Sequence conservation provides the best prediction of the role of proline residues in p13suc1

ARTICLE *in* JOURNAL OF MOLECULAR BIOLOGY · SEPTEMBER 2000

Impact Factor: 4.33 · DOI: 10.1006/jmbi.2000.3958 · Source: PubMed

3 AUTHORS:

Joost Schymkowitz
University of Leuven
107 PUBLICATIONS **5,412** CITATIONS
SEE PROFILE

Frederic Rousseau
University of Leuven
105 PUBLICATIONS **5,218** CITATIONS
SEE PROFILE

Laura S Itzhaki
University of Cambridge
77 PUBLICATIONS **3,779** CITATIONS
SEE PROFILE

doi:10.1006/jmbi.2000.3958 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2000) 301, 199–204

JMB

Sequence Conservation Provides the Best Prediction of the Role of Proline Residues in p13suc1

J. W. H. Schymkowitz[1,2], F. Rousseau[1,2] and L. S. itzhaki[1]*

[1]*MRC Centre for Protein Engineering, University Chemical Laboratory, Lensfield Road, Cambridge, CB2 1EW UK*

[2]*Departement of Ultrastructure Vlaams interuniversitair Instituut voor Biotechnologie Vrije Universiteit Brussel Paardenstraat 65, B-1640, Sint-Genesius-Rode, Belgium*

**Corresponding author*

The unique nature of the proline side-chain imposes severe constraints on the polypeptide backbone, and thus it seems likely that it plays a special structural or functional role in the architecture of proteins. We have investigated the role of proline residues in suc1, a member of the cyclin-dependent kinase (cks) family of proteins, whose known function is to bind to and regulate the activity of the major mitotic cdk. The effect on stability of mutation to alanine of all but two of the eight proline residues is correlated with their conservation within the family. The remaining two proline residues are located in the hinge loop between two β-strands that mediates a domain-swapping process involving exchange of a β-strand between two monomers to form a dimer pair. Mutation of these proline residues to alanine stabilises the protein. cdk binding is unaffected by these mutations, but dimerisation is altered. We propose, therefore, that the double-proline motif is conserved for the purpose of domain swapping, which suggests that this phenomenon plays a role in the function of cks proteins. Thus, the conservation of the proline residues is a good indicator of their roles in suc1, either in the stabilisation of the native state or in performing functions that are as yet unknown. In addition, the strain resulting from two of the proline residues was relieved successfully by mutation of the preceeding residue to glycine, suggesting a general method for designing more stable proteins.

Keywords: proline; protein engineering; protein stability; domain swapping

Introduction

Proline is a unique amino acid, because its side-chain is covalently linked to the backbone, thereby imposing rigidity on the backbone and removing the hydrogen bonding potential of the amide group (MacArthur & Thornton, 1991). It was proposed that mutation to proline could increase the level of protein stability by this reduction in the configurational entropy of unfolding (Matthews *et al*., 1987). Several experimental studies have since complemented the original work on bacteriophage T4 lysozyme and have shown that this idea can sometimes be applied to design more stable proteins (Watanabe & Suzuki, 1998; Suzuki, 1999, Zhu *et al*., 1999). However, the effect of such mutations is highly context dependent and the opposite effect on stability is sometimes observed. A mutational investigation of nine conserved proline residues in the tryptophan synthase alpha subunit showed that three of the residues do not contribute significantly to the stability of the protein, whereas the others do (Yutani *et al*., 1991). In human lysozyme, Pro71 is conserved for stability whilst Pro103 is not (Herning *et al*., 1992). The conserved Pro40 in *Escherichia coli* thioredoxin is required for the stability of the protein and mutation to Ala greatly destabilises the protein by 2.9 kcal mol^{-1} (de Lamotte *et al*., 1997). The higher level of thermostability of alcohol dehydrogenase from a thermophilic strain of *Bacillus stearothermophilus*, compared with a less thermostable homologue, can be partly explained by the introduction of Pro242 in the former protein (Fiorentino *et al*., 1998). Residues in the solvent-exposed 63-69 loop of thioredoxin from *Bacillus stearothermophilus* were replaced by proline. The mutations caused large positive (Ser65, Ala69) or negative (Thr63, Tyr66) changes in thermostability (Hardy *et al*., 1993). In the *Aspergillus awamori* protein, five residues were replaced individually by proline, and

Abbreviations used: cdk, cyclin dependent kinase.

