

Cyclophilin active site mutants have native prolyl isomerase activity with a protein substrate

Christian Scholz^a, Thomas Schindler^a, Kara Dolinski^b, Joseph Heitman^b, Franz X. Schmid^{a,*}

^aBiochemisches Laboratorium, Universität Bayreuth, D-95440 Bayreuth, Germany

^bDepartments of Genetics and Pharmacology, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710, USA

Received 14 July 1997

Abstract The prolyl isomerase activity of cyclophilins is traditionally measured by an assay in which prolyl *cis/trans* isomerization in a chromogenic tetrapeptide is coupled with its isomer-specific cleavage by chymotrypsin. Two variants of mitochondrial cyclophilin with substitutions in the presumed active site (R73A and H144Q) are inactive in the protease-coupled assay, but show almost wild-type activity in an assay that is based on the catalysis of a proline-limited protein folding reaction. This prolyl isomerase assay is preferable, both because coupling with proteolysis is avoided and because an intact protein instead of a short peptide is used as a substrate. Possibly, some earlier conclusions about the catalytic mechanism and the involvement of the prolyl isomerase activity in the cellular function of immunophilins may need reevaluation.

© 1997 Federation of European Biochemical Societies.

Key words: Cyclophilin; Immunophilin; Prolyl isomerization; Protein folding; Mitochondria

1. Introduction

Cyclophilins are ubiquitous proteins that are enzymatically active as prolyl isomerases and bind with high affinity to the immunosuppressive drug cyclosporin A (CsA). The first cyclophilin (cyclophilin A) was co-discovered in 1984 by Fischer and coworkers [1], who developed a protease-coupled assay to identify enzymes that might catalyze proline-limited protein folding reactions¹, and by Handschumacher and coworkers [2], who employed affinity chromatography to identify a receptor for CsA. In 1989 it became clear that the proteins identified by the two groups were identical [3,4], and it was immediately speculated that the function of cyclophilins in immunosuppression might be linked to prolyl isomerase activity. Subsequent work, however, indicated that this is not the case. First, yeast mutants lacking cyclophilin A are viable and cyclosporin resistant, indicating that cyclophilin is not essential, and both the protein and the drug are required for drug action [5,6]. Second, the binary complex between CsA and cyclophilin was found to bind to and inhibit the phosphatase calcineurin [7,8]. A similar mechanism is used by FK

506, another immunosuppressive drug that also inhibits calcineurin when bound to its receptor FKBP, which, surprisingly, is also a prolyl isomerase [9–11].

Cyclophilins are probably involved in many processes in the cell [12–20]. Whether the prolyl isomerase activity of a particular cyclophilin is involved in these functions is usually examined by comparing the effects of site-directed mutations on the *in vivo* function and on the *in vitro* prolyl isomerase activity towards a tetrapeptide in the protease-coupled assay. Several mutations that apparently impaired the prolyl isomerase activity *in vitro* seemed to affect the cellular functions of cyclophilins in some processes but not in others. This led to conflicting views about the role of this enzymatic activity for the function of cyclophilins *in vivo* [21–24].

The conformational specificity of a protease (usually chymotrypsin) for all-*trans* peptides is used to follow prolyl isomerization in the standard prolyl isomerase assay. The protease must be present at high concentration in these assays, and therefore a mutation that increases the protease susceptibility of a cyclophilin would erroneously be classified as inactivating. We therefore employed an alternative assay for PPI activity, which avoids coupling with proteolysis and uses a protein instead of a short peptide as the substrate. In this assay the catalysis of a folding reaction is measured, which is limited in rate by prolyl *cis/trans* isomerization.

We show here that based on the protease-coupled assay two mutations at the presumed active site of cyclophilin have falsely been classified as inactivating. In fact, the prolyl isomerase activity is retained in these variants, and they catalyze proline-limited protein folding almost as efficiently as the wild-type protein. This indicates that the results from the traditional protease-linked prolyl isomerase assay may have led to invalid conclusions about the enzymatic mechanism and the *in vivo* function of the prolyl isomerase activity of cyclophilins.

