

# A new twist for an Hsp70 chaperone

Joanna F. Swain and Lila M. Gierasch

**A new study has demonstrated that the *E. coli* Hsp70, DnaK, can catalyze *cis-trans* isomerization of non-prolyl peptide bonds.**

It has long been recognized that isomerization of Xaa-Pro peptide bonds can cause slow phases in protein folding<sup>1,2</sup>, and many helper proteins that catalyze the isomerization, called peptidyl prolyl isomerases, have been characterized<sup>3</sup>. Recent studies have shown that isomerization of non-prolyl (secondary) peptide bonds can also significantly slow the refolding of certain proteins<sup>4,5</sup>, but relevant isomerases have not been identified until now. On page 419 of this issue of *Nature Structural Biology*, Schiene-Fischer *et al.*<sup>6</sup> provide unexpected evidence that the *Escherichia coli* Hsp70 molecular chaperone, DnaK, is capable of catalyzing secondary peptide bond isomerization, albeit modestly. This is indeed an intriguing observation; whether DnaK has evolved to perform this function in the cell remains an open question.

## Secondary amide isomerization during folding

Due to the double bond character of the peptide bond, a significant energy barrier prevents free rotation<sup>7-9</sup> (Fig. 1). Of the two available conformations, the *trans* state is energetically favored by ~15 kJ mol<sup>-1</sup> over the *cis* state for secondary peptide bonds because of the close approach of the two C $\alpha$  atoms in the *cis* form<sup>9</sup>. For Xaa-Pro peptide bonds, however, the two conformers are nearly identical in energy<sup>8</sup>. As a result, in native protein structures, *cis* Xaa-Pro bonds are more commonly found than are *cis* non-prolyl peptide bonds (5.2% versus 0.03%, respectively)<sup>7</sup>.

Recently, the  $\alpha$ -amylase inhibitor tenamistat was observed to display a slow folding phase that was proposed to arise from isomerization of non-prolyl peptide bonds<sup>5</sup>. In this case, the slow phase appears to be contributed by a very small proportion of non-prolyl peptide bonds that equilibrate to the *cis* form in the unfolded state and must isomerize to *trans* in the native state. Since all peptide bonds are synthesized in the *trans* configuration on the ribosome<sup>10</sup>, one might argue whether proteins have sufficient time between synthesis and folding (or

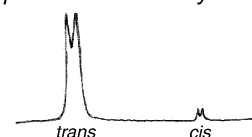
binding to chaperones) for significant equilibration to *cis*. On the other hand, native protein structures containing a *cis* peptide bond must isomerize from the *trans* form, and catalysis of this process would be advantageous.

The study by Schiene-Fischer *et al.*<sup>6</sup> demonstrates that, in addition to its established role in chaperoning nascent protein chains, the *E. coli* Hsp70 DnaK has the potential to accelerate isomerization of non-prolyl peptide bonds. These authors first used an Ala-Ala dipeptide in an absorbance-based isomerization assay<sup>11</sup> to purify isomerase activity from *E. coli* extracts, and DnaK was identified by N-terminal sequencing as the active component. The sequence specificity for the isomerase activity of DnaK, determined using a panel of Ala-Xaa dipeptides, is surprisingly different from its binding preferences for unfolded polypeptides. Whereas DnaK prefers to bind hydrophobic and positively charged residues, such as Leu, Ile, Val, Tyr, Phe, Arg and Lys<sup>12-14</sup>, the isomerase activity was highest for Met, Ala and Ser, and unobservable for many residues, including Val, Tyr, Phe and Arg.

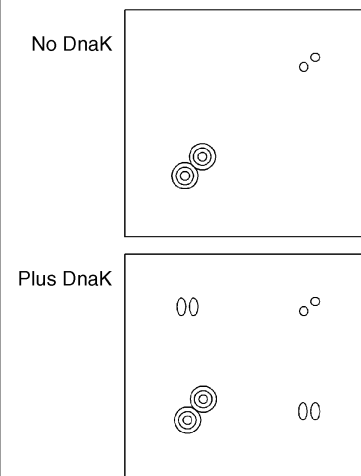
Advantageous signal dispersion for the *cis* isomer allowed the use of NMR to quantify isomerization rates<sup>9</sup> for the Ala-Tyr and Tyr-Ala peptide bonds in an Ala-Ala-Tyr-Ala-Ala peptide (Box 1). Interestingly, DnaK only catalyzes isomerization of the Ala 2-Tyr 3 peptide bond, and the assisted isomerization is blocked by addition of substance P, a neurotransmitter peptide that is known to bind in the peptide-binding pocket of DnaK<sup>14</sup>. This suggests that the catalysis site and the peptide-binding pocket are one and the same. Since ATP binding to DnaK decreases peptide-binding affinity 100-fold, with increases in both on and off rates<sup>15</sup>, it is perplexing that DnaK-catalyzed isomerization is ATP-independent. In order to show that the isomerase activity of DnaK has relevance for protein folding, the authors followed refolding of an RNase T1 mutant that contains a native state *cis* non-prolyl peptide bond<sup>4</sup>.

