Destabilization of the complete protein secondary structure on binding to the chaperone GroEL

Ralph Zahn*, Claus Spitzfaden†, Marcel Ottiger†, Kurt Wüthrich† & Andreas Plückthun*‡

* Max-Planck-Institut für Biochemie, Protein Engineering Group, Am Klopferspitz, D-82152 Martinsried, Germany
† Institut für Molekularbiologie und Biophysik, ETH-Hönggerberg, CH-8093 Zürich, Switzerland

PROTEIN folding *in vivo* is mediated by helper proteins, the molecular chaperones[1–3], of which Hsp60 and its *Escherichia coli* variant GroEL are some of the best characterized. GroEL is an oligomeric protein with 14 subunits each of M_r 60K[4–6], which possesses weak, co-operative ATPase activity[7–9] and high plasticity[10]. GroEL seems to interact with non-native proteins, binding one or two molecules per 14-mer[11–19] in a 'central cavity'[20], but little is known about the conformational state of the bound polypeptides. Here we use nuclear magnetic resonance techniques to show that the interaction of the small protein cyclophilin[21,22] with GroEL is reversible by temperature changes, and all amide protons in GroEL-bound cyclophilin are exchanged with the solvent, although this exchange does not occur in free cyclophilin. The complete secondary structure of cyclophilin must be disrupted when bound to GroEL.

Exchange of cyclophilin amide protons (Fig. 1) in the presence of GroEL was studied under conditions in which cyclophilin could bind to GroEL, that is, at 30 °C and pH 6.0 (see below). An equimolar mixture of ^{15}N-labelled cyclophilin and GroEL was heated in D_2O to 30 °C for 8 h to induce cyclophilin binding, followed by cooling to 6 °C for 14 h, to induce protein–chaperone dissociation (Fig. 2*a*). This cycle was repeated three times to ensure that most cyclophilin molecules were bound at least once even if the turnover of bound cyclophilin was slow (at pH 6.0 and 30 °C, only 50% of cyclophilin is bound to GroEL at equilibrium). Before the nuclear magnetic resonance (NMR) experiments, cyclophilin and GroEL were separated by cation-exchange chromatography in D_2O at 6 °C. The resulting cyclophilin fractions were pooled and concentrated to a protein concentration of 0.4 mM. A two-dimensional [^{15}N, ^{1}H]-correlation ([^{15}N, ^{1}H]-COSY) spectrum of this solution was completely empty (not shown), demonstrating that all amide protons had been exchanged with deuterium.

To show that the cyclophilin recovered in D_2O from the GroEL–cyclophilin complex is in the native folded form, it was

‡ To whom correspondence should be addressed at: Biochem. Institut, Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland.

re-exchanged into H_2O and kept at 26 °C for 2 weeks. A [^{15}N, ^{1}H]-COSY spectrum of this sample (Fig. 2*d*) had the same appearance as a spectrum of native cyclophilin (Fig. 2*b*), except that the signals corresponding to the 39 most slowly exchanging amide protons (Fig. 1) were very weak. A [^{15}N, ^{1}H]-COSY spectrum of cyclophilin treated as before (Fig. 2*a*) in the absence of GroEL showed that the 39 backbone amide protons which exchange most slowly in free cyclophilin[23] gave strong peaks. Residual peak intensities were observed for some residues with medium–fast exchange (Figs 1 and 2*c*), corresponding to the behaviour of free cyclophilin in D_2O solution without the special treatment used in Fig. 2*a*. Experiments with cross-sections[24] support these findings. The cross-section Fig. 2*b*′ contains peaks from a rapidly and a slowly exchanging amide proton. In cross-section Fig. 2*c*′ only the peak of the slowly exchanging amide proton is left. In cross-section Fig. 2*d*′ the rapidly exchanging proton has been fully re-exchanged, whereas less than 10% of the slowly exchanging protons have been re-exchanged. A complete survey of the backbone amide proton exchange in free and GroEL-bound cyclophilin is given in Fig. 2*e*, *f*, showing that residues that were strongly protected against exchange in a solution of free cyclophilin in D_2O re-exchange only to a limited extent in the experiment shown in Fig. 2*d*.

To investigate the kinetic stability of the complex formed between cyclophilin and GroEL, a cyclophilin solution containing only 0.1 equivalents of GroEL (but otherwise identical to the solutions used in Fig. 2*c* and *d*) was subjected to the treatment of Fig. 2*a*. If the GroEL–cyclophilin complex is relatively stable under these conditions, one would predict a 14% reduction in the intensity of the cross peaks corresponding to slowly exchanging amide protons, since in each cycle of Fig. 2*a* only about half of GroEL would bind cyclophilin (Fig. 3*d*), leading to exchange. A much bigger intensity loss could result if cyclophilin exchanged in and out of the GroEL binding site. The [^{15}N, ^{1}H]-COSY spectrum from this experiment (not shown) was very similar to that in Fig. 2*c*. Only a small exchange enhancement can thus be attributed to cyclophilin turnover, and the GroEL–cyclophilin complex at 30 °C and pH 6.0 must be kinetically rather stable.

