Molecular Chaperone Machines: Chaperone Activities of the Cyclophilin Cyp-40 and the Steroid Aporeceptor–Associated Protein p23

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Molecular chaperones are essential proteins that participate in the regulation of steroid receptors in eukaryotes. The steroid aporeceptor complex contains the molecular chaperones Hsp90 and Hsp70, p48, the cyclophilin Cyp-40, and the associated proteins p23 and p60. In vitro folding assays showed that Cyp-40 and p23 functioned as molecular chaperones in a manner similar to that of Hsp90 or Hsp70. Although neither Cyp-40 nor p23 could completely refold an unfolded substrate, both proteins interacted with the substrate to maintain a nonnative folding-competent intermediate. Thus, the steroid aporeceptor complexes have multiple chaperone components that maintain substrates in an intermediate folded state.

Although the general biochemical properties of certain molecular chaperones are well established, much less is known about how different chaperones interact with nonnative proteins in transient or stable complexes (*1*). Within the eukaryotic cytosol, heteromeric complexes containing chaperones and other accessory proteins have been identified, although their function remains uncharacterized. Perhaps the best studied of these complexes are steroid aporeceptors that contain the heat shock proteins Hsp90 and Hsp70, a DnaJ protein (Hdj-1), p60 (Sti1), p48 (HiP), p23, and the immunophilins (FKBP54, FKBP52, or Cyp-40) (*2*). The association of the chaperones Hsp90 and Hsp70 with glucocorticoid and progesterone aporeceptors serves to maintain an inert high-affinity hormone-binding state, which is activated by the appropriate hormone signal (*3*). The p23 protein, which interacts with Hsp90, is also required for high-affinity hormone binding; in the presence of the benzoquinoid ansamycin geldanamycin, interactions between p23 and Hsp90 are disrupted (*4*). Although it has been established that Hsp90, p48, and Hsp70 can function as chaperones or co-chaperones (*5*, *6*) and that the immunophilins are peptidylprolyl cis-trans isomerases (*7*), the functional properties of the aporeceptor-associated proteins p60 and p23 remain uncertain.

To establish whether Cyp-40, p23, or p60 exhibited properties of molecular chaperones, we purified these proteins to homogeneity and examined their activities in the refolding of a denatured protein substrate alone or in conjunction with Hsp70 and Hdj-1 (Fig. 1) (*8*). A step in the chaperone-dependent folding reaction in which Hsp90 exhibits a preferential ability to interact with a denatured protein substrate to maintain a nonnative folding-competent intermediate has been characterized (*6*). This intermediate state corresponds to a functional nonnative protein that can undergo additional folding events (promoted by Hsp70 and Hdj-1) that lead to the appearance of the enzymatically active native protein. Guanidine hydrochloride–denatured β-galactosidase (β-Gal) did not spontaneously refold, even in the presence of Hsp90, Hsp70, Cyp-40, p23, p60, or Hdj-1 (<6% activity; Fig. 1A). However, in combination with Hsp70, Hdj-1, and nucleotide, ~54% of the native β-Gal activity was recovered (Fig. 1B). The addition of Hsp90, p60, Cyp-40, or p23 neither enhanced nor inhibited the refolding activity of Hsp70 and Hdj-1 (Fig. 1B). As these experiments were performed at a high molar excess (500:1) of Hsp70 and Hdj-1 relative to the unfolded β-Gal, the effects of Cyp-40, p23, and p60 may not be apparent. Therefore, additional experiments were performed with elevated concentrations of β-Gal (up to 12:1) in which we observed a 2.5-fold stimulatory effect of p23 on the refolding of β-Gal, whereas Hsp90, p60, and Cyp-40 had no effect (*9*).

