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The N terminus of the serpin, tengpin, functions to trap the metastable native state

Qingwei Zhang[1*], *Ashley M. Buckle*[1*], *Ruby H.P. Law*[1*], *Mary C. Pearce*[1], *Lisa D. Cabrita*[1], *Gordon J. Lloyd*[1], *James A. Irving*[1], *A. Ian Smith*[1,2], *Katya Ruzyla*[1], *Jamie Rossjohn*[1,2], *Stephen P. Bottomley*[1+] *& James C. Whisstock*[1,2++]

[1]Department of Biochemistry and Molecular Biology and [2]ARC Centre of Excellence for Structural and Functional Microbial Genomics, Monash University, Clayton, Melbourne, Victoria, Australia

Serpins fold to a metastable native state and are susceptible to undergoing spontaneous conformational change to more stable conformers, such as the latent form. We investigated conformational change in tengpin, an unusual prokaryotic serpin from the extremophile *Thermoanaerobacter tengcongensis*. In addition to the serpin domain, tengpin contains a functionally uncharacterized 56-amino-acid amino-terminal region. Deletion of this domain creates a variant—tengpinΔ51—which folds past the native state and readily adopts the latent conformation. Analysis of crystal structures together with mutagenesis studies show that the N terminus of tengpin protects a hydrophobic patch in the serpin domain and functions to trap tengpin in its native metastable state. A 13-amino-acid peptide derived from the N terminus is able to mimick the role of the N terminus in stabilizing the native state of tengpinΔ51. Therefore, the function of the N terminus in tengpin resembles protein cofactors that prevent mammalian serpins from spontaneously adopting the latent conformation.

Keywords: folding; conformational change; chaperone; extremophile; protease inhibitor

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INTRODUCTION

Serpins are the largest and most widely distributed family of protease inhibitors (Irving *et al*, 2000). The native fold of inhibitory serpins is metastable and conformationally labile (Cabrita & Bottomley, 2004). Following interaction with a target protease, the serpin molecule is cleaved within the reactive centre loop (RCL) and the molecule switches to a more stable 'cleaved' conformation; typically, the T_m for cleaved serpins is greater than 120 °C, compared with less than 60 °C for the native state (Kaslik *et al*, 1997). This conformational rearrangement results in the protease being trapped, at the acyl intermediate stage of the catalytic cycle, in a distorted conformation (Lawrence, 1997; Huntington *et al*, 2000). The serpin conformational change is commonly termed the stressed-to-relaxed (S to R) transition (Stein & Chothia, 1991; Whisstock *et al*, 2000a,b) and involves a change in topology, with the RCL forming an additional, fourth β-strand (Lobermann *et al*, 1982) in the A β-sheet in the cleaved state.

Certain serpins are able to undergo the S to R transition in the absence of RCL cleavage to form the 'latent' conformation, which represents the most stable monomeric conformation of the serpin chain (Mottonen *et al*, 1992; Perry *et al*, 1995; Beauchamp *et al*, 1998). Most notably, plasminogen activator inhibitor-1 (PAI-1) folds to the native state, but in the absence of the cofactor vitronectin spontaneously converts to the latent inactive state (Hekman & Loskutoff, 1985). This represents an elegant mechanism that controls the inhibitory activity of this serpin.

It is unclear why serpins fold to a native metastable state and do not fold to the latent state. Similarly, the molecular mechanism of spontaneous conformational change of disease-linked variants of serpins remains to be fully understood (Whisstock & Bottomley, 2006). We have begun to investigate this problem by studying a group of serpins from thermophilic prokaryotes. These molecules are able to function as normal inhibitory serpins, but have developed strategies to fold and function at high temperatures (Irving *et al*, 2003; Fulton *et al*, 2005). Here, we investigated the structure of the serpin, tengpin, from the extremophilic prokaryote *Thermoanaerobacter tengcongensis* (Xue *et al*, 2001).

