Recombinant soluble human tissue factor secreted by *Saccharomyces cerevisiae* and refolded from *Escherichia coli* inclusion bodies: glycosylation of mutants, activity and physical characterization

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Tissue factor (TF) is the cell-surface transmembrane receptor that initiates both the extrinsic and intrinsic blood coagulation cascades. The abilities of TF to associate with Factor VIIa and Factor X in a ternary complex and to enable proteolytic activation of Factor X by Factor VIIa reside in the extracellular domain of TF. We describe the expression of the surface domain of TF (truncated TF, tTF) in both *Saccharomyces cerevisiae* and *Escherichia coli* and the biochemical and physical characterization of the recombinant proteins. Wild-type tTF and several glycosylation-site mutants were secreted efficiently by *S. cerevisiae* under the control of the yeast prepro-α-signal sequence; the T13A,N137D double mutant was the most homogeneous variant expressed in milligram quantities. Wild-type tTF was expressed in a non-native state in *E. coli* inclusion bodies as a fusion protein with a poly(His) leader. The fusion protein could be fully renatured and the leader removed by proteolysis with thrombin; the correct molecular mass (24729 Da) of the purified protein was confirmed by electrospray mass spectrometry. Recombinant tTFs from yeast, *E. coli* and Chinese hamster ovary cells were identical in their abilities to bind Factor VIIa, to enhance the catalytic activity of Factor VIIa and to enhance the proteolytic activation of Factor X by Factor VIIa. Furthermore, CD, fluorescence emission and NMR spectra of the yeast and *E. coli* proteins indicated that these proteins are essentially identical structurally.

INTRODUCTION

Tissue factor (TF) is the cell-surface transmembrane receptor that initiates the extrinsic and intrinsic blood coagulation cascades (reviewed by Nemerson [1], Bach [2], Edgington et al. [3] and Davie et al. [4]. TF binds Factor VII, promoting its proteolytic conversion into Factor VIIa. Subsequently, the active complex of TF and Factor VIIa converts Factors IX into IXa and X into Xa by limited proteolysis, thus triggering the downstream coagulation pathways.

The biochemistry of TF is relevant to haemostasis and to various pathological events. Under normal physiological conditions, it is expressed by extravascular and perivascular cells but not by vascular endothelial cells or leucocytes [5]. On damage to a blood vessel, coagulation factors circulating in the blood come into contact with TF in the perivascular layer, allowing initiation of the coagulation cascades. Although such activation is clearly important in the event of vascular injury, the expression of TF within the vasculature can have catastrophic consequences. Intravascular TF expression has been implicated in thrombosis after atherosclerotic plaque rupture [6] and in disseminated intravascular coagulation (DIC) associated with Gram-negative septic shock [7]. TF antagonists have been demonstrated in animal models to prevent arterial thrombosis [8], to prevent arterial reclusion after thrombolytic reperfusion therapy [9] and to attenuate DIC [10,11]. In addition, recent evidence implicates TF as a participant in mediating tumour metastasis in a mouse model [12].

All recognized physiological and pathological effects of TF are attributable to its interactions with Factor VII/VIIa to form the functional binary complex, then with substrate zymogens, Factors IX and X in the ternary complex. Thus inhibition of these interactions may be an effective therapeutic strategy to intervene in vascular thrombosis, DIC and tumour metastasis. In order to design such inhibitors, a detailed structural model for the interactions of TF is required.

The deduced amino acid sequence of TF indicates a mature protein of 263 amino acid residues consisting of three distinct domains: an extracellular domain, residues 1–219; a hydrophobic transmembrane domain, residues 220–242; and a cytoplasmic domain, residues 243–263 [13–16]. The extracellular domain contains two disulphide bonds and three potential N-linked glycosylation sites. The single cysteine of the cytoplasmic tail of human TF carries stearate or palmitate covalently linked via a thioester bond [2]. The abilities of TF to associate with Factor VIIa and to enhance the catalytic and proteolytic activities of Factor VIIa reside primarily in the TF extracellular domain [17–21].

Recently, two X-ray-crystal structures of the TF extracellular domain (truncated TF, tTF) have been described [22,23]. However, no data are yet available on the solution structure and dynamics of tTF and its complex with Factor VIIa. As an initial step towards obtaining such information, we have investigated the utility of both *Saccharomyces cerevisiae* and *Escherichia coli* expression systems for obtaining milligram quantities of tTF suitable for structural analysis by NMR spectroscopy. We

Abbreviations used: TF, tissue factor; tTF, TF extracellular domain; $tTF_{T13A,N137D}$, T13A,N137D double mutant of tTF; DIC, disseminated intravascular coagulation; YPD, yeast extract/peptone/dextrose growth medium; LB, Luria broth growth medium; PGK, phosphoglycerate kinase; IPTG, isopropyl β-D-thiogalactopyranoside; TEA triethanolamine; TBS, tris-buffered saline; Ni–NTA, nickel nitrilotriacetic acid; CHO, Chinese hamster ovary; HSQC, heteronuclear single-quantum coherence.

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report here the expression of wild-type tTF and a series of glycosylation-site mutants by yeast and the expression of the wild-type protein in *E. coli*; efficient incorporation of ^{15}N labels was achieved in both systems. In addition, we describe an efficient method for *in vitro* renaturation of the *E. coli* material, and detailed biochemical and physical characterization of proteins from both sources.

EXPERIMENTAL

Materials

Strains of *S. cerevisiae* used were GRY67 (MATα trp1Δ ura3-52) and HMY401 (MATα leu2-3, 112 can1^R) which were provided by Dr. David Higgins (Invitrogen, San Diego, CA, U.S.A.). *E. coli* strains DH5α (Gibco–BRL) and BL21 (Novagen) were used for cloning and fusion protein expression respectively. The *E. coli–S. cerevisiae* shuttle vector pMFα8 was obtained from American Type Culture Collection, and *E. coli–S. cerevisiae* shuttle vector pMA91 [24] was provided by Dr. Ian Campbell (Oxford University, Oxford, U.K.); both of these vectors contain genes for replication and maintenance in both *E. coli* and *S. cerevisiae*. The *E. coli* expression vector pTrcHisC was obtained from Invitrogen. Restriction endonucleases, *Taq* polymerase and DNA ligase were purchased from commercial sources. DNA oligonucleotides were synthesized by The Scripps Research Institute Molecular Biology Core facility. Thrombin was produced by cleavage of prothrombin with *Echis carinatus* venom and purified by ion-exchange chromatography, as described by Ruf et al. [25]. Growth media used for yeast were YPD and omission media, both described by Rothstein [26], and minimal medium [1.7 g/l yeast nitrogen base without amino acids or $(NH_4)_2SO_4$, 20 g/l glucose, 0.5 g/l $(^{15}NH_4)_2SO_4$, 5 g/l Na_2SO_4]. Growth media used for *E. coli* were LB (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 50 mg/l ampicillin) and minimal medium [6.7 g/l Na_2HPO_4, 3.0 g/l KH_2PO_4, 1.5 g/l NaCl, 8 g/l glucose, 0.75 g/l $(^{15}NH_4)_2SO_4$, 0.75 g/l $^{15}NH_4Cl$, 10 mg/l thiamine, 10 mg/l biotin, 50 mg/l ampicillin, 2.86 mg/l boric acid, 25 mg/l $CaCl_2,2H_2O$, 0.04 mg/l $CoCl_2,6H_2O$, 0.2 mg/l $CuSO_4,5H_2O$, 208 mg/l $MgCl_2,6H_2O$, 0.19 mg/l $MnCl_2,4H_2O$, 0.002 mg/l MoO_3, 0.208 mg/l $ZnCl_2$, 3.24 mg/l $FeCl_3$].

