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Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)-domain co-chaperones

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The *in vivo* function of the heat shock protein 90 (Hsp90) molecular chaperone is dependent on the binding and hydrolysis of ATP, and on interactions with a variety of co-chaperones containing tetratricopeptide repeat (TPR) domains. We have now analysed the interaction of the yeast TPR-domain co-chaperones Sti1 and Cpr6 with yeast Hsp90 by isothermal titration calorimetry, circular dichroism spectroscopy and analytical ultracentrifugation, and determined the effect of their binding on the inherent ATPase activity of Hsp90. Sti1 and Cpr6 both bind with sub-micromolar affinity, with Sti1 binding accompanied by a large conformational change. Two co-chaperone molecules bind per Hsp90 dimer, and Sti1 itself is found to be a dimer in free solution. The inherent ATPase activity of Hsp90 is completely inhibited by binding of Sti1, but is not affected by Cpr6, although Cpr6 can reactivate the ATPase activity by displacing Sti1 from Hsp90. Bound Sti1 makes direct contact with, and blocks access to the ATP-binding site in the N-terminal domain of Hsp90. These results reveal an important role for TPR-domain co-chaperones as regulators of the ATPase activity of Hsp90, showing that the ATP-dependent step in Hsp90-mediated protein folding occurs after the binding of the folding client protein, and suggesting that ATP hydrolysis triggers client-protein release.

Keywords: ATP/chaperone/Hsp90/protein folding/TPR domain

Introduction

Heat shock protein 90 (Hsp90) is a ubiquitous and abundant molecular chaperone with essential functions in thermotolerance and the folding and activation of many signal transduction and cell regulatory proteins. Although isolated Hsp90 has passive anti-aggregant properties *in vitro* (Wiech *et al*., 1992), its activity *in vivo* depends on association with a set of co-chaperones which are components in a series of multiprotein complexes involving Hsp90 and a folding client protein. The direct role of ATP in the mechanism of Hsp90-mediated protein folding, has been contentious (Jakob *et al*., 1996). Recently, several studies have identified an ATP-binding site in the N-terminal domain of Hsp90 (Grenert *et al*., 1997; Prodromou *et al*., 1997a) and demonstrated a specific ATPase activity *in vitro* (Panaretou *et al*., 1998; Scheibel *et al*., 1998) sensitive to inhibition by the antibiotic geldanamycin, which binds to the ATP-binding site (Prodromou *et al*., 1997a; Stebbins *et al*., 1997). With further demonstration of the essential role of ATP binding and hydrolysis in the function of Hsp90 *in vivo* (Panaretou *et al*., 1998), Hsp90 must now be considered as an 'active' chaperone.

In the other ATP-dependent chaperone families, Hsp60/GroEL and Hsp70/DnaK (Bukau and Horwich, 1998), the ATPase activity is modulated by interactions with co-chaperones (Jordan and McMacken, 1995; McCarty *et al*., 1995; Theyssen *et al*., 1996), which enhance hydrolysis of ATP and/or promote nucleotide exchange. We have now analysed the interaction of Hsp90 with the tetratricopeptide repeat (TPR)-domain co-chaperones Sti1 and Cpr6, and investigated the effect of binding these co-chaperones on the inherent ATPase activity of yeast Hsp90.

Results

TPR-domain co-chaperone binding to Hsp90

Both Sti1 and Cpr6 displayed sigmoidal binding curves on titration with Hsp90, in isothermal titration calorimetry (ITC) experiments, consistent with simple two-state association reactions. Dissociation constants calculated from these curves were 0.24 μM for Cpr6 and 0.33 μM for Sti1 (Figure 1). The isolated TPR domain of human protein phosphatase 5 (PP5) showed even higher affinity for Hsp90 in ITC; however, as the TPR-PP5 domain had a tendency to aggregate, the titration curves are not simple sigmoids and a reliable estimate of the binding constant and contingent parameters was not obtainable.

Although the affinities of Cpr6 and Sti1 for Hsp90 are similar, the binding reactions are accompanied by very different contributions from enthalpy and entropy. For Cpr6 binding, the change in enthalpy approximates to the free energy of the reaction (ΔG = –8.9 kcal/mol; ΔH = –9.2 kcal/mol), indicating a very small entropic contribution to binding ($T\Delta S$ = –0.3 kcal/mol). In marked contrast, binding of Sti1 to Hsp90 displays a very large favourable enthalpic contribution (ΔH = –27.9 kcal/mol) offset by a large unfavourable change in entropy ($T\Delta S$ = –19.0 kcal/mol). The large favourable enthalpy accompanied by a large unfavourable entropy is characteristic of a major change in the conformation of one or both of the components in the binding reaction.