[1]Department of Biochemistry and Molecular Biology and [2]ARC Centre of Excellence for Structural and Functional Microbial Genomics, Monash University, Clayton, Melbourne, Victoria 3800, Australia
*These authors contributed equally to this work
[+]Corresponding author. Tel: +613 9905 3747; Fax: +613 9905 3703;
E-mail: steve.bottomley@med.monash.edu.au
[++]Corresponding author. Tel: +613 9905 3747; Fax: +613 9905 4699;
E-mail: james.whisstock@med.monash.edu.au

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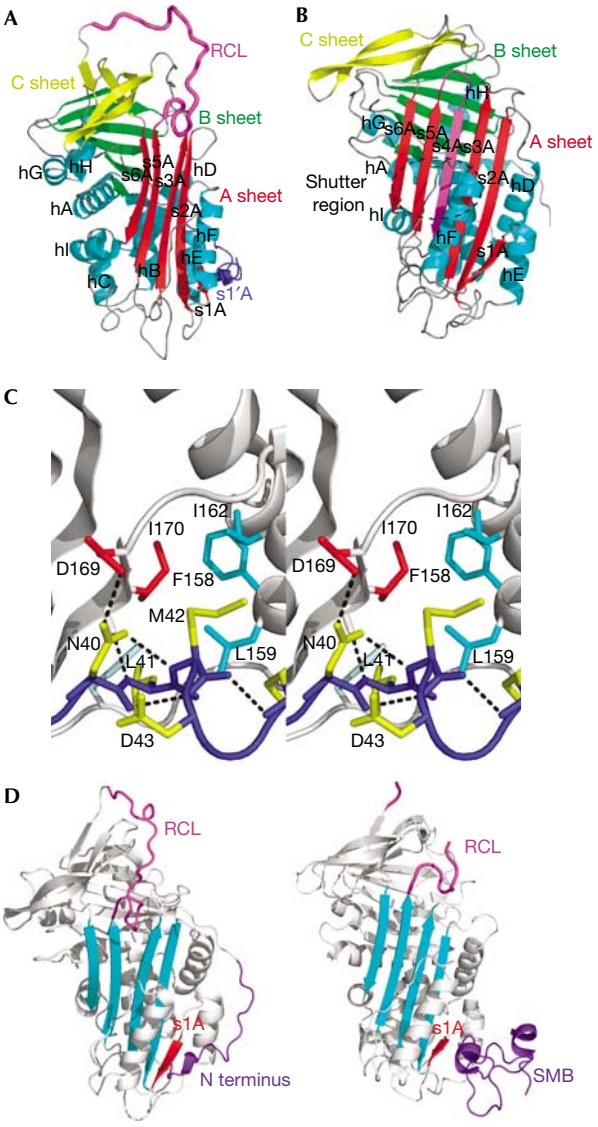

Fig 1 | Crystal structures of tengpin. (**A**) Structure of native tengpinΔ31. Elements of secondary structure are labelled. The A β-sheet is in shown in red; B β-sheet in green; C β-sheet in yellow and α-helices are in cyan; the reactive centre loop (RCL) is in magenta and the N-terminal region is shown in purple. Tengpin contains 42 of the 51 highly conserved residues present in most serpins; substitutions at these positions are generally conservative. The number of salt bridges of the surface of tengpin (78) is also comparable with other mesophilic and thermophilic counterparts (Fulton *et al*, 2005). Notably, 21 amino acids of the amino terminus of the serpin domain could be fully resolved in electron density; these residues adopt an extended conformation and form several interactions with the D-helix (supplementary Table 1 online). (**B**) Structure of latent tengpinΔ51. Colouring as for (**A**). A structural comparison of the native and latent conformations of tengpin shows that strands s3A, s2A and s1A, together with the E- and F-helix, shift to accommodate the RCL as a fourth strand in the A β-sheet. Conformational changes in strands s3C and s4C are apparent as a result of the transition to the latent state and the repositioning of s1C. The shutter region is indicated. (**C**) The contacts between the N terminus, helices E, F and A sheet strands 1 and 2 of tengpinΔ31. Side chains from the A-sheet are in red, from α-helices in cyan and from the N terminus in yellow. Dashed lines indicate hydrogen bonds. (**D**) Comparison of the binding sites of the N terminus (purple) and tengpin (left) with that of the somatomedin B (SMB) domain of vitronectin (purple) and plasminogen activator inhibitor-1 (PAI-1, right; Zhou *et al*, 2003).