When 0.005 stoichiometric equivalents of GroEL were present during the NMR observation of cyclophilin (much more would not be soluble at these high molar concentrations), the exchange of the slowly exchanging protons was not measurably affected (see above). There was significant line broadening in the NMR spectra however, which persisted after eightfold dilution of the protein sample, but disappeared on addition of 6 mM Mg–ATP (Fig. 2*g*). Under the conditions of these experiments (pH 6.0, 26 °C), the GroEL present contains some stably bound cyclophilin but is probably not saturated. Cyclophilin must thus interact transiently with either the free GroEL or the GroEL–cyclophilin complex, or both. While they are bound, these cyclophilin molecules would be restricted to the slow rotational tumbling of the GroEL–cyclophilin complex, causing the observed line broadening. The broadening in turn indicates that the cyclophilin stays bound for more than about 1 ns. Mg–ATP suppresses this interaction, presumably by causing a change in GroEL conformation[9]. The fact that this type of binding induces no exchange of the most stable amide protons (Fig. 1) indicates that it does not involve unfolding or destabilization of native cyclophilin, setting it apart from that seen in Fig. 2*d*.

TABLE 1 Kinetics of cyclophilin folding

	pH	T-shift (°C)	[Cyclophilin] (μM)	[GroEL] (μM)	Rate constant
Aggregation	7.0	25→54	25	—	$1.3\times10^{3}\ s^{-1}\ M^{-1}$
Unfolding	7.0	25→48	25	25	$2.3\times10^{-3}\ s^{-1}$
	7.0	25→46	25	25	$1.9\times10^{-3}\ s^{-1}$
	6.0	6→30	1	5	$7.6\times10^{-5}\ s^{-1}$
Refolding	7.0	46→1	25	25	$2.9\times10^{-4}\ s^{-1}$
	7.0	46→5	25	25	$5.5\times10^{-4}\ s^{-1}$
	6.0	30→6	1	5	$1.8\times10^{-5}\ s^{-1}$

The folding kinetics were measured after shifting the temperature of a cyclophilin-containing solution to the value indicated and measuring the enzymatic activity as a function of time. The rate constants were determined by a three-parameter fit (starting activity, final activity, rate constant) to first-order kinetics (for GroEL-assisted unfolding and refolding) or second-order kinetics (for irreversible aggregation in the absence of GroEL).

The conditions for these experiments were selected on the basis of biochemical measurements of the kinetics and equilibria of cyclophilin folding in the presence of GroEL. Gel chromatography at pH 7.0 (data not shown) showed that micromolar concentrations of cyclophilin do not give rise to a stable complex with equimolar amounts of GroEL at room temperature, but that a significant proportion of cyclophilin co-elutes with GroEL once the temperature of the column is raised to 58 °C. If the solution is subsequently cooled to 25 °C, the complex of GroEL and cyclophilin dissociates again.

Measurements of enzymatic activity (Fig. 3*a*) show that there is concentration-dependent, irreversible unfolding of cyclophilin in the absence of GroEL, the kinetics of which are consistent with a second-order reaction (Table 1) as is typical of aggregation processes. In the presence of equivalent concentrations of GroEL, most of the cyclophilin is protected from irreversible

