Intersubunit linker length as a modifier of protein stability: Crystal structures and thermostability of mutant TRAP

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Abstract

The ability of proteins to self-assemble into complex, functional nanoscale structures is expected to become of significant use in the manufacture of artificial nanodevices with a wide range of novel applications. The bacterial protein TRAP has potential uses as a nanoscale component as it is ring-shaped, with a central, modifiable cavity. Furthermore, it can be engineered to make a ring of 12-fold symmetry, which is advantageous for packing into two-dimensional arrays. The 12mer form of TRAP is made by linking multiple subunits together on the same polypeptide, but the usefulness of the 12mers described to date is limited by their poor stability. Here we show that, by altering the length of the peptide linker between subunits, the thermostability can be significantly improved. Since the subunit interfaces of the different 12mers are essentially identical, stabilization arises from the reduction of strain in the linkers. Such a simple method of controlling the stability of modular proteins may have wide applications, and demonstrates the lack of absolute correlation between interactions observable by crystallography and the internal energy of a complex.

Keywords: self-assembly; nanoengineering; ring protein; RNA-binding; strain

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From the beginning of nanotechnology, proteins have been heralded as ideal candidates for nanodevices (Astier et al. 2005), not least because they already carry out just such a role in nature. However, designing proteins to fulfill specific functions that do not occur naturally is a

major challenge in synthetic biology. Obstacles include biological complexity, the time and work required to construct and characterize synthetic systems, and spontaneous variations in the behavior of biological systems (Endy 2005). Advances have been made in the use of massively parallel computing to simulate and predict protein folding for small proteins (Shirts and Pande 2001; Zagrovic et al. 2001), but proteins of normal size remain far beyond current computational limits.

Until it becomes possible to predict tertiary structure from amino acid sequence alone, one of the most promising approaches to engineering new, artificial protein structures is a “building block” technique, where protein domains with desired structural or functional properties

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are chosen from existing, characterized proteins and connected together. This approach has been used to make a number of artificial proteins including a novel chimaeric DNA binding protein (Chevalier et al. 2002), a biotin-modified aldolase linked together by streptavidin to form a two-dimensional grid (Ringler and Schulz 2003), a backward-moving myosin made from three preexisting protein domains (Tsiavaliaris et al. 2004), a charge-trapping chimaeric protein made by a fusion of cytochrome b_{562} and green fluorescent protein (Choi et al. 2006), and a ball-and-stick supramolecule (Sugimoto et al. 2006).

Similarly, linking tandem copies of a protein on the same polypeptide chain is another potentially powerful technique that can be used to alter the stability and functional properties of a protein. The native 11mer TRAP has been engineered in this way to form an artificial 12-membered ring (Heddle et al. 2006, 2007a). TRAP is an RNA-binding protein involved in the control of tryptophan synthesis in various species of *Bacillus* (Gollnick 1994; Babitzke 2004). The TRAP monomer is ~ 8.2 kDa in size and associates to form a ring-shaped homo-11mer that is highly thermostable (Baumann et al. 1997; Heddle et al. 2006). The ring is ~ 8.4 nm in diameter and 2.5 nm in height, with the central hole being ~ 2.4 nm in diameter. TRAP is able to bind cognate single-stranded RNA (ssRNA) once it has bound tryptophan. In vivo and in vitro studies show that TRAP will bind ssRNA carrying 11 copies of the sequence NAG (Babitzke et al. 1994, 1995, 1996; Antson et al. 1999) with an optimal spacing of two nucleotides between triplet repeats.

Ring-shaped proteins and peptides are of particular interest as potential components of nanodevices. They can be used to create arrays (McMillan et al. 2002) or tubes (Hartgerink et al. 1998) of metals or semiconductors. TRAP also offers the potential to arrange materials such as gold nanodots. The protein surrounding the nanodot can be utilized to organize the dots in a desired pattern such as a tightly packed array. Recently, a modified TRAP protein was used to place gold nanodots on a titanium oxide surface (Heddle et al. 2007a).