RESULTS

The N terminus affects inhibitory activity and folding

Tengpin contains a serpin domain preceded by a 56-amino-acid amino-terminal region of unknown structure and function (supplementary Fig 1 online; Irving *et al*, 2002). Attempts to express full-length material were unsuccessful and resulted in small amounts of insoluble material; therefore, we initially expressed two constructs: tengpinΔ51 that represents the serpin domain alone—that is, lacking the N-terminal region—and tengpinΔ31 that includes 20 amino acids of the N terminus. Bioinformatic analysis suggested that tengpin would be expected to function as an authentic protease inhibitor (Irving *et al*, 2002). Inhibitory data showed that tengpinΔ31 was an effective inhibitor of the chymotrypsin-like protease human leucocyte elastase (SI = 2.1, $k_{ass} = 1.35 \times 10^5\,M^{-1}\,s^{-1}$) and formed the SDS-stable complex typical of native serpins (supplementary Fig 2 online). By contrast, we were unable to measure any inhibitory activity of tengpinΔ51 against a range of target proteases (data not shown). Furthermore, biophysical studies showed that tengpinΔ51 did not undergo thermal denaturation even in the presence of a denaturant, suggesting that it is in the latent conformation. Thus, the N-terminal region seems to have a crucial role in the folding and inhibitory activity of tengpin. To understand the structural basis of these contrasting characteristics, we determined the crystal structures of the tengpinΔ31 and tengpinΔ51 constructs.

Structural characterization of native and latent forms

The 2.7 Å crystal structure of tengpinΔ31 shows two molecules in the asymmetric unit. Both molecules are essentially identical with the exception of minor differences in the RCL, indicating the flexibility of this region (supplementary Fig 3 online). The overall structure of the molecules adopts a 'partly inserted' native serpin conformation (Fig 1A), in which a gap in the β-sheet hydrogen bonding at the top of strands s3A and s5A allows the insertion of two residues of the RCL (Fig 1A). By contrast, the 1.6 Å crystal structure of tengpinΔ51 shows that the molecule adopts the latent, inactive conformation (Fig 1B; Table 1).

The structure of tengpinΔ31 shows that the N terminus adopts an extended conformation spanning the D- and E-helices, followed by a short β-strand (residues 38–41) termed S1A (Fig 1A). With the exception of the first four residues of the N terminus, all residues are resolved in the electron density (supplementary Fig 4 online). N-terminal to s1A′, Q37 and A38 pack against the F-helix and form hydrogen bonds with D182. In total, residues 37–56 of tengpinΔ31 make 20 hydrogen bonds and 37 van der Waals contacts, and bury 170 $Å^2$ accessible surface area at the interface with the serpin domain (supplementary Table 1 online; Fig 1C).

A structural comparison of the native and latent conformations of tengpin shows that strands s3A, s2A and s1A, together with the

Table 1 | Data and refinement statistics

	TengpinΔ51	TengpinΔ31
Data collection		
Space group	$P2_12_12_1$	$I23$
Molecules in AU	1	2
Cell parameters *a*, *b*, *c* (Å)	44.7, 44.9, 159.2	$a=b=c=217.9$
Resolution (Å)	79.0–1.6	154.3–2.7
Total reflections	233,332	137,140
Unique reflections	42,797	45,951
Multiplicity	5.4 (1.6)	3.0 (2.7)
Completeness (%)	98.4 (94.0)	97.8 (98.1)
$\langle I/\sigma \rangle$	20.1 (3.4)	14.4 (2.2)
R_{merge} (%)*	7.6 (35.1)	7.7 (66)
Refinement		
Resolution (Å)	79.0–1.6	154.3–2.7
R_{free} (%)‡	26.0	25.1
R_{factor} (%)‡	21.2	21.3
R.m.s.d. bonds (Å)	0.006	0.007
R.m.s.d. angles (deg)	1.47	0.95
R.m.s.d. improper	1.29	1.28
R.m.s.d. dihedral	25.36	26.16
Ramachandran plot		
Most-favoured and allowed regions (%)	99.7	99.9
B-factors (Å²)		
Average main chain	10.3	52.5 (A), 4.2 (B)
Average side chain	12.0	52.3 (A), 53.7 (B)
Average water molecule	23.1	54.6

Values in parentheses are for the highest resolution bin.
*Agreement between intensities of repeated measurements of the same reflections and can be defined as $\sum(I_{h,i}-\langle I_h\rangle)/\sum I_{h,i}$, where $I_{h,i}$ are individual values and $\langle I_h\rangle$ is the mean value of the intensity of reflection *h*; ‡$R_{factor}=\sum_{hkl}||F_o|-|F_c||/\sum_{hkl}|F_o|$ for all data except for 5%, which were used for R_{free} calculation.

E- and F-helix, shift to accommodate the RCL as a fourth strand in the A β-sheet (supplementary Fig 5A online). Furthermore, substantial conformational change in strands s3C and s4C is apparent as a result of the transition to the latent state and the repositioning of s1C (supplementary Fig 5B online). Together, these structural data explain the lack of inhibitory activity of tengpinΔ51 and indicate that the additional 20 amino acids at the N terminus of tengpinΔ31 have a crucial role in maintaining the metastable native state.

