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The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding

Brian C.Freeman and Richard I.Morimoto[1]

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL 60208, USA

[1]Corresponding author

The properties of molecular chaperones in protein-assisted refolding were examined *in vitro* using recombinant human cytosolic chaperones hsp90, hsc70, hsp70 and hdj-1, and unfolded β-galactosidase as the substrate. In the presence of hsp70 (hsc70), hdj-1 and either ATP or ADP, denatured β-galactosidase refolds and forms enzymatically active tetramers. Interactions between hsp90 and non-native β-galactosidase neither lead to refolding nor stimulate hsp70- and hdj-1-dependent refolding. However, hsp90 in the absence of nucleotide can maintain the non-native substrate in a 'folding-competent' state which, upon addition of hsp70, hdj-1 and nucleotide, leads to refolding. The refolding activity of hsp70 and hdj-1 is effective across a broad range of temperatures from 22°C to 41°C, yet at extremely low (4°C) or high (>41°C) temperatures refolding activity is reversibly inhibited. These results reveal two distinct features of chaperone activity in which a non-native substrate can be either maintained in a stable folding-competent state or refolded directly to the native state; first, that the refolding activity itself is temperature sensitive and second, that hsp90, hsp70 (hsc70) and hdj-1 each have distinct roles in these processes.

Keywords: heat shock protein/hsp90/hsp70/molecular chaperone/protein folding

Introduction

Molecular chaperones, some of which are also heat shock proteins, participate in the biogenesis of proteins including their synthesis, folding, assembly, disassembly and translocation (Lindquist and Craig, 1988; Gething and Sambrook, 1992; Georgopoulos and Welch, 1993; Hendrick and Hartl, 1993; Morimoto *et al.*, 1994). Despite the apparent diversity of these activities, molecular chaperones may serve a common function to influence the conformational state of many proteins.

The role of molecular chaperones in protein folding has been established primarily by *in vitro* assays using idealized protein substrates or by genetic approaches (Bochkareva *et al.*, 1992; Langer *et al.*, 1992; Horwich *et al.*, 1993; Schroder *et al.*, 1993; Craig *et al.*, 1994; Georgopoulos *et al.*, 1994; Freeman *et al.*, 1995). For example, the *Escherichia coli* hsp70 homolog, dnaK, can reactivate heat inactivated RNA polymerase (Skowyra *et al.*, 1988; Ziemienowicz *et al.*, 1993), furthermore, the reactivation of RNA polymerase is stimulated by the co-chaperones dnaJ and grpE (Skowyra *et al.*, 1988; Ziemienowicz *et al.*, 1993). Additional studies have suggested that the dnaK/dnaJ/grpE (hsp70/hsp40) chaperone machine may function in concert with groEL/ES (TriC) (Langer *et al.*, 1992; Frydmann *et al.*, 1994). dnaK (hsp70) and/or dnaJ (hsp40) have been shown to interact with the substrate early in the (re)folding reaction (i.e. immediately upon dilution from a chaotrope or during translational elongation) such that the substrate is then passed to the groEL/ES (TriC) chaperone machine for completion of the (re)folding reaction (Langer *et al.*, 1992; Frydmann *et al.*, 1994). Although a sequential chaperone refolding pathway can occur, separately the dnaK and groEL chaperone machines can refold substrate proteins (Goloubinoff *et al.*, 1989; Skowyra *et al.*, 1990; Buchner *et al.*, 1991; Martin *et al.*, 1993; Ziemienowicz *et al.*, 1993; Freeman *et al.*, 1995; Mayhew *et al.*, 1996; Weissman *et al.*, 1996).

The cytoplasm of eukaryotic cells contains a number of prominent chaperones including the homologs of dnaK (hsp70 family), dnaJ, groEL (TriC), htpG (hsp90), hsp100 and small heat shock proteins (Morimoto *et al.*, 1994). The eukaryotic 70 kDa chaperones, in conjunction with the eukaryotic homolog of dnaJ, have also been shown to efficiently refold denatured substrate proteins *in vitro* (Freeman *et al.*, 1995; Levy *et al.*, 1995). Their role in protein biogenesis has been demonstrated by both biochemical and genetic assays (Brodsky and Schekman, 1994; Craig *et al.*, 1994; Frydmann and Hartl, 1994; Langer and Neupert, 1994). Association of the 70 kDa chaperones with nascent polypeptides and other components of the translation machinery has been reported (Beckmann *et al.*, 1990; Nelson *et al.*, 1992; Frydmann *et al.*, 1994). The eukaryotic dnaJ homologs interact with the translational machinery (Zhong and Arndt, 1993; Frydmann *et al.*, 1994), however, direct *in vitro* demonstration that hdj-1 functions as a chaperone has not been reported. Hsp90 has not been detected as a component of the translational machinery (Frydmann *et al.*, 1994) however, hsp90 has been shown to function as a chaperone *in vitro* (Wiech *et al.*, 1992; Jakob *et al.*, 1995). *In vivo*, the chaperone activities of hsp90 include interactions with steroid aporeceptors, tubulin, oncogenic tyrosine kinases and cellular serine-threonine kinases (Rose *et al.*, 1987; Sanchez *et al.*, 1988; Miyata and Yahara, 1992; Doyle and Bishop, 1993; Smith and Toft, 1993; Xu and Lindquist, 1993; Stancato *et al.*, 1993; Cuttforth and Rubin, 1994; Pratt and Welsh, 1994; Wartmann and Davis, 1994; Nathan and Lindquist, 1995).

In this study, we have investigated the biochemical properties of the cytosolic human chaperones hsp90, hsp70, hsc70 and hdj-1, by monitoring their effects individually and in various combinations, on the fate of the denatured substrate β-galactosidase. Although hsp90

