Unusual stability of a multiply nicked form of *Plasmodium falciparum* triosephosphate isomerase

Soumya S Ray[1], Hemalatha Balaram[2] and P Balaram[1]

Background: The limited proteolytic cleavage of proteins can result in distinct polypeptides that remain noncovalently associated so that the structural and biochemical properties of the 'nicked' protein are virtually indistinguishable from those of the native protein. The remarkable observation that rabbit muscle triosephosphate isomerase (TIM) can be multiply nicked by subtilisin and efficiently religated in the presence of an organic solvent formed the stimulus for our study on a homologous system, *Plasmodium falciparum* triosephosphate isomerase (PfTIM).

Results: The subtilisin nicked form of PfTIM was prepared by limited proteolysis using subtilisin and the major fragments identified using electrospray ionization mass spectrometry. The order of susceptibility of the peptide bonds in the protein was also determined. The structure of the nicked form of TIM was investigated using circular dichroism, fluorescence and gel filtration. The nicked enzyme exhibited remarkable stability to denaturants, although significant differences were observed with the wild-type enzyme. Efficient religation could be achieved by addition of an organic cosolvent, such as acetonitrile, in the presence of subtilisin. Religation was also demonstrated after dissociation of the proteolytic fragments in guanidinium chloride, followed by reassembly after removal of the denaturant.

Conclusions: The eight-stranded β8/α8 barrel is a robust, widely used protein structural motif. This study demonstrates that the TIM barrel can withstand several nicks in the polypeptide backbone with a limited effect on its structure and stability.

Addresses: [1]Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560012, India. [2]Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur Campus, Jakkur P.O., Bangalore 560004, India.

Correspondence: P Balaram
E-mail: pb@mbu.iisc.ernet.in

Key words: folding, ligation, mass spectrometry, triosephosphate isomerase

Received: **28 April 1999**
Revisions requested: **1 June 1999**
Revisions received: **14 June 1999**
Accepted: **18 June 1999**

Published: **11 August 1999**

Chemistry & Biology September 1999, **6**:625–637
http://biomednet.com/elecref/1074552100600625

1074-5521/99/$ – see front matter

Introduction

Protein three-dimensional structures are stabilized by a sophisticated network of tertiary interactions between amino acids that are distant in the primary sequence [1–3]. The role of tertiary interactions is best illustrated in the case of 'nicked proteins', in which, despite cleavage of an amide bond, the two fragments remain complexed, yielding a structure virtually indistinguishable from the parent protein [4,5]. Since the seminal work of Richards and Vithayathil [6] on the ribonuclease-S system, several fragment complementation systems have been reported in the literature (Table 1) [7–20]. In most cases the number of interacting fragments in the nicked protein is two; multiply nicked proteins are rare [5].

The β8/α8 barrel is one of the most well-characterized protein folds, with triosephosphate isomerase (TIM) being the first member of the family for which a three-dimensional structure was determined [18]. This fold has therefore been referred to as the TIM barrel fold. Vogel and Chmielewski [14] made the remarkable discovery that rabbit muscle TIM upon proteolysis with subtilisin yielded eight fragments that could be religated in the presence of an organic cosolvent such as acetonitrile to yield a native-like enzyme. The most striking feature about this observation is that, in rabbit TIM, despite several nicks in the polypeptide chain the fragments remain noncovalently held, resembling the native structure, and resulting in high fidelity of resynthesis. In this report we extend the work of Vogel and Chmielewski [14] to a homologous protein *Plasmodium falciparum* TIM (PfTIM) [19], with a view to characterizing the sites of cleavage, and to provide a comparison of the structure and stability of the intact and multiply nicked TIM. Sequence alignment of PfTIM and rabbit muscle TIM indicates that the two proteins are about 42% homologous. Figure 1 shows the sequence alignment of some representative triosephosphate isomerases with the cleavage sites from the study of Vogel and Chmielewski [14] and this study marked on the aligned sequences. It is noteworthy that the cleavage sites on the two homologous proteins are different.

PfTIM is a homodimeric 27 kDa (monomer weight) protein for which a classical TIM barrel fold has been determined in a crystal structure at 2.2 Å resolution [20]. Extensive unfolding studies indicate that the protein has a robust architecture and is substantially resistant to unfolding by urea (C_m ≈ 6.5 M, where C_m is defined as the

Table 1

Systems for which fragment complementation systems have been generated.

Protein	Number of fragments	Method of cleavage	Reference
Staphylococcal nuclease	2	Tryptic digest	[4]
Barnase	2	Cyanogen bromide	[7]
Thermolysin	2	Subtilisin, chymotrypsin	[8]
Cytochrome c	2	Trypsin	[9]
RNAse A	2	Subtilisin	[6]
Streptococcal G protein	2	Chemically synthesized fragments	[10]
Phosphoglycerate kinase	2	Thio-cyanobenzoic acid	[11]
Phosphoribosyl anthrinilate isomerase	2	Genetically engineered, cyanogen bromide	[12]
Maltose-binding protein	2	Genetically engineered	[13]
Triosephosphate isomerase	8	Subtilisin	[14]
Lysozyme	3	Subtilisin	[15]
Chymotrypsin inhibitor II	2	Cyanogen bromide	[16]
Chloride channel	2	Genetically engineered	[17]

denaturant concentration at the mid-point of the unfolding transition) and other forms of denaturation such as heat (T_m = 68°C) [21,22].

Results

Proteolysis of PfTIM and religation

PfTIM (100 μM) was incubated with subtilisin Carlsberg in a ratio of 100:1 (w/w) at 37°C for 15 minutes in a reaction volume of 100 μl. The reaction was quenched with the addition of loading buffer that contained sodium dodecylsulfate (SDS) and β-mercaptoethanol and analyzed on 15% SDS–polyacrylamide gel electrophoresis (PAGE) as shown in lane 2 of Figure 2a. A poorly resolved set of fragments is obtained in the molecular weight range of 10–12 kDa. Lane 1 shows intact PfTIM (~27 kDa). Lane 2 does not show this band, indicating that the starting material was digested completely. The partially digested sample was used for the religation experiments

Figure 1

```
TPIS_RABIT      APSRKFFVGGNWKMNGRKKNLGELITTLNAAKVP-ADTEVVCAPPTAYIDFARQKLD-PK
TPIS_HUMAN      APSRKFFVGGNWKMNGRKQSLGELIGTLNAAKVP-ADTEVVCAPPTAYIDFARQKLD-PK
TPIS_TRYBB      MSKPQPIAAANWKCNGSQQSLSELIDLFNSTSIN-HDVQCVVASTFVHLAMTKERLS-HP
TPIS_PLAFA      -MARKYFVAANWKCNGTLESIKSLTNSFNNLDFDPSKLDVVVFPVSVHYDHTRKLLQ-SK
TPIS_ECOLI      --MRHPLVMGNWKLNGSRHMVHELVSNLRKELAGVAGCAVAIAPPEMYIDMAKREAEGSH

TPIS_RABIT      IAVAAQNCYKVTNGAFTGEISPGMIKDCGATWVVLGHSERRHVFGESDELIGQKVAHALS
TPIS_HUMAN      IAVAAQNCYKVTNGAFTGEISPGMIKDCGATWVVLGHSERRHVFGESDELIGQKVAHALA
TPIS_TRYBB      KFVIAAQNAIAKSGAFTGEVSLPILKDFGVNWIVLGHSERRAYYGETNEIVADKVAAAVA
TPIS_PLAFA      FSTGIQNVSKFGNGSYTGEVSAEIAKDLNIEYVIIGHFERRKYFHETDEDVREKLQASLK
TPIS_ECOLI      IMLGAQNVDLNLSGAFTGETSAAMLKDIGAQYIIIGHSERRTYHKESDELIAKKFAVLKE

TPIS_RABIT      EGLGVIACIGEKLDEREAGITEKVVFEQTKVIADNVK--DWSKVVLAYEPVWAIGTGKTA
TPIS_HUMAN      EGLGVIACIGEKLDEREAGITEKVVFEQTKVIADNVK--DWSKVVLAYEPVWAIGTGKTA
TPIS_TRYBB      SGFMVIACIGETLQERESGRTAVVVLTQIAAIAKKLKKADWAKVVIAYEPVWAIGTGKVA
TPIS_PLAFA      NNLKAVVCFGESLEQREQNKTIEVITKQVKAFVDLID--NFDNVILAYEPLWAIGTGKTA
TPIS_ECOLI      QGLTPVLCIGETEAENEAGKTEEVCARQIDAVLKTQGAAAFEGAVIAYEPVWAIGTGKSA

TPIS_RABIT      TPQQAQEVHEKLRGWLKSNVSDAVAQSTRIIYGGSVTGATCKELASQPDVDGFLVGGASL
TPIS_HUMAN      TPQQAQEVHEKLRGWLKSNVSDAVAQSTRIIYGGSVTGATCKELASQPDVDGFLVGGASL
TPIS_TRYBB      TPQQAQEAHALIRSWVSSKIGADVAGELRILYGGSVNGKNARTLYQQRDVNGFLVGGASL
TPIS_PLAFA      TPEQAQLVHKEIRKIVKDTCGEKQANQIRILYGGSVNTENCSSLIQQEDIDGFLVGNASL
TPIS_ECOLI      TPAQAQAVHKFIRDHIAKVDAN-IAEQVIIQYGGSVNASNAAELFAQPDIDGALVGGASL

TPIS_RABIT      KP-EFVDIINAKQ-----
TPIS_HUMAN      KP-EFVDIINAKQ-----
TPIS_TRYBB      KP-EFVDIIKATQ-----
TPIS_PLAFA      KE-SFVDIIKSAM-----
TPIS_ECOLI      KADAFAVIVKAAEAAKQA

                                                    Chemistry & Biology
```

Alignment of representative triosephosphate isomerase sequence from six sources. The sites of cleavage obtained in PfTIM (TPIS_PLAFA; this study) have been shaded in green and the sites obtained in rabbit muscle TIM (TPIS_RABIT) [14] have been shaded in red.