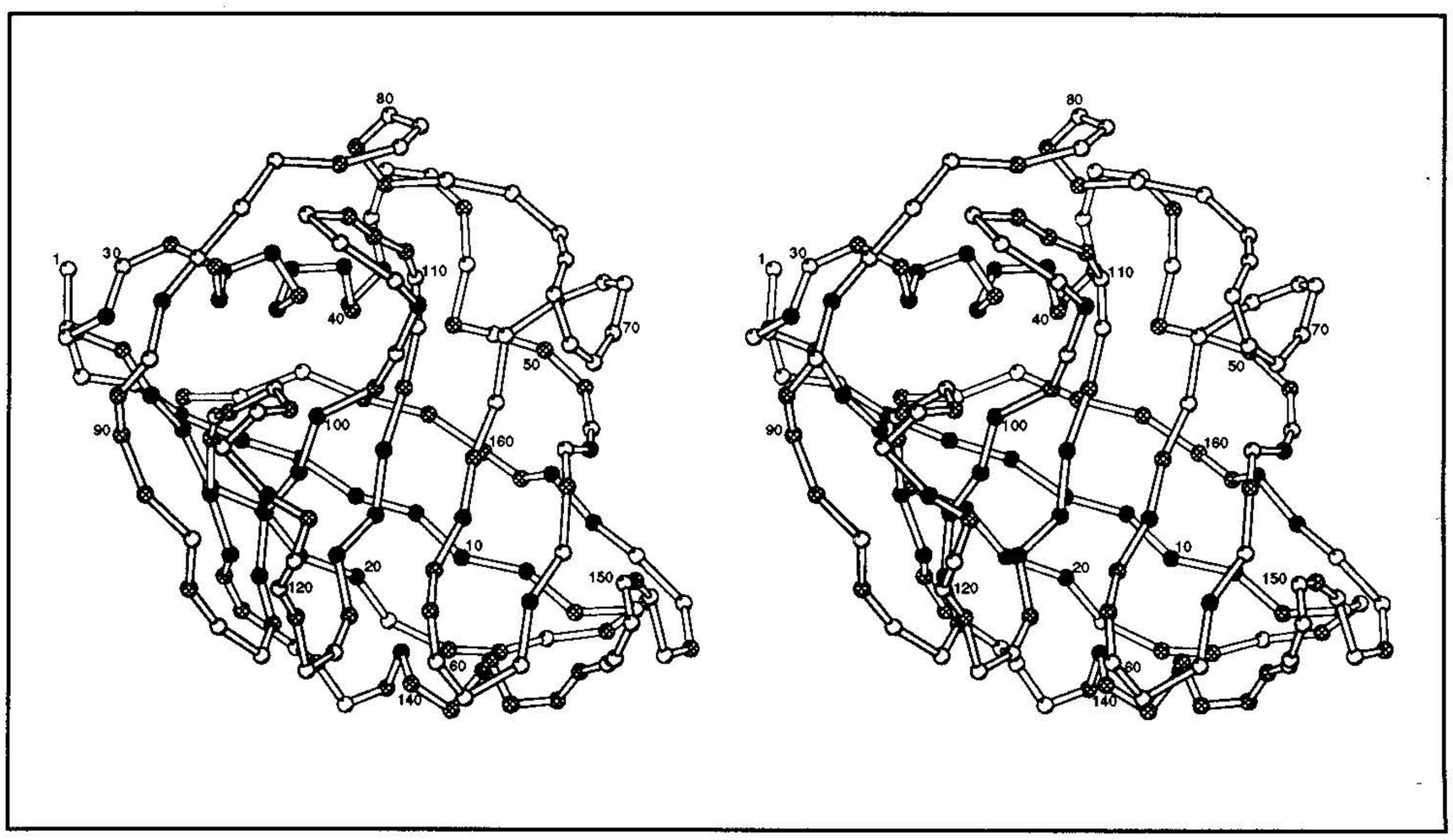
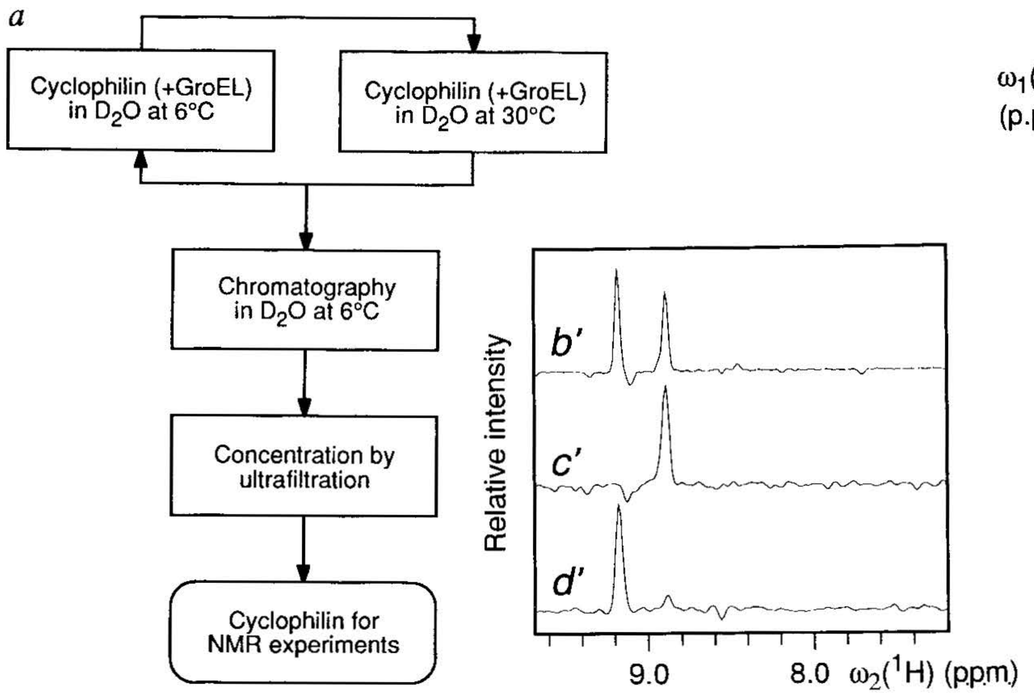