Construction of yeast expression vectors

The expression vector pMFα8-tTF for the secretion of tTF by yeast under the control of the α-factor promoter was constructed as follows. The cDNA coding for tTF (residues 1–218) was extended and amplified by PCR using the primers 5′-GGGGTATCTTTGGATAAAAGATCAGGCACTACAAATACTGTG-3′ (5′-primer) and 5′-GCAGCCAAGCTGGCCTCGAAGTTATCTGAATTCCCCTTTCTCCTG-3′ (3′-primer). The PCR product was cloned into the *Stu*I restriction site of the *E. coli–S. cerevisiae* shuttle vector pMFα8 by *in vivo* homologous recombination in DH5α *E. coli* [27]. The *S. cerevisiae* strain GRY697 was transformed with the recombinant plasmid pMFα8-tTF (see Figure 1) using the LiCl transformation procedure [26], and selection of transformants on tryptophan-deficient media gave MATα haploids that expressed and secreted tTF.

The expression vector pMA91-tTF for the secretion of tTF by yeast under the control of the phosphoglycerate kinase (PGK) promoter was constructed as follows. The prepro-α-tTF fragment of pMFα8-tTF was amplified by PCR using the primers 5′-ATATAAGGATCCAAAAGAATGAGATTTCC-3′ (5′-primer) and 5′-CTTTGACCTGGATCCAAGCTGGCCTCG-3′ (3′-primer). The PCR product was cleaved by the restriction enzyme *Bam*HI and ligated into the unique *Bgl*II restriction site on the shuttle vector pMA91; recombinant plasmids were isolated after DH5α *E. coli* transformation. The recombinant vector, pMA91-tTF (see Figure 1), was used to transform *S. cerevisiae* strain HMY401 using the LiCl transformation procedure and transformants were selected on leucine-deficient media.

Site-directed mutagenesis

Site-directed mutants in the TF coding sequence cloned into CDM8 were generated by oligonucleotide-directed mutagenesis, as described in detail [19]. Mutants were identified by newly introduced restriction sties. The mutated coding sequence was then subcloned in pMFα8, as described above.

Expression and purification of tTF from yeast

Inoculant cultures of GRY697 transformed with the vector pMFα8-tTF or HMY401 transformed with the vector pMA91-tTF were grown by shaking in tryptrophan- or leucine-omission medium respectively at 30 °C overnight. Unlabelled samples were prepared using YPD growth medium and ^{15}N-labelled samples were prepared using minimal medium. Inoculant cultures (5 ml) were added to growth medium (1 litre) which were then grown with shaking at 30 °C until the cultures reached stationary phase (about 40 h). The cultures were centrifuged [4000 ***g***, 2 h] and the supernatants were filtered consecutively through 1.2 μm and 0.45 μm membranes. The pH of the filtrate was adjusted to 7.4–7.5 and PMSF was added to a final concentration of 0.75 mM. The resultant solution was passed at about 1.5 ml/min over an immunoaffinity column loaded with AVIDGEL (BioProbe International, Tustin, CA, U.S.A.) conjugated to the anti-TF monoclonal antibody TF8-5G9. The column was washed successively with Tris-buffered saline (TBS, pH 7), 1 M NaCl and 0.1 M glycine (pH 4.5), and the protein was eluted with 0.1 M glycine (pH 2.7). Fractions containing protein were pooled, dialysed against 20 mM triethanolamine (TEA, pH 7.5) and loaded on to an HR 10/10 Mono Q anion-exchange column. The protein was eluted using a 20 mM TEA buffer containing NaCl at a concentration increasing linearly from 0 to 100 mM over 40 min (flow rate 3 ml/min).

Construction of the *E. coli* expression vector

The cDNA coding for tTF (residues 1–218) was amplified by PCR using the primers 5′-GAAGAAGGGATCCTGGTGCCTCGTGGTTCTGGCACTACAAATACT-3′ (5′-primer) and 5′-CTGGCCTCAAGCTTAACGGAATTCACCTTT-3′ (3′-primer) which allowed the addition of the coding sequence for a thrombin cleavage site upstream of the cDNA. The PCR products were cleaved using *Bam*HI and *Hin*dIII and ligated between the *Bam*HI and *Hin*dII sites of the expression vector pTrcHisC (Invitrogen). DH5α cells were transformed with the ligation mixture and recombinant plasmids were isolated after selection in the presence of ampicillin. The *E. coli* strain BL21 was transformed with the recombinant plasmid pTrcHisC-tTF (Figure 1), and the resultant transformants were used for fusion protein expression.

Expression, refolding and purification of tTF from *E. coli*

The poly(His)–tTF fusion protein was expressed using BL21 cells transformed with pTrcHisC-tTF. Inoculant cultures (10 ml in LB medium) were grown overnight with shaking at 37 °C. Unlabelled samples were prepared using LB medium, and ^{15}N-labelled samples were prepared using minimal medium. Inoculant

cultures were added to growth medium (1 litre) which were then grown with shaking at 37 °C. When the absorbance at 550 nm had reached about 0.5, 10 ml of 100 mM isopropyl β-D-thiogalactopyranoside (IPTG) was added. Shaking was continued at 37 °C for about 20 h (to stationary phase).

The cells were harvested by centrifugation (10000 *g*; 20 min) and the inclusion bodies were isolated as follows (quantities of reagents are per gram of cell paste). The cell paste was suspended in 4 ml of 10 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 1 mM $MgCl_2$, 0.17 mg/ml PMSF and 2 mg/ml hen's-egg white lysozyme (Sigma). Benzonase (250 units; EM Science) was added and the suspension was mixed gently at room temperature for 1.5 h then centrifuged at 12000 *g* for 15 min. The pellet was resuspended in 10 mM Tris/HCl, pH 7.5, containing 1 mM EDTA and 3% Nonidet P40 (2 ml), sonicated for 1 min at 50% power and centrifuged at 12000 *g* for 20 min. The pellet was resuspended in water, sonicated for 20–30 s at 50% power and centrifuged at 12000 *g* for 20 min. The water wash was repeated and the final pellet, highly enriched for the inclusion bodies, was suspended in buffer containing 6 M guanidinium chloride, 0.5 M NaCl, 20 mM phosphate and 10 mM 2-mercaptoethanol, pH 8 (9 ml/g of inclusion bodies) by gentle mixing at room temperature overnight. The suspension was centrifuged at 12000 *g* for 20 min and the supernatant was loaded onto a nickel nitrilotriacetic acid (Ni–NTA; Qiagen) column. The column was washed successively with the same 6 M guanidinium chloride buffer at pH 8 then pH 7, then the protein was eluted by decreasing the pH to 4.