E-mail address of the corresponding author: lsi@mrc-lmb.cam.ac.uk

0022-2836/00/010199–6 $35.00/0

only one resulted in an increase in the degree of thermostability (Li *et al.*, 1997). Human triosephosphate isomerase was stabilised by introducing a proline residue at the N-cap of an alpha helix (Mainfroid *et al.*, 1996).

An examination of more than 1600 protein-protein interaction sites found that proline is the residue most commonly found near interaction sites. The unique nature of proline helps to protect the integrity, and present the sites, thus promoting protein-protein interactions by lowering the entropic cost of association (Kini *et al.*, 1995). Significantly, proline residues are frequently observed in domain-swapping proteins in the hinge loop region that links the swapped domain with the rest of the protein. It was proposed that by conferring rigidity on the backbone, proline residues favour the extended conformation of the hinge loop and thereby stimulate oligomerisation (Bergdoll *et al.*, 1997).

$p13^{suc1}$ (referred to subsequently as suc1) is a member of the cyclin-dependent kinase subunit (cks) family of essential eukaryotic cell cycle regulatory protein that function by binding to and modifying the activity of the major mitotic cyclin dependent kinase (cdk) (Hayles *et al.*, 1986; Hindley *et al.*, 1987). It has been observed in two forms from crystallographic studies, a globular monomer (Endicott *et al.*, 1995) and a domain-swapped dimer (Bourne *et al.*, 1995). The dimer is formed by exchange of a central β-strand, β4, between two monomers, and this process is mediated by the loop between β3 and β4 that acts as a molecular hinge. The hinge loop sequence, HVPEPH, adopts a β-turn conformation in the monomer and is in an extended conformation in the dimer. The human cks was shown to bind to cdk in the monomeric form, and residues involved in cdk binding are located in the β-hinge and in β2 and β4 (Bourne *et al.*, 1996). This surface is buried in the domain-swapped dimer and therefore it is unlikely that the cks proteins can bind to cdk in the dimeric form. The members of the cks family vary in length from 79 to 150 residues with insertions occurring between secondary structure elements, and there is very high level of sequence conservation among the family members.

An extensive set of point mutations of the cks protein from *Schizosaccharomyces pombe*, suc1, has been used to dissect the folding pathway of the protein (Schymkowitz *et al.*, 2000). Here, we investigate the role of proline residues in protein structure using suc1 as a model system. There are eight proline residues, all of which adopt the *trans* conformation in the native state. We have mutated them individually to Ala. Pro90 and Pro92 (located in the β-hinge) have been mutated to Ala and to Gly and a double mutant cycle analysis performed also. The effects of mutation on the free energy of unfolding of the protein were determined and interpreted in terms of the residue conservation across the cks family.

Results

Conservation of the proline residues in the cks family

A sequence alignment of the cks family is shown in Figure 1. The proline residues in suc1 can be divided into two categories. Those in the first group are either completely conserved or very highly conserved across the family, whilst those in the second group are not.

Location of the proline residues in suc1

The structure of suc1 is shown in Figure 2 with the proline side-chains highlighted. Pro7 is located in the N-terminal region of the protein, which is absent in most of the other homologues. Pro20 is located towards the C-terminal of α1 that is absent in most of the other homologues, and kinks the helix. Pro29 is located in the middle of β1 and kinks this β-strand. As is commonly observed (Barlow *et al.*, 1988), neither Pro20 nor Pro29 cause significant disruption of the hydrogen bonding pattern in the respective secondary structure elements. Pro44 is located between β2 and α2. Pro52 and Pro58 are located in the long loop between α2 and α3. Pro90 and Pro92 are located in the hinge loop between β3 and β4 that mediates exchange of β4 in the domain-swapped dimer. The second proline in a Pro-X-Pro motif often lies at position $i + 1$ of a β-turn, slightly favouring type I turns. This is the case with Pro92 in suc1. Although the hinge loop is involved in cdk binding, the two proline residues do not directly contact the cdk, and their mutation to Ala does not affect the binding constant (G. Divita, CNRS, Montpellier, France, personal communication). The solvent accessibilities and *B*-factors for the proline side-chains are listed in Table 1.