### Box 1 NMR method to measure amide bond isomerase activity of DnaK.

1D spectrum: Ala methyls

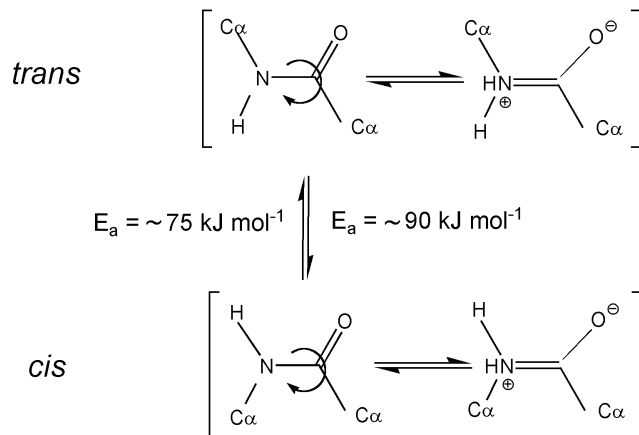


2D proton spectrum



Interconversion between the *cis* and *trans* isomers of the peptide bond is slow on the NMR time scale due to the high barrier to amide bond rotation. As a consequence, separate signals are usually observed for the two isomeric states at ambient temperatures (top). During a 2D NMR experiment with typical mixing times, slow interconversion precludes the transfer of magnetization during the experiment and only very weak to unobservable off-diagonal peaks are present (middle). Schiene-Fischer *et al.*<sup>6</sup> exploit the fact that enhancement of the rate of interconversion between *cis* and *trans* isomers leads to an observable crosspeak due to the now significant magnetization transfer in the course of the experiment (bottom). This provides an assay for the rate enhancement due to addition of a potential *cis-trans* isomerase, and was used to identify and quantify this catalytic activity in DnaK.

DnaK addition caused a dose-dependent increase in the first order rate constant of folding, which could be reversed by addition of a peptide (NR) that binds to the peptide-binding site of DnaK<sup>16</sup>.



**Fig. 1** Resonance forms of *trans* (top) and *cis* (bottom) amide bonds. Considerable double bond character leads to a high barrier to rotation about the amide bond. Reduction of this barrier and consequent enhancement of the rate of interconversion between isomers can be achieved by geometric distortion from planarity or destabilization of the charge-separated resonance form.

### Complementary roles for trigger factor and DnaK?

In many ways, it makes physiological sense for DnaK to be a secondary amide isomerase, based on its functional similarity to trigger factor, a known peptidyl prolyl isomerase<sup>17</sup>. Both are present at the ribosome during protein synthesis and cooperate in the handling of nascent protein chains<sup>18,19</sup>. *E. coli* can survive loss of either the gene encoding DnaK or that encoding trigger factor, but deletion of both at once results in synthetic lethality. Whereas trigger factor tends to bind smaller proteins and can catalyze isomerization at Xaa-Pro bonds, DnaK preferentially binds to proteins >30 kDa in size<sup>18</sup>, and we now learn that it can speed isomerization of the other peptide bonds. Because large proteins have many more peptide bonds, one might imagine that they have even more need for a secondary amide isomerase. However, trigger factor is a much better catalyst. In the native state of RNase T1, the Tyr 38-Pro 39 peptide bond is *cis*; trigger factor (0.5  $\mu\text{M}$ ) enhances the folding rate of RNase T1 45-fold<sup>17</sup>. Mutation of Pro 39 to Ala results in retention of the *cis* peptide bond (now Tyr-Ala) in the native state<sup>4</sup>, but an even higher amount of DnaK

(2  $\mu\text{M}$ ) can only accelerate folding of this mutant by a factor of 2.5.