To determine whether Cyp-40, p23, and p60 exhibited the maintenance activity common to Hsp90 and Hsp70, we performed order-of-addition experiments in which unfolded β-Gal was diluted into buffer containing various chaperones at 37°C. The ability of p60, Cyp-40, or p23 to interact productively with a nonnative protein was determined by dilution of the unfolded β-Gal into refolding buffer supplemented with Hsp90, Hsp70, p60, Cyp-40, Hdj-1, or p23. After incubation at 37°C, the reaction was supplemented with Hsp70, Hdj-1, or both and the recovery of β-Gal activity was monitored. Hsp90 was highly effective in this chaperone maintenance and refolding assay (Fig. 2). In the presence of Hsp90, β-Gal was maintained in a folding-competent state throughout a 2-hour period at 37°C; likewise, both Cyp-40 and p23 were effective in maintaining β-Gal in a folding-competent state (at a 25:1 or 100:1 ratio, respectively), albeit at an intermediate efficiency relative to that of Hsp90 at equivalent concentrations (Fig. 2) (*6*). This maintenance by Cyp-40 and p23 of the intermediate folded state was not dependent on nucleotide. Moreover, the ability of Cyp-40 to interact productively with the denatured substrate was not affected by the immunosuppressant drug cyclosporin A, which suggests that the Cyp-40 chaperone activity is not dependent on peptidylprolyl cis-trans isomerase activity (*9*). In contrast to the chaperone activities of Cyp-40 and p23, p60 (at concentrations up to ~1000:1 molar excess) did not interact productively with denatured β-Gal (Fig. 2). Thus, p60 and Hdj-1 did not exhibit any activity as a

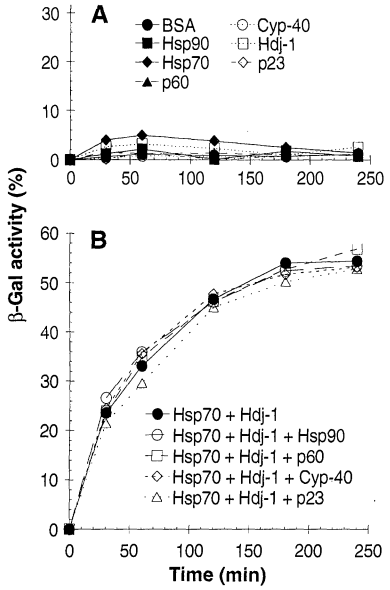

Fig. 1. Cyp-40, p60, or p23 neither independently refold denatured β-Gal nor stimulate the refolding activity of Hsp70 and Hdj-1. (**A**) Incubation of denatured β-Gal with individual components (3.2 μM Hsp90, Hsp70, Cyp-40, p60, Hdj-1, or p23) is insufficient to promote recovery of native β-Gal activity. (**B**) Addition of Hsp90, Cyp-40(GST), p60, or p23 to a refolding reaction does not stimulate the refolding activity of Hsp70 and Hdj-1. The effect of the various components on refolding mediated by Hsp70 and Hdj-1 was determined by diluting the denatured β-Gal into refolding buffer containing only Hsp70 (1.6 μM) and Hdj-1 (3.2 μM) or buffer supplemented with 3.2 μM Hsp90, p60, Cyp-40(GST), or p23.

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molecular chaperone in this refolding assay.

We next investigated the nature of the interaction between Cyp-40 or p23 and unfolded β-Gal in the maintenance chaperone activity. Dilution of the unfolded substrate into buffer containing Hsp90, Hsc70, or Hsp70 prevents aggregation of the nonnative β-Gal and mediates an apparent collapse of the unfolded β-Gal to a premonomer state (6). The relative effectiveness of the molecular chaperones Hsp90, Hsp70, Hsc70, Cyp-40, and p23 in maintaining the nonnative β-Gal in a folding-competent state may indicate that each protein interacts in a distinct manner with the denatured substrate. We resolved the chaperones and β-Gal by native polyacrylamide gel electrophoresis (PAGE) or treated the reaction mixture with chymotrypsin. The resulting full-length β-Gal or proteolytic fragments of β-Gal were detected by protein immunoblot analysis (Fig. 3) (*10*). Dilution of unfolded β-Gal into Hsc70, for example, maintained β-Gal in a soluble nonnative folded state that could enter a native gel (Fig. 3A) and was relatively resistant to protease digestion (Fig. 3B) (6). Interaction between Cyp-40 and unfolded β-Gal also resulted in a soluble substrate that was relatively resistant to proteolysis. In contrast, β-Gal coincubated with either p23 or p60 did not enter the native gel matrix and was sensitive to protease digestion (Fig. 3, A and B). These results are consistent with the idea that the denatured β-Gal, in the presence of p23 and p60, aggregates to a high–molecular mass complex that cannot enter the native gel. To test this hypothesis, we incubated denatured β-Gal for 2 hours at 37°C in the presence of bovine serum albumin (BSA), Hsc70, p60, Cyp-40, Hdj-1, or p23 and clarified the reactions by centrifugation to separate the insoluble aggregated protein. Samples from the supernatant and pellet fractions were resolved by SDS-PAGE, and the β-Gal was detected by protein immunoblot analysis (Fig. 3C). Native β-Gal was found entirely in the supernatant, whereas the denatured β-Gal aggregated in the presence of BSA, p60, or Hdj-1 and was recovered in the pellet. In the presence of Hsc70, Cyp-40, or p23, the β-Gal was detected in the supernatant fraction (Fig. 3C), consistent with the partial maintenance of the nonnative β-Gal in a folding-competent state (Fig. 2) (6). Upon addition of Hsp70 and Hdj-1, β-Gal enzymatic activity was recovered only from the Hsc70, Cyp-40, and p23 supernatant fractions (9).