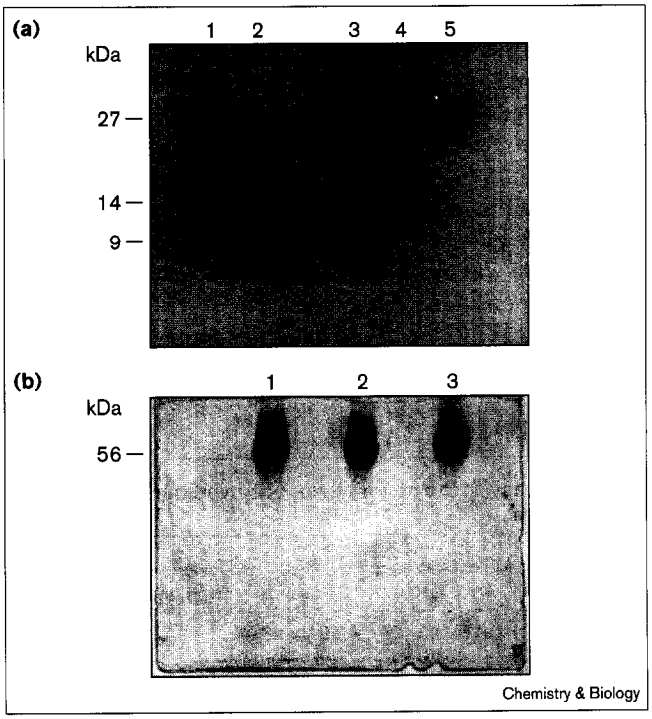
by direct addition of organic solvent. Different concentrations of acetonitrile (v/v) were added to the proteolyzed mixture. It was observed that, at 60% (v/v) acetonitrile concentration, the fragments spontaneously religated to yield a band at 27 kDa (lane 3) that comigrates with PfTIM. Incubation of TIM with subtilisin for longer than 30 minutes leads to a complex mixture of short fragments, with diminishing religation efficiency as the extent of proteolytic cleavage increases. Qualitative inspection of the band intensities on the gel suggests that religation efficiency is > 90% for samples of TIM, which were incubated with subtilisin for 10–15 minutes. Following this, a large number of organic solvents were investigated that included dioxan, 2,2,2-trifloroethanol (TFE) and acetonitrile. In every case there was substantial amount of religation observed as shown in lanes 3, 4 and 5 of Figure 2a. Concentrations of organic solvents at which maximum religation was observed were: dioxan, 70% v/v; TFE, 50%; and acetonitrile, 60%. Religation carried out as a function of time revealed that complete resynthesis could be achieved within 1 minute, indicating that under these conditions the cleavage reaction was significantly slower, requiring about 15 minutes for complete digestion of TIM as judged from SDS–PAGE. This has also been observed in the case of rabbit muscle TIM, which requires 24 hours for complete digestion to occur as compared with the religation, which occurs in a much shorter time period. Further analysis was carried out on nondenaturing PAGE (without SDS). Figure 2b shows co-migrating bands of native TIM (dimer ~56 kDa), proteolyzed fragments and religated TIM, indicating that the TIM fragments do not fall apart after digestion with subtilisin and are held together by noncovalent interactions. This also indicates that the organic cosolvent merely facilitates peptide bond synthesis and does not play a role in promoting interaction between TIM fragments.

Characterization of TIM fragments by mass spectrometry

Electrospray ionization mass spectrometry (ES–MS) was used to analyze the fragments that were generated from the limited subtilisin digestion. For this, high-performance liquid chromatography (HPLC)-ES–MS was carried out using a reverse phase C18 column using an acetonitrile:water system. When the digest was injected into a reverse-phase column a single peak was obtained on the mass detector that had a molecular mass of 27,831 Da corresponding to the mass of intact PfTIM (Figure 3a). Surprisingly, it was found that the fragments do not separate from each other on the HPLC column and, in fact, are religated under HPLC conditions. The digested protein, when injected directly into the electrospray source, yielded a spectrum characteristic of a peptide mixture clearly demonstrating that the TIM was indeed digested into fragments (data not shown). This observation is identical to the one made by Vogel and Chmielewski [14] on rabbit muscle TIM.

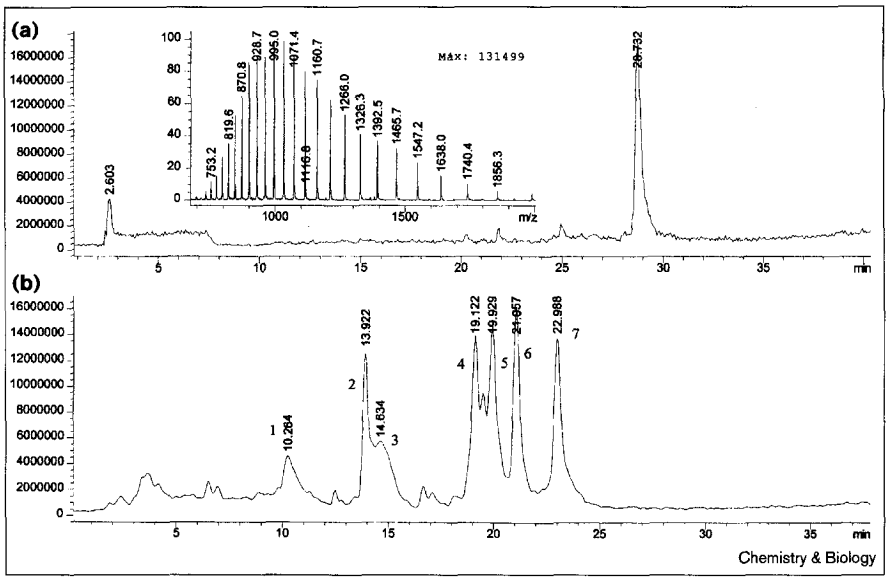
Figure 3a shows the mass spectral total ion chromatogram (TIC) obtained during the course of HPLC and the inset shows the charge state distribution corresponding to native PfTIM (27,831 Da). As a control, the subtilisin in the digest was inactivated by adding a large excess of phenylmethylsulfonyl fluoride (PMSF) and injected on the reverse-phase HPLC column. A single peak was obtained indicating that the fragments did not separate under HPLC conditions, suggesting that the fragments formed a tight noncovalent complex. SDS–PAGE analysis of the subtilisin-inactivated proteolyzed mixture revealed that fragments did not religate, even after extensive incubation in organic solvent. This suggests that the fragments form a relatively strong complex and that denaturing conditions might be necessary to separate the fragments prior to HPLC-ES–MS. The fragments were unfolded in 6 M guanidinium chloride (GdmCl) and then injected onto the column. This procedure indeed leads to separation of the fragments on the column as judged from the multiple peaks obtained on the mass spectral detector (Figure 3b).

Figure 2



(a) SDS–PAGE analysis of PfTIM, subtilsin digest and religated products in various organic solvents. Lane 1, wild-type TIM migrating as a 27 kDa protein; lane 2, the subtilisin digest of wild-type TIM resulting in fragments that migrate at around 9–12 kDa; lanes 3, 4 and 5, the religation of the subtilisin generated fragments that yields a band co-migrating with native TIM in the organic solvents dioxan, 2,2,2-trifluoroethanol and acetonitrile, respectively. **(b)** Analysis of subtilisin-derived fragments and the religated TIM on a nondenaturing PAGE. Lane 1, native TIM; lane 2, the subtilisin digest; lane 3, the religated TIM in acetonitrile. It is important to note that TIM is a dimer and migrates as a ~56 kDa protein (dimer) under nondenaturing PAGE conditions.