An artificial 12-membered version of the TRAP ring was produced by fusing together three or four copies of the TRAP gene (to make fusions called "TRAP3" and "TRAP4," respectively). In these constructs the copies of the monomers are connected by three alanine residues (Heddle et al. 2006). Three molecules of TRAP4 and four molecules of TRAP3 self-assemble to form ring proteins with subunit contacts essentially identical to the wild-type protein in the crystal structures, but with 12 instead of 11 subunits in the ring (Heddle et al. 2006). However, the linker peptide was not visible in the resulting electron density maps, and it was unclear if they were located

inside or outside the ring (Fig. 1). Simplistic linear measurements of the two possible paths suggest a loop length of ~ 47 Å for the inside route and 66 Å for the outside route (including not just the linker peptide but residues disordered in previous TRAP crystal structures). This might suggest that the inside path would be preferable but does not take into account the possible effects of crowding eight copies of the linker within the cavity. It is also possible that a linker taking the outside path might pack more closely with the existing structures, reducing its path length. Both of these cases would significantly promote the outside path, and only by solving the crystal structure, revealing part of the linker, can the actual path be verified. Although the hydrogen bonds and apolar interactions between subunits are almost identical to those of the wild-type protein, the 12mer TRAP was found to be significantly less stable, with differential scanning calorimetry suggesting that initial peaks in heat capacity occur at more than 10°C lower than for the wild-type protein (Heddle et al. 2006). The 12-membered ring was apparently destabilized significantly by the short length of the linker peptide, which introduces mechanical strain into the system. In this paper we have investigated the position of the linker peptides in the ring structure and the effect of the linker length on the stability of the engineered 12mer ring. The crystal structures of two 12mer TRAP proteins with increased linker length are described, showing for the first time part of the linker and N-terminal region of the protein pointing toward the center of the ring. Increasing the length of the linker increases the stability of the protein, although the crystal structures of the different 12mers are essentially identical.

Figure 1. Schematic figure comparing wild-type 11mer TRAP and artificial 12mer TRAPs showing possible positions of linker peptides. (A) A wild-type TRAP monomer. Folding and assembly (indicated by the arrow) gives the 11mer wild-type TRAP ring. The RNA-binding region around the outside of the ring is indicated by the blue stripe. (B) The artificial monomer TRAP, T3A7, consisting of three wild-type monomers that are covalently linked by seven-alanine linker peptides (shown in red). Four of these linked proteins assemble into a "12mer" ring. The linker connecting the C and N termini of the linked subunits could do so via a path around the outside of the ring (external linker) or through the inside of the ring (internal linker). In the former case, the linker would obstruct the RNA-binding site.

Results

In the original 12mer protein (T3A3), two linker sequences (L) of three alanine residues join three copies of TRAP (T) in the pattern “T-L-T-L-T.” The newly produced 12mers in this work have linkers of five or seven alanines (T3A5 and T3A7, respectively). Transmission electron microscopy (TEM) was used to confirm that the mutant proteins form rings, and all of the mutant proteins showed characteristic doughnut-like rings, indistinguishable from TEM images of the wild-type protein (see Fig. 4).

RNA-binding assays

The previously determined crystal structures of 12mer forms of TRAP gave no indication of the position of the linker peptides connecting the subunits. To see if the linker peptide runs around the outside of the protein or through the central cavity, we tested the RNA binding of wild-type 11mer and mutant 12mer forms of TRAP. If the linker peptide runs around the outside of the 12mer ring, it would block RNA binding to the outer surface of the ring (Yang et al. 1997). If the linkers run through the center of the ring, then RNA binding affinity is expected to be close to that of wild-type TRAP. Gel retardation assays (Fig. 2) showed all of the mutant TRAP proteins were able to bind RNA, with affinity close to that of wild-type protein, strongly suggesting that the linker peptides run through the center of the ring. Interestingly, while wild-type TRAP protein gave a sharp band of the protein–RNA complex, the mutant TRAPs all gave a more smeared band at higher molecular weights. This may be because the 12mer TRAP is not completely enclosed by the 55mer RNA, and so excess RNA may lead to some higher order complexes forming.