Ni–NTA column fractions containing the fusion protein were combined and dithiothreitol was added to 50 mM. The solution was held at room temperature overnight then diluted to a protein concentration of about 1 mg/ml in 6 M urea/50 mM Tris/HCl/0.02% NaN_3, pH 8 and dialysed at 4 °C overnight against 10–20 vol. of the same buffer. The buffer was changed to one containing 2 M urea, 50 mM Tris/HCl, 300 mM NaCl, 2.5 mM GSH, 0.5 mM GSSG and 0.02% NaN_3, pH 8 (folding buffer). Dialysis was continued for 2 days; the buffer was then replaced by fresh folding buffer and dialysis was continued for 2 more days. The solution was then dialysed extensively against 20 mM TEA, pH 7.5, removed from the dialysis bag, treated with human thrombin (about 1 part per 500 parts recombinant protein, w/w) overnight at room temperature, and loaded on to an HR 10/10 Mono Q anion-exchange column. tTF protein was eluted using a 20 mM TEA buffer containing NaCl at a concentration increasing linearly from 0 to 150 mM over 30 min (flow rate 3 ml/min).

Expression and purification of tTF from Chinese hamster ovary (CHO) cells

tTF (residues 1–219) was expressed in CHO cells and purified as described previously [17,28].

DNA sequencing

Sequences of coding DNA in the expression plasmids were confirmed using dideoxy fluorescent terminator cycle sequencing technology performed using an Applied Biosystems (Foster City, CA, U.S.A.) model 373A DNA sequencer.

N-Terminal amino acid sequencing

N-Terminal amino acid sequences of proteins were confirmed by Edman degradation performed on Beckman 8 mm protein supports on a Porton 2090 gas phase sequencer.

Amino acid analysis

Protein samples (5–10 μg) were hydrolysed in 6 M HCl/0.01% phenol/0.05% 2-mercaptoethanol at 110 °C for 20–24 h, dried *in vacuo*, then reconstituted in 250 μl of 0.2 M sodium citrate, pH 2.2. Aliquots (50 μl) were analysed, by the method of Spackman et al. [29], on a Beckman model 6300 high-performance analyser modified with a 25 cm-long cation-exchange column for improved resolution.

Electrospray MS

Electrospray MS experiments were performed on an API III Perkin–Elmer SCIEX triple-quadruple mass spectrometer. Samples, in 5–10% acetonitrile, were introduced into the analyser at a rate of 4 μl/min. The positive ions generated by the evaporation process entered the analyser through an interface plate and a 100 μm orifice, and the declustering potential was maintained at 100 V to control the collision energy of the entering ions. The instrument was scanned from m/z 500 to 1500.

ELISA

The concentrations of wild-type or mutant tTF secreted into the culture media by yeast were assessed by an ELISA in which a defined aliquot of cell-free culture supernatant was serially diluted and subjected to immunoassay based on two monoclonal antibodies to non-overlapping epitopes [19].

SDS/PAGE

SDS/PAGE was performed on 12% polyacrylamide gels at 30 mA using the buffer system described by Laemmli [30]. Gels were stained with 0.08% (w/v) Coomassie Brilliant Blue G (Sigma), 3.5% (w/v) $HClO_4$ and destained in 5% (v/v) acetic acid/10% (v/v) methanol.

Western-blot analysis

Proteins separated by SDS/PAGE (using a Novex 8–16% Tris/glycine gel) were transferred to nitrocellulose using a Janssen SemiDry Electroblotter. After blocking with 5% non-fat dry milk in TBS, membranes were incubated with goat anti-TF (5 μg/ml) polyclonal IgG fraction for 1 h. After washing in TBS, bound antibody was allowed to react with horseradish peroxidase-conjugated secondary antibody. Addition of the ECL Western-blotting detection reagents (Amersham) gave rise to chemiluminescence which was detected by a 1 min exposure of blue-light-sensitive autoradiography film.

Spectrozyme FXa hydrolysis

The dissociation constant for the interaction of tTF samples with Factor VIIa was measured using Spectrozyme FXa (American Diagnostica, Greenwich, CT, U.S.A.) as the chromogenic substrate for the tTF–Factor VIIa complex [17,18]. To each well in a microtitre plate was added: (a) 26 μl of 20 mM $CaCl_2$ in TBS containing 0.1% BSA; (b) 26 μl of tTF diluted in TBS containing 0.1% BSA to a concentration ranging from 24 μM to 307 pM; and (c) 26 μl of 8 nM Factor VIIa in TBS containing 0.1% BSA. After 15 min at room temperature, 26 μl of Spectrozyme FXa was added and the initial reaction rate was monitored by the

development of colour at 405 nm. Rates were converted into concentrations of tTF–Factor complexes based on the maximum rate observed at saturation of the fixed concentration of Factor VIIa. Based on the calculated free and bound ligand, the apparent dissociation constant $K_{d_{app.}}$ was determined by fitting these data to the single-site-binding equation

$$[\text{Bound ligand}] = (\text{number of binding sites} \times [\text{free ligand}])/([\text{free ligand}] + K_{d_{app.}}).$$

Factor X activation

The ability of tTF to enhance the specific proteolytic activation of Factor X by Factor VIIa was assessed as described by Ruf et al. [17].

CD spectroscopy

CD spectra were generated on an Aviv 61DS spectropolarimeter in 1 mm cells at 25 °C using a protein concentration of 50 μM in 50 mM NaCl/2.5 mM phosphate/50 μM EDTA, pH 7. Data were collected at 0.5 nm intervals over the range 260–205 nm with a collection time of 4 s per point and a 1.5 nm bandwidth. Data shown are the average of five scans smoothed using a third-order polynomial function over a four-point range.

Fluorescence emission spectroscopy

Samples for fluorescence emission spectra were dissolved at 4 mM in 20 mM phosphate/0.5 M NaCl, pH 7.5, containing concentrations of guanidinium chloride ranging from 0 to 5.85 M. Spectra were generated at 19 °C on a SLM Aminco SPF-500C spectrofluorimeter (SLM Instruments) using an excitation wavelength of 290 nm and excitation and emission slit widths of 2 and 7.5 nm respectively. Data were recorded at 1 nm intervals over the range 300–450 nm and were smoothed (three passes) after acquisition. Equilibrium unfolding data were fitted to the two-state equation:

$$\lambda_{max.}(D) = \frac{\{\lambda_N + \lambda_U \exp[-(\Delta G^\circ_{N-U} + m_G[D])/RT]\}}{\{1 + \exp[-(\Delta G^\circ_{N-U} + m_G[D])/RT]\}}$$

in which $\lambda_{max.}(D)$ is the wavelength of the fluorescence emission maximum at the denaturant concentration [D], λ_N and λ_U are the wavelengths of the fluorescence emission maxima for the native and fully denatured states respectively, ΔG°_{N-U} and m_G are the intercept and slope respectively of the ΔG versus [D] curve [31], and R and T are the universal gas constant and the absolute temperature respectively. This equation assumes that λ_N and λ_U do not vary significantly as a function of [D] and that ΔG varies linearly as a function of [D].