Figure 3



(a) The mass spectral total ion chromatogram obtained during the course of HPLC separation of the subtilisin-derived fragments. The retention time is indicated above the chromatogram peak. A single peak with a retention time of 28 min was obtained on a C18 reverse phase column. The peak yields a mass of 27831.2 Da (inset), which corresponds to the molecular mass of the native TIM. **(b)** The mass spectral total ion chromatogram obtained after guanidinium chloride denaturation of the fragments prior to injection on the HPLC reverse-phase column. Seven major peaks were obtained and the masses corresponding to each peak are given in Table 2.

From the TIC, mass spectra under individual peaks could be derived and the determined masses are shown in Table 2, in which both 10 minute and 30 minute digests are compared. As PfTIM has a nonrepetitive sequence, each mass corresponds to a unique portion of the TIM sequence. Furthermore, in case of ambiguities the charge state distributions obtained for the fragment were used to distinguish the composition of the fragment from the various possibilities. For example, a fragment with a mass of 9438.2 Da was obtained, which yielded two possibilities for its composition: namely, Ile92–Gly172 (9437.8) or Val162–Met247 (9438.7). Analysis of the charge state distribution revealed that the fragment had distribution from +5 to +12 positive ions. From the sequence information it may be noted that the former fragment (Ile92–Gly172) can have a maximum of ten positive charge states (nine basic residues and the amino terminus), whereas the latter fragment (Val162–Met247) can have up to 12 positive charges (11 basic residues and the amino terminus). It is observed that the charge state distribution for the 9438 Da fragment ranges from +5 to +12 to confirm that the mass indeed corresponds to the Val162–Met247 fragment.

Table 2

Molecular mass of the major peaks observed during HPLC of the TIM digest.

Time	No.	Molecular mass (Da)	Sequence	Calc'd molecular mass from the sequence*
10 min	1	18,410	Ala2–Ile161	18,410
	2	9438	Leu162–Met248	9438
	3	13,907	Val124–Met248	13,907
	4	4580	Leu162–Gln202	4580
30 min	1	5930	Leu30–Ala83	5930
	2	2675	Val91–Glu111	2675
	3	3963	Ile88–Asn119	3963
	4	4601	Asp194–Leu236	4601
	5	4903	Ala2–Leu36	4093
	6	6638	Thr174–Gly231	6638
	7	4829	His29–Val41	4829

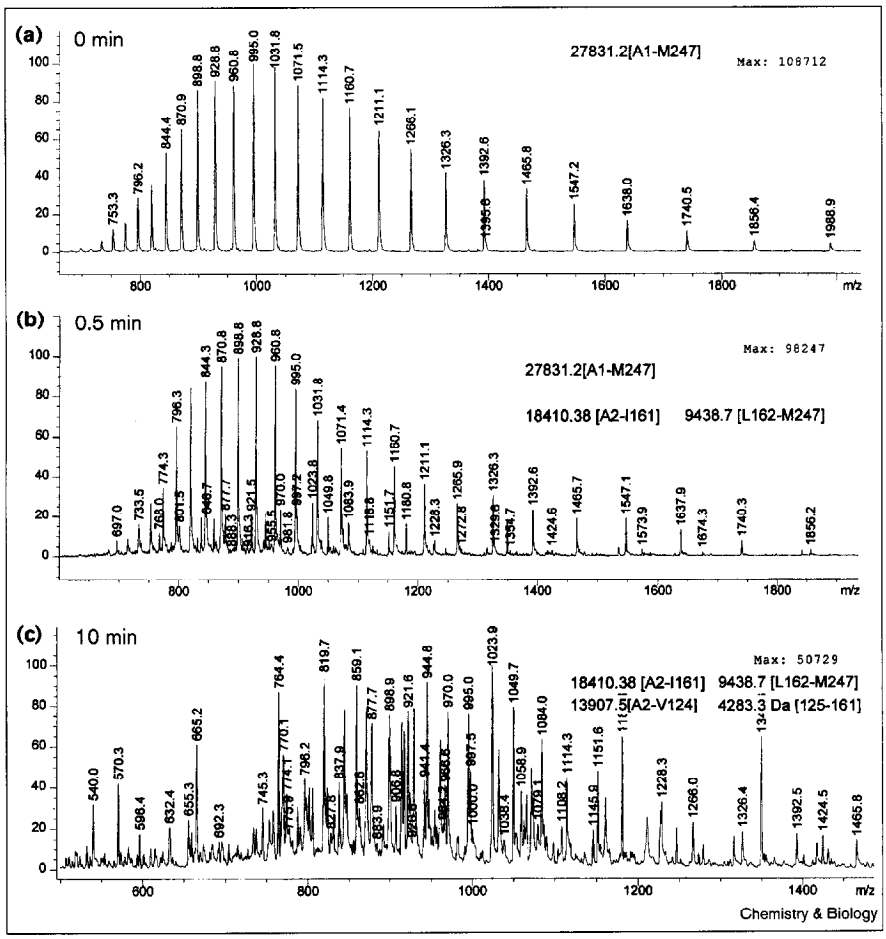
*The PfTIM protein sequence after expression in *Escherichia coli* does not have the amino-terminal methionine, whereas the genomic sequence has methionine. The genomic sequence is used for numbering of amino acids assuming Met1 as the first residue.

To determine the relative susceptibility of peptide bonds to subtilisin cleavage, a time course of digestion was carried out and was followed by ES–MS. Figure 4a shows the charge states obtained from native TIM, which yields a molecular weight of 27,831 Da. In the first 30 seconds of the reaction, PfTIM was cleaved at a single peptide linkage between residues Ile161–Val162 leading to the formation of a large fragment of 18,410 Da and a smaller fragment of 9438 Da (Figure 4b). After a period of 10 minutes the mass spectrum reveals a mixture of polypeptides, the major ones being 13907 Da (1–124), 9438 Da (162–247), 4283 Da (125–161) and a minor component 4380 Da (162–202; Figure 4c). The mixture of polypeptides resulting from multiple nicks in the protein obtained after 30 minutes does not religate efficiently. We therefore used the digestion time of 10 minutes to obtain a nicked PfTIM sample for further structural characterization. At this stage, the nicked protein largely consists of three distinct polypeptides.

Spectroscopic characterization of the nicked PfTIM

The subtilisin-nicked form of PfTIM was prepared by limited proteolysis of PfTIM with subtilisin for 10 minutes at 37°C and the reaction was stopped by

Figure 4

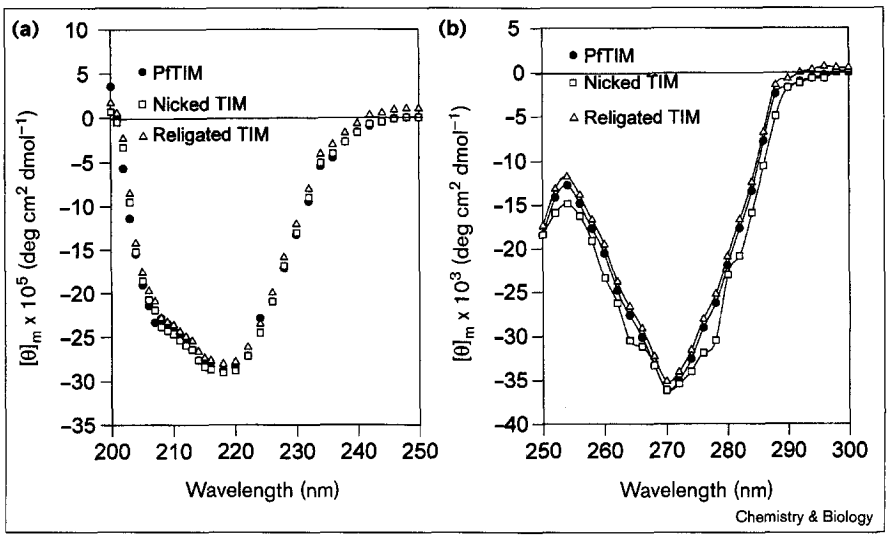


(a) The charge states obtained from the intact native TIM, which yields a molecular mass of 27831.2 Da. **(b)** The mass spectrum obtained after 30 s of digestion with subtilisin. Clearly, three distinct sets of charge states with varying intensity are observed. The charge states are deconvoluted to the molecular masses shown in the inset. The most dominant charge states are from native TIM, suggesting that substantial amount of TIM is still undigested after 30 s. **(c)** The mass spectrum obtained after 10 min of digestion with subtilisin. The mass spectrum is a complex mixture of a number of small polypeptide chains, some of which could be identified by deconvolution of the raw data. Relatively few peaks are observed corresponding to native TIM, suggesting that most of the protein is in fact digested.

adding PMSF. For the purpose of generating religated TIM, the subtilisin digested form was added to 60% acetonitrile solution and incubated for 2 minutes at 37°C. The samples were checked on SDS–PAGE prior to use in spectroscopic experiments. UV spectra were recorded for the subtilisin-nicked form over a range of 220–300 nm and compared with that of native TIM (data not shown). The spectra were identical, showing a maximum at 280 nm. CD spectra for the native, nicked and religated TIM were nearly identical in both the near-UV (Figure 5b) and far-UV regions (Figure 5a) for all three samples, indicating that the proteins have retained almost all the secondary and tertiary structure. Near identical fluorescence spectra (data not shown) were obtained for all the three forms, further confirming that there was very little change in the environment of the tryptophan residues (Trp11/Trp168). It has been well established that in TIM association of subunits to form dimers is very important for its structural integrity [23,24]. Gel filtration studies were carried out which revealed that all the three proteins had the same elution volume and therefore had the same quaternary structure (data not shown). Enzymatic activity measurements were carried out on all the three forms using a coupled enzyme assay system using α-glycerophosphate dehydrogenase and NADH [20]. Similar rates of consumption of NADH, as judged from a decrease in absorbance at 340 nm, indicated that all the three forms had near equal catalytic activity (wild type = 8974 units/mg, nicked form = 8798 units/mg and religated form = 8912 units/mg). This clearly demonstrates that fact that subtilisin ligation did not lead to rearrangement of the TIM fragments leading to non-native sequences.