Heat stability tests

The thermostability of different forms of TRAP was tested by incubating the protein, with or without added tryptophan, in a heating block and raising the temperature in steps of 10°C. Wild-type TRAP and the three mutant TRAPs showed no visible precipitation after a 5-min incubation at 70°C in the presence of saturating amounts of tryptophan. T3A3 however showed precipitation in absence of tryptophan (Fig. 3A). At 80°C T3A5 showed significant precipitation in the absence of tryptophan (Fig. 3B), and at 100°C both T3A3 and T3A5 showed precipitation both in the presence and absence of tryptophan (Fig. 3C). At 110°C T3A7 started to show very slight precipitation in the presence but not absence of tryptophan (Fig. 3D). Even at 110°C, wild-type TRAP remained soluble whether or not tryptophan was added.

Figure 2. RNA binding to wild-type and mutant TRAPs. (A) Mixtures of 2.5 μ M single-stranded RNA with wild-type or mutant TRAPs were applied to an agarose gel that was stained with ethidium bromide for the presence of protein (*top*) and RNA (*bottom*). Protein concentrations were 0 μ M, 2.5 μ M, and 5 μ M. All of the 12mer TRAPs show very high apparent molecular weight in the presence of RNA, suggesting the 55-base-long RNA binds more than one 12mer ring. (B) Mixtures of 100 pM single-stranded RNA and increasing amounts of wild-type (*left-hand gel*) or T3A7 (*right-hand gel*) were run on a 5% native-PAGE TBE gel. Lanes are marked with the picomolar concentration of protein used. Lane C is a control containing RNA only. 3A, 5A, and 7A denote TRAP proteins T3A3, T3A5, and T3A7, respectively.

In summary, wild-type TRAP appears the most stable. Generally, TRAP stability is increased by the addition of tryptophan (with the exception of T3A7 at 110°C), and mutant TRAPs increase in thermostability as the linker length increases from three to seven alanine residues. Both T3A5 and T3A7 also retain the expected ring shape (Fig. 4).

Differential scanning calorimetry

As previously found with T3A3 (Hedde et al. 2006), the DSC results showed broad, noisy peaks indicative of complex interactions rather than a two-state process and a reduced thermostability compared to wild-type protein. However, each DSC trace contains a sharp trough that corresponds to the irreversible denaturation of the protein

Figure 3. Heat stability of the mutants. Wild-type and mutant proteins (1 mg/mL) were heated to 70°C (A), 80°C (B), 100°C (C), and 110°C (D) for 5 min before cooling to room temperature. Precipitation at 110°C in the case of T3A7 was too slight to be readily visible. Denatured protein formed a white precipitate. (+) Added tryptophan, (–) no added tryptophan. 3A, 5A, and 7A denote TRAP proteins T3A3, T3A5, and T3A7, respectively.

(see Supplemental Fig. S1). The temperature at which this trough occurs is significantly higher in T3A5 and T3A7 than in T3A3 and corresponds closely to the denaturation temperature observed in the heat stability tests (Table 1). This confirms that the mutant proteins with longer linkers are significantly more stable than the protein with the short linker, but less stable than the wild-type protein.