The N terminus prevents folding to the latent state

We investigated whether the N terminus of tengpin is required for initial folding to the native state or to maintain the serpin in a native conformation. Equilibrium refolding of tengpinΔ31 and tengpinΔ51 shows a two-state transition with midpoints centred around 1 M guanidinium thiocyanate (Fig 2A). Critically, both of the refolded tengpin constructs—rΔ51 and rΔ31—were able to inhibit target proteases (Fig 2B). However, monitoring the inhibitory activity over time at 37 °C showed that rΔ51 rapidly and spontaneously lost inhibitory activity ($t_{1/2}\sim5$ h; Fig 2B). By contrast, minimal loss of inhibitory activity was observed for rΔ31 ($t_{1/2}>800$ h; Fig 2B). Together, these data suggest that the N terminus of tengpin is not required for initial folding to the native state, but is required to stabilize and maintain the native conformation, thus preventing the subsequent folding of the molecule into the inactive latent conformation.

An N-terminal peptide analogue stabilizes the native state

Next, we investigated whether the N-terminal region in isolation could perform the same function and stabilize the native state of tengpinΔ51. We refolded denatured tengpinΔ51 in the presence of a peptide, corresponding to residues 39–51 of the N-terminal region (Ac-ANLMDRIKANPVS), and monitored the inhibitory activity of the refolded material. Our data showed that, although approximately 30% of tengpinΔ51 lost activity, the peptide was able to maintain the native state of approximately 70% of tengpinΔ51 (Fig 2C). It is unclear why 30% of the refolded material was not stabilized by the peptide. We suggest that this is most probably the result of competition between the rate of peptide binding and the rate of conformational change during folding or the transition to the latent state. However, these data strongly indicate that the peptide forms an analogous interaction with the body of the molecule, which prevents tengpinΔ51 from undergoing the transition to the latent conformation.

Dissection of the interactions that stabilize the native state

To define crucial interactions made by the N terminus that are important for tengpin metastability, we subjected the N terminus to a combination of truncation and mutagenesis. We constructed nine tengpin mutants and investigated whether each construct adopted the native or latent conformation (Table 2). We used three criteria to determine the conformation: (i) we were able to distinguish between native and latent material by using phenyl-*S*epharose chromatography (tengpinΔ31 and tengpinΔ51 eluted at approximately 1.1 M and approximately 0.35 M of ammonium sulphate, respectively; supplementary Fig 6 online); (ii) native tengpin unfolds completely in 6 M guanidine hydrochloride, but latent tengpin does not; and (iii) all proteins were tested for inhibitory activity and ability to form SDS-stable complexes with elastase, and the half-life of each variant was calculated.

Initially we truncated the molecule from the N terminus; these data showed that it was possible to remove the N-terminal sequence up to but not including amino acid N40 (tengpinΔ39) and form a stable native conformation (tengpinΔ39 $t_{1/2}=594$ h; Fig 2C; Table 2). Examination of the structure shows that N40, L41 and M42 make substantial interactions with the body of the serpin (Fig 1C). Indeed, mutation of any one of these three residues in tengpinΔ39 resulted in more rapid formation of the inactive, latent state ($t_{1/2}$ of the native state 25–30 h; Fig 2C; Table 2). The side chain of D169 forms a hydrogen bond with the ND2 atom of N40 (Fig 1C; supplementary Table 1 online); therefore, we were able to define further interactions made by N40 by generating D169A.