The presence of native-like structure in the subtilisin-nicked form of PfTIM suggests that the TIM fragments are held together after digestion by noncovalent interactions. The sites of cleavage can be mapped on the three-dimensional structure of the protein (Figure 6), revealing that most of the early cleavage sites are in the loop regions of the protein, the initial sites being Val124–Val125 (loop 4) and Ile161–Leu162 (loop 5). The structurally equivalent cleavage sites obtained in the study of Vogel and Chmielewski [14] on rabbit muscle are also shown in Figure 6 for comparison (Ala31–Lys32, Leu93–Gly94 and

Figure 5



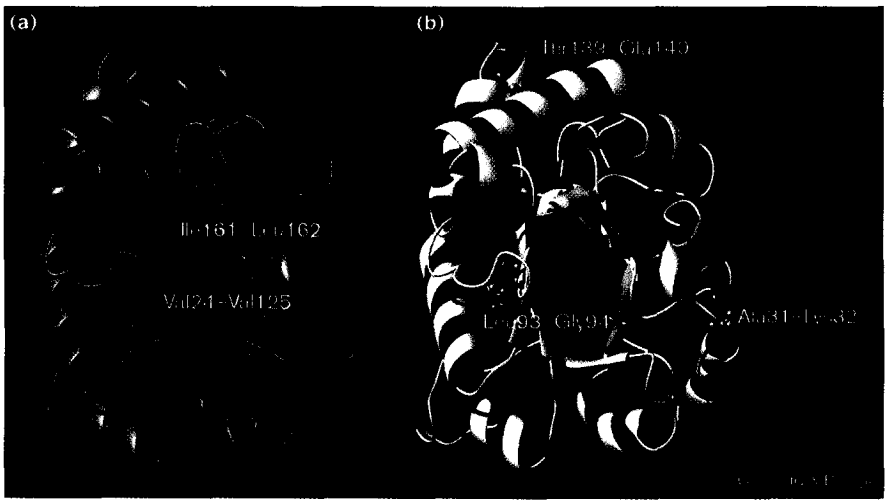
(a) Far-UV CD and **(b)** near-UV CD spectra of the native TIM, fragments and religated TIM, respectively. The spectral characteristics of all three forms look virtually identical suggesting that there is no appreciable loss of structure after digestion of TIM with subtilisin.

Thr139–Glu140). The cleavage sites Val124–Val125 (PfTIM) and Leu93–Gly94 (rabbit muscle TIM) are close to each other in the three-dimensional structure, although the sites are distant on the linear sequence. It is noteworthy that the loop 6 (residues 167–174) of PfTIM, which has the highest mobility, is not nicked by subtilisin; the reasons for this are not clearly understood. It is well known that proteases prefer exposed loops for cleavage rather than secondary structure elements [25]. Figure 7 shows a stick plot of the number and distribution of contacts between the various fragments A (Ala2–Val124), B (Val124–Ile161) and C (Ile162–Met248). The three fragments make extensive contacts with each other, forming a tight interlocked network of interactions, which might be the reason for the remarkable stability of the nicked form in spite of multiple cleavage. The loop regions of the protein are solvent exposed and make very few contacts with the protein interior. It is also noteworthy that the cleavage sites are in close proximity (Figure 6) in the three-dimensional structure.

Stability of nicked TIM

As subtilisin-nicked TIM seemed to have most of its structure intact, it was of interest to investigate the stability of this noncovalent complex to denaturing agents. Unfolding of the subtilisin-nicked form was carried out using CD and fluorescence measurements. Thermal unfolding was carried out by Rayleigh scatter measurements as a function of temperature. It was observed that both the forms exhibit nearly equal thermal melting

Figure 6



A ribbon representation of the PfTIM crystal structure. **(a)** The sites of early cleavage (Val124–Val125 and Ile161–Leu162) are marked. The active-site residues are away from the sites of cleavage. The labile sites are located on the loop regions of the protein with high B-factors. The regions in the vicinity of the active sites are not nicked by subtilisin. **(b)** The structurally equivalent residues at the sites of cleavage on rabbit muscle TIM are also marked for comparison.

temperatures of 70°C (Figure 8a). Stability of both forms were compared in urea and GdmCl solutions using fluorescence and CD spectroscopy. Changes in the intrinsic tryptophan fluorescence of PfTIM and the nicked form were monitored as a function of urea concentration. Very small wavelength changes are observed for both forms in the region of 0–6 M urea, with the emission maximum shifting from 331 nm to 333 nm. Above 6 M urea, there is a sharp shift in the spectral maximum for PfTIM, with a limiting value of 340 nm being obtained at 8 M urea. In the case of the nicked form, the shift is more pronounced with the emission maximum at 8 M being 344 nm. Figure 6b shows that PfTIM up to 6 M urea retains 80% of the original emission intensity. There is a sharp fall in the emission yields, however, between 6 and 8 M urea. In contrast the nicked form appears to be less stable with a C_m of 5 M (Figure 8b). Significant structural changes are evident even at urea concentrations as low as 3 M. Even the nicked form does not exhibit typical 'two-state' behavior, however, but a broad transition similar to native TIM (Figure 8b).

Both the native protein and the nicked form have a prominent negative CD band at 220 nm. With increasing urea concentration there is a reduction in the ellipticity of the CD bands, without any significant change in the shape of the spectrum. Interestingly, even at 8 M urea there is significant far-UV CD ellipticity at 220 nm, suggesting that appreciable secondary structure is still retained. Figure 8c shows the ellipticity at 220 nm plotted as a function of urea concentration. It is clearly seen that there is no sharp unfolding transition detected by far-UV CD, indicative of a complex multistep process. Again, it is observed that the nicked form exhibits a sharper unfolding transition than the native form, with a marginal decrease in C_m (Figure 8c).

Figure 7

A stick plot for the interfragment contacts between individual residues of the major fragments obtained after subtilisin cleavage. A cut off value of 4 Å was used to define an interatom contact. The top panel shows the contact between the fragment Ala2–Val124 and Val125–Ile161; the middle panel below it shows the contacts between Ala2–Val124 and Leu162–Met248 and the bottom panel shows the contacts between Val125–Ile161 and Leu162–Met248.