NMR spectroscopy

Samples for NMR spectroscopy were dissolved at concentrations of 0.5–2 mM in 90 % H_2O/10 % ^2H_2O buffer containing 20 mM phosphate, 50 mM NaCl and 0.02 % NaN_3. NMR spectra were recorded at 308 K on Brüker AMX500 and AMX600 spectrometers equipped with three-channel interfaces. ^1H–^{15}N heteronuclear single quantum coherence (HSQC) spectra were recorded using the pulse sequence described by Bodenhausen and Ruben [32] and using low-power presaturation for solvent suppression. The ^1H carrier was placed on the H_2O signal and 1 K complex points were acquired in ω_2, with a spectral width of 12.5 kHz. In ω_1, the ^{15}N carrier was placed at 118.0 p.p.m., and 256 real points were collected, with a spectral width of 1600 Hz on the AMX500 or 2000 Hz on the AMX600 spectrometer. Frequency discrimination in ω_1 was achieved by time-proportional phase incrementation [33]. Spectra were processed using a modified version of the FTNMR software (Hare Research). A low-pass filter was applied to suppress residual solvent signal. Window functions used were 10 Hz line-broadening in ω_2 and a 90°-shifted sine bell in ω_1.

RESULTS AND DISCUSSION

Expression of wild-type tTF in yeast

Wild-type tTF was produced, using the *E. coli–S. cerevisiae* shuttle plasmid pMFα8-tTF (Figure 1), in a *S. cerevisiae* expression system under the control of prepro-α, the leader and signal sequence for yeast α-mating factor. In this expression system, prepro-α directs the glycosylation and secretion of native tTF into the culture medium; during the secretory process, the prepro-α sequence is cleaved from the tTF polypeptide by the KEX2 protease. The prepro-α sequence has been used previously

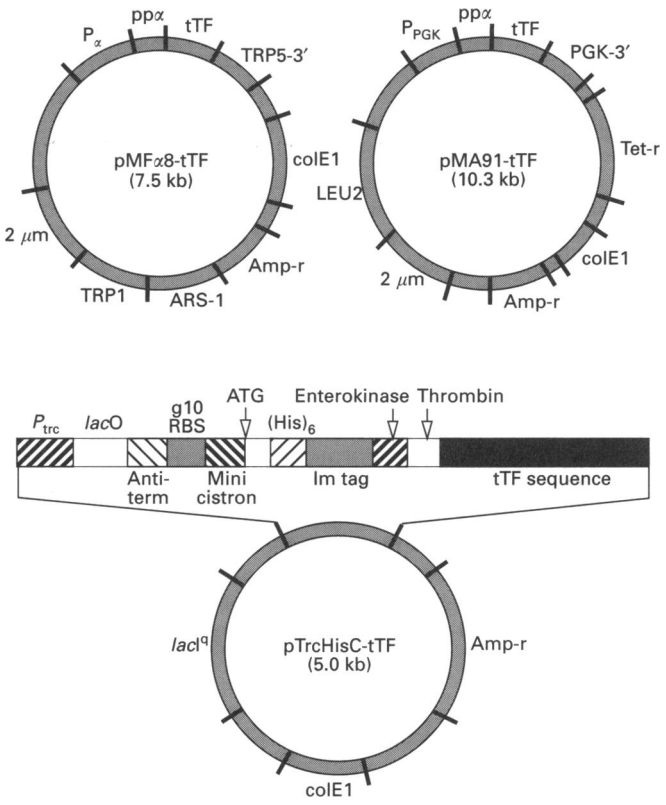

Figure 1 tTF expression vectors

Vectors constructed for the expression of tTF in yeast (pMFα8-tTF and pMA91-tTF) and *E. coli* (pTrcHisC-tTF) are shown. ppα is the prepro signal sequence of yeast α-mating factor. tTF indicates the coding sequence for TF, residues 1–218. P_α and P_{PGK} are the α-factor and phosphoglycerate kinase promoters respectively; TRP5-3′ and PGK-3′ are transcription termination sequences; colE1 is the origin of replication in *E. coli*, and 2 μm and ARS-1 are origins of replication in *S. cerevisiae*; Amp-r and Tet-r are ampicillin- and tetracycline-resistance genes for selection in *E. coli*, and TRP1 and LEU2 are tryptophan- and leucine-biosynthesis genes for selection in *S. cerevisiae* strains containing defects in these genes. The initiation codon (ATG) in pTrcHisC-tTF is preceded by a hybrid *trp/lac* promoter (P_{trc}), the binding site (*lac*O) for the *lac* repressor (encoded by the *lacI*^{q} gene), a transcription anti-termination sequence (anti-term), the bacteriophage T7 gene 10 ribosome-binding site (g10 RBS) and a mini-cistron to promote efficient initiation of translation, and followed by a poly(His) tag, an immunoreactive tag (Im tag, the T7 gene 10 translational enhancer; [34]), an enterokinase-cleavage site, a thrombin-cleavage site and the tTF sequence.

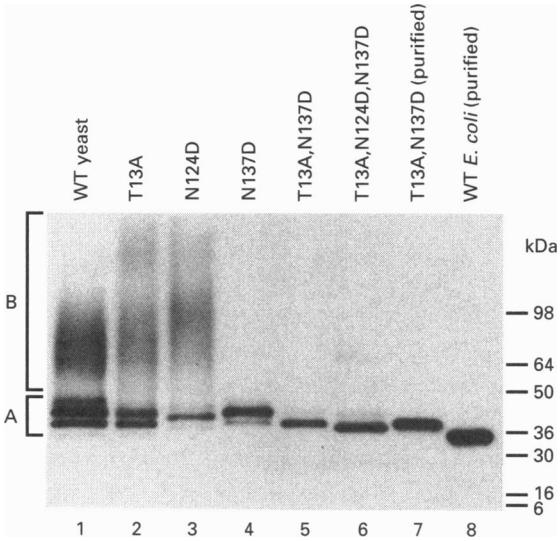

Figure 2 Western-blot analysis of recombinant tTF

The results of Western-blot analysis of tTF expressed in yeast and *E. coli* are shown. Lanes 1–6, wild-type (WT) and selected glycosylation-site mutants expressed in yeast. Cultures were grown for 24 h in tryptophan-omission medium. tTF was isolated from the culture medium by binding to anti-TF antibody TF8-5G9 conjugated to AVIDGEL beads and was eluted from the beads with 100 mM acetic acid, lyophilized and assayed by ELISA. Lane 7, $tTF_{T13A,N137D}$ expressed in yeast and purified as described in the Experimental section; lane 8, tTF expressed in *E. coli* and purified as described in the Experimental section. Some 25–50 ng of reduced protein was loaded in each lane. Region A indicates protein that either is non-glycosylated or contains only core glycosylation; region B indicates protein containing outer-chain glycosylation.

to direct the secretion of a variety of other foreign proteins at expression levels ranging from 10 μg/l to 10 mg/l [35–37]. In the case of tTF the foreign protein was secreted into the culture medium at levels of 5–15 mg/l (see Table 1) as assessed by an ELISA using two anti-TF monoclonal antibodies.