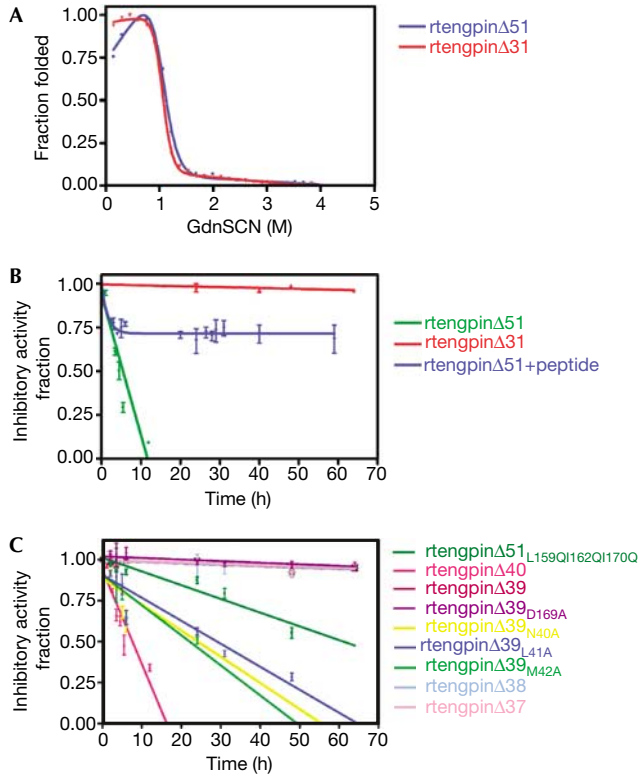

Fig 2 | Refolding and inhibitory activity of tengpin. **(A)** Equilibrium refolding of tengpinΔ51 and Δ31 monitored by fluorescence. The fluorescence signal is shown as a function of guanidinium thiocyanate (GdnSCN) concentration in 50 mM Tris–HCl (pH 7.5) at 25 °C. **(B)** Loss of inhibitory activity as a function of time at 37 °C for refolded tengpinΔ31, refolded tengpinΔ51 and refolded tengpinΔ51 in the presence of synthetic peptide. **(C)** Loss of inhibitory activity as a function of time at 37 °C for all the other tengpin variants.

Analysis of the mutant protein tengpinΔ39$_{D169A}$ shows that this variant adopts the native conformation ($t_{1/2}$ = 526 h; Fig 2C; Table 2). Together, these data define the minimum contacts required to prevent native tengpin folding to the latent conformation. Furthermore, it is possible to estimate the additional interactions made between the body of the serpin and the N terminus in tengpinΔ39$_{D169A}$ (native) in comparison with those present in tengpinΔ39$_{N40A}$ (latent): these comprise one hydrogen bond and three van der Waals interactions. Mutating L41 and M42 to alanine, thus truncating the side chains of these residues, would be predicted to result in the loss of two and four van der Waals interactions, respectively. Together the three residues form a cap protecting the underlying hydrophobic core that includes I162, L159 and I170. A structural comparison of the native and latent state shows that, following the S to R transition, strand s1A of tengpinΔ51 adopts a position similar to the region occupied by the N terminus in tengpinΔ31. In particular, I170 moves to partly cover I162 and L159 (Fig 3).

Mutations in s1A and the E-helix stabilize tengpinΔ51

We were interested in probing further the role of the N-terminal region in stabilizing the native state. We reasoned that mutations of hydrophobic residues ordinarily covered by the N terminus might abrogate the requirement to undergo transition to the latent state. Thus, we mutated the three hydrophobic residues (I162, L159 and I170) contacted by L41 and M42 of the N terminus. The mutations were made in tengpinΔ51 and all three residues were changed to the polar, uncharged residue glutamine. Interestingly, these mutations did not abolish conformational change, although the native state of tengpinΔ51$_{L159QI162QI170Q}$ was substantially stabilized ($t_{1/2}$ = 60 h) in comparison with tengpinΔ51 ($t_{1/2}$ = 5 h) (Table 2).

Table 2 | Conformation and half-life of mutants

Mutants	Conformation	Half-life (h)
Truncated mutants		
TengpinΔ31	Native	800
TengpinΔ37	Native	649.5
TengpinΔ38	Native	614
TengpinΔ39	Native	594.5
TengpinΔ40	Latent	8.16
TengpinΔ41	Latent	7.52
TengpinΔ51	Latent	5
Single amino-acid mutants		
TengpinΔ39$_{D169A}$	Native	526
TengpinΔ39$_{N40A}$	Latent	27.72
TengpinΔ39$_{L41A}$	Latent	32.34
TengpinΔ39$_{M42A}$	Latent	24.64
Triple amino-acid mutants		
TengpinΔ51$_{L159QI162QI170Q}$	Latent	60.3

DISCUSSION

Our data show that, despite its extremophilic source, the serpin domain of tengpin readily undergoes conformational change to the latent state. We have shown that the N terminus of tengpin functions to trap the serpin domain in the native metastable state and to prevent the spontaneous transition to the latent conformation. The function of the N terminus of tengpin is thus strikingly similar to the role of the plasma protein vitronectin in stabilizing the metastable state of the mammalian PAI-1 (Declerck *et al*, 1988). Furthermore, similarly to the crucial residues 40–42 of the N terminus of tengpin, structural studies have shown that the somatomedin B domain of vitronectin binds to strand s1A of serpins (Zhou *et al*, 2003; Fig 1D). However, the N terminus of tengpin does not adopt the same fold as that of the somatomedin B domain of vitronectin, and therefore this is consistent with convergent, rather than divergent evolution.