X-ray crystallography

T3A5 and T3A7 were crystallized in space groups $P4_212$ and $P4$, respectively (Table 2). The crystal packing of the T3A5 rings is identical to that previously found for T3A3 with one TRAP3 unit (equivalent to three wild-type monomers) per asymmetric unit. In the case of T3A7 there are two. In both cases the new structures closely resemble the previous model in that four such units combine to form a single ring facing the crystallographic fourfold axis. These rings form continuous tubes running through the crystal, unlike the previously solved wild-type TRAP structures (Antson et al. 1994, 1999; Chen et al. 1999; Hopcroft et al. 2002). The models of T3A5 and T3A7 are essentially identical in tertiary structure to T3A3 and wild-type TRAP and subunit interface hydrogen bonds are also essentially unchanged but in the case of T3A7 additional residues of the linker peptide are visible (Figs. 5–7; Table 3). Overlaying the $C\alpha$ backbones of T3A3 and T3A5 (Fig. 7) gives an RMS deviation of 0.16 Å. The one significant difference with T3A7 is that several additional linker residues are now visible, protruding into the central cavity. These regions are not ordered in T3A3 and T3A5, which more closely resemble

the wild-type TRAP structures also missing the first six N-terminal and two C-terminal residues. In T3A7 the C-terminal residues form a hairpin turn between Glu73 and Gly74 and the chain forming a β -strand running up the central cavity of the ring adjacent to residues 67–71. The visible region of the linker is stabilized by a number of hydrogen bonds with surrounding residues (Supplemental Table S1). The region of the hole close to the C-terminal face of T3A7 is significantly narrowed due to the presence of the linker and at the N-terminal face due to the presence of N-terminal residues that were not visible in previous structures (Figs. 5, 7).

As expected, the hydrophobic tryptophan-binding pocket of the mutants is identical to that seen in the wild-type protein. The buried surface area between protein domains as calculated by AREAIMOL (Collaborative

Figure 4. Transmission electron micrographs of mutant TRAPs deposited on a carbon-coated copper mesh. (A) T3A5. (B) T3A7. We applied 0.05mg/mL of each protein in the presence of tryptophan. The white scale bar is 50 nm.

Table 1. Melting temperature of different TRAP proteins

Protein	Phase transition minimum (°C)
Wild-type TRAP + or – trp	>115 ^a
T3A3 – Trp	82
T3A3 + Trp	99
T3A5 – Trp	92
T3A5 + Trp	100
T3A7 – Trp	99
T3A7 + Trp	115

Denaturation temperature was assessed by DSC with and without added tryptophan. Unlike wild-type 11mer TRAP, however, the 12mer forms cannot be refolded easily, making the removal of copurified ligand more difficult. In each case, the addition of small amounts of tryptophan (final concentration 0.5 mM) substantially improved the thermostability, so the samples without added ligand must be significantly unliganded.

^aWild-type result is from previously published results (Heddle et al. 2007b).

Computational Project, Number 4 1994) is 1700, 1780, and 1860 Å², for T3A3, T3A5, and T3A7, respectively, compared to 1630 Å² for the wild-type protein. The slightly larger area for T3A7 is mainly due to the presence of extra C-terminal residues in the crystal structure.

Previous examination of the contacts in the wild-type and T3A3 mutants showed that hydrogen bonds and hydrophobic interactions across the monomer interface were unchanged (Heddle et al. 2006). Similar examination of contacts between monomer interfaces in T3A5 and T3A7 using CONTACT, part of the CCP4 package (Collaborative Computational Project, Number 4 1994), again showed no significant deviations (Table 3). The crystal structures alone therefore give no immediately obvious explanation for the change in thermostability such as lost hydrogen bonds or other interactions.

Discussion

As a thermostable ring protein, TRAP has considerable potential as a component of nanoscale devices and/or as a biomineralization agent. Biomineralization using cage proteins such as ferritin and viral capsids has been shown to be possible if suitable mutations are introduced into the protein cavity (Douglas et al. 2002; Miura et al. 2006). We have shown it is possible to alter the inner cavity of the TRAP ring in the same way (Heddle et al. 2007a). To form ordered arrays on surfaces, however, 12-fold symmetry is much more suitable than 11-fold symmetry. We have previously constructed artificial 12mer TRAP rings by linking together TRAP monomers with three alanine residues (Heddle et al. 2006), but these proteins were found to have low thermostability. The crystal structures showed that the tri-alanine linker peptides in T3A3 must be tightly stretched between the N and C termini of the subunits, but offered no other obvious structural cause for the reduced stability. Here we

have shown that increasing linker length does indeed significantly stabilize 12mer TRAP.