From a study of 963 *trans* proline residues, it was shown that the backbone adopts two distinct conformations that are almost evenly divided, and that the two groups are tightly clustered around their mean values of $(\Phi,\psi) = -61^\circ,-35^\circ$ for the region and $(\Phi,\psi) = -63^\circ,150^\circ$ for the region (MacArthur & Thornton, 1991). The proline residues in suc1 do not deviate from this behaviour, as shown in the Ramachandran plot, and there are four residues in each region (Ramachandran & Sasiekharan, 1968). Proline side-chains also influence the conformation of the preceding residue, through steric clashes between the CH2 attached to the imide nitrogen and NH and CH2 atoms of the other amino acid residue. The residues preceeding the proline in suc1 are also within the expected regions of the Ramachandran plot.

Mutation of the proline residues does not cause any gross structural changes

The absorbance, fluoresence and CD spectra of the mutants do not deviate significantly from those

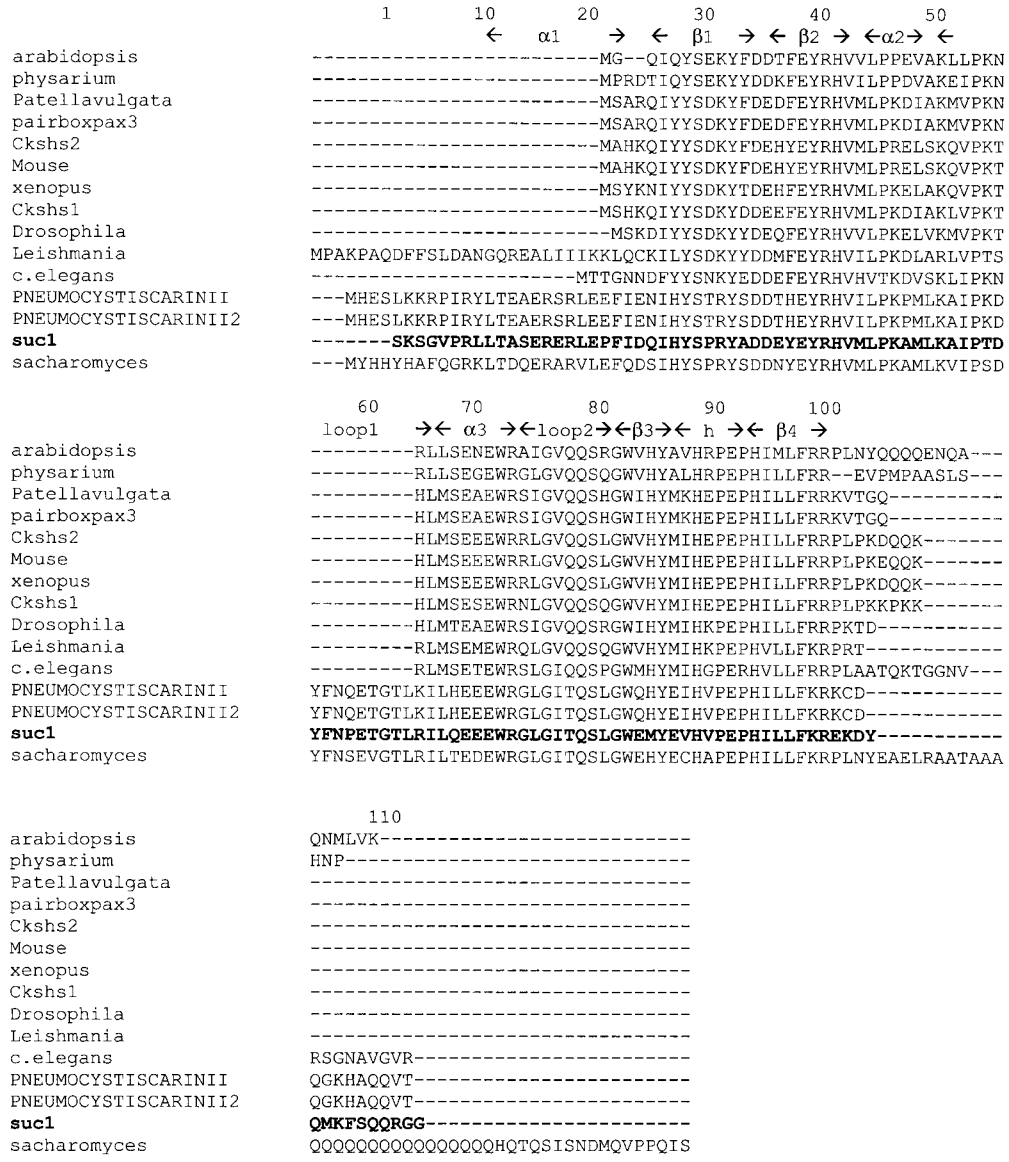

Figure 1. Sequence alignment of suc1 from *S. pombe* and its homologues. suc1 numbering and secondary structure are indicated above the sequences. The hinge loop (h) mediates strand exchange.

of the wild-type, and the elution patterns from gel filtration and ion-exchange columns used in the purification protocol are identical. Typical urea-induced equilibrium denaturation profiles are shown in Figure 3. The *m* values, midpoints of