2. Materials and methods

2.1. Materials

(S54G,P55N)-RNase T1² was purified and carboxymethylated as described [25]. The concentrations of RCM-(S54G/P55N)-RNase T1 were determined spectrophotometrically by using an absorption coefficient of $\epsilon_{278} = 21\,060\text{ M}^{-1}\text{ cm}^{-1}$ [26].

2.2. Expression and purification of wild-type and mutated forms of the yeast *Cpr3* protein

The R73A and H144Q mutations in the mitochondrial cyclophilin *Cpr3* of yeast are analogous to the R55A and H126Q mutations in human cyclophilin A, respectively [21]. They were engineered and cloned in the bacterial His⁶-expression plasmid pTrcHisB (Invitrogen), as described (Dolinski et al., submitted). The wild-type and the mutant proteins lack the mitochondrial leader sequence but carry a hexahistidine tag at the amino-terminus.

Cpr3-producing cells were harvested by centrifugation (10 000 × g,

*Corresponding author. Fax: (49) (921) 553661.
E-mail: franz-xaver-schmid@uni-bayreuth.de

Abbreviations: RNase T1, ribonuclease T1; RCM-(S54G/P55N)-RNase T1, disulfide-reduced and *S*-carboxymethylated form of a variant of RNase T1 with Ser⁵⁴ and Pro⁵⁵ replaced by Gly and Asn, respectively; *Cpr3*, mitochondrial cyclophilin of *Saccharomyces cerevisiae*; GdmCl, guanidinium chloride

¹ To facilitate reading we use the expression 'proline-limited' protein folding for a folding reaction that involves the isomerization of a Xaa-Pro peptide bond as the rate-limiting step.

20 min, 4°C) and lysed by a 1-h incubation at room temperature in 50 mM Tris-HCl, pH 7.9, containing 6.0 M GdmCl, 0.25 M NaCl and 5 mM imidazole (5 ml per gram of cell pellet), followed by sonication. The lysate was cleared by centrifugation (10 000 × g, 20 min, 4°C). The supernatant containing the denatured protein was bound to a Ni-NTA Superflow column (Qiagen, Hilden, Germany) and washed with 300 ml of the lysis buffer. To refold the proteins a linear gradient from 6.0 to 0 M GdmCl in 180 ml 50 mM Tris-HCl, pH 7.9, 10 mM imidazole was applied over a period of 90 min at 4°C. Then the refolded protein was eluted by a linear gradient from 10 to 100 mM imidazole in 100 ml of 50 mM Tris-HCl, pH 7.9. Protein-containing fractions were assayed for prolyl isomerase activity in the protease-coupled and in the protein folding assay, pooled, and concentrated by ultrafiltration (Ultrafree-15, Millipore Corp.). The Cpr3 proteins showed purities higher than 95% as judged by denaturing gel electrophoresis and Coomassie staining.

2.3. Measurements of prolyl isomerase activity in the peptide assays

In the first procedure the chromogenic peptide succinyl-Ala-Leu-Pro-Phe-4-nitroanilide in a coupled assay with chymotrypsin was used [1]. 910 µl of 0.1 M Tris-HCl (pH 8.0) and 50 µl of a 600 µM solution of α -chymotrypsin (Boehringer-Mannheim, Germany) were mixed in the spectrophotometer cell and preincubated at 15°C for 10 min. Then 30 µl of the cyclophilin solution to be assayed was added and after 5 min the assay was initiated by adding 10 µl of a 7.8 mM solution of the assay peptide in trifluoroethanol that additionally contained 0.45 M LiCl [27]. The *cis*→*trans* isomerization of the Leu-Pro bond, coupled with the chymotryptic cleavage of the *trans* peptide was followed by the increase in absorbance at 390 nm in a HP 8452 diode array spectrophotometer. Monoexponential functions were fit to the progress curves, and the activity was calculated from the observed rate constants [1].