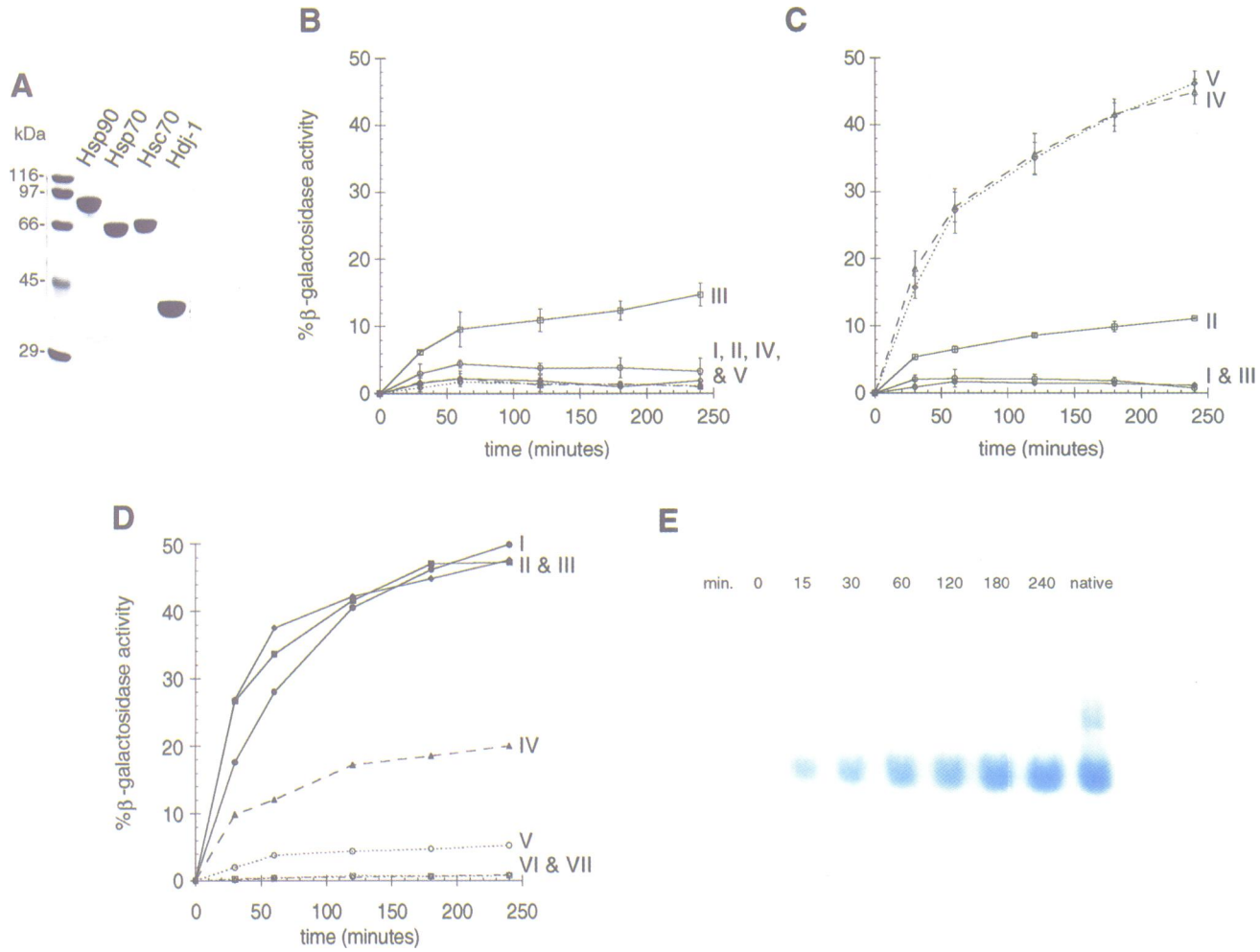

Fig. 1. The 70 kDa chaperones in cooperation with hdj-1 can efficiently refold guanidinium hydrochloride-denatured β-galactosidase. Recombinant hsp90, hsp70, hsc70 and hdj-1 (2 μg of each) were resolved on a 10% SDS–PAGE and stained with Coomassie Blue (**A**). (**B**) Guanidinium hydrochloride-denatured β-galactosidase was diluted 125-fold to a final concentration of 3.4 nM in refolding buffer supplemented with either 1.6 μM hsp70 (I), 1.6 μM hsc70 (II), 3.2 μM hdj-1 (III) or 3.2 μM hsp90 (IV), and incubated at 37°C for the indicated times. The percent activities were derived relative to the activity of native β-galactosidase (3.4 nM) in refolding buffer supplemented with BSA (3.2 μM) utilizing ONPG as the chromogenic substrate. Error bars are indicated. Dilution of the denatured β-galactosidase into refolding buffer supplemented with concentrations of hsp90, hsp70 or hdj-1 up to 25.6 μM resulted in equivalent reactivation of β-galactosidase as shown in (B). (**C**) The denatured β-galactosidase was also diluted into various combinations of the chaperones at the above concentrations, hsp70 + hsp90 (I), hsc70 + hsp90 (II) or hsp90 + hdj-1 (III), hsp70 + hdj-1 (IV) or hsc70 + hdj-1 (V). Error bars are indicated. (**D**) To examine the chaperone:substrate stoichiometry the β-galactosidase was diluted into refolding buffer supplemented with 1.6 μM hsp70 and 3.2 μM hdj-1 to final concentrations of either 3.4 (I), 34 (II), 68 (III), 136 (IV), 340 (V), 680 (VI) or 1360 (VII) nM. Aliquots from individual refolding reactions containing β-galactosidase, 68 nM final concentration, hsp70 (3.2 μM) and hdj-1 (6.4 μM) in refolding buffer were incubated at 37°C for the indicated times and then resolved on a native 4% acrylamide (50:1) sodium borate gel at 4°C. The gel was then stained utilizing X-gal (**E**).

neither refolds nor stimulates the refolding activities of hsp70 and hdj-1, hsp90 is highly effective in converting the denatured substrate in the absence of nucleotide to a 'folding-competent' state, which can subsequently be refolded upon addition of hsp70, hdj-1 and nucleotide. This ability of certain chaperones to affect the fate of a denatured substrate is reversibly temperature dependent which suggests a possible regulatory role in the heat shock response.

Results

Refolding of denatured β-galactosidase requires hsp70, hdj-1 and nucleotide

We examined the activities of the recombinant human molecular chaperones hsp90, hsp70, hsc70 and hdj-1 (Figure 1A) to mediate the refolding of denatured β-galactosidase. We selected β-galactosidase as a model substrate as it has a readily monitored enzyme activity over a broad range of temperatures and pH values (Hill and Huber, 1971; Tenu *et al.*, 1971; Huber *et al.*, 1979, 1994). β-galactosidase is composed of 1023 amino acids which fold to monomeric subunits that assemble to form the enzymatically active tetramer for which an atomic structure has been determined (Jacobson *et al.*, 1994). The refolding reaction of β-galactosidase was optimized for dependence on chaperone, K^+, Mg^{2+}, pH and nucleotide concentration. To establish these parameters, β-galactosidase was denatured in 6 M guanidinium hydrochloride, a condition that converts the substrate to a random coil, as determined by circular dichroism (data not shown). The denatured β-galactosidase was subsequently diluted 125-

fold into refolding buffer containing either BSA or chaperones and the recovery of β-galactosidase activity was measured by hydrolysis of ONPG relative to native β-galactosidase at the same concentration.

Individual chaperones at a range of concentrations from 0.4 to 25.6 μM hsp70, hdj-1 or hsp90 did not result in refolding of β-galactosidase (Figure 1BII, IV and V and data not shown). Additionally, the denatured β-galactosidase did not refold spontaneously in the presence of 0.4–25.6 μM BSA (Figure 1BI and data not shown). In contrast, ~50% of the denatured β-galactosidase re-acquires enzyme activity in a time-dependent reaction in the presence of both hsp70 (hsc70) and hdj-1 at concentrations of 1.6 and 3.2 μM respectively (Figure 1CIV and V). The requirement of hsp70 (hsc70) and hdj-1 is specific as other combinations of chaperones did not enhance the refolding activity. The stoichiometry of the chaperone–substrate reaction was established by dilution of various concentrations of denatured β-galactosidase (3.4–1360 nM) into 1.6 μM hsp70 and 3.2 μM hdj-1 (Figure 1D). Efficient refolding of β-galactosidase was detected at initial substrate concentrations of 3.4, 34 and 68 nM. At higher concentrations of 136 nM β-galactosidase the level of refolding was reduced to 20% with even less refolding of β-galactosidase occurring at higher substrate concentrations. These results establish that efficient protein refolding is obtained at a ratio of hsp70:substrate of 24:1, which is within the range of studies employing the hsp70 chaperones (Freeman *et al.*, 1995; Hohfeld *et al.*, 1995; Levy *et al.*, 1995). For technical reasons, in the majority of experiments presented here, we utilized a final concentration of 3.4 nM β-galactosidase to allow direct quantitative analysis of refolding activity without the need for further dilution of the reaction mixture.

Additional evidence to support our contention that the denatured β-galactosidase had refolded and assembled to the native tetramer was provided by resolution of refolding reactions on native PAGE and direct activity staining with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-actopyranoside (X-gal). The appearance of native tetrameric β-galactosidase activity occurs in a time-dependent manner in the presence of hsp70 and hdj-1 (Figure 1E). The chaperone-dependent step in reactivation

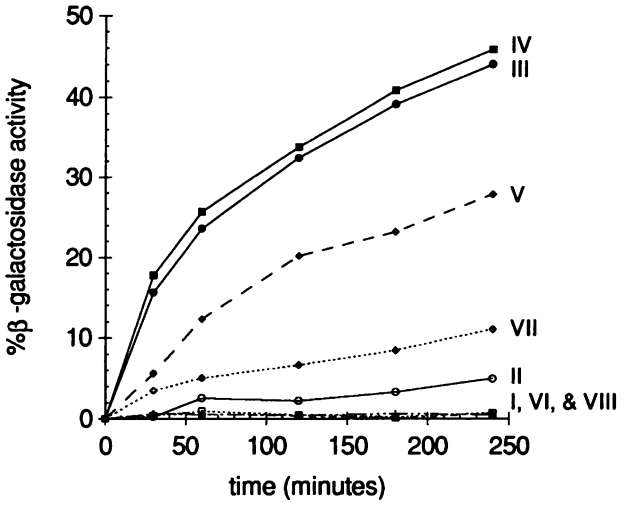

Fig. 2. Refolding of β-galactosidase by hsp70 and hdj-1 efficiently occurs in the presence of either ATP or ADP. The requirement for nucleotide on the hsp70 (1.6 μM) and hdj-1 (3.2 μM) refolding activity was examined by diluting the β-galactosidase (3.4 nM final) into refolding buffer lacking exogenous nucleotide (I) or containing either 1 μM (II), 100 μM (III) or 1 mM (IV) ATP, 100 μM ADP (V), 100 μM AMP (VI), 1 mM ATP-γS (VII) or 1 mM AMP-PCP (VIII).