The expression vector pMFα8-tTF uses the α-factor promoter and terminator sequences for transcription control. In an attempt to increase expression levels, we also expressed tTF using the vector pMA91-tTF (Figure 1) in which transcription is controlled by the PGK promoter and terminator sequences, considered to promote high levels of transcription in yeast. Changing the promoter did not result in any change in expression levels. Others have reported similar expression levels for tTF (residues 1–217) in yeast using the yeast acid phosphatase promoter and yeast invertase signal sequence [38]. Thus the expression levels appear to be limited not by transcription but rather by some aspect of the translation or secretion processes.

tTF contains three potential N-linked glycosylation sites of the form Asn-Xaa-Ser/Thr (Asn^{11}-Leu^{12}-Thr^{13}, Asn^{124}-Val^{125}-Thr^{126} and Asn^{137}-Asn^{138}-Thr^{139}). N-linked glycosylation in yeast typically involves an inner core of about ten mannose residues, linked to the asparagine via two GlcNAc residues, and a branched outer chain of 50–100 mannose residues [39]. Therefore N-linked glycosylation could potentially add as many as 300 mannose residues to tTF, an increase in molecular mass from about 25 to about 80 kDa. It is also possible that several mannose residues could be attached to various O-linked glycosylation sites. Although SDS/PAGE is not thought to yield accurate mass estimates for TF (see below), it does allow approximate masses and mass differences to be evaluated. The wild-type tTF that we expressed in yeast was highly and heterogeneously glycosylated (Figure 2). Low-molecular-mass species (apparent molecular masses 37–45 kDa), comprising about 50% of total expressed protein, were probably due to various configurations of core glycosylation only. These were distributed among four discrete bands, presumably corresponding to core glycosylation at different numbers of sites (see discussion of mutants below). The remaining approx. 50% of the protein was distributed over the apparent molecular-mass range 50–100 kDa, indicating heterogeneous outer-chain glycosylation.

In contrast with our results, tTF (residues 1–217) secreted by yeast strain AH22 (MATa, leu2, his4, $can1^R$, cir^+) under the control of the inducible yeast acid phosphatase promoter and the yeast invertase signal sequence [38] was purified as two forms with apparent molecular masses of 150 and 37 kDa; the former was a broad band on SDS/PAGE whereas the latter was highly homogeneous. The protein produced in our system is more heterogeneous than the Shigematsu protein, particularly with respect to the different configurations of core glycosylation. The difference is probably attributable to the different signal sequences and yeast strains used in the two systems. We note that most commonly available yeast strains, including AH22, contain multiple amino acid synthesis gene mutations. However, in order to obtain isotopically labelled samples for NMR spectroscopy (see below) it is necessary to grow the transformed yeast on minimal medium, i.e. the transformed yeast must have the ability to biosynthesize all amino acids. Therefore we used a strain of yeast containing a single amino acid gene mutation which is complemented by a gene on the expression plasmid (TRP1 or LEU2).

tTF was isolated from the culture medium by immunoaffinity chromatography using an anti-TF monoclonal antibody column. Subsequent ion-exchange chomatography allowed residual antibody to be removed. The ion-exchange elution trace indicated a high level of apparent charge heterogeneity (results not shown). Although glycosylation is not expected to change the actual charge on the protein in this system, this result suggests that heterogeneous glycosylation results in protein surface-charged groups being differentially protected in differently glycosylated molecules.

Expression of glycosylation-deficient tTF mutants in yeast

The high degree of glycosylation and heterogeneity of wild-type tTF secreted by yeast made this material unsuitable for high-resolution structural studies by NMR spectroscopy. Therefore several strategies were considered to reduce the level of glycosylation. Complete inhibition of N-linked glycosylation using either tunicamycin or strains of yeast defective in the addition of GlcNAc to proteins was not feasible because N-linked glycosylation within the prepro-α signal sequence is essential for protein processing and secretion [40–42]. An attempt to reduce the degree of glycosylation by expression of tTF in a yeast strain with the *mnn9* mutation, which prevents formation of certain outer-chain mannose–mannose linkages [43], was unsuccessful. Treatment of immunopurified tTF with jack bean α-mannosidase [44] resulted in a slight decrease in molecular mass. However, efficient removal of high-molecular-mass mannose chains could not be achieved by this method. Therefore a series of ten mutants was constructed in which one or more of the N-linked glycosylation sites were abolished.

The mutants and their expression levels, as measured by ELISA of the culture supernatants, are listed in Table 1. There was significant variation in expression levels measured in independent experiments. However, the following trends are evident. (1) The wild-type construct expressed at the highest levels, generally in the range 5–15 mg/l, and mutation of one or

Table 1 Expression levels of tTF glycosylation-site mutants in yeast

Given are the expression levels of wild-type and glycosylation-site mutant tTF proteins secreted into the culture medium in the yeast expression system. For each measurement, 5 ml of tryptophan-omission medium was inoculated with an individual colony of GRY697 yeast transformed with the appropriate pMFα8-derived expression vector. The culture was grown for 40 h at 30 °C (i.e. to stationary phase), and the cell-free medium was assayed by ELISA. Values shown are the means ± S.D. of 6–11 independent experiments, as indicated. For the N11Q,N137Q mutant, expression levels were always less than the limit of detectability by this method.

Construct	Expression level (mg/l)	Number of experiments
Wild-type	11.9 ± 9.1	8
N11Q	1.2 ± 0.4	7
T13A	1.9 ± 2.6	10
N124D	3.8 ± 1.7	7
N137D	8.5 ± 6.8	8
N137Q	4.0 ± 1.9	6
N11Q,N137D	0.6 ± 0.3	9
N11Q,N137Q	< 0.1	6
T13A,N137D	2.0 ± 0.9	10
T13A,N137Q	1.0 ± 0.8	11
T13A,N124D,N137D	0.2 ± 0.1	8

more of the N-linked glycosylation sites resulted in decreased protein yields. (2) Expression levels were sensitive to the specific mutation introduced to abolish glycosylation of a site. In particular, substitution of asparagine with glutamine gave lower expression levels than other mutations at the same site. (3) The expression levels observed for multiple-site mutants were generally lower than those observed in the corresponding single mutants. For example, the N11Q,N137D double mutant expressed at lower levels than either the N11Q or the N137D single mutant.

A Western blot of the wild type and selected mutant tTF proteins is shown in Figure 2. Mutation of either the first or second glycosylation site (lanes 2 and 3) does not significantly reduce the level of outer-chain glycosylation although a clear redistribution of core glycosylation is evident. Mutation of the third site alone (lane 4) essentially eradicates all outer-chain glycosylation, resulting in a protein with two predominant core glycosylated isoforms with apparent molecular masses of approx. 42 (major form) and 38 (minor form) kDa, whereas mutation of the first site in addition to the third (lane 5) yields a protein with just one predominant glycoform of apparent molecular mass about 38 kDa. Simultaneous mutation at all three sites (lane 6) further reduces the apparent molecular mass of the predominant band to about 37 kDa, although the triple mutant expresses at a 10-fold lower level than the T13A,N137D double mutant. The persistence of a minor band at approx. 39 kDa in the triple mutant suggests that tTF contains O-linked as well as N-linked sugar.