These results indicate that Cyp-40 shares features in common with Hsp90, Hsc70, and Hsp70 by at least three criteria of chaperone function (protein refolding competency, solubility, and folding to a native-like proteolysis-resistant state). Although p23 was as effective as Cyp-40 in maintaining the intermediate nonnative state of β-Gal and slightly stimulated Hsp70 and Hdj-1 refolding activity, there were critical features of the substrate-p23 interaction that were distinct. In contrast to Cyp-40, Hsp90, and Hsp70, the interaction between p23 and denatured β-Gal did not lead to the collapse of β-Gal to a proteolysis-resistant form that could readily enter a native gel matrix despite its apparent soluble state. This may suggest that p23 forms a stable high–molecular mass complex with nonnative β-Gal that is incapable of entering a native gel matrix. The sensitivity of the nonnative β-Gal to protease digestion in the presence of p23 suggests that the substrate is in an extended conformation. To address whether maintenance of the nonnative β-Gal in an extended conformation by p23 requires direct association, we used the chemical crosslinkers glutaraldehyde and ethylene glycol-bis(succinimidyl) succinate (EGS) to demonstrate that the nonnative β-Gal formed a high–molecular mass complex with p23 (9).

Our observations establish a role for Cyp-40 and p23 as molecular chaperones, yet p23 interacts with the nonnative substrate in a manner distinct from that of Cyp-40 and other chaperones. In contrast, p60 does not function as a chaperone despite its proposed role in complex formation between Hsp90 and Hsp70 (2). Perhaps p60 serves to organize the molecular chaperones into a functional unit and thereby enhances their combined activities. However, we do not detect p60-dependent synergistic effects on Hsp90 and Hsp70 chaperone activities (9). Thus, we propose that interactions between a nonnative protein and the molecular chaperones can have at least three distinct fates. Dilution of denatured β-Gal at permissive temperatures (22° to 41°C) into Hsp70, Hdj-1, and nucleotide results in

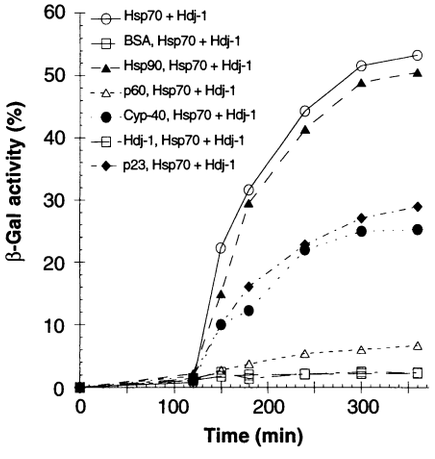

Fig. 2. Cyp-40 and p23 can maintain nonnative β-Gal in a folding-competent nonnative state over an extended period of time at 37°C. Denatured β-Gal (final concentration, 3.4 nM) was diluted 1:125 into refolding buffer containing 1.6 μM p60, Cyp-40(GST), Hdj-1, or p23, and incubated at 37°C. After 2 hours, Hsp70 (1.6 μM) and Hdj-1 (3.2 μM) were added and β-Gal activity was measured. As a positive control for folding activity, a β-Gal refolding reaction was initiated at the 2-hour time point by dilution of denatured β-Gal directly into refolding buffer supplemented with Hsp70 (1.6 μM) and Hdj-1 (3.2 μM). As controls for chaperone maintenance activity, the denatured β-Gal (final concentration, 3.4 nM) was diluted into refolding buffer containing 1.6 μM BSA, Hdj-1, or Hsp90 and incubated at 37°C. After 2 hours, Hsp70 (1.6 μM) and Hdj-1 (3.2 μM) were added and β-Gal activity was measured at the indicated time points.