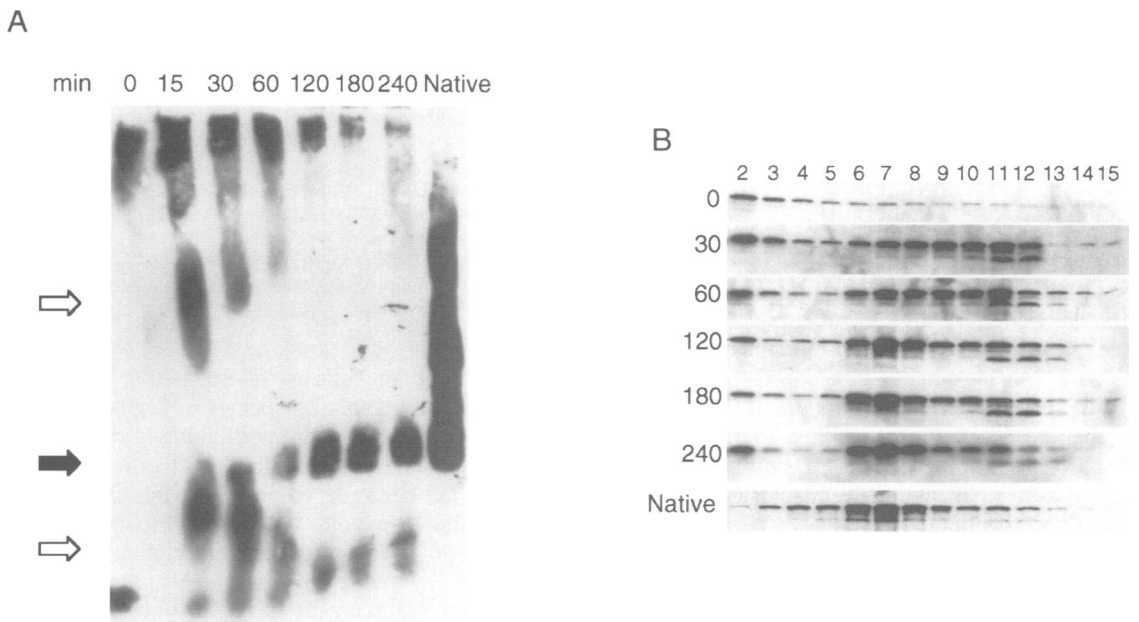

Fig. 3. Refolding of β-galactosidase mediated by hsp70 and hdj-1 proceeds through intermediates. Denatured β-galactosidase (68 nM final) was incubated in refolding buffer supplemented with hsp70 (3.2 μM) and hdj-1 (6.4 μM) at 37°C for the indicated times and resolved on a 4% acrylamide Na–borate gel at 4°C. The resolved fractions were transferred to nitrocellulose and the β-galactosidase was detected by Western blot analysis utilizing a polyclonal antiserum to β-galactosidase (**A**). The closed arrow indicates the position of the enzymatically active tetramer, whereas the open arrows indicate the position of apparent folding intermediates. Additionally, individual refolding reactions containing β-galactosidase (68 nM), hsp70 (3.2 μM) and hdj-1 (6.4 μM) were resolved by gel filtration. The β-galactosidase was detected by Western blot analysis from the individual fractions following trichloroacetic acid precipitation and resolution on a 10% SDS–PAGE (**B**).

of β-galactosidase is in the refolding of the β-galactosidase monomer and not on formation of the tetramer. This was established by obtaining monomer subunits of β-galactosidase, formed upon incubation of the native tetramer in 0.5 M sodium carbonate (Cohn, 1957) and by the demonstration that monomers diluted in refolding buffer assemble rapidly into active tetramers (data not shown).

The optimal environment in the refolding reaction for monovalent cations (25 mM KCl), divalent cations (5 mM $MgCl_2$) and pH (7.2–7.6) was established. These optima are similar to the previously established requirements for K^+ and Mg^{2+} for β-galactosidase activity (Ullmann and Monod, 1969; Hill and Huber, 1971; Strom *et al.*, 1971). The chaperone-dependent refolding reaction is dependent on nucleotide, with ATP (0.01–1 mM) having the greatest stimulatory effect on chaperone refolding activity, followed by ADP (optimal concentration is 0.1 mM), whereas AMP, γS-ATP or AMP-PCP were ineffective (Figure 2). In the absence of exogenous nucleotide, protein refolding is not detected (Figure 2I). Although the ATP was cleaved to ADP during the course of the refolding reaction, the ADP was not further hydrolyzed to AMP as determined by thin layer chromatographic analysis utilizing [^{32}αP]-ATP or [^{32}αP]-ADP as cofactors. These data are consistent with the suggestion that chaperone-dependent refolding activity is more closely related to the ATP/ADP-induced conformational change in hsp70 rather than due to the energy from cleavage of the phosphate bond (Liberek *et al.*, 1991a; Palleros *et al.*, 1993; Buchberger *et al.*, 1995; Freeman *et al.*, 1995).

Refolding of non-native β-galactosidase proceeds through intermediates

Resolution of β-galactosidase refolding reactions by native gel electrophoresis followed by staining with X-gal allows detection of the enzymatically active form of β-galactosidase (i.e. the tetramer). As a complement to this assay, we performed Western blot analysis following native PAGE using antisera to β-galactosidase which allows detection of both enzymatically active and non-enzymatically active forms of β-galactosidase (Figure 3A). During the time course of the hsp70- and hdj-1-refolding reaction the β-galactosidase is initially detected as a slowly migrating species on a native PAGE with a mobility distinct from that of folded tetrameric β-galactosidase (Figure 3A). Early in the refolding reaction two more quickly migrating β-galactosidase species appear. One corresponds to the native enzymatically active tetrameric β-galactosidase and the other resolves more quickly than the native β-galactosidase. In a time-dependent manner the non-enzymatically active forms of β-galactosidase are not detected and the enzymatically active form (native tetramer) becomes the predominant species both in the in-gel X-gal assay and as determined by Western blot analysis.

These results were corroborated further using size exclusion chromatography (Figure 3B) and glycerol gradient analysis (data not shown). By size exclusion chromatography, a species of β-galactosidase which resolved more quickly than the native β-galactosidase was detected. In a time-dependent manner the fraction of more quickly resolving β-galactosidase decreases (fractions 9–12) relative to the enzymatically active β-galactosidase (fractions 6–8). In contrast to the native PAGE analysis, a more slowly resolving species of β-galactosidase was not apparent. Detection of a more slowly resolving species of β-galactosidase may be hindered by the resolving capacity of the gel filtration column (i.e. the slower species may be within the void volume of the column). However, overall the results obtained by fractionation of the refolding reaction by size exclusion chromatography shares many common features with the results obtained by fractionation using native PAGE.

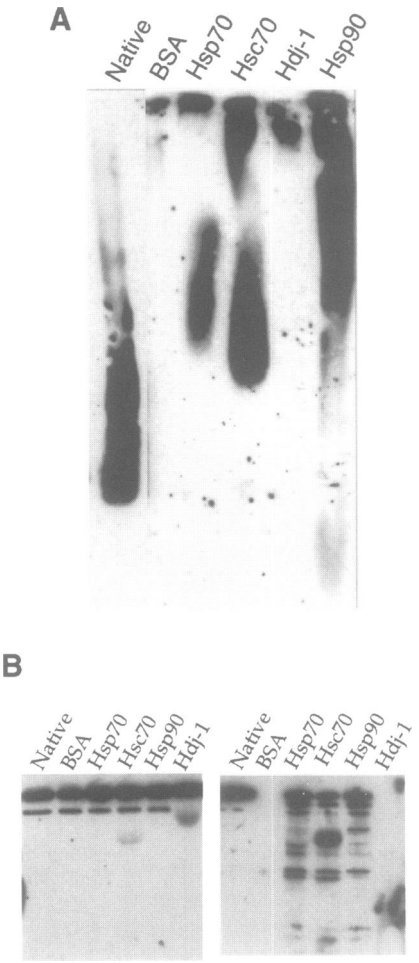

Fig. 4. The chaperones hsp90, hsc70 and hsp70 interact with the non-native β-galactosidase. Denatured β-galactosidase (68 nM) was incubated for 2 h at 37°C in refolding buffer supplemented with either BSA (3.2 μM), hsp70 (3.2 μM), hsc70 (3.2 μM), hdj-1 (3.2 μM) or hsp90 (3.2 μM) then resolved on a 4% acrylamide Na–borate gel. The resolved fractions were transferred to nitrocellulose and the β-galactosidase was detected by Western blot analysis using a polyclonal antiserum to β-galactosidase (**A**). Alternatively, chymotrypsin (1.25 μg) was added to the reactions following a 2 h incubation at 37°C. Aliquots were removed from each reaction prior to the addition of chymotrypsin and following a 10 min incubation at 37°C with the chymotrypsin. The aliquots were resolved on 10% SDS–PAGE, transferred to nitrocellulose, and the β-galactosidase was detected by Western blot analysis utilizing a polyclonal antiserum to β-galactosidase (**B**).

