13. Hybrid pigment genes were prepared as follows. Red or green pigment cDNA clones (hs7 or hs2, respectively, the latter reconstructed at its 5′ end to include the first 14 codons missing from the original cDNA clone) were inserted into the expression vector pCIS (*14*) as described (*1*), linearized at a restriction site adjacent to the cDNA insert, digested for a variable length of time with exonuclease III (Promega, Madison, WI), and then further digested with nuclease S1 (Promega). The partially digested red or green pigment cDNA fragment was used to prime synthesis on a single-stranded template that contained either the green or red pigment cDNA, respectively. After transfection of an *Escherichia coli* mutant deficient in mismatch repair (*mut L*), appropriate hybrids were identified by oligonucleotide hybridization and DNA sequencing. For those hybrids that contained a red pigment–derived exon 3, hybrids that contained a Ser at position 180 were constructed from the Ala-containing hybrids by oligonucleotide-directed site-specific mutagenesis. The 3′ untranslated region of each 5′red–3′green hybrid cDNA was replaced with the 3′ untranslated region of the red pigment cDNA hs7. The entire nucleotide sequence of each hybrid was determined on one strand both to confirm the identity of the hybrid and to rule out spurious mutations.
14. C. M. Gorman, D. R. Gies, G. McCray, *DNA Protein Eng. Tech.* **2**, 3 (1990).
15. The hybrid pigment cDNAs were used to transiently transfect 30 to 50 10-cm plates of a human embryonic kidney cell line (293S; American Type Culture Collection CRL 1573). Twenty-four hours after transfection, cells were harvested, and cell membranes that contained visual pigment apoprotein were isolated (*1*). After incubation with 11-*cis*-retinal, the membranes were solubilized in 2% CHAPS, and the soluble fraction was used for photobleaching difference absorption spectroscopy (*1*). Differences in yield of as much as tenfold were observed for different hybrid pigments (Table 1). Pigment yields after transient transfection were: green < 5′red–3′green ≤ 5′green–3′red < red (Table 1) (S. L. Merbs and J. Nathans, unpublished results). In general, the vast majority of the cone pigment apoprotein exists in an aggregated form as judged by protein immunoblot (S. L. Merbs and J. Nathans, unpublished results), which suggests that cone pigment apoproteins are unstable in tissue culture cells.
16. The most reproducible portion of the absorption spectrum is the long wavelength limb because of lower light scattering at long wavelengths and because some of the hybrid pigment curves show a distortion of their short wavelength limbs, centered at approximately 440 nm. The presence and size of this distortion are variable and increase with longer bleaching times, with an amplitude between 0 and 25% that of the visual pigment peak. It appears to have little or no effect on the long wavelength limbs of the hybrid pigment spectra. This distortion most likely represents formation of other photolabile Schiff's bases of 11-*cis*-retinal.
17. When measured on a wavelength scale, the slope of the long wavelength limb of the green pigment is steeper than that of either of the two red pigments. The values for λ_{max} of green and red-(Ala^{180}) or red(Ser^{180}) pigments differ by 22 or 27 nm, respectively, whereas their values for $\lambda_{1/2max}$ differ by 31 or 36 nm, respectively (*1*). This discrepancy between λ_{max} shifts and $\lambda_{1/2max}$ shifts can also be seen in a comparison of G4R5 and the green pigment, R4G5(Ala^{180}) and the red(Ala^{180}) pigment, or R4G5(Ser^{180}) and the red(Ser^{180}) pigment. This difference in slope of the long wavelength limbs is still evident when the logarithm of the pigment spectral sensitivity is plotted versus the logarithm of the pigment spectra wavenumber [D. A. Baylor, B. J. Nunn, J. L. Schnapf, *J. Physiol.* **390**, 145 (1987)].
18. M. Neitz, J. Neitz, G. H. Jacobs, *Science* **252**, 971 (1991). Neitz *et al.* found that the absorption maxima of ten primate visual pigments could be predicted by a model in which substitution of Ser for Ala, Tyr for Phe, and Thr for Ala at positions 180, 277, and 285, respectively, produces additive red shifts of 6, 9, and 15 nm, respectively.
19. R. E. Ibbotson, D. M. Hunt, J. K. Bowmaker, J. D. Mollon, *Proc. R. Soc. London Ser. B* **247**, 145 (1992); A. J. Williams, D. M. Hunt, J. D. Mollon, *EMBO J.* 11, 2039 (1992).
20. T. Chan, M. Lee, T. P. Sakmar, *J. Biol. Chem.* **267**, 9478 (1992).
21. A model in which the variant pigments underlying anomalous trichromacy correspond to the common allelic variants found in normal trichromats was first proposed by Alpern and colleagues [M. Alpern and J. Moeller, *J. Physiol. (London)* **266**, 647 (1977); M. Alpern and E. N. Pugh, *ibid.*, p. 613; M. Alpern and T. Wake, *ibid.*, p. 595; M. Alpern, *ibid.* **288**, 85 (1979)].
22. We thank C. Riley, A. Collector, and C. Waldron for synthesis of oligonucleotides; C. Gorman, A. Levinson, and R. Kline (Genentech) for making available the pCIS vector and 293S cell line; L. Stryer (Stanford University) and the Hoffman–La Roche Co. for gifts of 11-*cis*-retinal; and I. Chiu, J. Macke, and C. Weitz for comments on the manuscript. S.L.M. is a trainee of the Medical Scientist Training Program (NIH). Supported by the Howard Hughes Medical Institute and the National Eye Institute (NIH).