In the protease-free assay succinyl-Ala-Phe-Pro-Phe-4-nitroanilide was used as a substrate. 955 µl 0.1 M Tris-HCl (pH 8.0) and 30 µl of the Cpr3 enzyme under investigation were incubated in the spectrophotometer cell for 10 min. The assay was started by adding 15 µl of a 7.8 mM solution of the assay peptide in trifluoroethanol/0.45 M LiCl. The final peptide concentration was 120 µM. The *cis*/*trans* isomerization of the Phe-Pro peptide bond was followed by the small decrease of the absorbance at 330 nm of the nitroanilide moiety (G. Fischer and coworkers, personal communication).

2.4. Folding experiments

RCM-(S54G/P55N)-RNase T1 was unfolded by incubating the protein in 0.1 M Tris-HCl, pH 8.0 at 15°C for at least 1 h. Refolding at 15°C was initiated by a 40-fold dilution of the unfolded protein to final conditions of 2.0 M NaCl and the desired concentrations of the wild type protein and the variants of Cpr3 in the same buffer. The folding reaction was followed by the increase in protein fluorescence at 320 nm (10 nm band width) after excitation at 268 nm (1.5 nm band width). The small contribution of the Cpr3 proteins to the fluorescence was subtracted from the measured values in the individual experiments. At 2.0 M NaCl slow folding was a monoexponential process and its rate constant was determined by using the program Graft 3.0 (Erithacus Software, Staines, UK). Fluorescence was measured in a Hitachi F4010 fluorescence spectrophotometer.

3. Results and discussion

3.1. Cpr3 mutant cyclophilins are inactive when measured by the protease-coupled assay

The standard assay for prolyl isomerase activity [1] is based on the conformational specificity of chymotrypsin. This protease cleaves the 4-nitroanilide moiety from succinyl-Ala-Xaa-Pro-Phe-4-nitroanilides only when the Xaa-Pro peptide bond is in the *trans* conformation. Under our assay conditions about 60% of the substrate molecules are *trans* and are cleaved within the dead time of manual mixing by the 30 µM chymotrypsin present in the assay solution. The hydrolysis of the remaining 40% *cis* peptide is slow, limited in rate by the *cis*→*trans* conversion at the Xaa-Pro bond of the peptide. This assay is very sensitive. Prolyl isomerases can