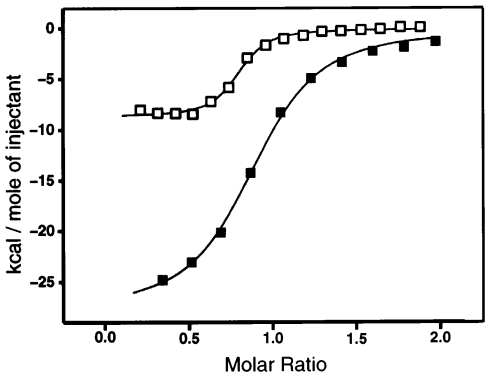

Fig. 1. ITC measurements of Hsp90 binding to Sti1 and Cpr6. Curves for the titration of Hsp90 into Cpr6 (open squares) and Sti1 (filled squares). Parameters derived from non-linear least-square fitting to these curves gives (for Cpr6) K_d = 0.24±0.05 μM; stoichiometry (Hsp90:Cpr6) = 1:1.33; ΔH = –8.6±0.2 kcal/mol, and (Sti1) K_d = 0.33±0.03 μM; stoichiometry (Hsp90:Sti1) = 1:1.19; ΔH = –27.9±0.1 kcal/mol.

We have also observed interaction between Hsp90 and Sti1, and between Hsp90 and Cpr6, using circular dichroism (CD) spectroscopy (Figure 2). Titration of Cpr6 or Sti1 into Hsp90 produces dose-dependent perturbations in the near-UV-CD (240–300 nm) region, and difference spectra, obtained by subtracting the spectrum of the co-chaperone (*n* molar equivalent) from the spectra of Hsp90 + co-chaperone (1:*n*) mixtures, show saturatable changes in the near-UV region consistent with changes in the environment of aromatic residues due to molecular interaction. In the far-UV-CD region, the observed CD spectrum of a mixture of Hsp90 + Cpr6 was superimposable on that obtained by linear combination of the individual CD spectra of Hsp90 and Cpr6 in the same ratio, indicating no change in the overall secondary structure content of either Hsp90 or Cpr6 resulting from their interaction (Figure 3A). In contrast, the observed CD spectrum of a Hsp90 + Sti1 mixture differed significantly from the linear combination of the individual CD spectra in the same ratio (Figure 3B), indicating an increase in overall secondary structure, consistent with significant conformational changes in one or both proteins on Sti1 binding to Hsp90, as suggested by the ITC analysis.

Stoichiometry of Hsp90–co-chaperone complexes

The interaction of immunophilins, Sti1 and its mammalian homologue p60/Hop, and PP5 with Hsp90 have been shown to be mutually competitive (Owens-Grillo *et al*., 1995; Silverstein *et al*., 1997). Further, the regions of the immunophilins and Sti1 which interact with Hsp90 have been localized to the TPR domains present in these proteins (Radanyi *et al*., 1994; Chen *et al*., 1996; Ratajczak and Carrello, 1996), implying the presence of a common TPR-domain-binding site in Hsp90 (Owens-Grillo *et al*., 1996). As immunoprecipitation of Hsp90 complexes via Hop/Sti1 does not co-precipitate immunophilins (Owens-Grillo *et al*., 1996; Chang *et al*., 1997), it is generally assumed that a single TPR-domain-binding site for these proteins is provided by the Hsp90 dimer. However, the ITC data for binding of Cpr6 or Sti1 to Hsp90 shows molar stoichiometries of ~1:1 in both cases, indicating that two co-chaperone molecules bind per Hsp90 dimer, and suggesting that each Hsp90 monomer provides a TPR-domain-binding site. The presence of two independent TPR-domain-binding sites in an Hsp90 dimer, and the comparable affinity of Cpr6 and Sti1, should allow for the existence of a Cpr6–$(Hsp90)_2$–Sti1 ternary complex. Such a complex has not been observed in immunoprecipitation from cells (Owens-Grillo *et al*., 1996; Chang *et al*., 1997), nor are we able to observe the formation of this complex from the pure components *in vitro* (data not shown). Thus, if two TPR-domain-binding sites do indeed exist, as the ITC data show, then the failure to observe a Cpr6–$(Hsp90)_2$–Sti1 ternary complex has one of two explanations: (i) there is a complicated coupling between the two TPR-domain-binding sites so that binding of Cpr6 to one site precludes binding of Sti1 to the other, whilst permitting binding of a second Cpr6, and vice versa; or (ii) Sti1 and/or Cpr6 are actually dimers themselves, binding to Hsp90 in a bidentate dimer–dimer interaction that simultaneously occupies both sites.