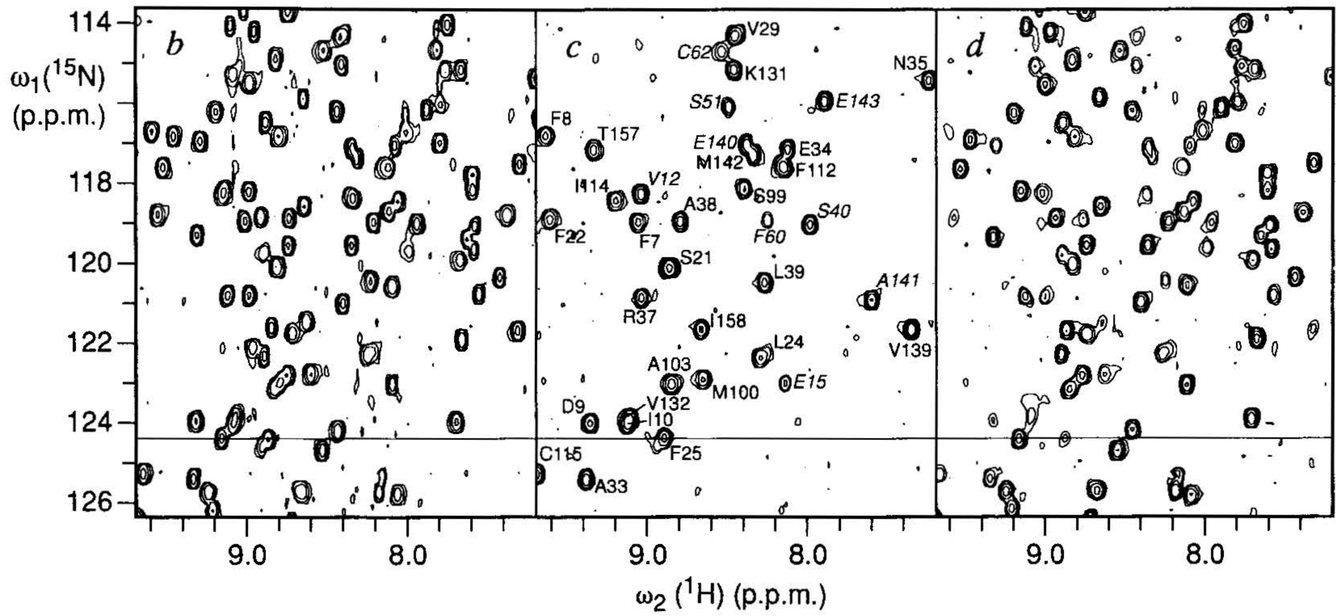
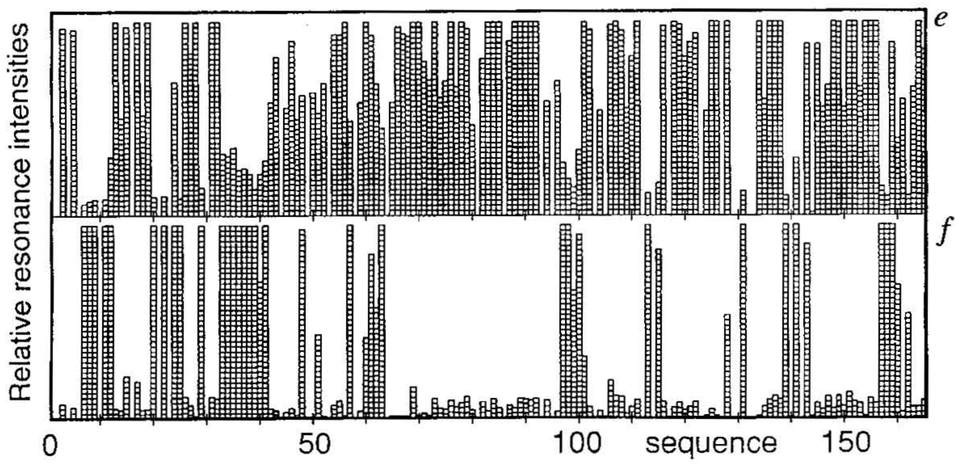