denaturation and the changes in the free energy of unfolding upon mutation, are listed in Table 1. The *m* values, which reflect the change in solvent accessibility of hydrophobic residues upon unfolding, are the same as that of the wild-type (within error). This indicates that no gross structural changes occur upon mutation.

Conservation of proline residues is dictated by stability outside of the functional motif

There does not appear to be any correlation between the changes in stability of suc1 upon mutation of the prolines to alanine and structural factors such as solvent accessibility or *B*-factors (Table 1). Mutation to alanine of the non-conserved proline residues, located at positions 7, 20, 29 and 58, has only a small effect on the stability (<0.4 kcal mol^{-1}). The mutations PA7, PA20 and PA58 destabilise the protein, whilst the mutant PA29 stabilises the protein slightly.

In contrast, mutation of the conserved proline residues at positions 44 and 52 has a large destabilising effect (2.5-3.0 kcal mol^{-1}), and therefore these residues are clearly selected for their role in stabilising the native state.

The conserved residues Pro90 and Pro92 are located in the hinge loop that mediates domain swapping which may be important for the function of suc1. The mutation PA92 has no effect on the stability of the protein. Mutation of Pro90 to Ala

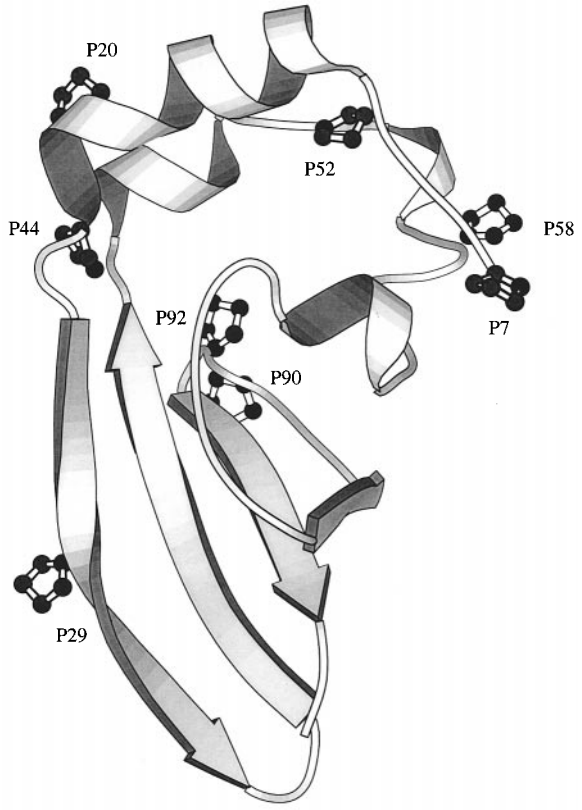

Figure 2. Schematic of the structure of suc1 showing the location of the proline residues.