On the basis of observed variations in glycosylation (Figure 2) and expression levels (Table 1), the T13A,N137D double mutant (denoted $tTF_{T13A,N137D}$) was selected as being most suitable for NMR structural studies. The predominant form of this mutant runs on SDS/PAGE at about 38 kDa, only slightly higher than the non-glycosylated *E. coli* protein (see below), and it is available in milligram quantities. This mutant was purified as described above (see Figure 2, lane 7 and Figure 3, lane 5); The N-terminal amino acid sequence was found to be SGTTNT, indicating that cleavage had occurred in the expected position, immediately after the KR sequence of the prepro-α leader, and the amino acid

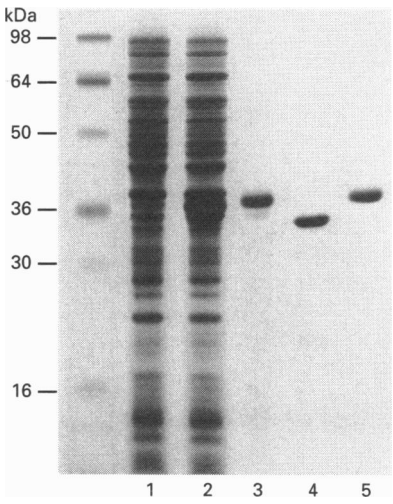

Figure 3 SDS/PAGE of recombinant tTF

Lanes 1 and 2, crude *E. coli* cell lysates. Cells were grown to an absorbance of 0.6, induced by addition of IPTG to 1 mM (lane 2) or not induced (lane 1), then grown for an additional 24 h. Lane 3, poly(His)–tTF fusion protein from *E. coli* after purification on a Ni–NTA column; lane 4, tTF from *E. coli* after cleavage from the leader sequence and ion-exchange purification; lane 5, $tTF_{T13A,N137D}$ expressed in yeast and purified as described in the Experimental section. All samples are reduced.

composition was concordant with the composition of tTF. The biological activity and biophysical properties of this mutant were characterized in detail (see below).

Expression of tTF in *E. coli* and *in vitro* renaturation

tTF was expressed as a fusion protein in *E. coli* using the expression vector pTrcHisC-tTF (Figure 1). The fusion protein (258 amino acids, 29.2 kDa) consisted of: (i) a $(His)_6$ sequence, to allow ready purification by nickel-affinity chromatography; (ii) an immunoreactive tag, to allow ready identification of the fusion protein; (iii) consecutive enterokinase- and thrombin-cleavage sites; and (iv) residues 1–218 of TF. This fusion protein was expressed at about 50 mg/l, the most abundant cellular protein (Figure 3, lane 2), and was localized in inclusion bodies. The inclusion bodies could be solubilized in denaturing buffer and purified to approx. 95% purity (Figure 3, lane 3) by nickel-affinity chromatography.

Efficient renaturation of the affinity-purified fusion protein was highly dependent on the buffer conditions used. Renaturation was expected to be limited by the rate of formation of the correct disulphide bonds (Cys^{29}-Cys^{57} and Cys^{186}-Cys^{209}). Thus folding was performed at pH 8 in the presence of a redox exchange buffer containing a mixture of GSH and GSSG, under which conditions disulphide exchange is expected to be rapid. Guanidinium chloride (1.4–2.0 M) or urea (2–5 M) provided conditions under which the denatured material remained soluble, but the native state was more stable than the denatured state (see Figure 6d). Under such conditions the protein does not aggregate, thereby allowing the system to reach equilibrium with the native structure predominant. Refolding was also favoured by low temperature (4 °C) and about 300 mM NaCl. This is the first description of conditions for the efficient *in vitro* renaturation of TF.

After refolding, the fusion protein could be cleaved by proteolysis with thrombin. It was necessary to perform the refolding

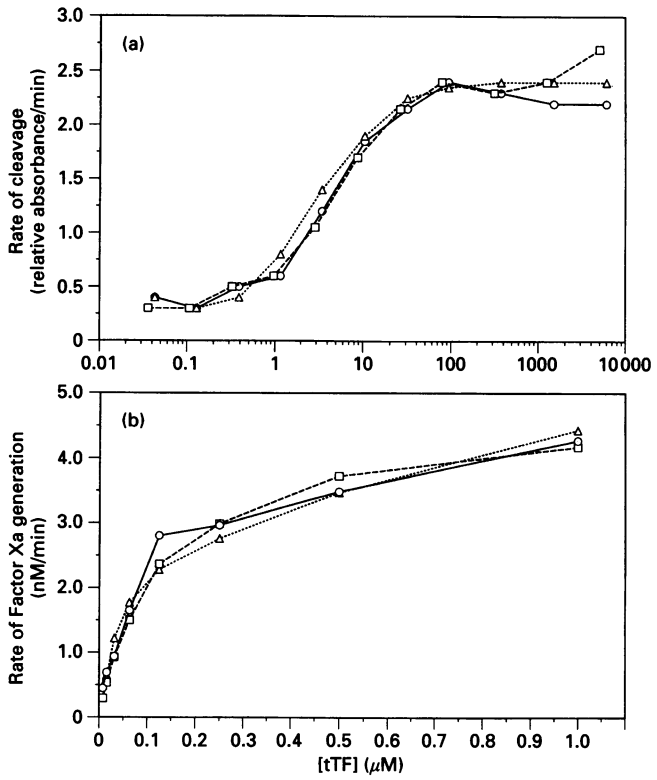

Figure 4 Activity assays of recombinant tTF

(**a**) Initial rates of cleavage of the chromogenic peptide substrate Spectrozyme FXa by 2 nM Factor VIIa in the presence of various concentrations of recombinant tTF from CHO cells (△), yeast (T13A,N137D mutant; □) or *E. coli* (○); (**b**) initial rate of Factor Xa generation from 1 μM Factor X in the presence of 100 nM Factor VIIa and various concentrations of recombinant tTF from CHO cells (△), yeast (T13A,N137D mutant; □) or *E. coli* (○). Factor Xa concentration was assayed by measuring the rate of cleavage of Spectrozyme FXa. Average uncertainities in the rates, evaluated from three independent experiments, are 19, 35 and 13% for the CHO cell, yeast and *E. coli* curves respectively.

before the cleavage because thrombin is denatured at the concentration of denaturant required to maintain solubility of the non-native fusion protein. The thrombin cleavage was highly efficient, being more than 90% complete after incubation of 1 part fusion protein with approx. 0.002 parts (w/w) thrombin at room temperature overnight. Furthermore, there was no evidence of any cleavage within the TF sequence, in line with predictions based on published guidelines for amino acid sequence susceptibilities to thrombin cleavage [45]. The thrombin cleaved between the Arg and Gly of the sequence Leu-Val-Pro-Arg-Gly-Ser*, in which Ser* is not only the final amino acid of the published thrombin-cleavage site [46] but also residue 1 of TF. Thus thrombin-cleavage leaves an additional Gly at the N-terminus of tTF. The cleavage site was confirmed by N-terminal amino acid sequencing of the ion-exchange-purified cleavage product; the N-terminal sequence was found to be GSGTTNTVA, as expected. The amino acid composition of the product was also concordant with the expected sequence.