Hsp70, hsc70 and hsp90 interact with non-native β-galactosidase

Given the ability to detect non-enzymatically active forms of β-galactosidase following native PAGE and Western blot analysis (Figure 3A), we examined the effect of the

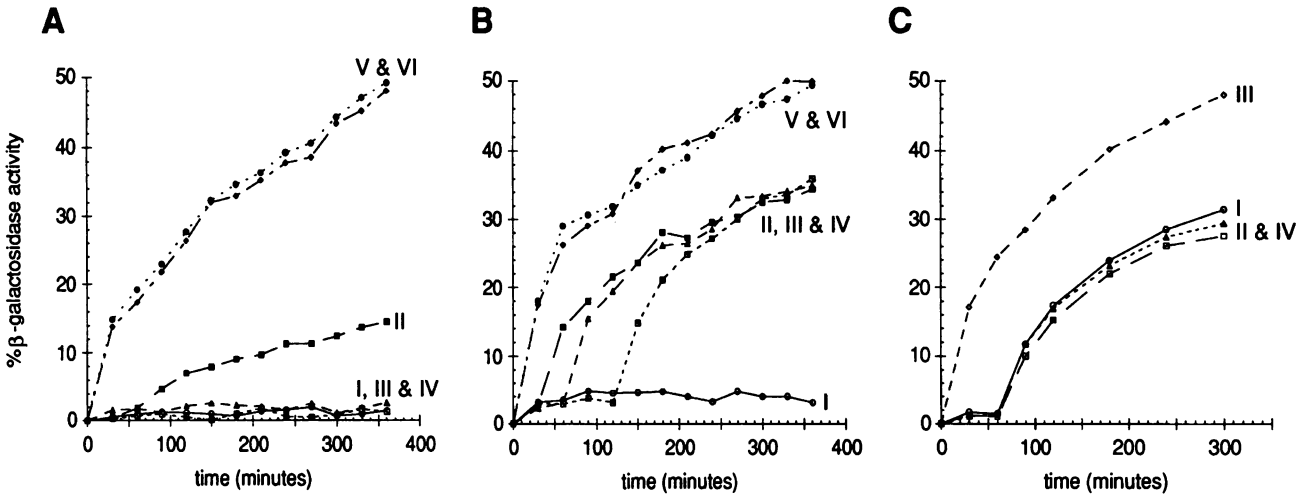

Fig. 5. Hsp90 maintains non-native β-galactosidase in a refoldable state over an extended period of time at 37°C, independent of nucleotide. (**A**) Denatured β-galactosidase (3.4 nM final) was diluted into refolding buffer supplemented with 3.2 μM BSA and incubated at 37°C (I) or following 30 (II), 60 (III) or 120 (IV) min incubation at 37°C, hsp70 (1.6 μM) and hdj-1 (3.2 μM) were added and the activity of β-galactosidase was monitored at the indicated time points. The denatured β-galactosidase was also diluted directly into refolding buffer supplemented with hsp70 (1.6 μM) and hdj-1 (3.2 μM; V) or hsp70 (1.6 μM), hdj-1 (3.2 μM) and hsp90 (3.2 μM; VI). (**B**) To test the effects of hsp90, the denatured β-galactosidase (3.4 nM) was incubated in refolding buffer supplemented with hsp90 (0.4 μM; I), or following 30 (II), 60 (III) or 120 (IV) min incubation at 37°C, hsp70 (1.6 μM) and hdj-1 (3.2 μM) were added and the activity of β-galactosidase was monitored at the indicated time points. The denatured β-galactosidase was also diluted directly into refolding buffer supplemented with hsp70 (1.6 μM) and hdj-1 (3.2 μM; V) or hsp70 (1.6 μM), hdj-1 (3.2 μM), and hsp90 (3.2 μM; VI). (**C**) To examine the effect of nucleotide on hsp90, the denatured β-galactosidase (3.4 nM) was incubated with hsp90 (0.4 μM) in refolding buffer containing either 1 mM ATP (I), 1 mM ADP (II) or no nucleotide (IV) for 2 h at 37°C and then hsp70 (1.6 μM), hdj-1 (3.2 μM) and 1 mM ATP were added and the activity of β-galactosidase was monitored at the indicated time points. As a control denatured β-galactosidase (3.4 nM final) was diluted directly into hsp70 (1.6 μM) and hdj-1 (3.2 μM; III).

individual chaperones on the non-native substrate. The more slowly migrating species of β-galactosidase were observed not only upon incubation with both hsp70 and hdj-1 but also upon incubation with either hsp90, hsc70 or hsp70 (Figure 4A). The ability to maintain a population of non-native β-galactosidase in a soluble state appears to be specific to certain chaperones, since β-galactosidase incubated with hdj-1 did not enter the gel matrix (Figure 4A). These results do not exclude the possibility that hdj-1, which is a highly basic protein, forms a stable complex with β-galactosidase and prevents the β-galactosidase from entering the gel matrix under the conditions employed for native gel analysis. To examine whether the chaperones are associated with the β-galactosidase, we used antisera to hsp90, hsp70 (hsc70) for Western blot analysis using the same filter shown in Figure 4A. Although chaperones were found in the same region as the slow-migrating species of β-galactosidase, this occurred in the absence or presence of β-galactosidase (data not shown). Taken together, these data suggest that hsp90, hsc70 and hsp70 interact with the denatured substrate to maintain a population of soluble non-native β-galactosidase.

To support our contention that hsp90, hsc70 and hsp70 interact with non-native β-galactosidase we utilized a protease protection assay (Figure 4B). The non-native β-galactosidase was incubated in the presence of either BSA or individual chaperones for 2 h at 37°C followed by the addition of chymotrypsin. The β-galactosidase was detected by Western blot analysis using a polyclonal antiserum to β-galactosidase. Intact β-galactosidase and proteolytic fragments were detected in those samples where the non-native β-galactosidase was diluted into hsp70, hsc70 and hsp90 whereas dilution into hdj-1 or BSA resulted in complete proteolysis of β-galactosidase. Under these limiting protease conditions the native β-galactosidase was not affected. Taken together these results reveal that hsp90, hsc70 and hsp70 can interact with denatured β-galactosidase to generate an intermediate non-native population which has features of the folded state as distinguished by protease sensitivity.

Hsp90 can maintain non-native β-galactosidase in a folding-competent state

We addressed whether initial interactions between the non-native substrate and individual chaperones influence subsequent interactions with the hsp70 chaperone machine leading to the folded state. Order-of-addition experiments were performed in which denatured β-galactosidase was incubated initially with either BSA, hsp90, hsp70 or hdj-1 and after various periods of time each reaction was supplemented with hsp70, hdj-1 and ATP, and allowed to incubate for an additional period prior to measuring β-galactosidase activity. Following a 30 min incubation in the presence of BSA, ~15% of the non-native β-galactosidase was refolded upon the addition of hsp70 and hdj-1 (Figure 5AII). Incubation of denatured β-galactosidase for 1 or 2 h with BSA prior to the addition of hsp70 and hdj-1 resulted in the complete loss of refoldable β-galactosidase (Figure 5AIII and IV). This experiment reveals that the aggregated state of β-galactosidase formed in the presence of BSA cannot be 'rescued' and refolded to the native state.

Next we examined whether hsp90 was effective in maintaining the non-native substrate in a hsp70 and hdj-1 responsive state as measured by the recovery of β-galactosidase activity. In the presence of hsp90 alone, there was little or no β-galactosidase activity (Figure 5BI),

however, upon addition of hsp70 and hdj-1 following either a 30, 60 or 120 min incubation with hsp90 there was rapid reactivation of β-galactosidase (Figure 5BII–IV). An important feature of this reaction is the ability of hsp90 to maintain β-galactosidase in a folding-competent state without a nucleotide requirement and distinct from the subsequent hsp70 and hdj-1 reaction, which requires nucleotide for refolding (Figures 1E and 5C). This was demonstrated by incubating the denatured β-galactosidase 2 h at 37°C with hsp90 in refolding buffer supplemented with ATP (Figure 5CI), ADP (Figure 5CII) or in the absence of nucleotide (Figure 5CIV) followed by the addition of hsp70 and hdj-1.

To investigate further the ability of individual chaperones to interact with and maintain the non-native β-galactosidase in a refoldable state, we established the absolute concentration requirements for this reaction. The denatured β-galactosidase was diluted directly into various concentrations of the individual chaperones ranging from 50 nM to 25.6 μM after which hsp70 and hdj-1 were supplemented (Figure 6). As expected, dilution of denatured β-galactosidase into BSA (Figure 6A) or hdj-1 (Figure 6B), regardless of concentration, was ineffective at maintaining the substrate in a folding-competent state, whereas both hsp70 (Figure 6C) and hsp90 (Figure 6D) were effective in maintaining the substrate in a state responsive to hsp70 and hdj-1. The concentration of hsp70 which was effective to maintain the non-native β-galactosidase in a folding-competent state was 1.6–6.4 μM (Figure 6CIII–V), whereas hsp90 was effective at concentrations >100 nM (Figure 6DII–VI).