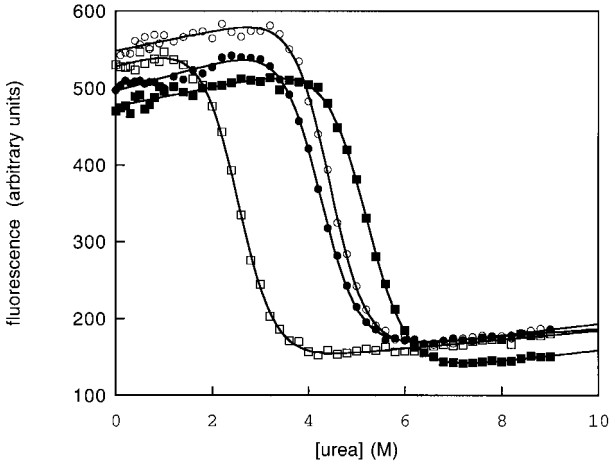

Figure 3. Plot of representative urea denaturation profiles of suc1 wild-type and mutants. Wild-type suc1 is shown in open circles, PA20 is shown in filled circles, PA44 is shown in open squares and PA90 is shown in closed squares.

stabilises the protein by 0.5 kcal mol^{-1}. Thus, although it is energetically costly to have a proline at position 90, it is completely conserved.

Double mutant cycles show that there is strain in the hinge

Double-mutant cycle analysis allows the magnitude of a specific, pairwise interaction between two side-chains to be measured, by mutating singly and then pairwise, each residue in a pair that interact, and thereby determining whether the effects of mutation are additive (Carter *et al.*, 1984; Horovitz, 1987). The two proline residues in the hinge loop, Pro90 and Pro92, were mutated to Ala and to Gly. The coupling energy, $\Delta\Delta G^{int}_{U\text{-}F}$, between the side-chains is calculated from:

$$\Delta\Delta^{int}_{U\text{-}F} = \Delta\Delta G^{E\text{-}XY\rightarrow E\text{-}Y}_{U\text{-}F} + \Delta\Delta G^{XY\rightarrow E\text{-}Y} - \Delta\Delta G^{E\text{-}XY\rightarrow E}_{U\text{-}F} \quad (1)$$

where the superscript E-XY → E-Y indicates the mutant P90 → A, E-XY → E-X indicates the mutant P92 → A and E-XY → E indicates the mutant P90 → A/P92 → A, etc. If the effect of the double mutant on the stability of the protein is equal to the sum of the effects of the two single mutants, then the effects of the two mutations are independent, and the side-chains do not interact. If these two are not equal, then the side-chains do interact and the discrepancy is the coupling energy between them. The coupling energy may be the result of direct or indirect interactions. The side-chains of Pro90 and Pro92 point away from each other in the crystal structure and do not appear to contact each other directly. In spite of this, the Ala and the Gly double-mutant cycles reveal that the mutations are not additive (Table 1). Furthermore, the calculated coupling energies between Pro90 and Pro92 are negative, indicating that the side-chains interact unfavourably. Thus, the hinge loop is strained in the native state of the wild-type protein. The results of the double-mutant cycle analysis are consistent with the stabilising effect that is observed for mutation singly of Pro90 and Pro92.