The apparent molecular mass of the purified protein by SDS/PAGE is 35 kDa (Figure 3, lane 4). However, the calculated molecular mass is 24.7 kDa. This difference is not due to glycosylation because *E. coli* does not glycosylate proteins. However, the unusually slow mobility for a protein of this size is identical with that observed by Paborsky et al. [47] for tTF expressed in *E. coli* and has also been observed in our laboratory and elsewhere for TF constructs expressed in yeast (see above), baculovirus, CHO cells and human kidney 293 cells. Direct evidence confirming the molecular mass of the tTF from *E. coli* was obtained by electrospray MS. By this method the molecular mass was observed to be 24729±2 Da (cf. calculated molecular mass 24729.6 Da).

The biological activity and biophysical properties of the refolded purified tTF from *E. coli* (Figure 3) were characterized in detail (see below).

Activity of recombinant tTF

The activity of recombinant TF extracellular domain expressed in both yeast and *E. coli* was compared with previously authenticated material, expressed in Chinese hamster ovary (CHO) cells, in two separate assays. First the affinity of recombinant protein for Factor VIIa was measured. tTF binds to Factor VIIa and activates it for cleavage of the peptidyl substrate Spectrozyme FXa [17,18]. Monitoring the rate of Spectrozyme FXa cleavage as a function of tTF concentration allows measurement of the apparent dissociation constant for the interaction of tTF and Factor VIIa [21] (Figure 4a). Using this assay, the $K_{d_{app.}}$ for tTF expressed in CHO cells, yeast (T13A,N137D mutant) and *E. coli* were found to be 2.8, 5.3 and 3.8 nM respectively, indicating that, within the accuracy of the experiments, these tTF molecules are identical in their abilities to bind to and activate Factor VIIa. Furthermore, the maximum catalytic rates of the tTF–Factor VIIa complexes at saturation were also identical for all three constructs (Figure 4a), suggesting that the essential components of the enzyme (Factor VIIa)–cofactor (tTF) interaction are retained in the proteins from the various expression systems.

A wide variation of dissociation constants for the TF–Factor VIIa interaction has been reported previously (summarized by O'Brien et al. [48]. In particular, Shigematsu et al. [38] have reported $K_{d_{app.}}$ values of 47.4 and 89.8 nM for low- and high-glycosylation forms respectively of tTF expressed in yeast. These values are more than an order of magnitude higher than the values reported here. Schullek et al. [21] have demonstrated recently that the measured $K_{d_{app.}}$ is highly dependent on the Factor VIIa concentration used in the assay if this concentration is higher than about 5 nM; below 5 nM the $K_{d_{app.}}$ is independent of Factor VIIa concentration. Our assays were performed using 2 nM Factor VIIa to ensure that the $K_{d_{app.}}$ determined was independent of Factor VIIa concentration. Shigematsu and co-workers employed 30 nM Factor VIIa, in the range at which the $K_{d_{app.}}$ is dependent on Factor VIIa concentration.

Second, the ability of the complex between tTF and Factor VIIa to convert Factor X into Factor Xa was assessed. Although the cleavage of Factor X is accelerated significantly by membrane association of both the catalytic complex and Factor X, the complex between tTF and Factor VIIa still cleaves Factor X at a significant rate [17,18]. Figure 4(b) shows the initial rate of Factor Xa generation from Factor X for preformed complexes between Factor VIIa and tTF expressed in CHO cells, yeast (T13A,N137D mutant) and *E. coli*. This comparison indicates that these three complexes are equally efficient at Factor X activation. Taken together with the Spectrozyme FXa assay, these results indicate that the recombinant tTF expressed in yeast and *E. coli* is fully active and therefore that biophysical studies of protein from either source are biologically relevant.

The glycosylation levels in tTF from *E. coli*, yeast and CHO cells vary significantly. Both the yeast and CHO cell proteins are heterogeneously glycosylated; for tTF from CHO cells

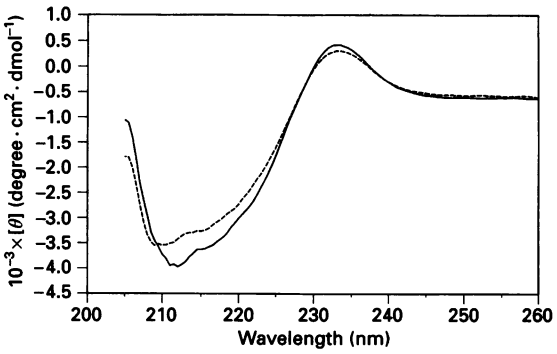

Figure 5 CD spectra of recombinant tTF

CD spectra of $tTF_{T13A,N137D}$ secreted from yeast (– – – –) and tTF refolded from *E. coli* (——) are shown.

glycosylation adds 10–20 kDa to the molecular mass [17]. In contrast, the *E. coli* material carries no sugar residues. The identical biological activity of these proteins indicates that glycosylation does not influence tTF interaction with Factor VIIa or Factor X. This is consistent with previous results reported for full-length TF [47]. The function, if any, of TF glycosylation remains unknown; possible functions include protection from degradation by proteases, facilitation of secretory mechanisms and protection from undesirable protein–protein interactions.

Physical characterization of recombinant tTF

tTF refolded from *E. coli* inclusion bodies and $tTF_{T13A,N137D}$ secreted by yeast were compared by three spectroscopic methods: CD, fluorescence emission and NMR.

CD spectra

tTF proteins from yeast and *E. coli* exhibit very similar CD spectra (Figure 5) with maxima at 233 nm, minima at 209–211 nm and negative shoulders at about 216 nm. The slight intensity difference observed (Figure 5) is within the experimental error which may be estimated on the basis of uncertainty of sample concentration measurements. These spectra are not typical of either α-helical or β-sheet proteins but the weak intensity suggests that there is minimal, if any, α-structure present [49], consistent with the prediction that TF is a distant member of the β-structured cytokine receptor superfamily [50]. This prediction was confirmed recently by X-ray-crystallographic analysis [22,23]. The source of the dominant CD absorbance is most likely either aromatic or disulphide groups in chiral environments, presumably in the hydrophobic protein core [51].