FIG. 1 Stereo view of the polypeptide backbone in the three-dimensional NMR solution structure of cyclophilin[28] with identification of residues with slowly exchanging amide protons. Backbone amide nitrogen positions represented by circles are connected by virtual bonds; every tenth residue is numbered. The black circles identify the previously identified[23] locations of the 39 residues for which complete amide proton exchange could not be achieved in free cyclophilin without irreversible denaturation of the protein. Shaded and open circles identify residues with medium and fast amide proton exchange, respectively[23]. The plot was made with the program MOLSCRIPT[29].







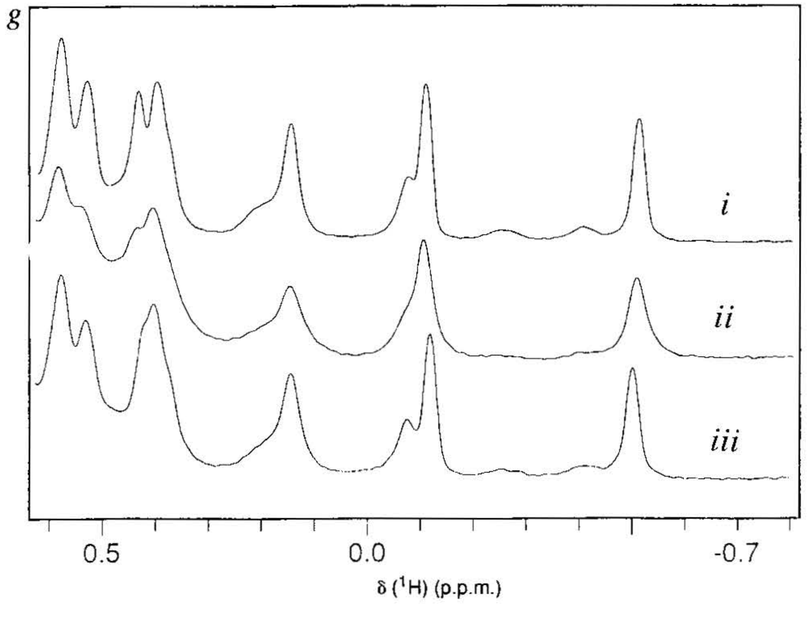


FIG. 2 *a*, Diagrammatic representation of the preparation of the protein solutions used to study GroEL-promoted amide proton exchange in cyclophilin by NMR. *b–d*, NMR spectra used to study the amide proton exchange of cyclophilin in the presence and absence of GroEL. The region ($\omega_1(^{15}N) = 114–126$ p.p.m., $\omega_2(^1H) = 7.2–9.7$ p.p.m.) of [^{15}N, ^{1}H]-COSY spectra (^{1}H frequency = 600 MHz, 26 °C, pH or pD 6.0) is shown. *b*, Cyclophilin (4 mM) in 90% H_2O/10% D_2O. *c*, A 0.5 mM D_2O solution of cyclophilin that had been subjected to the procedure outlined in *a* with no GroEL added. *d*, A 0.3 mM H_2O solution of cyclophilin that had been subjected to the procedure in *a* in the presence of a stoichiometric amount of GroEL, after subsequent re-equilibration in 90% H_2O/10% D_2O for 2 weeks at 26 °C. The cross peaks which correspond to very slowly exchanging amide protons[23] (Fig. 1) are identified in *c* with the amino acid one-letter symbol and the sequence position. Some additional peaks corresponding to amide protons with medium–slow exchange are similarly identified using italics. *b′–d′*, Cross-sections along $\omega_2(^1H)$ taken at the position of the horizontal line $\omega_1(^{15}N) = 124.4$ p.p.m. in the spectra *b*, *c* and *d*. e, *f*, Plots against the amino acid sequence of cyclophilin of the [^{15}N, ^{1}H]-COSY cross-peak volumes for the individual amino acid residues normalized by the corresponding peak intensities in the reference spectrum *b*, where *e* shows data from the experiment shown in *d* and *f* those for cyclophilin after amide proton exchange in D_2O for 37 h at 26 °C. The two data sets can be compared only qualitatively because of the different exchange periods of 336 h for *e* and 37 h for *f*. *g*, High-field region from −0.8 to 0.6 p.p.m. from 1D ^{1}H NMR spectra: (*i*), 0.3 mM cyclophilin; (*ii*), 0.3 mM cyclophilin +1.5 μM GroEL; (*iii*), same as (*ii*), 1.5 h after addition of 6 mM Mg–ATP (26 °C, ^{1}H frequency 500 MHz, solvent 90% H_2O/10% D_2O).

METHODS. Recombinant ^{15}N-labelled cyclophilin was over-expressed in *E. coli* and purified as described previously[30]. GroEL was purified from lysates of cells harbouring the multicopy plasmid pOF39, as described previously[19]. The concentration of GroEL is always given for the 14-subunit oligomeric form. For the NMR experiments *c* and *d*, a 20 μM solution of cyclophilin in D_2O (99.8% D_2O, 20 mM 2-[N-morpholino] ethane sulphonate (MES) pD 6.0, 2 mM DTT, 2 mM EDTA, 0.02% NaN_3) was subjected to the procedure in *c* without and in *d* with addition of 20 μM of GroEL. The two samples of 25 ml volume were subjected to three cycles of heating to 30 °C for 8 h and cooling to 6 °C for 14 h (see *a*). After standing at 6 °C for 40 h after the last temperature shift, cyclophilin was separated from GroEL on a HiLoad S-Sepharose column (Pharmacia) equilibrated with the same MES buffer in D_2O at 6 °C. The cyclophilin fractions were concentrated to about 0.4 mM by ultrafiltration at 6 °C.