Refolding activity of hsp70 and hdj-1 is reversibly inhibited at 4°C and temperatures >41°C

During the course of characterizing the *in vitro* folding properties of recombinant hsp70 and hdj-1, we examined the temperature dependence of this reaction. Efficient refolding of denatured β-galactosidase is observed over a broad temperature range that extends from 22°C to 41°C (Figure 7AII–VI). Incubation at lower (4°C; Figure 7AI) or higher temperatures (>41°C; Figure 7AVII–X) was found to inhibit the refolding of β-galactosidase. The effects of extreme temperature appear to be on the chaperone-mediated process as the enzyme activity of native β-galactosidase is itself unaffected by incubation at temperatures extending from 4°C through 45°C (data not shown).

Next we examined whether the inhibitory effect of elevated temperatures on protein refolding was reversible. A refolding reaction containing non-native β-galactosidase and the chaperones hsp70 and hdj-1 was incubated at 42°C which results in an 80% reduction in refolding activity (Figure 7AVII and BV). However, this inhibition was readily reversed upon shifting the reaction to 37°C (Figure 7BII–IV). The ability of hsp70 and hdj-1 to maintain the non-native β-galactosidase in a refoldable state during exposure to 42°C is dependent on ATP and does not occur in the presence of ADP (Figure 7BVI). These data also indicate that, despite the inability of the chaperones to refold β-galactosidase at 42°C, they are able to maintain the β-galactosidase in a foldable state at the elevated temperature.

While the observations presented in this section provide

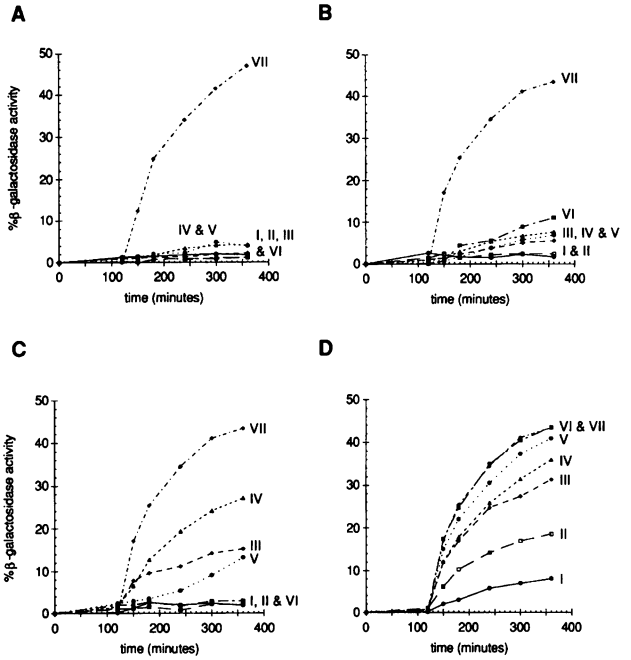

Fig. 6. The human molecular chaperones vary in their abilities to maintain denatured β-galactosidase in a refoldable state over a 2 h period at 37°C. (**A**) Denatured β-galactosidase was diluted 125-fold (3.4 nM final) into refolding buffer supplemented with either 0.4 (I), 0.8 (II), 1.6 (III), 3.2 (IV), 6.4 (V) or 12.8 μM (VI) BSA, incubated 2 h at 37°C, hsp70 (1.6 μM) and hdj-1 (3.2 μM) were added and the activity of β-galactosidase was measured utilizing the chromogenic substrate ONPG at the indicated time points. As a control a β-galactosidase refolding reaction was initiated at the 2 h time point by diluting denatured β-galactosidase directly into refolding buffer supplemented with hsp70 (1.6 μM) and hdj-1 (3.2 μM; AVII). (**B**) The ability of hdj-1 to maintain β-galactosidase in a refoldable state over a 2 h time period at 37°C was tested utilizing the above protocol except that the following concentrations of hdj-1 were used: 0.8 (I), 1.6 (II), 3.2 (III), 6.4 (IV), 12.8 (V) or 25.6 μM (VI). (**C**) and (**D**) Similar experiments were done utilizing 0.4 (CI), 0.8 (CII), 1.6 (CIII), 3.2 (CIV), 6.4 (CV) or 12.8 μM (CVI) hsp70 or 50 (DI), 100 (DII), 200 (DIII), 400 (DIV) 800 (DV) or 1600 nM (DVI) hsp90. For the experiments testing the ability of the hdj-1, hsp70 or hsp90 to maintain β-galactosidase in a refoldable state, a control (BVII, CVII or DVII respectively) for refolding was done by diluting the denatured β-galactosidase directly into refolding buffer supplemented with hsp70 (1.6 μM) and hdj-1 (3.2 μM).

ample evidence for the reversible effects of 42°C on protein folding, one question posed by this data is whether the effects of the extreme temperatures (i.e. 4°C or >42°C) are also reversible. Protein refolding activity was not substantially detected following incubation at any of these temperatures (Figures 7A, 8A and B). However, following a shift to 37°C, refolding of β-galactosidase resumed (Figure 8AIII, V and VII), albeit at a reduced level compared with incubation at 37°C alone or pre-incubation at 42°C (Figure 7B). Identical results were obtained either using the solution assay measuring ONPG hydrolysis or by native-PAGE and Western blot analysis using an antibody to β-galactosidase (Figure 8B). Next we examined whether the inhibitory effect of exposure to extreme temperatures is due to inactivation of the chaperones. Hsp70 and hdj-1 were incubated for various times at 45°C then shifted to 37°C prior to addition of denatured β-galactosidase. Prior incubation at 45°C did not interfere with the activity of hsp70 and hdj-1 to refold denatured β-galactosidase (Figure 8CII–V). These results suggest

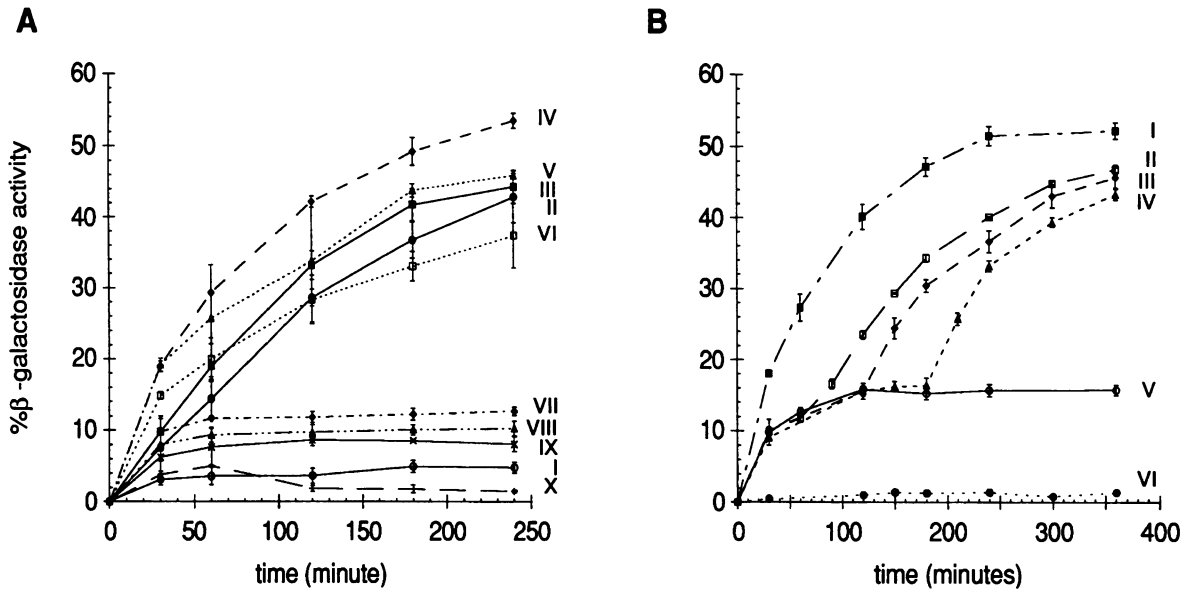

Fig. 7. Refolding of β-galactosidase by recombinant human hsp70 and hdj-1 is reversibly inhibited at 4°C or >41°C. (**A**) The ability of the hsp70 (1.6 μM) and hdj-1 (3.2 μM) to refold denatured β-galactosidase (3.4 nM final) at 4°C (I), 22°C (II), 30°C (III), 37°C (IV), 40°C (V), 41°C (VI), 42°C (VII), 43°C (VIII), 44°C (IX) or 45°C (X) for the indicated times was examined. Error bars are indicated. (**B**) Recovery of protein refolding activity was examined by diluting denatured β-galactosidase (3.4 nM final) into refolding buffer containing hsp70 (1.6 μM) and hdj-1 (3.2 μM), which was incubated at 37°C (I), at 42°C for 1 h then shifted to 37°C (II), 42°C for 2 h then shifted to 37°C (III), 42°C for 3 h then shifted to 37°C (IV) or 42°C for 6 h (V). Denatured β-galactosidase was also diluted into refolding buffer supplemented with hsp70 (1.6 μM), hdj-1 (3.2 μM) and 1 mM ADP (did not contain supplemented ATP) and was incubated 2 h at 42°C and then shifted to 37°C and ATP was added to 1 mM (VI). Error bars are indicated.

that the interaction between the non-native protein substrate and the hsp70 and hdj-1 chaperones must differ at 4°C or in the range 42–45°C compared with 22–41°C as evidenced by the contrast in the outcome of protein folding.