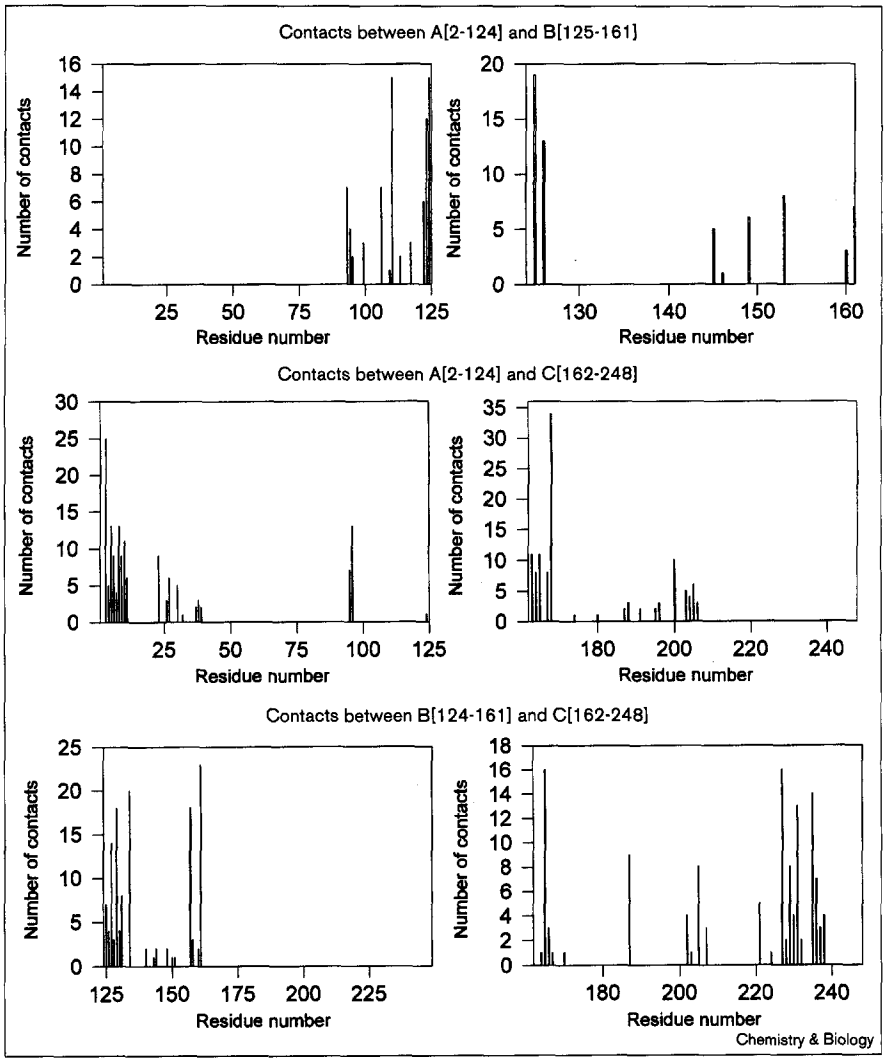
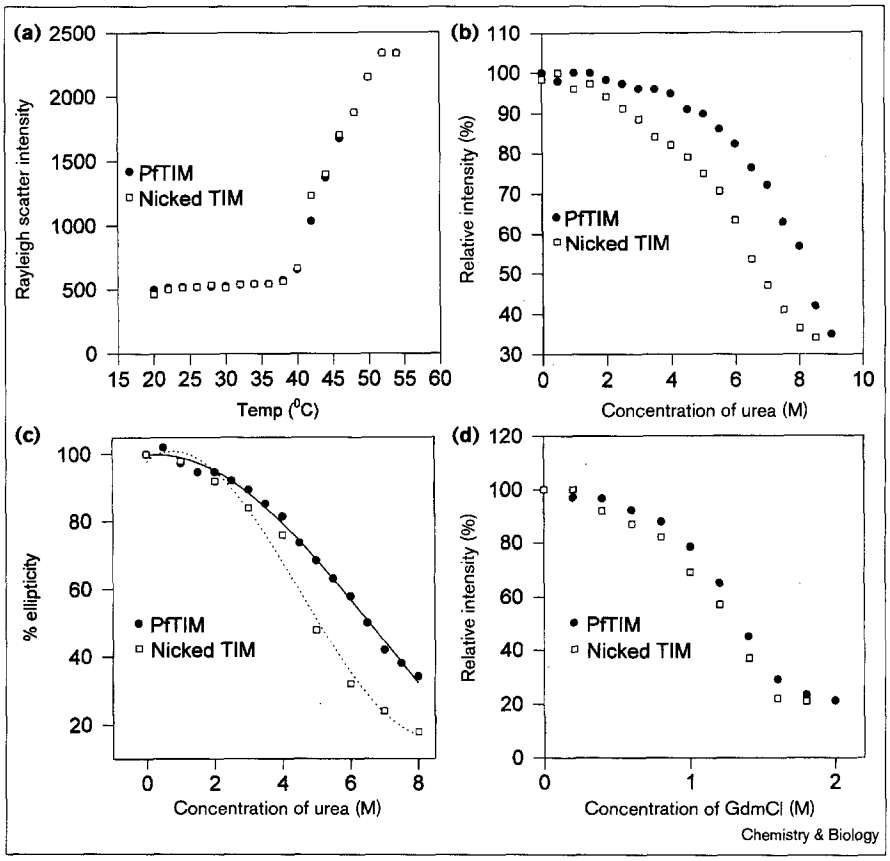


Figure 8



(a) Thermal melting curves for native TIM and for the subtilisin-nicked form monitored by Rayleigh scatter at 400 nm. Protein (2 μM) was equilibrated in 10 mM MOPS buffer pH 8.0 for 1 h. The temperature was ramped by 5°C every 10 min and the protein was incubated at the desired temperature for 5 min to allow equilibration prior to measurement of the scatter intensities. Both the forms of protein have a similar T_m of 65°C. **(b)** Urea unfolding of native TIM and for the subtilisin-nicked form monitored by following intrinsic tryptophan fluorescence at 25°C pH 8.0. Protein concentrations were 4 μM in 100 mM TrisCl pH 8.0. The plot is normalized by taking the fluorescence intensity at 0 M urea as 100%. **(c)** Dependence of CD ellipticity of the native TIM and the subtilisin-nicked form at 220 nm (θ_{220}) as a function of urea concentration at 25°C pH 8.0. Protein concentrations were 8 μM (100 mM Tris.Cl pH 8.0) and the path length was 0.1 mm. The ellipticity value in absence of denaturant was taken as 100%. **(d)** Unfolding of native TIM in GdmCl solution was monitored by intrinsic tryptophan fluorescence intensity. The other experimental conditions were identical to the urea unfolding experiments in (b). The fluorescence data for unfolding were normalized by taking the spectroscopic parameter to be 100% in absence of any denaturant.

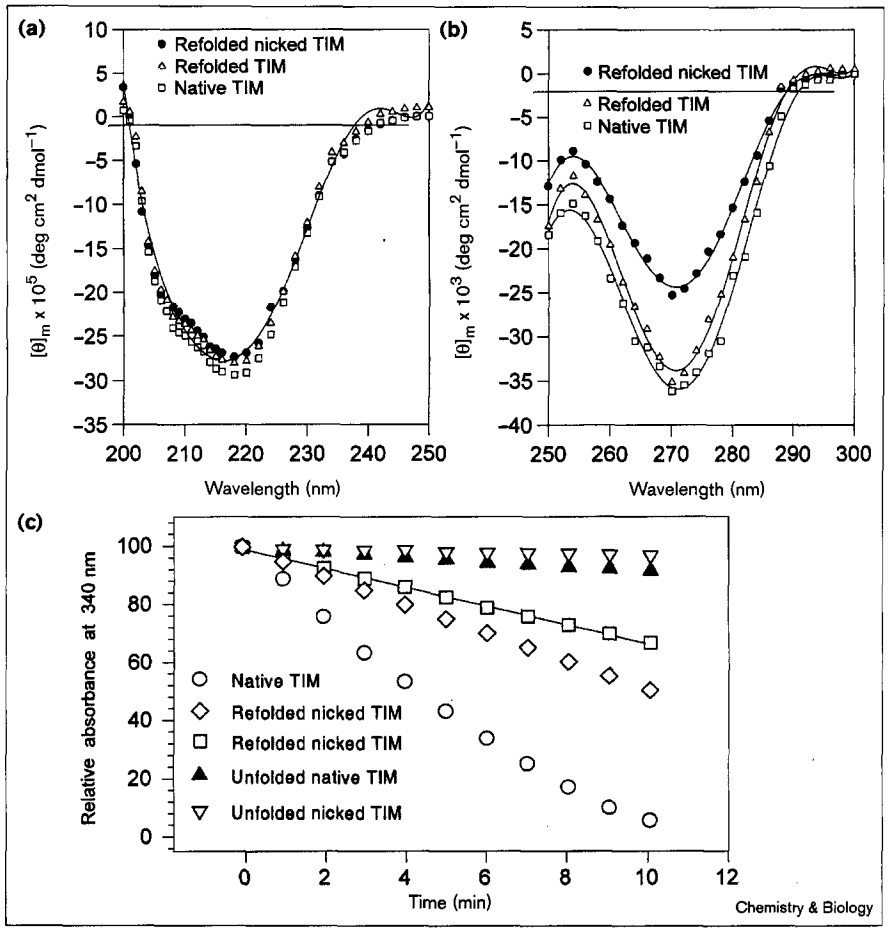
PfTIM is much less stable in GdmCl than in urea and is completely unfolded in 2 M GdmCl solutions [21]. Equilibrium unfolding studies of PfTIM and the nicked form were performed in GdmCl solutions to compare its conformational stability in a relatively strong denaturant. Figure 8d shows the unfolding profile obtained using intrinsic tryptophan fluorescence. PfTIM is completely unfolded by 2 M GdmCl. It is observed that the native protein and the nicked protein exhibit indistinguishable unfolding curves. Similar results were obtained from far-UV CD studies.

Refolding and association of nicked TIM from denaturant solutions

The remarkable stability of nicked TIM suggests the importance of noncovalent interactions in maintaining the folded structure. It is possible, however, that once the protein is unfolded the fragments do not reassociate to form a native-like TIM structure. The stability of the nicked protein could, in principle, be a result of 'kinetic trapping' in a metastable state, rather than a result of thermodynamic control. To investigate this, refolding studies were carried out using nicked TIM.