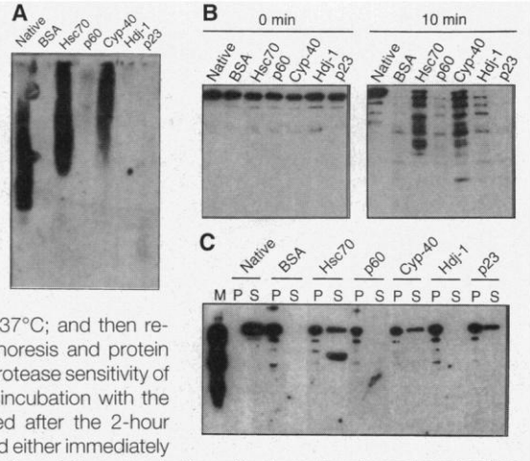

Fig. 3. Cyp-40 maintains β-Gal in a soluble and proteolysis-resistant state, whereas p23 does not have a similar effect. Denatured β-Gal was analyzed after incubation with BSA, Hsc70, p60, Cyp-40, Hdj-1, or p23 by native PAGE, limited proteolysis, or centrifugation to resolve the soluble and pelleted material. (**A**) Native gel analysis of the soluble fraction of nonnative β-Gal. Denatured β-Gal (final concentration, 68 nM) was diluted 1:125 into refolding buffer containing 3.2 μM BSA, Hsc70, p60, Cyp-40, Hdj-1, or p23; incubated 2 hours at 37°C; and then resolved on native acrylamide gel electrophoresis and protein immunoblot analysis with anti–β-Gal. (**B**) Protease sensitivity of the denatured β-Gal was determined by incubation with the protease chymotrypsin, which was added after the 2-hour incubation at 37°C. Samples were removed either immediately (0 min) before addition of chymotrypsin or after a 10-min incubation with chymotrypsin and resolved on 10% SDS-PAGE, and β-Gal was detected by protein immunoblot analysis with anti–β-Gal. (**C**) Separation of the nonnative β-Gal into soluble and pellet fractions by centrifugation. As a third assay of the folded state of the nonnative β-Gal, the reactions were separated into soluble or pellet fractions and resolved on 10% SDS-PAGE, and β-Gal was detected by protein immunoblot analysis with anti–β-Gal. M, molecular weight marker; P, pellet fraction; and S, supernatant fraction.

the recovery of the native enzymatically active tetramer (6). Alternatively, interaction with the individual chaperones Hsp90, Hsc70, Hsp70, or Cyp-40 does not lead to refolding of the denatured substrate to its native state, but rather leads to an apparent collapse of the denatured β-Gal to a stable proteolysis-resistant nonnative intermediate that is subsequently responsive to the refolding activity of Hsp70 and Hdj-1. We suggest that the interaction between p23 and denatured β-Gal represents a distinct activity that results in the maintenance of the β-Gal in a proteolysis-sensitive, yet soluble, nonnative state that can be converted to the native state upon addition of Hsp70 and Hdj-1. These studies identify new members of the family of proteins that act as molecular chaperones. The involvement of multiple proteins with apparently redundant chaperone activities in heteromeric complexes may provide diversity and specificity in the regulation of the biological activity of associated protein substrates. This may have implications for pathways of hormonal regulation, signal transduction, and immunosuppression (*11*).