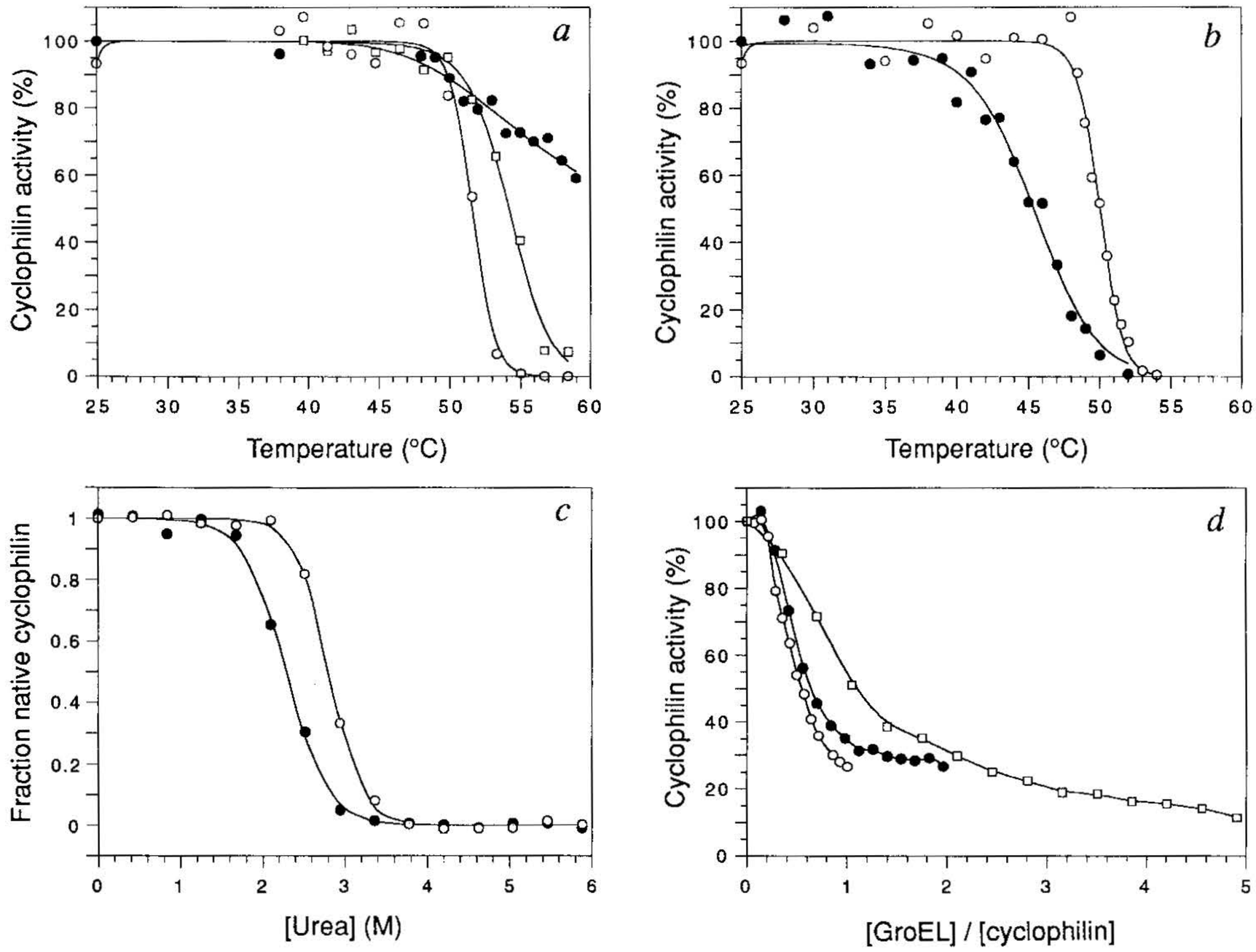


FIG. 3 Folding of cyclophilin in the absence and presence of GroEL. *a*, Irreversibly unfolded cyclophilin after heating at pH 7.0. □, low cyclophilin concentration (0.9 μM) in the absence of GroEL; ● and ○, high cyclophilin concentration (9 μM) in the presence or absence of GroEL (10 μM), respectively. *b*, Portion of total (reversibly and irreversibly) inactivated cyclophilin after heating at pH 7.0 in the presence (●) or absence (○) of GroEL. *c*, Urea denaturation curves of cyclophilin at 30 °C and pH 6.0 (●) or pH 7.0 (○). *d*, Titration of cyclophilin with GroEL at 46 °C and pH 7.0 (●, ○), and at 30 °C and pH 6.0 (□). The concentration of cyclophilin was 1 μM (●, □) or 10 μM (○).