Since the N- and C-terminal residues were not visible in the electron density maps (Heddle et al. 2006), it could not be determined previously whether the linkers passed through the cavity or around the outside of the ring. The crystal structure of T3A7 shows clearly the linker peptides pass through the central cavity. This result is confirmed by RNA-binding experiments that show RNA is capable of binding to the 12mer forms of TRAP. If the linkers lie on the outside of the protein they would block access to the RNA-binding site, which consists primarily of residues Glu36, Lys37, Asp39, Lys56, and Arg58 located around the circumference of the ring (Yang et al. 1997; Hopcroft et al. 2002).

While wild-type TRAP clearly prefers the 11mer form, there has been limited experimental evidence suggesting it can also make a 12mer ring (McCammon et al. 2004). Our results show that all forms of 12mer TRAP tested can bind single-stranded RNA with an appropriate sequence (Fig. 2A), suggesting that the artificial proteins could replace 11mer TRAP in vivo. Further testing showed that 12mer TRAP has a similar affinity for RNA as the wild-type

Table 2. Data collection and refinement statistics

	T3A5	T3A7
Data collection		
Space group	<i>P</i> 4 ₂ 1 ₂	<i>P</i> 4
Cell dimensions:	<i>a</i> = <i>b</i> = 109.6,	<i>a</i> = <i>b</i> = 110.1,
<i>a</i> , <i>b</i> , <i>c</i> (Å)	<i>c</i> = 36.7	<i>c</i> = 36.9
Resolution (Å)	50.0–2.5	50.0–1.8
(Outer shell, Å)	(2.59–2.50)	(1.86–1.80)
<i>R</i> _{merge} ^a		
(overall/outer shell, %)	6.1 (22.1)	8.0 (30.4)
<i>I</i> /σ (outer shell)	12.3 (11.7)	10.7 (7.4)
Completeness		
(overall/outer shell, %)	95.7 (93.6)	94.7 (70.3)
Redundancy		
(overall/outer shell)	5.0 (4.1)	4.3 (1.9)
Refinement		
Resolution (Å)	20.0–2.5	20.0–1.8
No. reflections		
(test reflections)	7503 (363)	37,466 (1975)
<i>R</i> _{work} / <i>R</i> _{free} (%)	19.6/25.6	19.4/24.5
No. atoms		
Protein	1522	3219
Ligand	45	90
Water	39	210
Mean B factors (overall)	27.4	14.2
Protein	27.6	13.8
Ligand	23.0	9.3
Water	28.9	22.8
RMS deviations		
Bond lengths (Å)	0.012	0.015
Bond angles (°)	1.4	1.5

^a*R*_{merge} = Σ(*I*_{*i*} – ⟨*I*⟩)/Σ*I*_{*i*}, where *I*_{*i*} is intensity of an observation and ⟨*I*⟩ is the mean value for that reflection. Summations are over all equivalents.

Figure 5. Comparison of the crystal structures of T3A3, T3A5, and T3A7. Each protein ring is shown in colored cartoon form with the molecular surface of the protein shown in semi-transparent gray. Images in the *top* row show the rings from “above.” The *middle* row is after a rotation of 90° about an axis parallel to the plane of the ring, and the *bottom* row after an additional 180° rotation about the same axis. In T3A7, the extra residues visible in the center of the ring are Thr5 and Asn6.