Hsp90 can maintain non-native β-galactosidase in a 'refoldable' state at 42°C independent of nucleotide

In our previous experiments, we had identified a novel activity of the molecular chaperone hsp90: to interact with the non-native state of β-galactosidase and to maintain the substrate in a folding-competent state. Having demonstrated that the hsp70 and hdj-1 chaperones were also capable of maintaining a non-native substrate in an intermediate folded state upon exposure to extreme temperatures, we examined whether hsp90 remained effective at elevated temperatures. β-galactosidase was diluted into hsp90, in either the presence (Figure 9IV) or absence (Figure 9V) of ATP at 42°C and the reaction was returned to 37°C in the presence of hsp70, hdj-1 and ATP. The results reveal that hsp90 remains effective at ambient and elevated temperatures in maintaining a non-native intermediate that can be further refolded to the native state. This feature of hsp90 is unique among the cytosolic human chaperones as hdj-1, hsp70 or hsc70 alone are incapable of maintaining the non-native substrate in a folding-competent state at 42°C (Figure 9II and III and data not shown). These results, in conjunction with the data in Figure 7B, indicate that maintenance of non-native β-galactosidase in a refoldable state at 42°C can occur by either of two chaperone-dependent mechanisms, requiring the combination of hsp70 and hdj-1, which is dependent on ATP, or hsp90 by a nucleotide-independent mechanism.

Discussion

This study provides evidence that the human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in the process of chaperone-mediated protein refolding. The general features of these chaperone-mediated refolding reactions are summarized in Figure 10. Our ability to distinguish between the hsp90 interactions that lead to the formation of a stable non-native intermediate rather than the direct refolding fostered by hsp70 and hdj-1 takes advantage of an unfolded protein substrate which we have denatured by means of the ionic chaotrope guanidinium hydrochloride. Whether chaperones encounter a substrate *in vivo* in this chemical state is uncertain; nevertheless, chaotrope denatured proteins have provided much information towards our understanding of *in vitro* (non-chaperone-mediated) protein refolding (Anfinsen and Scheraga, 1975) and is a widely used methodology with other substrates (e.g. rhodanese, firefly luciferase and citrate synthase) in the analysis of chaperone function (Bochkareva *et al.*, 1992; Langer *et al.*, 1992; Schroder *et al.*, 1993; Freeman *et al.*, 1995). Earlier studies demonstrated that guanidine-denatured β-galactosidase does not refold spontaneously upon dilution (Perrin and Monod, 1963) which suggests that refolding of guanidine-denatured β-galactosidase may require *in vivo* cofactors to attain its native state.

Interactions between guanidine-denatured β-galactosidase and the 70 kDa chaperones can result in either refolding of the non-native protein to its native state or maintenance of the substrate in a folding-competent state. It is well known that proteins collapse rapidly upon dilution from a chaotrope (Kim and Baldwin, 1990), which can lead to non-specific aggregation. Dilution of the guanidine-

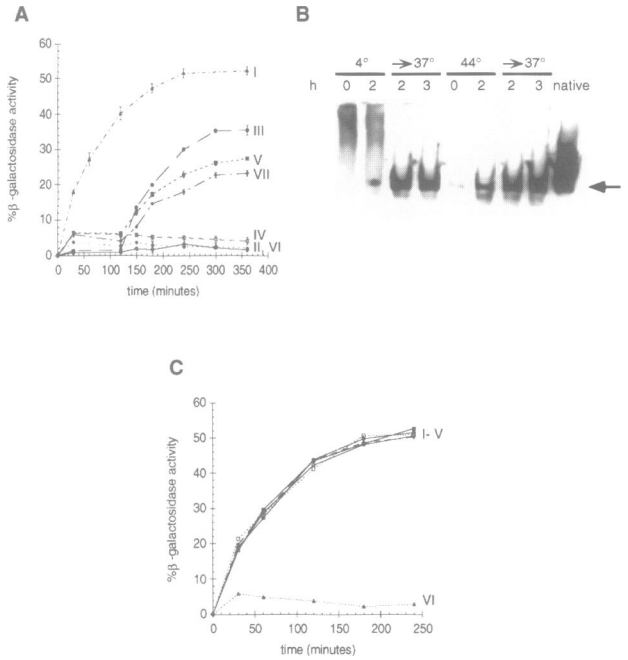

Fig. 8. The arrest and recovery of the hsp70 and hdj-1 protein folding activity following exposure to extreme temperatures. (**A**) Denatured β-galactosidase (3.4 nM) was incubated in refolding buffer supplemented with hsp70 (1.6 μM) and hdj-1 (3.2 μM) and incubated at 37°C (I), 4°C (II), 2 h at 4°C then shifted to 37°C (III), 44°C (IV), 2 h at 44°C then shifted to 37°C (V), 45°C (VI) or for 2 h at 45°C then shifted to 37°C (VII). The activity of β-galactosidase was determined at the indicated times. Error bars are indicated. Additionally, denatured β-galactosidase (68 nM) was incubated in refolding buffer supplemented with hsp70 (3.2 μM) and hdj-1 (6.4 μM) at the indicated temperatures for the indicated times, resolved on a 4% acrylamide Na–borate gel, transferred to nitrocellulose, and the β-galactosidase was detected by Western blot analysis utilizing a polyclonal antiserum to β-galactosidase (**B**). The arrow indicates the position of the enzymatically active tetramer. (**C**) Non-reversible effects of elevated temperature on hsp70 and hdj-1 were examined by incubating hsp70 (1.6 μM) and hdj-1 (3.2 μM) in refolding buffer at 45°C for 0 (I), 15 (II), 30 (III), 60 (IV) or 120 (V) min then shifted to 37°C, β-galactosidase (3.4 nM final) was added and the activity was monitored at the indicated time points. As a control the β-galactosidase was diluted into refolding buffer supplemented with hsp70 (1.6 μM) and hdj-1 (3.2 μM) and maintained at 42°C (VI).

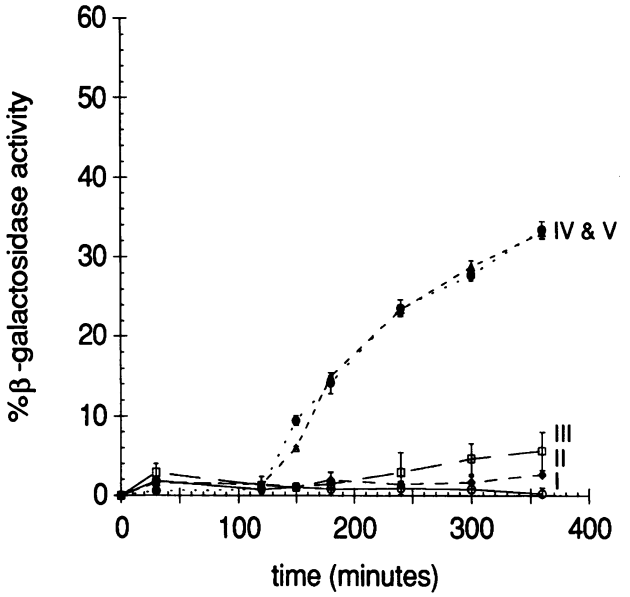

Fig. 9. Hsp90 maintains the non-native β-galactosidase in a refoldable state at 42°C independent of nucleotide. Denatured β-galactosidase was diluted into refolding buffer (3.4 nM final) supplemented with either 3.2 μM BSA (I), 3.2 μM hdj-1 (II), 1.6 μM hsp70 (III), 1.6 μM hsp90 with (IV) or without (V) 1 mM ATP in the refolding buffer, incubated at 42°C for 2 h, shifted to 37°C and either 1.6 μM hsp70 and 3.2 μM hdj-1 (I, IV and V) were added or just 1.6 μM hsp70 (II) or 3.2 μM hdj-1 (III) was added. ATP was also added to (V) to a final concentration of 1 mM after the 2 h incubation at 42°C. The activity of the β-galactosidase was monitored utilizing ONPG. Error bars are indicated.