METHODS. *a*, Cyclophilin was incubated for 20 min at the temperatures indicated in a buffer containing 100 mM K_2HPO_4/KH_2PO_4 pH 7.0 and 10 mM DTT. After cooling the samples to 10 °C and a subsequent waiting period of 1 h at 25 °C (to allow unfolded enzyme to refold), the enzymatic peptidyl-prolyl *cis-trans*-isomerase activity of cyclophilin was measured at 10 °C as described[31]. This experiment determines the sum of residual folded and refoldable cyclophilin. *b*, Solutions of 25 μM cyclophilin in 100 mM K_2HPO_4/KH_2PO_4 at pH 7.0 and 10 mM DTT were incubated at various temperatures in the presence of 25 μM GroEL or in the absence of GroEL. After 20 min the solutions were rapidly cooled to 1 °C by 100-fold dilution in the same buffer (to freeze the folding state of cyclophilin). The cyclophilin activity was immediately measured at 4.5 °C. In this experiment, only the amount of residual enzymatically active cyclophilin present in the incubation mixture is measured as a function of the incubation temperature. The remainder is reversibly or irreversibly inactivated. c, The enzymatic activity of a 1 μM cyclophilin solution, incubated for 22 h at 30 °C at different urea concentrations, was measured at 4.5 °C. The solution was either at pH 6.0 (100 mM MES, 10 mM DTT) or at pH 7.0 (100 mM K_2HPO_4/KH_2PO_4, 10 mM DTT). From these data, the fraction of native cyclophilin was calculated[32] (note that these are not true equilibrium curves, however, because cyclophilin cannot be refolded after denaturation in 8 M urea under these conditions). *d*, Solutions of cyclophilin containing different concentrations of GroEL were incubated for 20 min at 46 °C in a buffer containing 100 mM K_2HPO_4/KH_2PO_4, 10 mM DTT, pH 7.0, or for 19 h at 30 °C in a buffer containing 90 mM MES/38 mM K_2HPO_4/KH_2PO_4, 10 mM DTT, pH 6.0. Immediately after heating, the enzymatic activity of cyclophilin, which is given relative to that of a GroEL-free cyclophilin solution, was measured at 4.5 °C.

denaturation (Fig. 3*a*), probably by forming a stable reversible complex with GroEL, as shown by the gel filtration experiments. In the absence of GroEL, the total unfolded portion of cyclophilin after heating at pH 7.0 (Fig. 3*b*) is similar to that in Fig. 3*a*, indicating that once cyclophilin is unfolded by heat, it cannot be folded back under these conditions. In the presence of GroEL, however, a reduction of cyclophilin activity occurs far below the denaturation temperature for free cyclophilin at a rate consistent with first-order kinetics (Table 1.) When the temperature was subsequently shifted back from 46 °C to 1 °C, cyclophilin recovered enzymatic activity with slow first-order kinetics (Table 1).

Urea denaturation curves show that cyclophilin is less stable at pH 6.0 than at pH 7.0 (Fig. 3*c*). The titration curves of cyclophilin with GroEL at 30 °C show that the net interaction between the chaperone and the substrate is stronger at pH 6.0 than at pH 7.0 (Fig. 3*d*), probably because of the lower thermodynamic stability of cyclophilin at pH 6.0, where it can be unfolded at 30 °C (Table 1). The close similarity of the titration curves obtained at pH 7.0 with different absolute concentrations of GroEL and cyclophilin indicates that most of the cyclophilin molecules may already be transiently bound to GroEL at the concentrations used; the observed transition in enzymatic activity would thus occur in the complex and be independent of concentration. Even at the highest GroEL concentrations used, the residual cyclophilin activity exceeded 20%.

These data can be interpreted in terms of the model:

$$EL + N \underset{\text{fast}}{\overset{\text{fast}}{\rightleftharpoons}} EL\text{–}N \underset{\text{slow}}{\overset{\text{medium}}{\rightleftharpoons}} EL\text{–}U \underset{\text{fast}}{\overset{\text{slow}}{\rightleftharpoons}} EL + U$$

where EL is GroEL, N is folded, enzymatically active cyclophilin with 39 exchange-protected amide protons, U is unfolded, enzymatically inactive cyclophilin with no exchange-protected amide protons, EL–N is the complex of EL and N, and EL–U is the complex of EL and U. The rates shown apply to the unfolding reaction observed at high temperature.

The exchange experiment of Fig. 2*d*, which started with native cyclophilin and led to tightly GroEL-bound cyclophilin, would thus start on the left, go to EL–U, and return to the left after the temperature had been lowered. In these experiments, cyclophilin was destabilized by lowering the pH, allowing unfolding to occur even at 30 °C (Table 1). At pH 7.0, cyclophilin remains stable at this temperature, but can be reversibly unfolded by GroEL at around 45 °C (Fig. 3*a*, *b*). By contrast, in the experiment with transiently bound cyclophilin (Fig. 2*g*) only the state EL–N is transiently occupied in the absence of ATP. The transient binding of native cyclophilin may be analogous to the previously reported stabilization of helical structure in a GroEL-bound peptide with a short residence time[25,26]. *In vivo*, the reaction would presumably start on the right-hand side with U and EL, and as the EL–N↔EL–U equilibrium in the cell will normally be on the left, the reaction would proceed all the way from right to left, the last step being aided by ATP.