protein (Fig. 2B), consistent with the linker peptide being present in the central cavity rather than crossing the RNA-binding site. The binding results do, however, reveal one obvious difference in RNA binding between the wild-type and 12mer TRAPs: The 12mer proteins give higher molecular weight complexes that appear to precipitate. In fact, sensitive mass spectrometry studies have shown that wild-type protein also forms small amounts of higher order complexes of two or three TRAP rings bound to RNA (McCammon et al. 2004). This wild-type complex, however, is not visible in the gel-shift studies, suggesting that the 12mer complex is able to form complexes of even higher molecular weight that precipitate. This is confirmed by our own mass spectrometry studies (results not shown). This may be due to the fact that the 12mer ring has 12 RNA binding sites but the RNA used in these binding experiments has only 11 copies of the NAG binding sequence. Each 12mer therefore has one “free” binding site to which a second RNA strand could bind while simultaneously binding a second TRAP. In this case, some portion of the RNA would not be bound to the protein and therefore it would be easier to stain than protein-bound nucleotides. Consistent with this interpretation, we find that the RNA-12mer complexes could be stained with either ethidium bromide or SYBR-Gold, whereas the RNA complexed with

the wild-type protein could only be stained successfully with SYBR-Gold, which has a stronger affinity for single-stranded nucleic acid.

Linking different domains by incorporating them into the same polypeptide is an obvious method for producing bifunctional proteins, whether for nanoscale protein-based devices or other purposes. Our results show that the linker region can play an important role in the properties of the resulting protein, and linker length should be considered carefully when designing such proteins. Given the essential identity between the intersubunit contacts of the TRAP subunits in the different mutant structures, these proteins are also an example of the difficulty of predicting protein stability from crystallographic models. The differences in stability of the different proteins described here largely arise from parts of the structure invisible in the electron density map, and the strain induced in the visible parts has no measurable effect on the atomic coordinates of the refined models.

Materials and Methods

Construction of genes encoding mutant proteins

The plasmid encoding modified TRAP 12mer protein consisting of a fusion of three TRAP monomer genes was previously described (Heddle et al. 2006). The three wild-type monomers in

Figure 6. T3A7 linker residues, with a final, weighted *2Fo-Fc* electron density map, calculated to 1.8 Å resolution, displayed with CCP4mg (Potterton et al. 2002). The map is contoured at 1.0 σ . The first three linker residues (Ala77–Ala79) and Lys76 as well as residues Thr5, Asn6 are not visible in the electron density maps of earlier 11mer and 12mer TRAP crystal structures. The model shown here unambiguously demonstrates that the intersubunit linkers pass through the central hole of the ring.

Figure 7. (A) Peptide backbone overlay of T3A3 (red), T3A5 (green), and T3A7 (blue). (B) Peptide backbone overlay of TRAP3A3 (red), TRAP3A5 (green), and TRAP3A7 (blue), showing the N-terminal region of one wild-type monomer equivalent and the C-terminal of the neighboring monomer equivalent. The residue shown as cyan sticks is residue 79, the third alanine of the T3A7 linker, which points into the central hole of TRAP. (C) Close-up view of part of the peptide backbone overlay of T3A3 (red), T3A5 (green), and T3A7 (blue) with the same orientation as in B. The dashed line connects the last visible C-terminal residue (A79, the fourth linker residue) with the first visible N-terminal residue of the next monomer (T5). The dashed line indicates the connection of these two residues via the remaining four alanine residues, which are not visible in the crystal structure.

this protein are linked via a linker peptide consisting of three alanines. In this report, this protein is referred to as T3A3. The T3A3 coding sequence was further modified by the stepwise addition of the six bases, 5'-GCGGCG-3', encoding two addi-

tional alanine residues, to both linker regions to produce a new gene encoding T3A5. A second mutant (called T3A7) was constructed by further addition of the same six-base sequence to T3A5, lengthening the linker regions by a further two alanine residues. Insertions were made using the Quickchange mutagenesis kit (Stratagene).