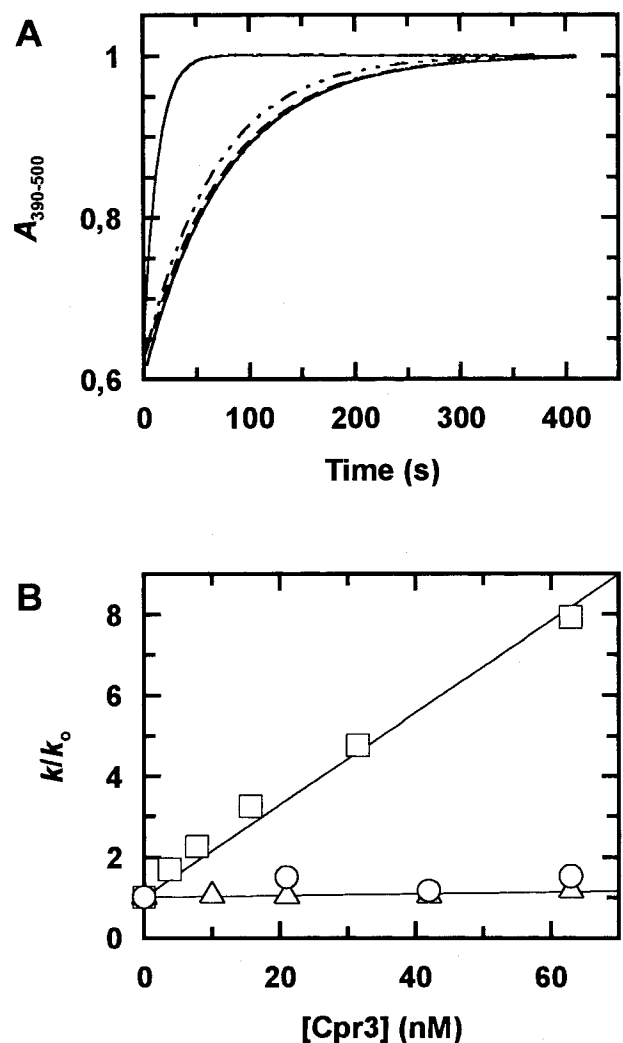


Fig. 1. Activity of wild-type and mutant Cpr3 proteins in the protease coupled prolyl isomerase assay. (A) Time course of the *cis*→*trans* isomerization of the Leu-Pro peptide bond in the assay peptide succinyl-Ala-Leu-Pro-Phe-4-nitroanilide, as measured by the increase in A_{390} after the coupled hydrolysis by chymotrypsin. The uncatalyzed *cis*→*trans* isomerization in the absence of Cpr3 is shown by the lower continuous line, isomerization catalyzed by 31 nM wild-type Cpr3 by the upper continuous line, the isomerizations in the presence of 100 nM R73A variant and 100 nM H144Q variant are shown by the dashed and dash-dotted lines, respectively. (B) The measured prolyl isomerase activities as a function of the concentration of wild-type Cpr3 (□), the H144Q (○) and the R73A (△) variants. The activity is shown as k/k_0 , where k and k_0 are the rate constants of isomerization in the presence and in the absence of these proteins, respectively. The activities were measured at 15°C described in the Methods (see Section 2).

be detected at nanomolar concentration, and many prolyl isomerases were discovered using this assay [14,28].

To test the reliability of the protease-coupled prolyl isomerase assay we employed the mitochondrial cyclophilin Cpr3 from *Saccharomyces cerevisiae* and its R73A and H144Q variants. This cyclophilin is essential for mitochondrial metabolism at elevated temperature [15], and it is involved in the folding of proteins imported into mitochondria [17,18] (Dolinski et al., submitted). The residues equivalent to R73 and H144 in human cyclophilin (R55 and H126) are in the presumed active site [29–33], and the respective mutations in the

human protein (R55A and H126Q) led to an almost complete loss of the prolyl isomerase activity as measured by the protease-coupled assay [21]. We observed such a loss of activity in the protease-coupled assay also for the R73A and H144Q variants of the yeast Cpr3 enzyme (Fig. 1). The wild-type protein showed a high activity, and the isomerization of the assay peptide was accelerated 5-fold in the presence of 31 nM Cpr3 protein (Fig. 1A). In contrast, when 100 nM of the R73A variant or 100 nM of the H144Q variant were added, only marginal accelerations of prolyl isomerization could be detected (Fig. 1A). The very low activity of the mutant enzymes in the protease-coupled assay was confirmed when the Cpr3 concentration was varied (Fig. 1B).