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Isomerase and Chaperone Activity of Prolyl Isomerase in the Folding of Carbonic Anhydrase

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Several proteins have been discovered that either catalyze slow protein-folding reactions or assist folding in the cell. Prolyl isomerase, which has been shown to accelerate rate-limiting *cis-trans* peptidyl-proline isomerization steps in the folding pathway, can also participate in the protein-folding process as a chaperone. This function is exerted on an early folding intermediate of carbonic anhydrase, which is thereby prevented from aggregating, whereas the isomerase activity is performed later in the folding process.

For many proteins, in vitro protein folding is a spontaneous process that does not require input of energy or any assisting factors (*1*). Folding in vivo, however, seems for many proteins to be mediated by several protein factors. These helper proteins are divided into two classes: (i) folding catalysts, such as the enzymes protein disulfide isomerase and prolyl isomerase (PPIase) and (ii) molecular chaperones that bind transiently to folding intermediates and thereby prevent incorrect interactions leading to aggregation (*2–5*). We have noted that with the use of PPIase in the study of the rate-limiting step of the refolding of human carbonic anhydrase II (HCA II), which contains 15 *trans*- and 2 *cis*-prolines (residues 30 and 202) (*6*), PPIase has a positive effect on both the kinetics and the yield of active HCA II (*7*). Because aggregation has been shown to lead to incomplete recovery of native HCA II in unassisted refolding of denatured enzyme (*8*), two questions arise. First, is the higher HCA II yield obtained in the presence of PPIase the result of a reduction of the time of exposure of hydrophobic surfaces in folding intermediates that is caused by catalysis of slow *cis-trans* isomerization reactions? That is, does PPIase facilitate the folding process in the way that is generally believed (*9*), or alternatively, does PPIase act as a chaperone by binding to an early folding intermediate, which is thereby protected from unproductive interactions that otherwise lead to aggregation?

PPIase accelerates the reactivation of denatured HCA II [1-hour incubation in 5 M guanidine hydrochloride (GuHCl)] and also increases the recovery of active enzyme: with PPIase, the half-time ($t_{1/2}$) of reactivation is lowered from 9 to 4 min, and the yield of enzyme is increased from 70 to 100% (Fig. 1). These stimulating effects are completely abolished when the PPIase inhibitor cyclosporin A (CsA) is added at the onset of refolding. No effect on the yield or the kinetics of the reactivation was noted when bovine serum albumin was added to the same concentration as that of PPIase, which rules out the possibility that these effects are caused by general protein interactions.