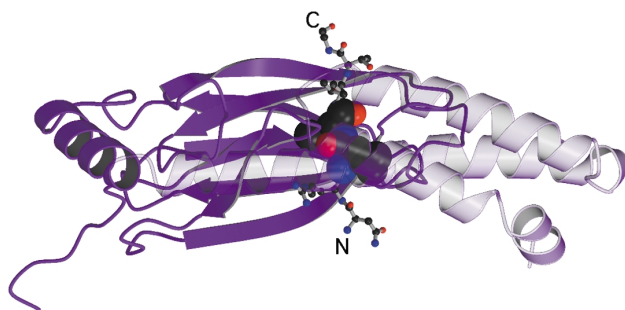
### Possible mechanisms of catalysis

Catalysis of peptide bond rotation could exploit transition state stabilization by physical distortion of the peptide group or by selection for the uncharged resonance form that rotates more freely (Fig. 1). The latter effect could arise either by destabilization of the charge-separated state *via* appropriate placement of charged side chains or by stabilization of the non-charged state with a hydrophobic binding pocket. Inspection of the crystal structure of the peptide-binding domain of DnaK bound to a seven-residue peptide<sup>16</sup> (NR, Asn-Arg-Leu-Leu-Leu-Thr-Gly, Fig. 2) demonstrates that only the middle five residues of the peptide interact with the chaperone, which limits the site of catalysis to one of four possible peptide bonds. All peptide bonds of NR are *trans*, and all are within 2° of 180° except for that between Leu 3 and Leu 4, which is 175°. While this is not a large deviation, refinement protocols used to generate protein structures typically favor a planar peptide bond, so a larger deviation may have been obscured during

refinement. If catalysis occurs by distortion, it would likely be a small effect and confined to the peptide bond between protein sites -1 and 0, which in the case of the NR peptide corresponds to the Leu 3-Leu 4 bond (Fig. 2). While there are no charges close to the bound peptide, the central peptide-binding pocket is highly hydrophobic, especially sites -1 and 0. Thus, by these two criteria, the peptide bond between protein sites -1 and 0 would be the most likely site of catalysis.

### Remaining conundrums

The physiological significance of the reported secondary amide isomerase activity of DnaK remains to be established. The fact that DnaK binds to nascent chains immediately after synthesis, when most peptide bonds have not had a chance to assume the *cis* isomer, as well as the fact that DnaK binds to discrete sites in each protein sequence<sup>13</sup>, suggests that DnaK probably does not facilitate isomerization of the small population of *cis* at many sites. Thus the most likely use of this isomerase activity would be to assist the folding of proteins that have one or more *cis* peptide bonds in the native state. Of the 34 proteins containing non-prolyl *cis* peptide bonds in a non-redundant set of protein structures from the Protein Data Bank, 20 are of bacterial origin<sup>7</sup> and could be substrates for the isomerase activity of DnaK. The high homology of substrate-binding pockets in different Hsp70 molecules<sup>20</sup> begs the question of whether this activity is conserved throughout the Hsp70 family and might play a role in eukaryotic protein folding as well. Nonetheless, the modest degree of catalysis by DnaK leaves open the possibility that its isomerase activity is simply an adventitious byproduct of its principal function: binding and stabilizing unfolded polypeptide chains.



**Fig. 2** Substrate binding to DnaK. The DnaK substrate-binding domain (ribbon) binds the NR peptide in an extended conformation (PDB entry 1DKZ). Here the peptide is represented by ball-and-stick, except for Leu 3 and Leu 4, which are in CPK representation. The  $\alpha$ -helical lid domain of the protein has been made transparent in order to see the peptide. Generated using MolScript<sup>21</sup> and rendered using Raster3D<sup>22</sup>.

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1. Brandts, J.F., Halvorson, H.R. & Brennan, M. *Biochemistry* **14**, 4953–4963 (1975).
2. Schmid, F.X. & Baldwin, R.L. *Proc. Natl. Acad. Sci. USA* **75**, 4764–4768 (1978).

3. Schiene, C. & Fischer, G. *Curr. Opin. Struct. Biol.* **10**, 40–45 (2000).
4. Odefey, C., Mayr, L.M. & Schmid, F.X. *J. Mol. Biol.* **245**, 69–78 (1995).
5. Pappenberger, G. *et al. Nature Struct. Biol.* **8**, 452–458 (2001).
6. Schiene-Fischer, C., Habazettl, J., Schmid, F.X. & Fischer, G. *Nature Struct. Biol.* **9**, 419–424 (2002).
7. Jabs, A., Weiss, M.S. & Hilgenfeld, R. *J. Mol. Biol.* **286**, 291–304 (1999).
8. Stein, R.L. *Adv. Protein Chem.* **44**, 1–24 (1993).
9. Scherer, G., Kramer, M.L., Schutkowski, M., Reimer, U. & Fischer, G. *J. Am. Chem. Soc.* **120**, 5568–5574 (1998).
10. Lim, V.I. & Spirin, A.S. *J. Mol. Biol.* **188**, 565–574 (1986).
11. Schiene-Fischer, C. & Fischer, G. *J. Am. Chem. Soc.* **123**, 6227–6231 (2001).