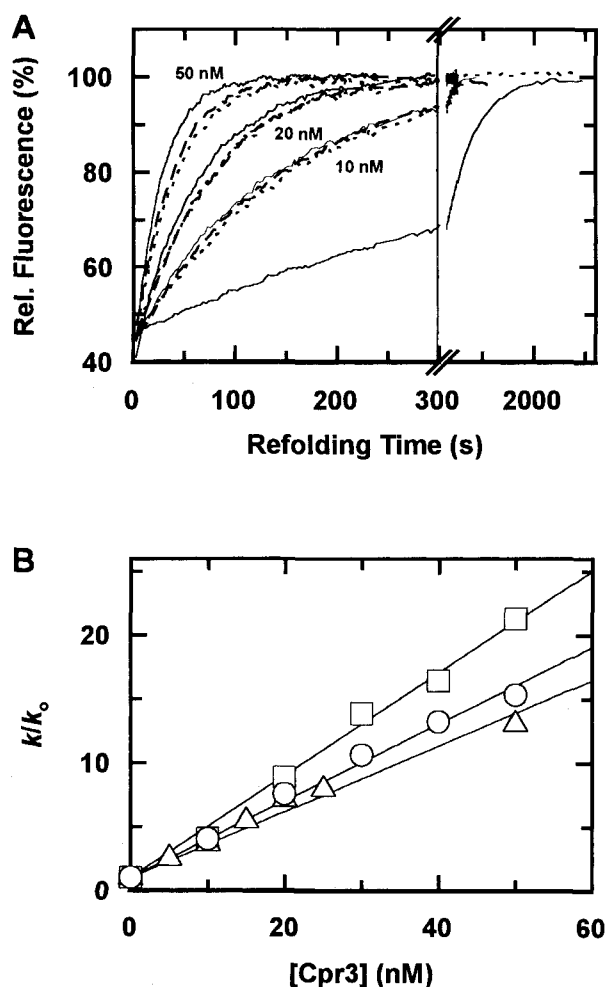


Fig. 2. Catalysis of RCM-(S54G/P55N)-RNase T1 refolding. (A) Kinetics of refolding of 0.35 μ M RCM-(S54G/P55N)-RNase T1 in the absence (lowest curve) and in the presence of 10, 20, and 50 nM wild-type and mutant Cpr3. The folding kinetics in the presence of the wild-type protein are shown by the continuous line, the folding reactions in the presence of the H144Q variant and the R73A variant are shown by the dashed and dotted lines, respectively. (B) Measured catalytic activities in folding as a function of the concentration of wild-type Cpr3 ($\square$), the H144Q ($\circ$) and the R73A ($\triangle$) variants. The activity is shown as k/k_0 , where k and k_0 are the rate constants of folding of RCM-(S54G/P55N)-RNase T1 in the presence and in the absence of the Cpr3 proteins, respectively. The folding kinetics were measured at 15°C in 0.1 M Tris-HCl, 2.0 M NaCl by the increase in fluorescence at 320 nm.

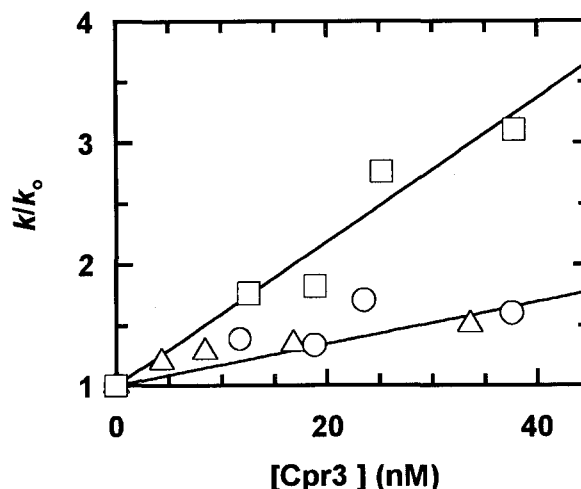


Fig. 3. Activity of wild-type and mutant Cpr3 proteins in the protease-free prolyl isomerase assay. The *cis/trans* isomerization of the Phe-Pro peptide bond in the assay peptide succinyl-Ala-Phe-Pro-Phe-4-nitroanilide was measured by the decrease in A_{330} in the absence of a protease. The measured prolyl isomerase activities are shown as a function of the concentration of wild-type Cpr3 ($\square$), the H144Q ($\circ$) and the R73A ($\triangle$) variants. The activity is shown as k/k_0 , where k and k_0 are the rate constants of isomerization in the presence and in the absence of these proteins, respectively. The activities were measured at 15°C as described in the Methods (see Section 2).

3.2. Mutated Cpr3 cyclophilins are active when measured by a protein folding assay

To measure the activities of the wild-type and the two mutant Cpr3 enzymes in a proline-limited protein folding reaction, we used a reduced and carboxymethylated variant of ribonuclease T1, RCM-(S54G/P55N)-RNase T1, as a substrate protein. Native wild-type RNase T1 contains two *cis* prolyl bonds at Pro³⁹ and Pro⁵⁵, and their isomerizations are the rate-limiting steps in the folding of RNase T1. The (S54G/P55N)-RNase T1 variant contains only a single *cys* prolyl bond (Tyr³⁸-Pro³⁹). Its folding mechanism is therefore particularly simple, and it is well characterized in molecular detail [34,35]. Moreover, in the disulfide-reduced and carboxymethylated RCM form it is an excellent substrate for a wide range of prolyl isomerases [36]. 85% of all unfolded molecules of RCM-(S54G/P55N)-RNase T1 contain an incorrect *trans* Pro³⁹ and fold in a monophasic and reversible reaction, which is controlled in its rate by the slow *trans-cis* isomerization of the Tyr³⁸-Pro³⁹ prolyl peptide bond. In the absence of a catalyst this folding reaction shows a time constant of 570 s (Fig. 2A). When 50 nM wild-type Cpr3 enzyme was added folding was a 22-fold accelerated and the time constant decreased to 26 s. Comparable acceleration factors of 14 and 16 were observed when 50 nM of the R73A or the H144Q mutant enzymes was added (Fig. 2A). In all three cases the acceleration factors were approximately linear functions of the Cpr3 enzyme concentration as expected for an enzyme-catalyzed reaction (Fig. 2B). The comparison of the slopes in Fig. 2B reveals that the R73A and H144Q variants are in fact 65% and 75%, respectively, as active as the wild-type protein when assayed with the protein substrate RCM-(S54G/P55N)-RNase T1.