Fluorescence emission spectra

tTF contains four tryptophan residues. Both *E. coli* tTF and $tTF_{T13A,N137D}$ from yeast give intense fluorescence spectra (Figure 6) typical of tryptophan-containing proteins [52]. For both proteins, the fluorescence emission maximum ($\lambda_{max.}$) of the native

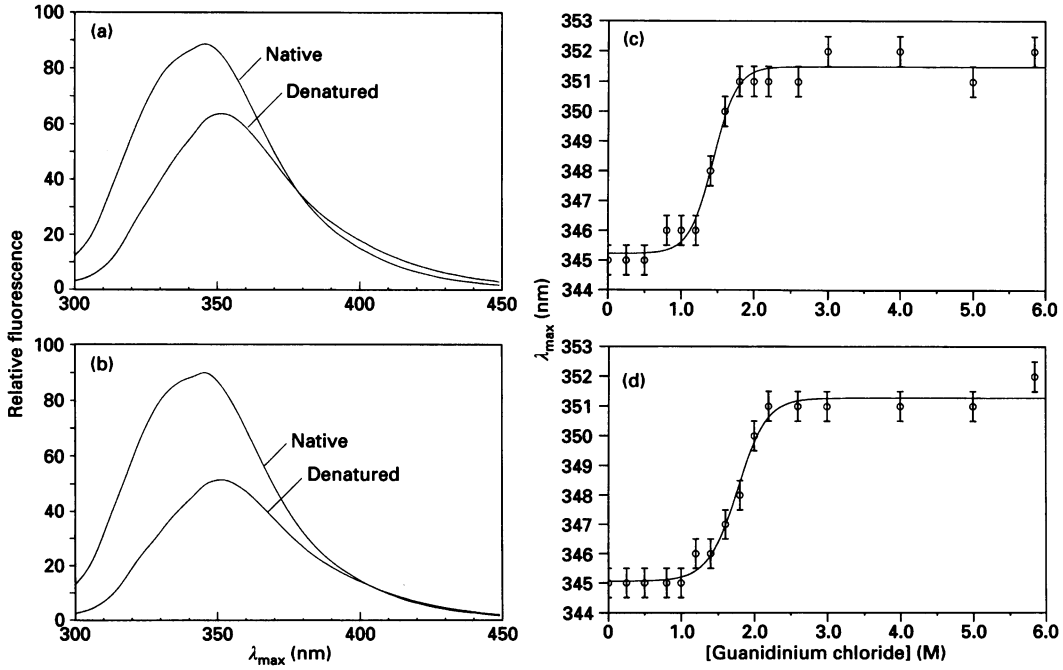

Figure 6 Fluorescence emission spectra of recombinant tTF

Fluorescence emission spectra of native (0.8 M guanidinium chloride) and denatured (2.2 M guanidinium chloride) samples of (**a**) $tTF_{T13A,N137D}$ secreted from yeast and (**b**) tTF refolded from *E. coli*. Also shown are the equilibrium unfolding profiles of (**c**) $tTF_{T13A,N137D}$ secreted from yeast and (**d**) tTF refolded from *E. coli*, measured by monitoring the fluorescence emission maxima at various concentrations of guanidinium chloride. The error bars shown represent the digital resolution of the instrument.

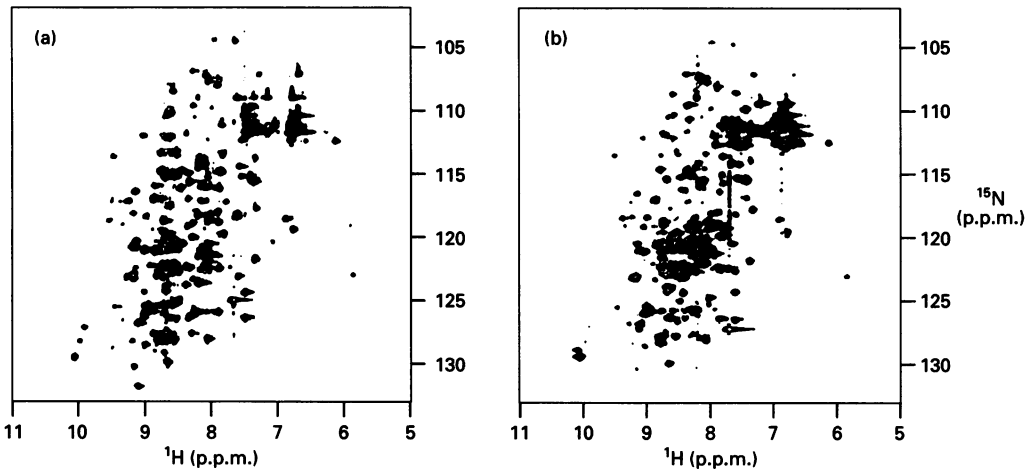

Figure 7 HSQC NMR spectra of recombinant tTF

^{1}H–^{15}N HSQC NMR spectra of (**a**) $tTF_{T13A,N137D}$ secreted from yeast and (**b**) tTF refolded from *E. coli*. Spectra were recorded at 600 MHz and 500 MHz respectively.

protein occurs at 345 nm, using an excitation wavelength of 290 nm. However, on denaturation with guanidinium chloride at a concentration above 2 M, the $\lambda_{max.}$ shifts to 351 nm (Figure 6); denaturation was performed without the reduction of disulphides. The guanidinium chloride-induced equilibrium unfolding curves determined by fluorescence (Figure 6) fit a two-state model with midpoints of 1.8 M guanidinium chloride for the wild-type protein from *E. coli* and 1.4 M guanidinium chloride for the yeast mutant protein. The thermodynamic parameters determined from these curves were $\Delta G^{\circ}_{N-U} = 21.4 \pm 4.6\ kJ \cdot mol^{-1}$ and $m_G = -12.1 \pm 2.5\ kJ \cdot mol^{-1} \cdot M^{-1}$ for tTF from *E. coli* and $\Delta G^{\circ}_{N-U} = 21.4 \pm 5.0\ kJ \cdot mol^{-1}$ and $m_G = -15.1 \pm 3.3\ kJ \cdot mol^{-1} \cdot M^{-1}$ for $tTF_{T13A,N137D}$ from yeast. These unfolding profiles are the same within experimental error.

NMR spectra

tTF produced in either the yeast or *E. coli* expressed system could be uniformly enriched with ^{15}N using expression media in which the sole source of nitrogen was ^{15}N-enriched ammonium salts. The ^{15}N-HSQC NMR spectra of uniformly ^{15}N-labelled tTF from *E. coli* and $tTF_{T13A,N137D}$ from yeast are shown in Figure 7. These spectra correlate directly bonded pairs of ^{1}H and ^{15}N nuclei, and the positions of the cross-peaks in such spectra provide a fingerprint for the tertiary protein structure. Thus the correspondence between peak positions in the two spectra provides strong evidence that tTF from *E. coli* and $tTF_{T13A,N137D}$ from yeast have essentially the same tertiary structure. The few minor differences observed are most likely due to minor structural differences at or adjacent to the two mutation sites or near any remaining glycosylation site(s) in the yeast protein. In addition, these spectra may be used to assess the feasibility of more sophisticated NMR structural studies of tTF. The amide ^{1}H linewidths are of the order of 20–25 Hz which is approximately as expected for a monomeric 25 kDa protein. Although this is considerably broader than the linewidths for most proteins whose structures have been determined by NMR, a number of new triple-resonance experiments are available which should allow resonance assignments and structure determination for this protein.

Conclusion

We have developed two new expression systems which allow access to milligram quantities of tTF from either yeast or *E. coli*, and have defined conditions for the efficient renaturation of the *E. coli* protein. Expression levels in yeast were highly sensitive to mutations at the three N-linked glycosylation sites. However, comparison of tTF from CHO cells, yeast and *E. coli* indicated that glycosylation is unnecessary for tTF to exhibit full biological activity. Comparison of the biochemical and spectroscopic properties of the recombinant proteins indicates that both the *E. coli* and yeast expression systems yield native tTF. Therefore future biophysical studies of these proteins will be relevant to the biological activity of TF.

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