12. Gragerov, A. & Gottesman, M.E. *J. Mol. Biol.* **241**, 133–135 (1994).
13. Rüdiger, S., Germeroth, L., Schneider-Mergener, J. & Bukau, B. *EMBO J.* **16**, 1501–1507 (1997).
14. de Crouy-Chanel, A., Kohiyama, M. & Richarme, G. *J. Biol. Chem.* **271**, 15486–15490 (1996).
15. Pierpaoli, E.V., Gisler, S.M. & Christen, P. *Biochemistry* **37**, 16741–16748 (1998).
16. Zhu, X. *et al. Science* **272**, 1606–1614 (1996).
17. Stoller, G. *et al. EMBO J.* **14**, 4939–4948 (1995).
18. Teter, S.A. *et al. Cell* **97**, 755–765 (1999).
19. Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A. & Bukau, B. *Nature* **400**, 693–696 (1999).
20. Rüdiger, S., Buchberger, A. & Bukau, B. *Nature Struct. Biol.* **4**, 342–349 (1997).
21. Kraulis, P. *J. Appl. Crystallogr.* **24**, 946–950 (1991).
22. Merritt, E.A. & Bacon, D.J. *Methods Enzymol.* **277**, 505–524 (1997).

## Mini-proteins Trp the light fantastic

Samuel H. Gellman and Derek N. Woolfson

**A new 20-residue peptide represents the smallest example to date of cooperatively folded tertiary structure. This achievement provides a new tool for elucidating protein conformational preferences. The mini-protein should serve as a fruitful platform for protein design.**

On page 425 of this issue, Neidigh *et al.*<sup>1</sup> describe a remarkable peptide that adopts a small but well-defined globular shape. Formation of discrete tertiary structure is generally thought to be the exclusive province of much longer polypeptides. Although other short peptides with stable tertiary structure have been reported, the present case stands out in that only natural amino acid residues are employed and there is no crosslinking *via* disulfide formation, metal ion chelation or stabilization through oligomerization. The success of Neidigh *et al.*<sup>1</sup> appears to be primarily due to a structural motif the authors dub the ‘Trp cage’, in which the side chain of a Trp residue is penned in by several other residues, notably the side chains of prolines.

Development of the Trp cage began during examination of a natural 39-residue peptide from Gila monster saliva. The C-terminal portion of this peptide showed promise for folding, but structure was observed only in the presence of the fold-promoting cosolvent 2,2,2-trifluoroethanol (TFE). Recognizing the prospect for structural optimization, the authors pursued a series of incremental sequence modifications to enhance the stability of the folded conformation. These shrewd efforts culminated in a 20-residue Trp cage that appears fully structured at low temperature in water and displays several hallmarks of cooperatively folded proteins.

### The Trp cage is cooperatively folded

The studies of protein structure and folding are mature fields of endeavor<sup>2</sup>, and one is led to ask two fundamental questions regarding the system reported by Neidigh *et al.*<sup>1</sup>: (i) Is the structure completely folded under accessible conditions? (ii) Is folding cooperative? This latter question is important because for most natural protein domains, all parts of the molecule undergo the folding transition in concert as conditions, such as temperature or denaturant concentration, are varied. This type of cooperativity is regarded as a hallmark of native protein structures. In such cases, only two ‘states’ of the protein — the folded and unfolded states — are present throughout the process. Both states are collections of conformations, also referred to as ensembles. The unfolded conformations are flexible and largely unrelated to one another, whereas the folded-state conformations are closely related. The work of Neidigh *et al.*<sup>1</sup> provides clear answers to these two questions.

Evidence for essentially complete folding into a specific tertiary structure near 0 °C comes from following independent global measures of folding — that is, characteristic CD signals and <sup>1</sup>H NMR chemical shifts — as a function of temperature. In both cases the measured parameters plateau at low temperature to values consistent with a highly folded state. In addition, amide protons of some buried residues are protected

from H/D exchange in the folded state, another classic sign of native-like folding. Further support for complete folding in water comes from the observation that there is very little change in the CD spectrum of the final Trp cage design when the sample contained 30% TFE. In contrast, intermediate designs showed substantial enhancements in folding upon addition of TFE. Although addition of TFE does not necessarily induce complete peptide folding<sup>3</sup>, it seems plausible that failure to see a TFE effect indicates that the extent of folding is already quite high.

What is the evidence for cooperative folding? Sigmoidal dependence of spectroscopic measurements with changes in temperature or denaturant concentration is a classic signature of cooperativity. The authors show such temperature dependencies for both CD and NMR signals. They also use a novel method<sup>4</sup> to test for cooperativity: they compare the rates of change of characteristic <sup>1</sup>H NMR chemical shifts through the thermal unfolding transition ( $\Delta\delta/\Delta T$ ) with the differences measured for these chemical shifts between the folded state and reference values for the unfolded state ( $\delta_{\text{folded}} - \delta_{\text{unfolded}}$ ). A linear correlation between these two parameters, which is observed for the Trp cage, fits a cooperative unfolding transition between a single folded state and an unfolded ensemble. These comparisons support the hypothesis that the final Trp cage peptide undergoes a concerted unfolding transition.