If HCA II is allowed to be in its denatured state (in 5 M GuHCl) for only 10 s, *cis-trans* isomerization of peptidyl-proline bonds is greatly limited ($t_{1/2}$ = ~2 min). However, within this 10-s incubation, full denaturation is achieved, as judged by activity and ultraviolet absorbance (absorbance = 292 nm) measurements. The reactivation of this short-time (10-s) denatured HCA II proceeds much faster ($t_{1/2}$ =

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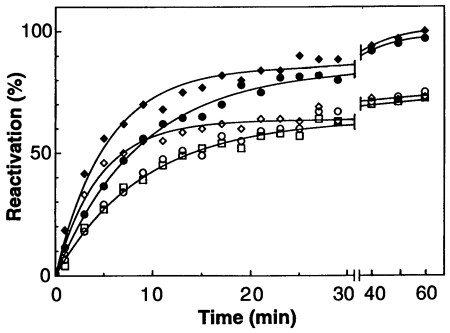

Fig. 1. Effect of PPIase on the time course of reactivation of long-time denatured HCA II. HCA II (14.0 μM, 0.42 mg/ml) was denatured for 1 hour in 5.0 M GuHCl. Reactivation was achieved by rapid dilution to 0.3 M GuHCl and a protein concentration of 0.83 μM (0.025 mg/ml). All solutions were buffered with 0.1 M tris-H_2SO_4 (pH 7.5) at 23°C. We monitored the reactivation process by measuring the CO_2-hydration activity (*21*). PPIase and CsA were used at concentrations of 8.8 μM and 27 μM, respectively. Reactivation of HCA II: □, without PPIase; ◆, with PPIase; ○, with PPIase and CsA; ●, with PPIase from the onset of reactivation and CsA added after 10 s of reactivation; and ◇, with PPIase added after 10 s of reactivation. HCA II was initially purified from erythrocytes by affinity chromatography (*22*) and further chromatographed on DEAE-cellulose (*23*). An extract of PPIase was prepared from pig kidney cortex according to Fischer *et al.* (*24*). The chromatographic purification was performed as described by Takahashi *et al.* (*25*) with minor modifications (*7*). The chromatographic separation resulted in several fractions with PPIase activity, as noted earlier (*26*). The PPIases tested (with molecular masses of 17 and 22 kD) gave identical results in all experiments performed in this study. To make sure that no impurity caused the chaperone effect observed, we chromatographed aliquots of PPIase by high-performance liquid chromatography on Fractogel TSK G2000SW (LKB, Bromma, Sweden) and MonoS (Pharmacia). In all instances, fractions that contained proline isomerase activity exhibited chaperone activity.

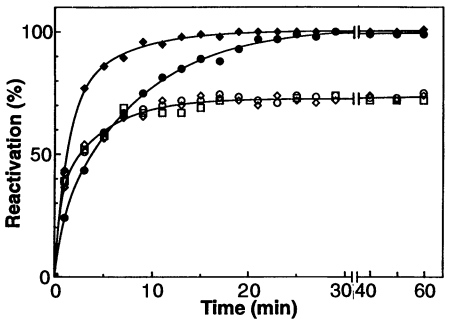

Fig. 2. Effect of PPIase on the time course of reactivation of short-time denatured HCA II. HCA II was denatured for 10 s in 5.0 M GuHCl. All other experimental conditions were as in Fig. 1. Reactivation of HCA II: □, without PPIase; ◆, with PPIase; ○, with PPIase and CsA; ●, with PPIase from the onset of reactivation and CsA added after 3 s of reactivation; and ◇, with PPIase added after 10 s of reactivation.