Stabilising suc1 by X→Gly mutations of X-Pro

There are two positions in suc1 where a proline residue is present at an energetic cost, Pro29 and Pro90. This strain must also be felt by the residue preceeding the proline, Gly being the only residue that does not have its Ramachandran plot much restricted by preceeding a proline. Mutation of Ser28 and Val89 to Ala destabilises the protein slightly (0.34 and 0.24, respectively) through removal of some weak interactions, but mutation to Gly results in a significant increase in stability of 0.8-0.9 kcal mol^{-1}.

Discussion

The eight proline residues in suc1 were mutated individually to alanine and the affect on protein stability determined. Mutation of the four, non-conserved proline residues, had very little effect on the stability of suc1. In contrast, mutation of two of the four proline residues that are completely con-

Table 1. Equilibrium unfolding of wild-type suc1 and mutant proteins and description of location of the prolines residues

Mutant	Sequence identity[a]	Secondary structure element	Side-chain % solvent accessibility[b]	*B*-factor[c]	$[Urea]_{50\%}$ (M)[d]	$m_{U\text{-}F}$ (kcal mol M^{-1})[e]	$\Delta\Delta G_{U\text{-}F\cdot\langle m\rangle}$ (kcal mol^{-1})[f]	$\Delta\Delta G^{int}_{U\text{-}F}$ (kcal mol^{-1})[g]
Wild-type					4.42 ± 0.01	1.63 ± 0.01	-	-
PA7	1	N-terminal	39	74	4.29 ± 0.01	1.57 ± 0.05	0.20 ± 0.03	-
PA20	1	α-helix 1	64	33	4.23 ± 0.01	1.50 ± 0.04	0.30 ± 0.03	-
PA29	1	β-strand 1	96	13	4.56 ± 0.03	1.39 ± 0.07	-0.23 ± 0.05	-
PA44	14	Turn	11	34	2.52 ± 0.02	1.56 ± 0.05	3.04 ± 0.04	-
PA52	15	Loop 1	17	47	2.81 ± 0.02	1.75 ± 0.08	2.57 ± 0.04	-
PA58	1	Loop 1	77	63	4.20 ± 0.03	1.57 ± 0.10	0.35 ± 0.04	-
PA90	15	β-hinge	78	28	5.16 ± 0.02	1.52 ± 0.05	-1.19 ± 0.03	-
PA92	14	β-hinge	30	33	4.46 ± 0.03	1.78 ± 0.09	-0.07 ± 0.04	-
PG90					5.25 ± 0.02	1.53 ± 0.05	-1.33 ± 0.04	-
PG92					4.46 ± 0.02	1.55 ± 0.07	-0.07 ± 0.04	-
SA28					4.21 ± 0.02	1.61 ± 0.07	0.34 ± 0.04	-
SG28					4.98 ± 0.02	1.62 ± 0.06	-0.89 ± 0.03	-
VA89					4.27 ± 0.01	1.60 ± 0.05	0.24 ± 0.03	-
VG89					4.93 ± 0.05	1.63 ± 0.14	-0.81 ± 0.07	-
PA90PA92					5.00 ± 0.01	1.40 ± 0.02	-0.93 ± 0.02	-0.33 ± 0.06
PG90PG92					4.97 ± 0.01	1.46 ± 0.03	-0.88 ± 0.02	-0.52 ± 0.06

[a] Number of homologues including suc1 with a proline residue at that position in the alignment (total number of homologues is 15).
[b] Calculated using the programme naccess (S. Hubbard, UMIST, UK).
[c] The *B*-factor listed is the average of all the atoms of the side-chain.
[d] $[Urea]_{50\%}$ is the concentration of urea at which the protein is 50% unfolded.
[e] $m_{U\text{-}F}$ is the variation in the free energy of unfolding with urea concentration.
[f] $\Delta\Delta G_{U\text{-}F\cdot\langle m\rangle}$ is the difference in the free energy of unfolding between wild-type and mutant suc1 at a urea concentration mid-way between the $[Urea]_{50\%}$ of the two proteins, calculated using an average *m* value of 1.60(±0.01) kcal mol M^{-1}. The *m* value is the average of all the mutant proteins and multiple runs of wild-type.
[g] $\Delta\Delta G^{int}_{U\text{-}F}$ is the coupling energy between the two side-chains mutated in the double-mutant cycle, calculated using equation (1).

