNF has similar specific activities in both these

assays. This finding is in agreement with the observations of Liang et al. (1986) with recombinant TNF purified from *E. coli*. It remains to be seen whether TNF produced in yeast exhibits the various other biological activities that have been attributed for recombinant TNF derived from *E. coli* (Beutler & Cerami, 1988).

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# Localization of the Fourth Membrane Spanning Domain as a Ligand Binding Site in the Human Platelet $\alpha_2$-Adrenergic Receptor†

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ABSTRACT: The human platelet $\alpha_2$-adrenergic receptor is an integral membrane protein which binds epinephrine. The gene for this receptor has been cloned, and the primary structure is thus known [Kobilka et al. (1987) *Science 238*, 650–656]. A model of its secondary structure predicts that the receptor has seven transmembrane spanning domains. By covalent labeling and peptide mapping, we have identified a region of the receptor that is directly involved with ligand binding. Partially purified preparations of the receptor were covalently radiolabeled with either of two specific photoaffinity ligands: [$^3$H]SKF 102229 (an antagonist) or *p*-azido[$^3$H]clonidine (an agonist). The radiolabeled receptors were then digested with specific endopeptidases, and peptides containing the covalently bound radioligands were identified. Lysylendopeptidase treatment of [$^3$H]SKF 102229 labeled receptor yielded one peptide of $M_r$ 2400 as the product of a complete digest. Endopeptidase Arg-C gave a labeled peptide of $M_r$ 4000, which was further digested to the $M_r$ 2400 peptide by additional treatment with lysylendopeptidase. Using *p*-azido[$^3$H]clonidine-labeled receptor, a similar $M_r$ 2400 peptide was obtained by lysylendopeptidase cleavage. This $M_r$ 2400 peptide corresponds to the fourth transmembrane spanning domain of the receptor. These data suggest that this region forms part of the ligand binding domain of the human platelet $\alpha_2$-adrenergic receptor.

$\alpha_2$-Adrenergic receptors bind endogenous epinephrine and norepinephrine and are involved in the regulation of a variety of physiological processes (Bylund & U'Prichard, 1983). At the cellular level, the signal transduction mechanism involves inhibition of the regulatory enzyme adenylyl cyclase (Jakobs, 1979). The $\alpha_2$-adrenergic receptor has been purified from human platelets (Regan et al., 1986a) and has been shown to functionally interact with the inhibitory guanine nucleotide binding protein, $G_i$ (Cerione et al., 1986). The gene for the human platelet $\alpha_2$-adrenergic receptor has also been cloned (Kobilka et al., 1987). The deduced primary structure of this receptor shows that it is a member of a larger family of G-protein-coupled receptors which includes the $\beta$-adrenergic receptors (Lefkowitz & Caron, 1988), the muscarinic receptors (Kubo et al., 1986; Peralta et al., 1987), the receptor for substance K (Masu et al., 1987), and rhodopsin (Nathans &

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