In 6 M GdmCl solutions the protein is completely unfolded and the fragments fall apart. Native PfTIM and nicked TIM were incubated in 6 M GdmCl solutions at pH 8.0 for 1 hour. Far and near-UV CD spectra demonstrate that protein is completely unfolded in 6 M GdmCl solution. The protein was refolded by addition of the refolding buffer (10 mM triethanolamine pH 7.5 + 1 mM DTT) to the unfolded protein. Far-UV and near-UV CD (Figure 9a) spectra were recorded for the final equilibrated form after 1 hour. The far-UV spectra of the refolded wild-type TIM was compared with a similar concentration of wild-type TIM recorded under identical conditions. CD spectra of refolded native TIM had 97% of the native TIM intensity, suggesting almost complete reversibility. Far-UV CD spectra recorded for the refolded nicked TIM under similar conditions showed as much as 92% of native-like intensity for the nicked TIM, suggesting that most of the fragments reassociated to form a native-like TIM structure (Figure 9a). Near-UV CD (Figure 9b) spectra for the refolded wild-type TIM was similar to native TIM, whereas the refolded nicked TIM regained only 70% of native-like near-UV CD. The near-UV CD spectrum of native TIM results from seven tyrosine and two tryptophan residues of the protein. One of the tryptophan residues (Trp168) is close to the cleavage site (161–162). It is possible that local disordering in this region results in the observed loss of intensity for near-UV

Figure 9

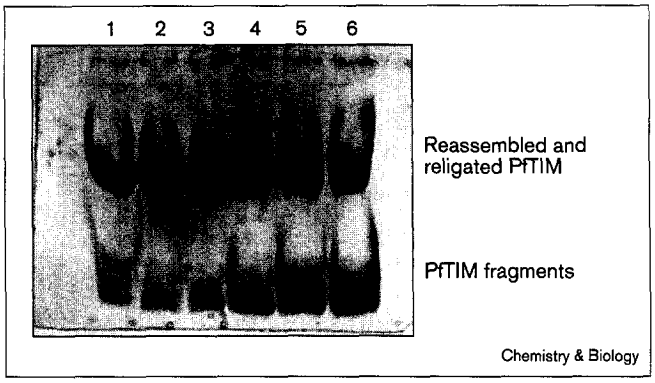


(a) Far-UV CD spectra and **(b)** near-UV CD spectra of native TIM, refolded TIM and refolded nicked TIM. **(c)** Comparison of enzymatic activity of native TIM, refolded TIM and refolded nicked TIM, monitored by a coupled enzyme assay using α-glycerophosphate dehydrogenase and NADH [20]. The decrease in absorption at 340 nm for NADH→NAD is monitored as a function of time. All reagents are added in excess so that the overall rate of reaction is proportional only to TIM concentration.

CD of refolded nicked TIM. Fluorescence spectra recorded for the various forms of the protein yield similar results (data not shown). The refolded nicked protein regains most of the secondary structure upon refolding, although tertiary structure is not completely regained. Furthermore, enzymatic activity was recorded for both refolded TIM and refolded nicked TIM, which revealed that the refolded TIM had only 60% of the native TIM activity (Figure 9c). This is probably because the TIM activity is sensitive to minor structural perturbation. It has been observed that, during refolding of trypanosomal TIM, complete enzymatic activity is never recovered, despite recovery of other spectroscopic properties such as fluorescence and CD [26]. The nicked TIM has lower activity upon refolding than refolded TIM.

It was of interest to see if the refolded nicked TIM can still be ligated by addition of organic solvent and subtilisin. The refolded nicked TIM was incubated in 60% acetonitrile in presence of subtilisin and the resulting product was analyzed on SDS–PAGE. Refolding, followed by addition of acetonitrile and subtilisin, was carried out from increasing concentrations of GdmCl (1–6 M). The products after religation were analyzed on SDS–PAGE (Figure 10). It is observed that up to 3 M GdmCl, efficient ligation could be achieved with relatively little intensity of peptide fragments as seen from SDS–PAGE analysis. The religation efficiency is somewhat lowered between 4–6 M GdmCl, although a significant amount of the 'native-like' form can still be obtained after refolding and ligation. This suggests that the nicked form of TIM behaves like a fragment complementation system, in which the fragments reassociate to a native TIM-like structure. The process of fragment association and ligation is summarized in Figure 11. It should be emphasized that the nicked TIM used in these experiment is heterogeneous with respect to the number of peptide bonds cleaved. HPLC and mass spectral analysis suggests that the major components are Ala2–Val124, Val125–Ile161, Leu162–Met248. It is possible that most of the religation may occur as a result of the association of the major fragments, which are cleaved initially at the Ile161–Leu162 bond. Further studies are necessary with isolated fragments in order to determine whether multiple components can be used to generate a

Figure 10



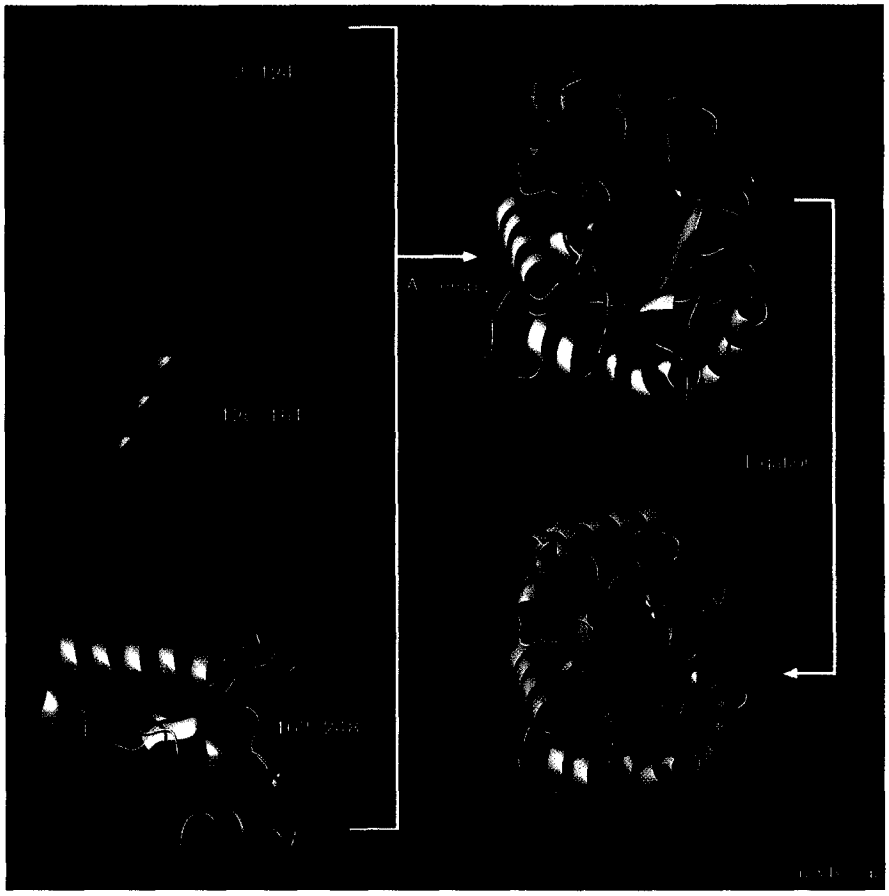
SDS–PAGE analysis of refolded and religated TIM obtained by diluting from initial GdmCl concentrations of 1–6 M (lanes 1–6, respectively). The final concentration of denaturant after dilution is less than 0.02 M. Refolding was carried out by addition of TEA buffer, pH 7.5. Religation was carried out by addition of 0.02 μM solution of subtilisin in 60% acetonitrile (v/v). Refolding and religation is more effective at GdmCl concentrations less than 5 M.

noncovalent assembly mimicking the native protein that can be smoothly religated by subtilisin.

Discussion

The conformational stability of a protein is largely determined by long-range noncovalent interactions. Proteolytic cleavage at surface loop regions might lead to minimal perturbation of the secondary structural elements and the tertiary interactions. Under such conditions the protein can, in principle, retain most of its structure even after the loss of some covalent peptide linkages. Such fragment complementation systems are rare and relatively few systems have been studied in detail. In most cases the number of interacting fragments are two, with a single nick in the polypeptide chain. The observation by Vogel and Chmielewski [14] that rabbit muscle TIM could be nicked by subtilisin and subsequently religated with great efficiency, formed the stimulus for our study. The β8/α8 barrel family, for which TIM is the prototype, is one of the most widely occurring folds among the crystal structures of proteins. Almost 10% of the protein structures in the Protein Data Bank (http://www.bnl.gov/) have this fold [27]. The TIM barrel is composed of a central core of eight parallel β strands, which forms the β barrel, surrounded by eight peripheral α helices covering the barrel exterior. Several surface-exposed loops provide potential sites for proteolytic cleavage. Our

Figure 11



A schematic representation of fragment assembly from a denaturant solution followed by ligation using subtilisin in acetonitrile. Only the major fragments obtained during the subtilisin cleavage of TIM are shown.

present study suggests that subtilisin cleavage occurs with great facility between residues Ile161–Leu162 and Val124–Val125 resulting in generation of at least three noncovalently held fragments after very brief exposure to subtilisin. Indeed, further cleavage can also be demonstrated at other sites.