served across the cks family destabilised the protein significantly, showing that they are important for the stability of the native structure. Mutation of the other two conserved proline residues, located in the hinge loop that mediates domain swapping, resulted in an increase in protein stability. The two side-chains are not necessary for binding to the cdk, but they are important for maintaining the ability to domain swap (Rousseau *et al*., 1998; J.W.H.S., F.R., L.S.I., unpublished results). Thus, the changes in stability of the proline residues on mutation to alanine appear to be correlated to the conservation of the proline residues, with the exception of the double-proline motif in the hinge. This suggests that the motif has a functional role in the cks proteins that may be related to domain swapping.

The strain that results from the proline side-chains at positions 29 and 90 can be relieved by mutation of the preceeding residue to glycine. This could be used as a strategy for designing more stable proteins.

Materials and Methods

Wild type and mutant proteins were prepared as described (Rousseau *et al*., 1998; Schymkowitz *et al*., 2000). Urea-induced denaturation experiments were performed at 25 °C in 50 mM Tris buffer (pH 7.5) and 1 mM EDTA (to prevent formation of zinc-mediated dimers) using urea as denaturant. The fluorescence of the protein at each urea concentration was measured on a Perkin Elmer LS5B luminescence spectrometer with excitation and emission wavelengths of 280 nm and 335 nm, respectively, and bandwidths of 5 nm. The data were fitted to a two-state transition as described (Rousseau *et al*., 1998; Schymkowitz *et al*., 2000).

Acknowledgements

This work was supported by the Medical Research Council of the UK (MRC). F.R. and J.W.H.S. were supported by awards from the Vrije Universiteit Brussel, and by Marie Curie Research Fellowships from the European Commission (Grant Numbers FMIBICT983049 and FMIBICT983050). L.S.I. was supported by a Beit Memorial Fellowship in Medical Research (UK) and a Career Development Award from the MRC of the UK.

References

Barlow, D. J. & Thornton, J. M. (1988). Helix geometry in proteins. *J. Mol. Biol.* **201**, 601-619.

Bergdoll, M., Remy, M. H., Cagnon, C., Masson, J. M. & Dumas, P. (1997). Proline-dependent oligomerization with arm exchange. *Structure*, **5**, 391-401.

Bourne, Y., Arvai, A. S., Bernstein, S. L., Watson, M. H., Reed, S. I., Endicott, J. A., Noble, M. E., Johnson, L. N. & Tainer, J. A. (1995). Crystal structure of the cell cycle-regulatory protein suc1 reveals a β-hinge conformational switch. *Proc. Natl Acad. Sci. USA*, **92**, 10232-10236.

Bourne, Y., Watson, M. H., Hickey, M. J., Holmes, W., Rocque, W., Reed, S. I. & Tainer, J. A. (1996). Crystal structure and mutational analysis of the human cdk2 kinase complex with cell cycle-regulatory protein CksHs1. *Cell,* **84**, 863-874.

Carter, P. J., Winter, G., Wilkinson, A. J. & Fersht, A. R. (1984). The use of double mutants to detect structural changes in the active site of the tyrosyl-tRNA synthetase (*Bacillus stearothermophilus*). *Cell,* **38**, 835-849.

de Lamotte-Guery, F., Pruvost, C., Minard, P., Delsuc, M. A., Miginiac-Maslow, M., Schmitter, J. M., Stein, M. & Decottignies, P. (1997). Structural and functional roles of a conserved proline residue in the alpha2 helix of *Escherichia coli* thioredoxin. *Protein Eng.* **10**, 1425-1432.

Endicott, J. A., Noble, M. E., Garman, E. F., Brown, N., Ramussen, R., Nurse, P. & Johnson, L. N. (1995). The crystal structure of p13suc1, a p34cdc2-interacting cell cycle control protein. *EMBO J.* **14**, 1004-1014.