1 min) than after 1 hour of denaturation, but the yield of active enzyme is not increased (Fig. 2). Hence, the incomplete yield is not a result of slow *cis-trans* isomerization of peptidyl-proline bonds during refolding because almost all of the native peptidyl-proline conformers should be maintained during the short period of time in the denatured state. Nevertheless, the presence of PPIase during the refolding, after denaturation for 10 s, increases the yield of active HCA II to 100%. In contrast, the reactivation kinetics are not influenced by PPIase in this case: $t_{1/2}$ (1 min) is the same as without PPIase (Fig. 2). If both PPIase and the inhibitor CsA are added at the onset of the refolding reaction, PPIase has no effect on the recovery of active HCA II (70%) and the kinetics of reactivation—that is, the reaction proceeds as if uncatalyzed (Fig. 2). From this result, it is evident that PPIase contributes to the higher yield of refolded HCA II not by accelerating any rate-limiting proline isomerization step, but by protecting an intermediate on the folding pathway, thereby eliminating competing side reactions such as aggregation. Thus, in addition to its well-documented function as an isomerase, PPIase can also act as a classical chaperone.

In order to gain insight into how and when PPIase mediates its chaperone effect during the course of HCA II folding, we studied the effect of adding PPIase and CsA at different times during the refolding process. If PPIase is added 10 s after the initiation of refolding of short-time (10-s) denatured HCA II, the yield of active enzyme is not improved as compared to the yield (70%) in the absence of PPIase (Fig. 2). This result implies that the chaperone effect is exerted by binding to an early folding intermediate (I_1; Fig. 3) and that the formed aggregates cannot be dissociated by PPIase. This result is in agreement with the action of the chaperone GroE, which apparently cannot rescue aggregated proteins and is active only during the early steps of protein folding (9). On the other hand, if PPIase is present from the start of the refolding process but is inhibited by the addition of CsA 3 s later, HCA II is fully reactivated (Fig. 2). This further demonstrates that it is both sufficient and necessary for PPIase to interact with a very early folding intermediate (I_1) to protect it from unfavorable interactions.

Another observed effect is that the $t_{1/2}$ of reactivation of the short-time denatured HCA II is increased from 1 to 5 min when PPIase is inhibited after 3 s of folding as compared to when PPIase is absent or is present throughout the folding process (Fig. 2). If PPIase is inhibited after 3, 50, or 150 s of refolding, the $t_{1/2}$ of reactivation is 1.3 min up to the point of addition of CsA, after which it is 5.0 min. Thus, the presence of active PPIase, even for only 3 s, seems to facilitate the *cis-trans* equilibration of a fraction of the prolines in HCA II (P^A; Fig. 3) and thereby increase the $t_{1/2}$ of reactivation from 1 to 5 min if PPIase is subsequently inhibited ($I_2 \rightarrow N$ in Fig. 3).

The $t_{1/2}$ of reactivation is 1 min if the conformations of the prolines are maintained, as is the case after 10 s of denaturation (Fig. 2). Conversely, if the prolines are allowed to *cis-trans* equilibrate (1 hour of denaturation), the $t_{1/2}$ of reactivation is 9 min without PPIase. When PPIase is present throughout the refolding, the $t_{1/2}$ of reactivation is 4 min (Fig. 1) instead of 1 min as would be expected if PPIase could isomerize all of the prolines. This result supports the conclusion above that not all prolines are equilibrated by PPIase during

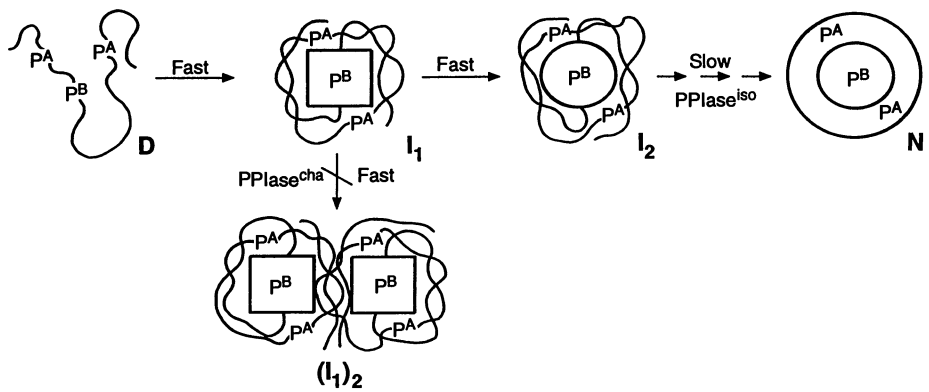

Fig. 3. Schematic drawings of proline folding intermediates of HCA II. D, denatured state; N, native state; I_1 and I_2, folding intermediates; $(I_1)_2$, aggregate. P^A and P^B denote accessible and buried prolines, respectively. The chaperone effect is illustrated by $PPIase^{cha}$, and the peptidyl-proline *cis-trans* isomerase activity is illustrated by $PPIase^{iso}$. By binding to the aggregation-prone intermediate I_1, PPIase lowers the concentration of this intermediate, and thus the formation of the aggregates $(I_1)_2$ will be minimized.