As cyclophilin is not bound instantaneously and quantitatively by GroEL under the conditions used (Fig. 3*d*), yet all protons are quantitatively lost, the protection factor of the amide protons labelled black in Fig. 1 must be reduced from $>10^7$ in native cyclophilin to $<10^3$ in GroEL-bound cyclophilin. By contrast, the protection factors of α-lactalbumin are 10^4 in the native state

and are lowered to 10^2 in the 'molten globule' state[27]. Cyclophilin bound to GroEL thus seems to be fully unfolded, or in a low-energy molten globule state. The apparent absence of any stable secondary structure may be essential for the observed substrate promiscuity of GroEL[11–19]. The chaperone may interact with interior side-chains to shift the equilibrium towards an unfolded state. By disrupting all native structure at least transiently, it may thus direct folding towards the native state by favouring stable structures and thereby avoiding aggregation, regardless of the final topology of the substrate. □

Received 22 September 1993; accepted 4 January 1994.

1. Ellis, R. J. & van der Vies, S. M. *A. Rev. Biochem.* **60,** 321–347 (1991).
2. Gething, M.-J. & Sambrook, J. *Nature* **355,** 33–45 (1992).
3. Jaenicke, R. *Curr. Opin. struct. Biol.* **3,** 104–112 (1993).
4. Hendrix, R. W. *J. molec. Biol.* **129,** 375–392 (1979).
5. Hohn, T., Hohn, B., Engel, A., Wurtz, M. & Smith, P. R. *J. molec. Biol.* **129,** 359–373 (1979).
6. Hemmingsen, S. M. *et al. Nature* **333,** 330–334 (1988).
7. Gray, T. E. & Fersht, A. R. *FEBS Lett.* **292,** 254–258 (1991).
8. Bochkareva, E. S., Lissin, N. M., Flynn, G. C., Rothman, J. E. & Girshovich, A. S. *J. biol. Chem.* **267,** 6796–6800 (1992).
9. Jackson, G. S. *et al. Biochemistry* **32,** 2554–2563 (1993).
10. Zahn, R., Harris, J. R., Pfeifer, G., Plückthun, A. & Baumeister, W. *J. molec. Biol.* **229,** 579–584 (1993).
11. Goloubinoff, P., Christeller, J. T., Gatenby, A. A. & Lorimer, G. H. *Nature* **342,** 884–889 (1989).
12. Laminet, A. A., Ziegelhoffer, T., Georgopoulos, C. & Plückthun, A. *EMBO J.* **9,** 2315–2319 (1990).
13. Badcoe, G. *et al. Biochemistry* **30,** 9195–9200 (1991).
14. Buchner, J. *et al. Biochemistry* **30,** 1586–1591 (1991).
15. Höll-Neugebauer, B., Rudolph, R., Schmidt, M. & Buchner, J. *Biochemistry* **30,** 11609–11614 (1991).
16. Martin, J. *et al. Nature* **352,** 36–42 (1991).
17. Mendoza, J. A., Rogers, E., Lorimer, G. H. & Horowitz, P. M. *J. biol. Chem.* **266,** 13044–13049 (1991).
18. Viitanen, P. V., Donaldson, G. K., Lorimer, G. H., Lubben, T. H. & Gatenby, A. A. *Biochemistry* **30,** 9716–9723 (1991).
19. Zahn, R. & Plückthun, A. *Biochemistry* **31,** 3249–3255 (1992).
20. Langer, T., Pfeifer, G., Martin, J., Baumeister, W. & Hartl, F.-U. *EMBO J.* **11,** 4757–4765 (1992).
21. Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J. & Speicher, D. W. *Science* **226,** 544–547 (1984).
22. Schreiber, S. L. *Science* **251,** 283–287 (1991).
23. Wüthrich, K., Spitzfaden, C., Memmert, K., Widmer, H. & Wider, G. *FEBS Lett.* **285,** 237–247 (1991).
24. Wüthrich, K. *NMR of Proteins and Nucleic Acids* (Wiley, New York, 1986).
25. Landry, S. J. & Gierasch, L. M. *Biochemistry* **30,** 7359–7362 (1991).
26. Landry, S. J. Jordan, R., McMacken, R. & Gierasch, L. M. *Nature* **355,** 455–457 (1992).
27. Chyan, C.-L., Wormald, C., Dobson, C. M., Evans, P. A. & Baum, J. *Biochemistry* **32,** 5681–5691 (1993).
28. Spitzfaden, C., Wider, G., Widmer, H. & Wüthrich, K. *Abstr. XV Int. Conf. Magnetic Resonance in Biological Systems*, Jerusalem, 192 (1992).
29. Kraulis, P. J. *J. appl. Crystallogr.* **24,** 946–950 (1991).
30. Weber, C. *et al. Biochemistry* **30,** 6563–6574 (1991).
31. Fischer, G., Bang, H. & Mech, C. *Biomed. biochim. Acta* **43,** 1101–1111 (1984).
32. Pace, C. N. *Trends Biotechnol.* **8,** 93–98 (1990).

ACKNOWLEDGEMENTS. We thank K. Memmert (Sandoz) for the cyclophilin expression plasmid and discussions about the production of ^{15}N-labelled protein. The works was supported by the Deutsche Forschungsgemeinschaft, the Schweizerischer Nationalfonds and Sandoz, and a predoctoral fellowship from the Fonds der chemischen Industrie (to R.Z.).