Fiorentino, G., Cannio, R., Rossi, M. & Bartolucci, S. (1998). Decreasing the stability and changing the substrate specificity of the *Bacillus stearothermophilus* alcohol dehydrogenase by single amino acid replacements. *Protein Eng.* **11**, 925-930.

Hardy, F., Vriend, G., Veltman, O. R., van der Vinne, B., Venema, G. & Eijsink, V. G. (1993). Stabilization of *Bacillus stearothermophilus* neutral protease by introduction of prolines. *FEBS Letters,* **317**, 89-92.

Hayles, J., Aves, S. & Nurse, P. (1986). suc1 is an essential gene involved in both the cell cycle and growth in fission yeast. *EMBO J.* **5**, 3373-3379.

Herning, T., Yutani, K., Inaka, K., Kuroki, R., Matsushima, M. & Kikuchi, M. (1992). Role of proline residues in human lysozyme stability: a scanning calorimetric study combined with X-ray structure analysis of proline mutants. *Biochemistry,* **31**, 7077-7085.

Hindley, J., Phear, G., Stein, M. & Beach, D. (1987). suc1 encodes a predicted 13-kilodalton protein that is essential for cell viability and is directly involved in the division cycle of *Schizosaccharomyces pombe*. *Mol. Cell Biol.* **7**, 504-511.

Horovitz, A. (1987). Non-additivity in protein-protein interactions. *J. Mol. Biol.* **196**, 733-735.

Kini, R. M. & Evans, H. J. (1995). A hypothetical structural role for proline residues in the flanking segments of protein-protein interaction sites. *Biochem. Biophys. Res. Commun.* **212**, 1115-1124.

Li, Y., Reilly, P. J. & Ford, C. (1997). Effect of introducing proline residues on the stability of Aspergillus awamori. *Protein Eng.* **10**, 1199-1204.

MacArthur, M. W. & Thornton, J. M. (1991). Influence of proline residues on protein conformation. *J. Mol. Biol.* **218**, 397-412.

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.

Matthews, B. W., Nicholson, H. & Becktel, W. J. (1987). Enhanced protein thermostability from site-directed mutations that decrease the entropy of unfolding. *Proc. Natl Acad. Sci. USA,* **84**, 6663-6667.

Ramachandran, G. N. & Sasiekharan, V. (1968). Title. *Advan. Protein Chem.* **23**, 283-437.

Rousseau, F., Schymkowitz, J. W. H. & Itzhaki, L. S. (1998). Stability and folding of the cell cycle regulatory protein, $p13^{suc1}$. *J. Mol. Biol.* **284**, 503-519.

Schymkowitz, J. W. H., Rousseau, F., Irvine, L. R. & Itzhaki, L. S. (1999). The folding pathway of the cell cycle regulatory protein p13suc1: clues for the mechanism of domain-swapping. *Structure Fold. Des.* **8**, 89-100.

Suzuki, Y. (1999). The proline rule. A strategy for protein thermal stabilization. *Proc. Japan Acad. ser. B - Phys. Biol. Sci.* **75**, 133-137.

Watanabe, K. & Suzuki, Y. (1998). Protein thermostabilization by proline substitutions. *J. Mol. Cat. B - Enzymatic,* **4**, 167-180.

Yutani, K., Hayashi, S., Sugisaki, Y. & Ogasahara, K. (1991). Role of conserved proline residues in stabilizing tryptophan synthase alpha subunit: analysis by mutants with alanine or glycine. *Proteins: Struct. Funct. Genet.* **9**, 90-98.

Zhu, G. P., Xu, C., Teng, M. K., Tao, L. M., Zhu, X. Y., Wu, C. J., Hang, J., Niu, L. W. & Wang, Y. Z. (1999). Increasing the thermostability of D-xylose isomerase by introduction of a proline into the turn of a random coil. *Protein Eng.* **12**, 635-638.

Edited by J. Thornton

(Received 7 April 2000; received in revised form 18 May 2000; accepted 13 June 2000)