Protein expression and purification

Plasmids encoding the mutant proteins were transformed into BL21 DE3 cells (Stratagene), which were grown in liquid LB medium at 37°C and induced with 500 μ M IPTG at an optical density at 695 nm of $\sim$ 0.5. Protein purification was carried out using a method similar to that described previously (Hedde et al. 2006). In brief, cell pellets of induced cells were resuspended in 50 mM Tris HCl (pH 8.5), 100 mM NaCl. Resuspended cells were lysed by sonication. Supernatant was applied to a Q-sepharose column (Amersham) equilibrated in 50 mM Tris HCl (pH 8.5) and eluted with an ascending salt gradient. Mutant TRAP proteins eluted from the column at $\sim$ 450 mM NaCl. Fractions containing TRAP protein were pooled and exchanged into 20 mM MES (pH 6.5), 100 mM NaCl, and applied to a heparin sepharose column (Amersham) equilibrated in the same buffer. Protein was eluted with an ascending salt gradient. Protein was found to elute at $\sim$ 1 M NaCl. As a final step, eluted protein was added directly to a Sephadex 200 16–60 gel filtration column (Amersham) equilibrated in 20 mM Tris HCl (pH 8.5), 100 mM NaCl. Protein collected from this final step was more than 95% pure.

Protein crystallization

Mutant TRAP proteins were crystallized using the hanging-drop vapor diffusion method using 2.5 mg/mL protein stocks in a total drop volume of 2 μ L. T3A5 was crystallized in buffer containing 0.09 M CAPS (pH 10.5), 30% (w/v) PEG 300, 0.15 M ammonium sulfate, and 10 mM L-tryptophan. T3A7 was crystallized in buffer containing 0.1 M sodium citrate (pH 5.5), 30% (w/v) MPD, 0.2 M ammonium acetate, and 10 mM L-tryptophan. Data for T3A5 and T3A7 were collected at beamlines NW12A and BL17A, respectively, at the Photon Factory, Tsukuba, Japan, and processed with HKL2000

Table 3. Comparison of subunit interface hydrogen bonds in T3A5, T3A7, and wild-type *B. stearothermophilus* TRAP

TRAP3A5/A7 Atoms		TRAP3 a–b		TRAP3 b–c		TRAP3 c–a		Wild-trap TRAP
		Bond distance (Å)						
		A5	A7	A5	A7	A5	A7	
R26NH ₂	Q47Oε1	2.7	2.6	2.9	2.7	2.7	2.8	2.5–3.2
I55N	I45O	2.7	2.8	2.8	2.8	2.7	2.9	2.7–3.0
I55O	I45N	2.6	2.7	2.9	2.8	2.8	2.9	2.7–3.0
H67Nε2	F9O	3.2	2.9	3.2	3.0	2.9	3.1	2.9–nb
K56Nζ	K37O	2.7	3.0	2.7	2.8	2.9	3.0	2.7–nb
R58Nε	E42Oε2	3.0	2.7	3.2	2.9	3.5	2.8	2.8–nb
S53O	Q47N	3.1	3.0	2.9	3.0	3.0	3.1	3.1–nb
K56Nζ	E36Oε2	2.6	2.8	2.8	2.7	2.5	2.6	2.5–nb

A–b, b–c, and c–a are the three TRAP subunit interfaces found in the TRAP 12mer crystal structures. Atoms are identified by single letter amino acid code, residue number, and atom symbol. (A5) TRAP3A5, (A7) TRAP3A7. Figures for the wild-type (11mer) protein are also shown (PDB 1QAW). Since there are multiple subunits in the asymmetric unit of this structure, a range of distances is given. (nb) No bond is formed in some subunits.

(Otwinowski and Minor 1997). T3A5 and T3A7 crystallized in space groups $P4_212$ and $P4$, respectively.