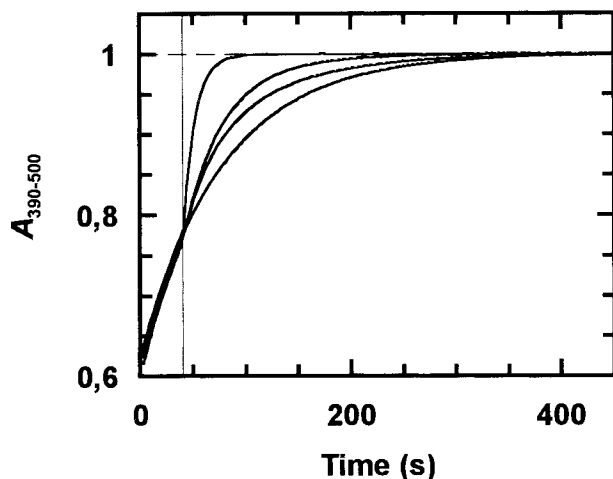


Fig. 4. Inactivation of the Cpr3 mutant proteins in the protease-coupled assay. The kinetics of the uncatalyzed *cis*→*trans* isomerization of the Leu-Pro peptide bond in the assay peptide succinyl-Ala-Leu-Pro-Phe-4-nitroanilide, as measured by the increase in A_{390} is shown by the lowest line. In separate experiments 100 nM of wild-type Cpr3 (upper continuous line), of the R73A mutant (second line from the bottom), or the H144Q mutant (third line), were added after 40 s into the ongoing assay (as indicated by the vertical line). Isomerization-coupled peptide hydrolysis was measured at 15°C as described in the Methods (see Section 2).

3.3. The R73A and H144Q mutants show partial activity in a protease-free peptide-based assay

Recently Fischer and coworkers developed a variant of the peptide-based assay for prolyl isomerase activity, which avoids the coupling with isomer-specific proteolysis (G. Fischer and coworkers, submitted). In this assay the slight decrease in absorbance at 330 nm upon *cis*→*trans* isomerization is used in combination with a jump in the solvent conditions to monitor the kinetics of Phe-Pro isomerization in the uncleaved assay peptide. Because of the small change in signal the sensitivity of the assay is limited, but it indicates that the two Cpr3 mutants show roughly 20% of the prolyl isomerase activity of the wild-type protein (Fig. 3). That the mutations affect the activity towards a peptide more strongly than towards a protein may be attributable to additional binding interactions that are available for long but not for short substrates.

3.4. The R73A and H144Q mutations sensitize Cpr3 to degradation by chymotrypsin

The simplest explanation for the apparent lack of prolyl isomerase activity in the protease-coupled assay is that the mutations increased the protease sensitivity of the Cpr3 protein. When the time of pre-incubation of the H144Q mutant with the protease is progressively decreased from 10 min as in the standard assay procedure to as short as 10 s, a marginal but significant prolyl isomerase activity is observed (data not shown).

The rapid degradation during the protease-coupled assay is clearly revealed in an experiment in which uncatalyzed prolyl isomerization in the peptide is initiated at time zero in the absence of a prolyl isomerase (Fig. 4). When after 40 s 100 nM of the wild-type protein is added, isomerization is strongly accelerated. The slope of the absorbance curve in Fig. 4 increases abruptly, and the final absorbance is reached after a further 50 s. When instead 100 nM of the H144Q mutant is

added after 40 s, the isomerization rate also increases immediately, but to a smaller extent than in the presence of 100 nM wild-type Cpr3 enzyme, and, moreover, after about a further 100 s it returns to the rate of the uncatalyzed reaction, because the protein is inactivated by the protease within this short period of time. Very similar results were obtained with the R73A mutant enzyme (Fig. 4).