the refolding of the short-time denatured enzyme, but that a fraction of the prolines must be very rapidly buried during the initiation of the refolding process (P^B in Fig. 3) and thus become inaccessible to PPIase. It has been reported (*10–12*) that an initiation structure of carbonic anhydrase is rapidly formed during refolding. When the prolines in this core of the enzyme structure (P^B in Fig. 3) are *cis-trans* equilibrated by 1 hour of denaturation, the isomerization of these residues to the native conformers limits the rate of reactivation ($t_{1/2}$ = 4 min) in the presence of PPIase. However, when the prolines in this core are in correct conformation from the beginning of refolding and all the other prolines are *cis-trans* equilibrated by the action of PPIase only during the first 3 s of refolding, uncatalyzed isomerization of the PPIase-accessible prolines (P^A; Fig. 3) is rate determining ($t_{1/2}$ = 5 min) in the reactivation process.

Thus, the action of PPIase on the refolding HCA II reveals the presence of two classes of prolines (accessible and buried), as evidenced by the impact of PPIase on the refolding kinetics. For ribonuclease T_1, the structural context of prolines is important for the efficiency of catalysis (*13*). That PPIase acts as a classical chaperone is clearly demonstrated also by its independent effect on the kinetics and the yield of the refolding reaction. This effect is exemplified in Fig. 1: after 10 s of refolding, inhibition of PPIase still gives rise to a 100% yield and a relatively slow $t_{1/2}$ (9 min; chaperone), whereas addition of PPIase after 10 s of refolding does not increase the yield but does result in a shorter $t_{1/2}$ (4 min; isomerase).

Two mechanisms proposed for the action of PPIase are catalysis by distortion (*14*) and catalysis by desolvation (*15–17*). The latter mechanism was suggested because binding of a peptide segment into a hydrophobic environment, as in the binding site of PPIase (*18*), promotes *cis-trans* isomerization by decreasing the charge separation of the peptide bond and thus creating a peptide that has a more single-bond character. Hence, if this in fact is the mechanism for PPIase activity, then other chaperones that bind peptide chains to apolar sites (*19, 20*) might also possess isomerase activity. Because PPIase (which is also cyclophilin) is known to be involved in T cell activation, it is possible that the chaperone function of this protein is important for essential protein-folding processes in the immune response.