denatured β-galactosidase into refolding buffer containing either 70 kDa chaperone or hsp90 results in the formation of an intermediate non-native substrate state. The ability of the 70 kDa chaperones or hsp90 to prevent aggregation is revealed by maintenance of β-galactosidase in a soluble state (Figure 4A). Additional evidence that β-galactosidase forms chaperone-dependent non-native intermediates follows from experiments using proteases to probe for the formation of folded structures (Figure 4B). We do not interpret our results to suggest that the chaperones form stable heteromeric complexes with the folding-competent state of non-native β-galactosidase; rather, our observations reveal that certain chaperones can facilitate the transition to this intermediate state and restrict the conversion of the substrate into an insoluble aggregate. Efficient refolding is obtained at a range of chaperone:substrate concentrations in which this ratio does not exceed 24:1. Furthermore, the absolute level of chaperones (hsp70 or hsp90) required in these studies is in the range 1–3 μM which corresponds to the *in vivo* concentration of these chaperones in human tissue culture cell lines (data not shown).

Although our data offer evidence for the reversible temperature dependence for protein refolding *in vitro*, we do not know whether our observations extend to events within the stressed cell. The oligomeric state of the 70 kDa chaperones is not significantly altered between 22°C and 45°C (data not shown) and interaction with the non-native β-galactosidase is apparently maintained since a population of the β-galactosidase is kept in a refoldable state over an extended period of time at the non-permissive folding temperatures (i.e. >41°C). One possibility for the temperature inhibition would be that the interaction between hsp70 and hdj-1 is restricted at temperatures >41°C, which could result in a loss of the chaperone refolding activity. It is well known that the dnaJ homologs can stimulate the ATPase rate of the 70 kDa members (Liberek *et al.*, 1991b; Freeman *et al.*, 1995), therefore we utilized this assay to test whether these chaperones, by at least one criterion, are interacting at temperatures >41°C. Between 37°C and 44°C the ATPase rate increases ~2- to 3-fold from 2.4 to 4.9 pmol/min/μg of hsp70 for the intrinsic rate and from 8.9 to 23.1 for the hdj-1-stimulated rate. This suggests that the chaperones are able to functionally interact at these temperatures. Additional studies will be necessary to establish how chaperones shift from a 'folding' activity to a 'maintenance' activity at temperatures over 41°C or at 4°C. We note that the elevated temperatures at which the *in vitro* chaperone refolding activity is inhibited (>41°C) correlates closely with the *in vivo* temperatures of the human heat shock response (>41°C). Whether these observations have a

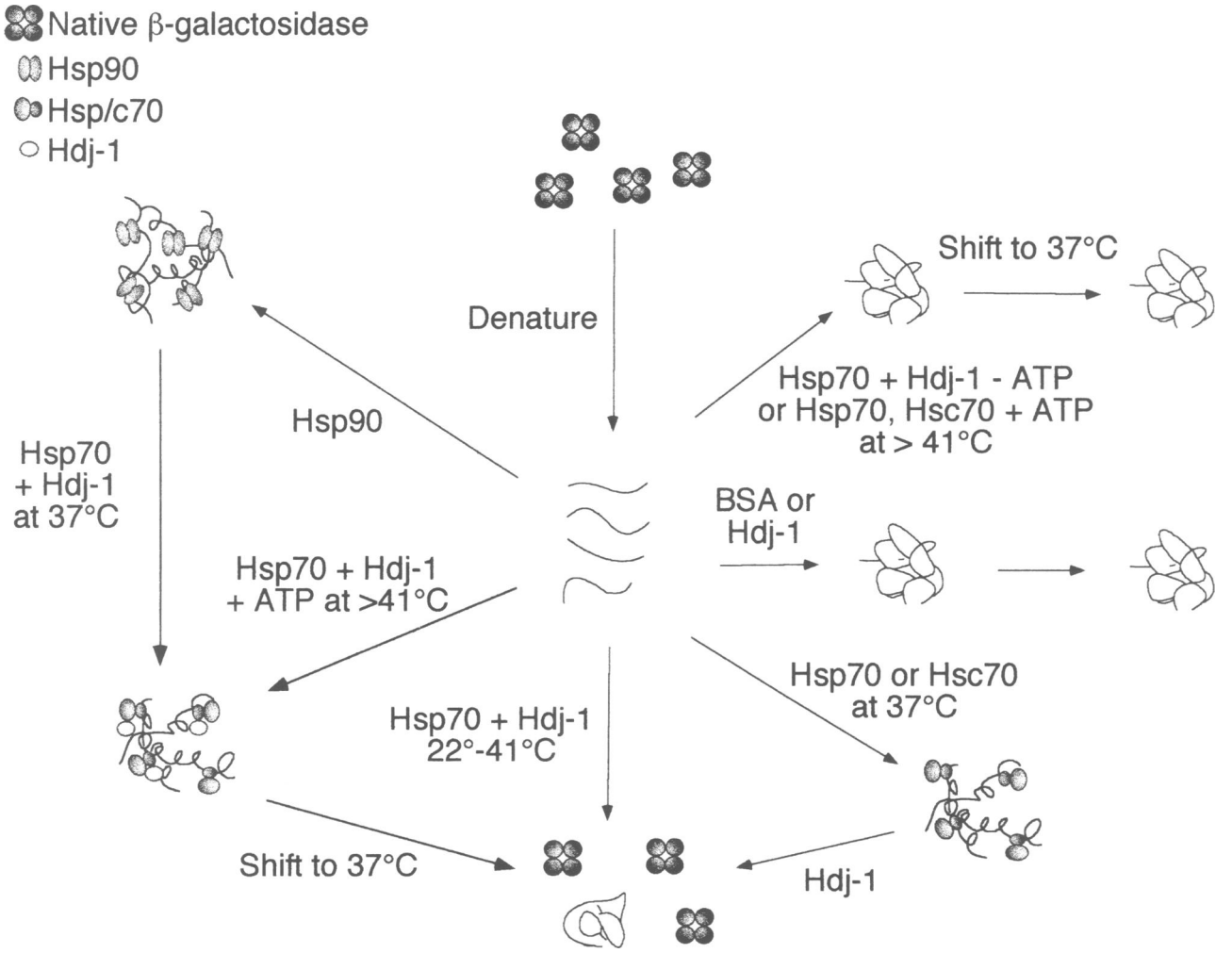

Fig. 10. Role of the human chaperones hsp90, hsp70 (hsc70) and hdj-1 in chaperone-mediated protein refolding. The fate of guanidine-denatured β-galactosidase is determined by the interaction with specific molecular chaperones. Interaction with hsp90, in the absence of nucleotide, leads to the maintenance of the β-galactosidase in a refoldable state which is subsequently responsive to hsp70 (hsc70), hdj-1 and ATP, leading to refolding to the native state. An alternative pathway is that the denatured β-galactosidase is refolded in the presence of hsp70 (hsc70), hdj-1 and ATP. However, this refolding activity of the chaperones is only detected between 22–41°C. At extremely low or high temperatures, the hsp70 (hsc70) and hdj-1 chaperones, in the presence of ATP, adopt a maintenance activity similar to that observed for hsp90.

causal relationship to link activation of HSF1 and transcriptional activation of the heat shock response to the chaperone activities at elevated temperatures will be of interest.

It is evident from the data presented here that hsp90, hsc70 and hsp70 can interact with the non-native β-galactosidase and maintain it in a refoldable state at 37°C. The ability of hsp90 to prevent the aggregation of a non-native protein has been previously reported (Wiech *et al.*, 1992; Jakob *et al.*, 1995), however, our studies have an important distinction in that hsp90 can interact with a protein denatured in guanidinium hydrochloride. Furthermore, our results also indicate that hsp90 does not refold a chaotrope-denatured substrate to the native state. The ability of hsp90 to maintain a substrate in a refoldable state may have additional significance at elevated temperatures where hsp70 and hsc70 no longer maintain the non-native β-galactosidase in the absence of hdj-1 and ATP. This distinction may take on added importance under conditions of heat shock in which ATP levels are drastically reduced (Beckmann *et al.*, 1992). We suggest that hsp90 may interact preferentially with non-native substrates to maintain them in a soluble state in the stressed cell. Upon return to permissive temperatures, hsp70 and hdj-1 would interact and refold substrates in concert with re-establishment of the intracellular ATP pool.