Together, our results suggest that the two mutations have only a marginal effect on the prolyl isomerase activity of the Cpr3 enzyme towards protein substrates. The activity towards a tetrapeptide is significantly reduced, and, moreover, this activity is hardly detectable in the standard protease-coupled assay, because unlike the wild-type protein the mutated proteins are rapidly destroyed by chymotrypsin at the onset of the assay.

4. Conclusions

The protease-coupled assay for prolyl isomerase activity provides meaningful results only for proteins that are resistant to proteolytic cleavage during the assay, which typically takes 1–5 min. The protease resistance of newly discovered prolyl isomerases or of mutated forms was not examined routinely in the past, and therefore it is possible that some of the mutations in cyclophilins that were reported to inactivate these proteins instead destabilized them such that they are rapidly degraded by the helper protease in the assay. Several non-proteolytic assays based on modified peptides have been developed, but are not used widely, due to their inherent complexity [37–39].

The catalysis of proline-limited protein folding provides a superior assay for prolyl isomerase activity because an intact protein (instead of a short peptide) is employed as substrate, and because coupling with proteolysis is avoided. Folding protein chains or native proteins with exposed prolyl bonds may be the natural substrates of prolyl isomerases, and therefore the folding assay reflects the cellular functions of these enzymes more closely. When using the protease-coupled peptide assay the proteolytic susceptibility of the prolyl isomerase under investigation must be examined (as suggested already when the first prolyl isomerase was discovered [1]).

Our results on two variants of a yeast mitochondrial cyclophilin indicate that some mutations in prolyl isomerases may have erroneously been classified as inactivating. The respective conclusions about the mechanism of catalyzed prolyl isomerization and about the involvement of the prolyl isomerase activity in the cellular function of immunophilins may therefore need reevaluation. It is clear that mutations, even when they are located at a presumed active site, may nevertheless impair the *in vitro* or *in vivo* function of a protein not because a catalytic residue is changed, but indirectly because the protein is destabilized or sensitized for proteolytic degradation by the mutation.

Acknowledgements: This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. Joseph Heitman is an assistant investigator of the Howard-Hughes Medical Institute.

References

- [1] Fischer, G., Bang, H. and Mech, C. (1984) Biomed. Biochim. Acta 43, 1101–1111.