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8. The substrate (β-Gal) was denatured at 30°C for 30 min in 6 M guanidinium hydrochloride, diluted 1:125 into refolding buffer [25 mM Hepes (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM adenosine triphosphate] to a final concentration of 3.4 nM β-Gal, and supplemented with BSA, Hsp90, Hsp70, p60, Cyp-40, Hdj-1, or p23, or various combinations of chaperones. The refolding reactions were incubated at 37°C for 240 min and aliquots (10 μl) were taken at various time points. The percent enzyme activity was determined after incubation with orthonitrophenyl galactoside and measurement of absorbance at 412 nm. The results are presented as percent enzyme activity relative to the same amount of native β-Gal (3.4 nM) in refolding buffer containing BSA (1.6 μM). The molecular chaperones Hsp90, Hsp70, and Hdj-1 were purified to homogeneity as described (*6*). Cyp-40 [L. J. Kieffer *et al.*, *J. Biol. Chem.* **268**, 12303 (1993)] was purified with a Resource Q column and used as a glutathione-S-transferase (GST) fusion protein. The p60 [D. F. Smith *et al.*, *Mol. Cell. Biol.* **13**, 869 (1993)] and p23 [J. L. Johnson, T. G. Bieto, C. J. Krco, D. O. Toft, *ibid.* **14**, 1956 (1994)] proteins were recombinantly expressed and purified by sequential passages over DEAE, Resource Q, and Superdex-200 columns (Pharmacia Biotech).
9. B. C. Freeman and R. I. Morimoto, unpublished observations.
10. The folded state of denatured β-Gal diluted into BSA, Hsc70, p60, Cyp-40, Hdj-1, or p23 was characterized by electrophoresis on native 4% polyacrylamide Na-borate (0.1 M sodium acetate and 0.1 M boric acid) gels at 4°C at 1 mm/min (*6*) or by separation of the soluble and insoluble fractions of β-Gal by centrifugation at 13,000 rpm for 5 min and separation with 10% SDS-PAGE. The β-Gal was detected by protein immunoblot analysis after electroblotting onto nitrocellulose, incubation with an antibody to β-Gal (anti–β-Gal), and detection by ECL (Amersham).
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Influence of Nitrogen Loading and Species Composition on the Carbon Balance of Grasslands

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In a 12-year experimental study of nitrogen (N) deposition on Minnesota grasslands, plots dominated by native warm-season grasses shifted to low-diversity mixtures dominated by cool-season grasses at all but the lowest N addition rates. This shift was associated with decreased biomass carbon (C):N ratios, increased N mineralization, increased soil nitrate, high N losses, and low C storage. In addition, plots originally dominated by nonnative cool-season grasses retained little added N and stored little C, even at low N input rates. Thus, grasslands with high N retention and C storage rates were the most vulnerable to species losses and major shifts in C and N cycling.

Humans have dramatically altered the cycling of nitrogen on Earth, doubling the natural rate of N fixation and causing atmospheric N deposition rates to increase more than tenfold over the last 40 years to current values of 0.5 to 2.5 g N m^{-2} $year^{-1}$ in eastern North America and 0.5 to 6.0 g N m^{-2} $year^{-1}$ in northern Europe (*1*). Because N is the primary nutrient limiting terrestrial plant production, N addition is causing shifts in plant species composition, decreases in species diversity, and changes in food-web structure in terrestrial ecosystems (*2–5*). This N-driven terrestrial eutrophication parallels phosphorus-driven eutrophication in lakes. Increased N deposition may lead to greater C storage in soil organic matter and vegetation, thus providing a sink for CO_2 and potentially explaining the globally "missing C" (*6*). Despite this, almost no experimental data exist on changes in ecosystem C in response to long-term N addition in nonagricultural ecosystems; rather, effects on C stores have been estimated from models, giving divergent predictions (*6*).

We present results of 12 years of experimental N addition to 162 grassland plots in three N-limited Minnesota grasslands that varied in successional age, total soil C, and plant species composition (*7, 8*). The youngest field (Field A) was dominated by vegetation with the C_3 photosynthetic pathway, primarily nonnative "cool-season" grasses and forbs, whereas the two older fields (Fields B and C) were dominated by native C_4 "warm-season" prairie grasses. Because other potentially limiting nutrients were supplied and soil pH was controlled, our study addresses the eutrophication effects of N loading while controlling for acidification and related biogeochemical effects that might also affect natural ecosystems (*9, 10*).

Nitrogen loading dramatically changed plant species composition, decreased species diversity, and increased aboveground productivity in these plots (*2, 7, 11*). After 12

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