REFERENCES AND NOTES

1. R. Jaenicke, *Prog. Biophys. Mol. Biol.* **49**, 117 (1987).
2. M.-J. Gething and J. Sambrook, *Nature* **355**, 33 (1992).
3. R. J. Ellis and S. M. van der Vies, *Annu. Rev. Biochem.* **60**, 321 (1991).
4. F. X. Schmid, *Curr. Opin. Struct. Biol.* **1**, 36 (1991).
5. G. Fischer and F. X. Schmid, *Biochemistry* **29**, 2205 (1990).
6. A. E. Eriksson, T. A. Jones, A. Liljas, *Proteins Struct. Funct. Genet.* **4**, 274 (1988).
7. C. Fransson *et al.*, *FEBS Lett.* **296**, 90 (1992).
8. J. L. Cleland and D. I. C. Wang, *Biochemistry* **29**, 11072 (1990).
9. J. Buchner *et al.*, *ibid.* **30**, 1586 (1991).
10. U. Carlsson, R. Aasa, L. E. Henderson, B.-H. Jonsson, S. Lindskog, *Eur. J. Biochem.* **52**, 25 (1975).
11. N. Bergenhem, U. Carlsson, J.-Å. Karlsson, *Int. J. Pept. Protein Res.* **33**, 140 (1989).
12. O. B. Ptitsyn, *FEBS Lett.* **285**, 176 (1991).
13. T. Kiefhaber, R. Quaas, U. Hahn, F. Schmid, *Biochemistry* **29**, 3061 (1990).
14. R. K. Harrison and R. L. Stein, *ibid.*, p. 1684.
15. R. Wolfenden and A. Radzicka, *Chemtracts Biochem. Mol. Biol.* **2**, 52 (1991).
16. T. Drakenberg, K.-I. Dahlqvist, S. J. Forsén, *J. Phys. Chem.* **76**, 2178 (1972).
17. R. Wolfenden, *Science* **222**, 1087 (1983).
18. J. Kallen *et al.*, *Nature* **353**, 276 (1991).
19. H. R. B. Pelham, *Cell* **46**, 959 (1986).
20. G. C. Flynn, J. Pohl, M. T. Flocco, J. E. Rothman, *Nature* **353**, 726 (1991).
21. P.-O. Freskgård, U. Carlsson, L G. Mårtensson, B.-H. Jonsson, *FEBS Lett.* **289**, 117 (1991).
22. R. G. Khalifah, D. J. Strader, S. H. Bryant, S. M Gibson, *Biochemistry* **16**, 2241 (1977).
23. L. E. Henderson and D. Henriksson, *Anal. Biochem.* **51**, 288 (1973).
24 G. Fischer, H. Bang, C. Mech, *Biomed. Biochim. Acta* **43**, 1101 (1984).
25. N. Takahashi, T. Hayano, M. Suzuki, *Nature* **337**, 473 (1989).
26. G. Fischer, B. Wittmann-Liebold, K. Lang, T. Kiefhaber, F. X. Schmid, *ibid.*, p. 476.

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Spontaneous Hypercholesterolemia and Arterial Lesions in Mice Lacking Apolipoprotein E

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Apolipoprotein E (apoE) is a ligand for receptors that clear remnants of chylomicrons and very low density lipoproteins. Lack of apoE is, therefore, expected to cause accumulation in plasma of cholesterol-rich remnants whose prolonged circulation should be atherogenic. ApoE-deficient mice generated by gene targeting were used to test this hypothesis and to make a mouse model for spontaneous atherosclerosis. The mutant mice had five times normal plasma cholesterol, and developed foam cell–rich depositions in their proximal aortas by age 3 months. These spontaneous lesions progressed and caused severe occlusion of the coronary artery ostium by 8 months. The severe yet viable phenotype of the mutants should make them valuable for investigating genetic and environmental factors that modify the atherogenic process.

Atherosclerotic cardiovascular disease, the major cause of death in Western society, results from complex interactions among multiple genetic and environmental factors (*1*). Among the factors that have been identified to date are changes in the genes involved in lipid metabolism, including the gene encoding apolipoprotein E (apoE) (*2*). ApoE is a glycoprotein with a molecular size of approximately 34 kD that is synthesized in the liver, brain, and other tissues in both humans and mice (*3*); it is a structural component of all lipoprotein particles other than low density lipoprotein (LDL). One of its most important functions is to serve as a high affinity ligand for the apoB and apoE(LDL) receptor and for the chylomicron-remnant receptor, thereby allowing the specific uptake of apoE-containing particles by the liver.

A frequent genetic variant of human apoE, apoE-2, differs from the most common form, apoE-3, by having cysteine at position 158 in place of arginine. This amino acid substitution in the LDL receptor binding region reduces the binding ability of apoE-2 to less than 2% relative to that of apoE-3. Homozygosity for the gene $Apoe^2$ is associated with type III hyperlipoproteinemia, which is characterized by increased plasma triglyceride and cholesterol levels, yellow lipid-laden xanthomatous skin nodules, and the early development of atherosclerosis (*4*). The complexities of the pathogenesis of this disease are, however, illustrated by the fact that only about 2% of $Apoe^2/Apoe^2$ individuals develop hyperlipoproteinemia. The majority of individuals

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