- [2] Handschumacher, R.E., Harding, M.W., Rice, J. and Drugge, R.J. (1984) *Science* 226, 544–547.
- [3] Fischer, G., Wittmann-Liebold, B., Lang, K., Kiefhaber, T. and Schmid, F.X. (1989) *Nature* 337, 476–478.
- [4] Takahashi, N., Hayano, T. and Suzuki, M. (1989) *Nature* 337, 473–475.
- [5] Tropschug, M., Barthelmess, I.B. and Neupert, W. (1989) *Nature* 342, 953–955.
- [6] Breuder, T., Hemenway, C.S., Movva, N.R., Cardenas, M.E. and Heitman, J. (1994) *Proc. Natl. Acad. Sci. USA* 91, 5372–5376.
- [7] Liu, J., Farmer, J.D., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L. (1991) *Cell* 66, 807–815.
- [8] Friedman, J. and Weissman, I. (1991) *Cell* 66, 799–806.
- [9] Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S. and Sigal, N.H. (1989) *Nature* 341, 755–757.
- [10] Harding, M.W., Galat, A., Ueling, D.E. and Schreiber, S.L. (1989) *Nature* 341, 758–760.
- [11] Griffith, J.P. et al. (1995) *Cell* 82, 507–522.
- [12] Schreiber, S.L. (1991) *Science* 251, 283–287.
- [13] Galat, A. and Metcalfe, S.M. (1995) *Prog. Biophys. Mol. Biol.* 63, 67–118.
- [14] Fischer, G. (1994) *Angew. Chem. Int. Ed.* 33, 1415–1436.
- [15] Davis, E.S., Becker, A., Heitman, J., Hall, M.N. and Brennan, M.B. (1992) *Proc. Natl. Acad. Sci. USA* 89, 11169–11173.
- [16] Franke, E.K., Yuan, H.E.H. and Luban, J. (1994) *Nature* 372, 359–362.
- [17] Matouschek, A., Rospert, S., Schmid, K., Glick, B.S. and Schatz, G. (1995) *Proc. Natl. Acad. Sci. USA* 92, 6319–6323.
- [18] Rassow, J., Mohrs, K., Koidl, S., Barthelmess, I.B., Pfanner, N. and Tropschug, M. (1995) *Mol. Cell. Biol.* 15, 2654–2662.
- [19] Schönbrunner, E.R., Mayer, S., Tropschug, M., Fischer, G., Takahashi, N. and Schmid, F.X. (1991) *J. Biol. Chem.* 266, 3630–3635.
- [20] Thali, M., Bukovsky, A., Kondo, E., Rosenwirth, B., Walsh, C.T., Sodroski, J. and Gottlinger, H.G. (1994) *Nature* 372, 363–365.
- [21] Zydowsky, L.D., Etzkorn, F.A., Chang, H.Y., Ferguson, S.B., Stolz, L.A., Ho, S.I. and Walsh, C.T. (1992) *Protein Sci.* 1, 1092–1099.
- [22] Ferreira, P.A., Nakayama, T.A., Pak, W.L. and Travis, G.H. (1996) *Nature* 383, 637–640.
- [23] Luban, J. (1996) *Cell* 87, 1157–1159.
- [24] Braaten, D., Ansari, H. and Luban, J. (1997) *J. Virol.* 71, 2107–2113.
- [25] Mücke, M. and Schmid, F.X. (1994) *J. Mol. Biol.* 239, 713–725.
- [26] Takahashi, K., Uchida, T. and Egami, F. (1970) *Adv. Biophys.* 1, 53–98.
- [27] Kofron, J.L., Kuzmic, P., Kishore, V., Gemmecker, G., Fesik, S.W. and Rich, D.H. (1992) *J. Am. Chem. Soc.* 114, 2670–2675.
- [28] Galat, A. and Metcalfe, S.M. (1995) *Prog. Biophys. Mol. Biol.* 63, 67–118.
- [29] Ke, H.M., Zydowsky, L.D., Liu, J. and Walsh, C.T. (1991) *Proc. Natl. Acad. Sci. USA* 88, 9483–9487.
- [30] Ke, H.M. (1992) *J. Mol. Biol.* 228, 539–550.
- [31] Kallen, J. and Walkinshaw, M.D. (1992) *FEBS Lett.* 300, 286–290.
- [32] Zhao, Y.D. and Ke, H.M. (1996) *Biochemistry* 35, 7356–7361.
- [33] Zhao, Y.D. and Ke, H.M. (1996) *Biochemistry* 35, 7362–7368.
- [34] Mücke, M. and Schmid, F.X. (1994) *Biochemistry* 33, 14608–14619.
- [35] Mayr, L.M., Odefey, C., Schutkowski, M. and Schmid, F.X. (1996) *Biochemistry* 35, 5550–5561.
- [36] Schmid, F.X., Mayr, L.M., Mücke, M. and Schönbrunner, E.R. (1993) *Adv. Protein Chem.* 44, 25–66.
- [37] Garcia-Echeverria, C., Kofron, J.L., Kuzmic, P., Kishore, V. and Rich, D.H. (1992) *J. Am. Chem. Soc.* 114, 2758–2759.
- [38] Garcia-Echeverria, C., Kofron, J.L., Kuzmic, P. and Rich, D.H. (1993) *Biochem. Biophys. Res. Commun.* 191, 70–75.
- [39] Schutkowski, M., Drewello, M., Wollner, S., Jakob, M., Reimer, U., Scherer, G., Schierhorn, A. and Fischer, G. (1996) *FEBS Lett.* 394, 289–294.