One well characterized example of heteromeric complexes containing molecular chaperones is the steroid aporeceptor which contains a number of chaperones and immunophilins including hsp90, hsp70, p60, p59, p54, p50 and p23 (Smith and Toft, 1993; Pratt and Welsh, 1994). Presumably the chaperones and immunophilins maintain the aporeceptor in an inert intermediate inactive state that can productively respond to the proper stimuli. For example, hsp90 is required to maintain several of the steroid receptors in a high affinity hormone binding, non-DNA binding and transcriptionally inactive state, which upon hormone binding translocate to the nucleus, bind DNA and regulate the transcription of specific genes (Bresnick *et al.*, 1989; Hutchison *et al.*, 1992; Bohen and Yamamoto, 1994; Kimura *et al.*, 1995; Nathan and Lindquist, 1995). It has been suggested that hsp70 is required to 'load' the receptors onto hsp90 following

synthesis (Hutchinson *et al.*, 1994). This requirement for hsp70 may reflect an 'unfolding' activity of hsp70, which has been suggested to occur during protein translocation (Chirico *et al.*, 1988). The ability of hsp90 to interact with denatured β-galactosidase leading to the formation of a stable non-native folded intermediate that is protease resistant (relative to its unfolded counterpart) suggests features that may be in common with the aporeceptor. Unlike the situation with steroid receptors, which contain both hsp90 and hsp70 in the inert state, we have shown that the native state of β-galactosidase is rapidly acquired upon addition of both hsp70, hdj-1 and ATP. *In vivo* this would invoke a requirement for accessory proteins that interfere with nucleotide binding to hsp70 or with the requirement for hdj-1. The order-of-addition experiments presented here with hsp90 and hsp70 provide an opportunity to identify other proteins that act to enhance or interfere with chaperone functions.

Materials and methods

Protein purification

The hsc70, hsp70 and hdj-1 were purified as described in Freeman *et al.* (1995). Hsp90 was expressed in a strain of baculovirus Sf9 cells containing the hsp90 gene (generous gift of Dr G.Litwack, Thomas Jefferson University). Five days post-infection a crude cell extract was prepared by Dounce homogenization and sonication followed by centrifugation at 12 000 *g* for 1 h. The crude extract was loaded onto a DEAE–sepharose column and eluted with a 50–500 mM NaCl gradient, the appropriate fractions were pooled, loaded onto a ResourceS column (Pharmacia-LKB), eluted with a 100–600 mM NaCl gradient, the appropriate fractions were pooled, the sample was equilibrated to 100 mM NaCl and loaded onto a heparin column, eluted with a 100–500 mM NaCl gradient and the appropriate fractions were pooled and concentrated. The final concentration of hsp90 was determined utilizing the BCA assay (Pierce).

β-galactosidase activity assays

The ONPG activity assay utilized was a modification of the assay from Cohn (1957). To determine the chaperone:substrate stoichiometry, the β-galactosidase (Sigma) was prepared at 50 mg/ml in 1 M glycylglycine (pH 7.4) and denatured by a 100-, 10-, 5- or 2.5-fold dilution into 1 M glycylglycine (pH 7.4) and a further 10- or 5-fold dilution into denaturation buffer (25 mM HEPES pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol and 6 M guanidine–HCl) and incubation for 30 min at 30°C. The ability of the chaperones to refold the denatured β-galactosidase was examined by dilution of the denatured β-galactosidase 125-fold or 62.5-fold for the 1360 nM final β-galactosidase concentration, into refolding buffer (25 mM HEPES pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 10 mM DTT and 1 mM ATP) at 4°C supplemented with indicated chaperones at the indicated concentrations and then incubated at the given temperatures for the given time periods. The activity of the β-galactosidase at the various time points was determined by diluting a 4 μl aliquot of the refolding reaction 400-, 200-, 100-, 40-, 20- or 10-fold into refolding buffer then mixing either 10 μl of the diluted reaction or 10 μl of the non-diluted refolding reaction (3.4 nM final β-galactosidase reaction) with 10 μl of refolding buffer supplemented with ONPG (0.8 mg/ml final) and incubating at 37°C for 15 min. For the remaining refolding assays monitored with ONPG the β-galactosidase (Sigma) was prepared at 10 mg/ml in 1 M glycylglycine (pH 7.4) and denatured by a 20-fold dilution into 1 M glycylglycine (pH 7.4) and a further 10-fold dilution into denaturation buffer and incubated for 30 min at 30°C. The activity of the β-galactosidase at the various points was determined as described above for the 3.4 nM final β-galactosidase concentration. The activity assays were terminated by the addition of 50 μl of 0.5 M sodium carbonate and the absorbance of each sample was determined at A_{412}. The percent refolding activity was calculated relative to the activity of native β-galactosidase diluted to the same extent into 1 M glycylglycine (pH 7.4) and then 125-fold into refolding buffer supplemented with BSA (3.2 μM).

Detection of β-galactosidase activity following resolution on native PAGE was accomplished by denaturing the β-galactosidase as above except that the 20-fold dilution into the 1 M glycylglycine was omitted. The denatured β-galactosidase was diluted 125-fold (68 nM final) into refolding buffer at 4°C supplemented with 3.2 μM hsp70 and 6.4 μM hdj-1 and incubated at 37°C for the given times, loaded onto a native 4% acrylamide (50:1) sodium borate (0.1 M NaOAc, 0.1 M boric acid pH 8.5; Gething *et al.*, 1989) gel at 4°C and resolved at 1 mm/min. The native β-galactosidase was incubated for 2 h at 37°C prior to resolution on the native PAGE. The location of the native β-galactosidase was detected by incubating the gel in 1× PBS supplemented with 100 mM KCl, 5 mM $MgCl_2$ and 40 μg/ml X-gal at room temperature.

Western blot analysis of the native PAGE

β-galactosidase (10 mg/ml) was diluted 10-fold into denaturation buffer and incubated for 30 min at 30°C. The denatured β-galactosidase (68 nM) was incubated for indicated times at given temperatures in refolding buffer supplemented with indicated chaperones. Following the incubation the samples were resolved on a 4% acrylamide (50:1) Na–borate gel at 4°C at 1 mm/min. The resolved samples were transferred to nitrocellulose and Western blot analysis was performed to detect the β-galactosidase. Following transfer the nitrocellulose was air dried, incubated 1 h in 3% non-fat dried milk in 1× PBS, washed 3×10 min with 1× PBS, incubated 12 h at 22°C with the anti-β-galactosidase antibody (generous gift of Dr R.Holmgren, Northwestern University), washed 3×10 min with 1× PBS + 0.2% Tween 20, incubated for 2 h with anti-rabbit antibody, washed 3×10 min with 1× PBS + 0.2% Tween 20 and detected utilizing ECL (Amersham).

Gel filtration analysis

Non-native intermediates and native β-galactosidase which appear during a refolding reaction were detected by gel sieving analysis and subsequent immunodetection of the substrate. β-galactosidase (10 mg/ml) was diluted 10-fold into denaturation buffer and incubated for 30 min at 30°C. The denatured β-galactosidase (68 nM) was incubated for indicated times at 37°C in refolding buffer supplemented with 3.2 μM hsp70 and 6.4 μM hdj-1, resolved over a Superdex-200 column (Pharmacia-LKB) at 4°C and 0.5 ml fractions were collected into tubes containing BSA (100 μg/ml final). Trichloroacetic acid was added to each fraction (20% final), the fractions were centrifuged for 30 min at 12 000 *g*, washed with 1 ml of acetone and reconstituted with 50 μl of 1× sample buffer. Aliquots (15 μl) of fractions 2–15 were resolved on 10% SDS–PAGE, transferred to nitrocellulose and Western blot analysis was performed with an antibody to β-galactosidase. The native size of hsc70 was established using a similar protocol and the hsc70 was detected by Western blot analysis using a monoclonal antibody to hsp70 (3a3).

Protease protection assay

The limited proteolysis of β-galactosidase was performed following a 2 h incubation at 37°C in refolding buffer (125 μl) after which 1.25 μg of chymotrypsin was added to each reaction. Aliquots (15 μl) were removed prior to and following a 10 min incubation with the chymotrypsin, mixed with 5× sample buffer within the Hamilton needle used for loading and immediately resolved by 10% SDS–PAGE. The resolved fractions were transferred to nitrocellulose and the β-galactosidase was detected by Western blot analysis using an antibody to β-galactosidase. The obtained proteolytic pattern was critically dependent upon the amount of chymotrypsin (1.25 μg) utilized with the given concentrations of BSA or chaperones (3.2 μM).

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