Studies on mammalian serpins have shown that numerous mutations causing conformational disease (or serpinopathies; Carrell & Lomas, 1997) localize on or around a mobile 'trigger point' in the central portion of the molecule, commonly termed the shutter region (see Fig 1B). Our structural, mutagenetic and biophysical data extend these studies and show that in tenpgin exposure of a relatively small hydrophobic patch on the surface of

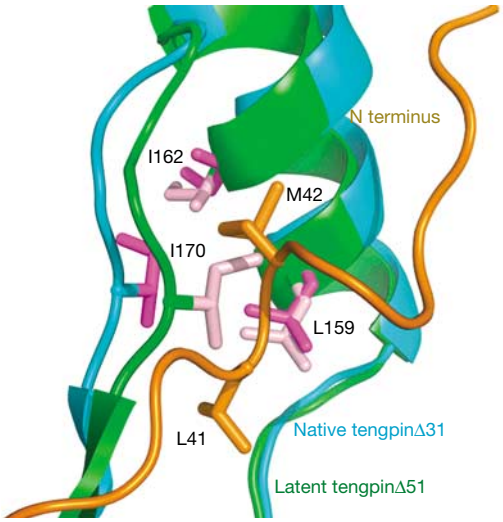

Fig 3 | Cartoon of latent tengpinΔ51 and tengpinΔ31. The figure shows how the side chains of residues L41 and M42, in the native state, protect the hydrophobic pocket formed by residues L159, I162 and I170.

the serpin domain, approximately 20 Å from the centre of the shutter, seems sufficient to promote conformational rearrangement. Consistent with this hypothesis, mutations in the hydrophobic patch can at least partly compensate for the lack of the N-terminal region and slow the transition to the latent state. Furthermore, we show that it is possible to stabilize the native state in the absence of the N terminus studies by using an exogenous peptide. Together, our research supports the therapeutic strategies that aim to prevent conformational change in mammalian serpins by targeting hydrophobic cavities in the mobile region of the molecule (Lomas & Mahadeva, 2002).

METHODS

Materials. The genomic DNA of *T. tengcongensis* was obtained from the Beijing Genomic Institute (Chinese Academy of Sciences, China; Xue *et al*, 2001). Details of the cloning, mutagenesis, expression, purification, peptide-binding studies, kinetic characterization, stability measurements and crystallization of tengpin are given in the supplementary information online.

Structure determination and analysis. Data were collected from cryo-cooled crystals at 100 K at the BIOCARS and IMCA-CAT beamlines at the Advanced Photon Source (Chicago, IL, USA). Structure elucidation was carried out using CCP4 software (1994), unless stated otherwise. The structure of tengpinΔ51 was determined by molecular replacement using AMORE (Navaza, 2001) and the structure of native thermopin (1SNG) as a search model. The structure of tengpinΔ31 was determined by molecular replacement using PHASER (McCoy *et al*, 2004) and an ensemble search probe consisting of structurally aligned molecules of thermopin (1SNG; Fulton *et al*, 2005) and tengpinΔ51. Tengpin contains two molecules in the asymmetric unit. The limited resolution of the data necessitated the use of strict non-crystallographic symmetry restraints throughout refinement; however, by using the R_{free} as a guide, we were able to model small differences between the RCL of each molecule by loosening restraints in this region. Structure refinement and building proceeded using the CCP4 (1994) suite, REFMAC (Murshudov *et al*, 1997) and O (Jones *et al*, 1991). Final refinement statistics (Table 1) for tengpinΔ31 and tengpinΔ51 are $R_{free}/R_{work} = 26.0/21.2\%$ and $R_{free}/R_{work} = 25.1/21.3\%$, respectively. Structures were superimposed using the program MUSTANG (Konagurthu *et al*, 2006). Accessible surface areas were calculated using the CCP4 program AREAIMOL. Figures were produced using PYMOL (Delano Scientific Pty Ltd, San Diego, CA, USA).

Data Deposition Statement. Coordinates have been deposited in the RCSB Protein Data Bank (www.rcsb.org; identifiers 2PEE and 2PEF).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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