Multiply nicked PfTIM displays remarkable stability in the presence of a wide variety of chemical denaturants and shows comparable thermal stability to that of the intact PfTIM. Efficient religation upon addition of an organic solvent further emphasizes the robustness of the TIM barrel, which appears to withstand cleavage at multiple sites. Reassociation of the cleaved fragments under refolding conditions demonstrates that the stability of the nicked TIM is indeed a consequence of thermodynamically favorable tertiary interactions and not a consequence of kinetic trapping. The three major fragments obtained contain all the helical secondary structural elements of TIM. The eight-stranded β barrel is formed only upon reassociation of the fragments, suggesting that the order of events may have some relevance to the mechanism by which the TIM barrel is formed during the folding process. Interestingly, the dimer interface is completely contained within the 1–124 fragment, suggesting that the necessary elements for the subunit assembly might, in fact, be formed prior to development of the β barrel.

Although the characterization of a fragment complementation system involving multiple fragments maybe valuable in future studies addressing the mechanism of folding and assembly, the observation of efficient ligation of peptide bonds is of great importance to protein semisynthesis approaches. Semisynthesis of many proteins have been achieved through the use of genetically engineered 'subtiligases' [26–28] and through chemoselective ligations [29–31]. In either approach, high fidelity can be achieved if the reactant fragments are held together by noncovalent interactions bringing the two reactive end groups in close proximity. This feature is exemplified by the recent work of Vogel *et al.* [15] on lysozyme and in this study. The use of organic solvent mediated chemical ligation has also been employed successfully in many cases for complete synthesis of proteins [32–35]. Work carried out by Klibanov and coworkers [36] has established that proteases and lipases are active in organic solvents such as DMF and acetonitrile. In fact, a significant increase in thermal stability and enhancement of catalytic rate is observed in organic solvents [37]. Organic solvents do not seem to significantly alter protein structures [38]. It has been suggested that protein active sites become rigid and retain the conformational memory of its aqueous milieu in the organic phase [39]. PfTIM might serve as a model system for the investigation of organic solvent mediated, protease catalyzed peptide bond ligation.

Significance

Protein structures are stabilized by a network of tertiary interactions between amino acids that are far apart in the primary sequence. 'Nicked' proteins that have been proteolytically cleaved can retain some stability because of these interactions. Here we investigate the sites of cleavage and stability of a subtilisin-cleaved form of *Plasmodium falciparum* triosephosphate isomerase (PfTIM). The results obtained in this study suggest that the β8/α8 TIM barrel can be proteolytically nicked and efficiently religated by organic solvent mediated peptide bond formation in presence of subtilisin. The demonstration that this religation process is effective in the case of both rabbit muscle TIM [14] and *P. falciparum* TIM, which have a homology of only 42%, suggests that the three-dimensional structure is overwhelmingly determined by long-range interactions and is capable of surviving multiple proteolytic nicks. The robustness of the β8/α8 barrel is demonstrated by the unusually high stability of the nicked PfTIM despite cleavage at two or more sites. The unusual stability might point towards an evolutionary reason for nature's choice of β/α barrel in almost 10% of known enzymes. The unfolded mixture of fragments can be reassembled to a native-like structure by refolding and the polypeptide chains can be religated by subtilisin in presence of organic solvents in fairly high yields. These observations further emphasize the importance of tertiary interactions in contributing to the stability of complex folds. The characterization of a fragment complementation system in which the amino-terminal fragment contains the complete dimer interface might be valuable in probing the process of subunit assembly and polypeptide chain folding during the formation of the β8/α8 barrel.

Materials and methods

Reagents

All chemicals were purchased from Sigma (St Louis, MO). PfTIM was purified from an overexpressed clone in *E. coli* as described in [20]. Urea was recrystallized from hot ethanol and fresh stock solutions were made for unfolding experiments.

SDS–PAGE and nondenaturing PAGE

SDS–PAGE and nondenaturing PAGE were carried out using standard procedures.

Proteolysis

Proteolysis was carried out in phosphate buffer pH 7.5. The substrate TIM (200 μM) was incubated with the protease (subtilisin) in a ratio of 100:1 (w/w) in a final volume of 100 μl. Samples at regular time intervals were withdrawn for analysis. The proteolytic digestion was carried out at 37°C. The reaction was quenched either by addition of 10% formic acid in case of mass spectrometry or by addition of gel loading buffer (1% SDS + 50% glycerol + bromophenol blue) in case of SDS–PAGE analysis.

Religation

Religation of the proteolyzed fragments was carried out by direct addition of organic solvent to the proteolyzed TIM without the removal of subtilisin. Organic solvent (6 μl) was added to the proteolyzed TIM

(4 µl) and incubated at 37°C for 15 minutes. These samples were then analyzed by mass spectrometry or SDS–PAGE.

Mass spectrometry

Mass spectrometric analysis was carried out on a HP1100 (Hewlett Packard) mass spectrometer interfaced with an HPLC system. Gradients of acetonitrile (80%) and water were used as solvents for elution of protein and peptides from a reverse phase C18 column. Intact protein samples and some proteolytic fragments were analyzed by direct injection bypassing the HPLC column. A flow rate of 0.1 ml/min was used and electrospray was carried out with pneumatic assistance using a nebulizer pressure of 15 psi. For separation and analysis of the fragments generated by proteolysis, LC–MS was carried out using an acetonitrile water system using a flow rate of 0.5 ml/min. To get a stable electrospray the nebulizer pressure was increased to 45 psi and the capillary voltage was raised to 5000 V. The masses for each fragment was calculated from the charge state information and the masses were allocated to various parts of the protein using the program PAWS (ProteoMetrics).

UV absorbance

UV measurements were carried out on a JASCO UV spectrophotometer using 4 µM protein, equilibrated in 100 mM TrisCl pH 8.0 prior to measurements.

Circular dichroism

Partially proteolyzed TIM (8 µM) was incubated for at least 1 h at the required urea/GdmCl. concentration in 100 mM TrisCl buffer pH 8.0, before recording the spectrum. Preliminary studies confirmed that equilibrium was reached within 0.5 h of incubation. Circular dichroism measurements were carried out on a JASCO J500A spectropolarimeter. Ellipticity changes at 220 nm were monitored to follow the far-UV unfolding transition using a path length of 1 mm. The near-UV band was followed using a 5 mm path length cuvette, at a protein concentration of 28 µM. Spectra were averaged over 2–4 scans at a scan speed of 10 nm/min. Unfolding transitions were followed by monitoring ellipticity at 280 nm.

Fluorescence spectroscopy

Emission spectra were recorded on a Hitachi 650–60 spectrofluorimeter. Intrinsic tryptophan fluorescence was monitored at varying concentrations of urea in 100 mM TrisCl (pH 8.0). Equilibration was achieved within 0.5 h of incubation. Proteolyzed TIM samples were excited at 280 nm and the emission was monitored at 331 nm. Excitation and emission band pass were set at 5 nm. Urea and guanidinium chloride unfolding studies were carried out using a protein concentration of 4 µM.

Size-exclusion gel chromatography

Gel filtration experiments were carried out on a Superose 6B column with an exclusion limit of 4×10^6 Da for proteins using a Pharmacia FPLC system. The void volume of the column was 10 ml. The elution of the proteolyzed TIM was detected at 280 nm. 20 µM of the protein was incubated for 1 h in 100 mM Tris.Cl (pH 8.0). An aliquot (50 µl) of this sample was injected on the column with 100 mM Tris (pH 8.0) at the required denaturant concentration. A uniform concentration of 150 mM NaCl was used for column equilibration at all denaturant concentrations to prevent nonspecific binding of protein to the column material. The flow rate was adjusted to 0.4 ml/min.

Refolding of TIM

PfTIM was nicked using subtilisin as described above. Following digestion, the mixture of fragments were incubated in the desired concentration of GdmCl (pH 8.0) for 2 h after inactivation of subtilisin. Fluorescence spectra at 331 nm were recorded to ensure complete unfolding of the fragments. Fragments were refolded in a single jump by rapid dilution by mixing 50 µl to 1000 µl using 5 mM triethanolamine (TEA; pH 7.5) and 1 mM DTT as the refolding buffer. The protein was incubated for 1 h at 25°C. After 1 h no appreciable change was noticed in the fluorescence spectrum suggesting that the system had reached equilibrium.