Refinement

General data handling was carried out with the CCP4 package (Collaborative Computational Project, Number 4 1994), and the structures were solved by molecular replacement using Phaser (McCoy et al. 2005). Manual adjustment of the models was carried out with TURBO-FRODO (Roussel and Cambillau 1989), and refinement using CNS (Brunger et al. 1998) and REFMAC5 (Murshudov et al. 1999). Water molecules were added to the models by manual inspection of the $2Fo-Fc$ and $Fo-Fc$ maps. Ramachandran plots for the T3A5 and T3A7 models showed 97.7% and 93.5% of the residues, respectively, to be in the most favored regions, with the remaining 2.3% and 6.5% of the residues in additional allowed regions. Data collection and refinement statistics are shown in Table 2.

Differential scanning calorimetry

Wild-type TRAP, T3A3, T3A5, and T3A7 in 50 mM MOPS (pH 7.8), 250 mM NaCl, 500 μ M L-tryptophan were used at a final concentration of 1.0 mg/mL. A VPDS instrument (Microcal) was used, and the scanning speed was 60°C per hour. Before each sample run at least 10 control runs in the absence of protein were carried out. DSC scans in the presence of protein were performed two or three times for each protein used. Results were analyzed using the manufacturer's software based on the Origin graphing program.

Heat stability tests

The heat stability of wild-type and mutant TRAPs was assessed by heating 1.0 mg/mL protein in 100 μ L of 50 mM Tris HCl (pH 8.5), 100 mM NaCl, in the presence or absence of 0.5 mM L-tryptophan. The samples were placed in a heat block, and the temperature raised from 50°C in steps of 10°C. Incubation was continued for 5 min at each temperature, and the temperature at which visible precipitation occurred was recorded.

RNA-binding assays

RNA binding was assessed by gel-shift assays. To see if the mutant proteins were able to bind RNA, varying amounts of TRAP, in the presence of excess tryptophan, were added to 2.5 μ M of the 55-base single stranded RNA consisting of 11 GAGAA repeats (Dharmacon), known to bind strongly to TRAP (Babitzke et al. 1996). Reaction mixtures were incubated at 37°C for 10 min. The presence or absence of RNA binding was assessed by running 15 μ L of the reaction mixture on a 0.5% Tris-borate agarose gel run at a constant current of 20 mA for 75 min. The position of RNA and protein on the gels were visualized using SYBR-Gold stain (Invitrogen) and CBB stain, respectively, and visualized under UV and visible light. The migration of RNA with TRAP bound through the gel was significantly retarded. A more sensitive binding assay with T3A7 was to the same RNA sequence except that an extra three bases (GCC) were added to the 5' end and the terminal 5' base was labeled with FAM (Hokkaido System Science). TRAP-binding to RNA was measured quantitatively by titrating increas-

ing amounts of TRAP into a solution of 100 pM RNA. Reaction mixtures were incubated at 37°C for 15 min. The presence or absence of RNA binding was assessed by running 20 μ L of the reaction mixture on a 5% native-PAGE_TBE gel in 1 $\times$ TBE buffer. Gels were run at 150 V for 30 min and imaged with a Typhoon 9400 multifunction imager. The approximate K_d was calculated as the amount of protein required to cause 50% of the RNA to be shifted.

Transmission electron microscopy

Transmission electron microscopy was carried out using a 200-keV JEM-2200 (JEOL). TRAP proteins were used at ~0.05 mg/mL in 50 mM Tris-HCl (pH 8.5), 100 mM NaCl buffer, 0.5 mM L-tryptophan. Protein was added to a carbon-coated copper grid and stained with 3% potassium tungsten acetate.

Accession codes

Coordinates for T3A5 and T3A7 have been deposited in the Protein Data Bank with the accession codes 2ZD0 and 2ZCZ, respectively.

Electronic supplemental material

The electronic supplemental material contains one figure and one table. The figure shows thermostability of T3A3 in the absence of tryptophans, measured via DSC. The table shows hydrogen bonds to the residues of the linker visible in TRAP3A7.

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