Acknowledgements

This research was supported by a grant from the Council of Scientific and Industrial Research, Government of India. The mass spectrometry facility is funded by a program support in the area of 'Drug and Molecular Design' by the Department of Biotechnology. We wish to thank S. Kumar Singh for his help with the generation of the contact plot.

References

1. Jaenicke, R. & Böhm, G. (1998). The stability of proteins in extreme environments. *Curr. Opin. Struct. Biol.* **8**, 738-748.
2. Munoz, V., Cronet, P., Lopez-Hernandez, E. & Serrano, L. (1996). Analysis of the effect of local interactions on protein stability. *Fold. Des.* **1**, 167-178.
3. Matthews, B.W. (1995). Studies on protein stability with T4 lysozyme. *Adv. Protein Chem.* **46**, 249-278.
4. Taniuchi, H., Parker, D. S. & Bohnert, J. L. (1977). Study of equilibration of the system involving two alternative, enzymically active complementing structures simultaneously formed from two overlapping fragments of staphylococcal nuclease. *J. Biol. Chem.* **252**, 125-140.
5. Taniuchi, H., Parr, G.R. & Juillerat, M.A. (1986). Complementation in folding and fragment exchange. *Methods Enzymol.* **131**, 185-217.
6. Richards, F.M & Vithayathil, R.J (1959). The preparation of subtilisin-modified ribonuclease and the separation of its peptide and protein components. *J. Biol. Chem.* **241**, 4389-4396.
7. Matthews, J.M. & Fersht, A.R. (1995). Folding and complementation of barnase fragments. *Biochemistry* **34**, 6805-6814.
8. Dalzoppo, D., Vita, C. & Fontana, A. (1985). Folding of thermolysin fragments. Identification of the minimum size of a carboxyl-terminal fragment that can fold into a stable native-like structure. *J. Mol. Biol.* **182**, 331-340.
9. Parr, G.R. & Taniuchi, H. (1981). Ordered complexes of cytochrome c fragments. Kinetics of formation of the reduced (ferrous) forms. *J. Biol. Chem.* **256**, 125-132.
10. Kobayashi, N., Honda, S., Yoshii, H., Uedaira, H. & Munekata, E. (1995). Complement assembly of two fragments of the streptococcal protein G B1 domain in aqueous solution *FEBS Lett.* **366**, 99-103.
11. Pecorari, F., Guilbert, C., Minard, P., Desmadril, M. & Yon, J.M. (1996). Folding and functional complementation of engineered fragments from yeast phosphoglycerate kinase. *Biochemistry* **35**, 3465-3476.
12. Eder, J. & Kirschner, K. (1992). Stable substructures of eightfold beta alpha-barrel proteins: fragment complementation of phosphoribosylanthranilate isomerase. *Biochemistry* **31**, 3617-3625.
13. Betton, J.M. & Hofnung, M. (1994). *In vivo* assembly of active maltose binding protein from independently exported protein fragments. *EMBO J.* **13**, 1226-1234.
14. Vogel, K. & Chmielewski, J. (1994). Rapid resynthesis of triosephosphate isomerase. *J. Am. Chem. Soc.* **3**, 295-299.
15. Vogel, K., Cook, J. & Chmielewski, J. (1996). Subtilisin-catalyzed religation of proteolyzed hen egg-white lysozyme: investigation of the role of disulfides. *Chem. Biol.* **3**, 295-299.
16. de Prat, G.G. & Fersht, A.R. (1994). Generation of a family of protein fragments for structure-folding studies. 1. Folding complementation of two fragments of chymotrypsin inhibitor-2 formed by cleavage at its unique methionine residue. *Biochemistry* **33**, 7957-7963.
17. Schmidt-Rose, T. & Jentsch, T.J. (1997). Reconstitution of functional voltage-gated chloride channels from complementary fragments of CLC-1. *J. Biol. Chem.* **272**, 20515-20521.
18. Knowles, J.R. (1991). Enzyme catalysis: not different, just better. *Nature* **350**, 121-124.
19. Ranie, J., Kumar, V.P. & Balaram, H. (1993). Cloning of the triosephosphate isomerase gene of *Plasmodium falciparum* in *Escherichia coli*. *Mol. Biochem. Parasitol.* **61**, 159-170
20. Velanker, S.S., Ray, S.S., Gokhale, R.S., Balaram, H., Balaram, P. & Murthy, M.R.N (1997). Triosephosphate isomerase from *Plasmodium falciparum*: the crystal structure provides insights into antimalarial drug design. *Structure* **5**, 751-761.
21. Gokhale, R.S., Ray, S.S., Balaram, H. & Balaram, P. (1999). Unfolding of *Plasmodium falciparum* triosephosphate isomerase in urea and guanidinium chloride: evidence for a novel disulfide exchange reaction in a covalently cross-linked mutant. *Biochemistry* **38**, 423-431.
22. Gopal, B., Ray, S.S., Gokhale, R.S., Balaram, H., Murthy, M.R.N & Balaram, P. (1999). Cavity-creating mutation at the dimer interface of *Plasmodium falciparum* triosephosphate isomerase: restoration of stability by disulfide cross-linking of subunits. *Biochemistry* **38**, 478-486.

23. Mainfroid, V., *et al.*, & Goraj, K. (1996). Three hTIM mutants that provide new insights on why TIM is a dimer. *J. Mol. Biol.* **257**, 441-456.
24. Mainfroid, V., Mande, S.C., Hol, W.G., Martial, J.A. & Goraj, K. (1996). Stabilization of human triosephosphate isomerase by improvement of the stability of individual alpha-helices in dimeric as well as monomeric forms of the protein. *Biochemistry* **35**, 4110-4117.
25. Fontana, A., Fassina, G., Vita, C., Dalzoppo, D., Zamai, M. & Zambonin, M. (1986). Correlation between sites of limited proteolysis and segmental mobility in thermolysin. *Biochemistry* **25**, 1847-1851.
26. Sepulveda-Becerra, M.A., *et al.*, & Darszon, A. (1996). Refolding of triosephosphate isomerase in low-water media investigated by fluorescence resonance energy transfer. *Biochemistry* **35**, 15915-15922.
27. Reardon, D. & Farber, G.K. (1995). The structure and evolution of the α/β barrel proteins. *FASEB J.* **9**, 497-503.
28. Braisted, A.C., Judice, J.K. & Wells, J.A. (1997). Synthesis of proteins by subtiligase. *Methods Enzymol.* **289**, 298-313.
29. Chang, T.K., Jackson, D.Y., Burnier, J.P. & Wells, J.A. (1994). Subtiligase: a tool for semisynthesis of proteins. *Proc. Natl Acad. Sci. USA* **91**, 12544-12548.
30. Welker, E. & Scheraga, H.A. (1999). Use of benzyl mercaptan for direct preparation of long polypeptide benzylthio esters as substrates of subtiligase. *Biochem Biophys. Res. Commun.* **254**, 147-151.
31. Wilkew, J & Kent, S.B.H (1998). Chemical synthesis of proteins. *Curr. Opin. Biotech.* 9, 412-426
32. Dawson, P.E., Muir, T.W., Clark-Lewis, I. & Kent, S.B.H. (1994). Synthesis of proteins by native chemical ligation. *Science* **266**, 776-779.
33. Muir, T.W., Dawson, P.E & Kent, S.B.H (1997). Protein synthesis by chemical ligation of unprotected peptide segments in aqueous solutions. *Methods Enzymol.* **289**, 266-298.
34. Jackson, D.Y., Burnier, J., Quan, C., Stanley, M., Tom, J. & Wells, J.A. (1994). A designed peptide ligase for total synthesis of ribonuclease A with unnatural catalytic residues. *Science* **266**, 243-247.
35. Chen, S.T., Chen, S.Y., Hsiao, S.C. & Wang, K.T. (1991). Application of industrial protease "Alcalase" in peptide synthesis. *Biomed. Biochim. Acta.* **50**, 181-186.
36. Russell, A.J., Trudel, L.J., Skipper, P.L., Groopman, J.D., Tannenbaum, S.R. & Klibanov, A.M. (1989). Antibody-antigen binding in organic solvents. *Biochem. Biophys. Res. Commun.* **158**, 80-85.
37. Zaks, A. & Klibanov, A.M. (1988). Enzymatic catalysis in nonaqueous solvents. *J. Biol. Chem.* **263**, 3194-3201.
38. Fitzpatrick, P.A., Steinmetz, A.C., Ringe, D. & Klibanov, A.M. (1993). Enzyme crystal structure in a neat organic solvent. *Proc. Natl Acad. Sci. USA* **90**, 8653-8657.
39. Burke, P.A., Griffin, R.G. & Klibanov, A.M. (1992). Solid-state NMR assessment of enzyme active center structure under nonaqueous conditions. *J. Biol. Chem.* **267**, 20057-20064.

Because *Chemistry & Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published via the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cmb – for further information, see the explanation on the contents pages.