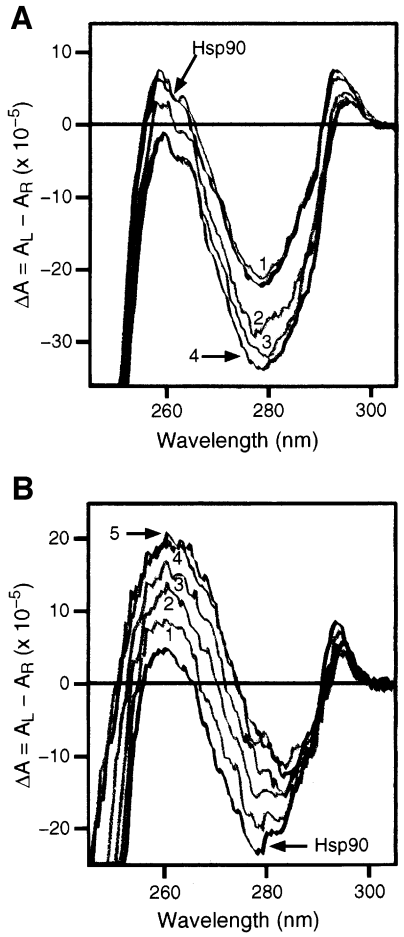

Fig. 2. Near-UV difference CD spectra for Hsp90–co-chaperone interactions. Near-UV difference CD spectra for titration of co-chaperones into Hsp90, obtained by subtracting the spectra of the free co-chaperone at *n* molar equivalents, from the spectra of Hsp90/co-chaperone mixtures at Hsp90: co-chaperone molar ratios of 1:*n*. (**A**) Cpr6–Hsp90 difference spectra. The spectrum of Hsp90 alone is indicated, curves 1–4 are from Hsp90/Cpr6 mixtures in the molar ratio 1:*n* (*n* = 0.38, 1.49, 2.20, 2.90). (**B**) Sti1–Hsp90 difference spectra. The spectrum of Hsp90 alone is indicated, curves 1–5 are from Hsp90/Sti1 mixtures in the molar ratio 1:*n* (*n* = 0.39, 0.77, 1.15, 1.53, 2.64).

Gel-filtration chromatography of Cpr6 (M_r = 43 500) shows a single species eluting with an apparent molecular weight of 47 000, indicating the presence of a monomer.

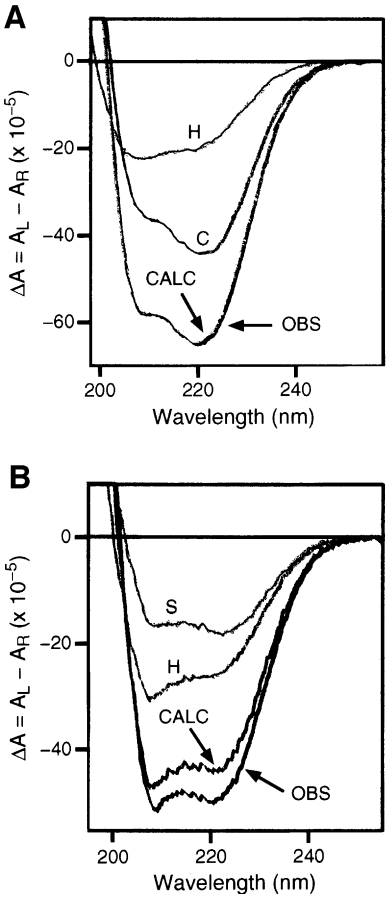

Fig. 3. Far-UV CD spectra for Hsp90–co-chaperone interactions. (**A**) Far-UV spectra for Hsp90, H; Cpr6, C; Hsp90 + Cpr6 observed spectrum, OBS; and linear combination of the separate spectra for Hsp90 and Cpr6 in the same proportion as the molar ratio of Hsp90 and Cpr6 in the complex, CALC. The observed and calculated spectra for the complexes are essentially identical, indicating no change in the overall secondary structure of either protein on binding. (**B**) as (A) but for Sti1, S. The observed spectrum for the complex cannot be reproduced by linear combination of the separate spectra for Hsp90 and Sti1, indicating a net change in the overall secondary structure of one or both proteins on binding.

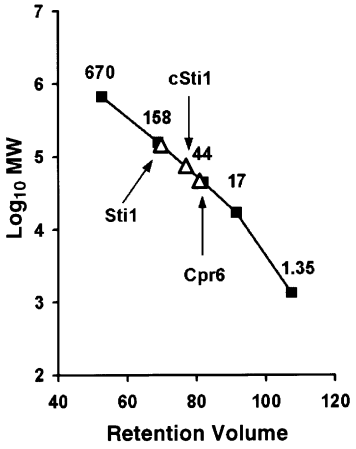

Fig. 4. Size determination of Sti1, cSti1 and Cpr6 by gel-filtration chromatography. Full-length Sti1, a truncated Sti1 construct lacking regions associated with Hsp70 binding (cSti1) and Cpr6, were run on a Superdex 200 PG 16/60 column calibrated with molecular weight standards (solid-line and squares) (see Materials and methods). Sti1, cSti1 and Cpr6 (open triangles) eluted at volumes corresponding to molecular weights of 141, 76 and 41 kDa, respectively.

However, Sti1 (M_r = 67 600) elutes as a single species with an apparent molecular weight of 141 000 suggesting that Sti1 is a dimer (Figure 4). Gel filtration does not always provide a reliable estimate of molecular size, and molecules with aspect ratios far from unity or those with cavities, can display anomalously high apparent molecular weights. Indeed, Hsp90 itself elutes with an apparent molecular weight of >450 000 on gel filtration. The oligomeric state of Cpr6 and Sti1 were therefore analysed by sedimentation equilibrium in an analytical ultracentrifuge. Analysis of Cpr6 sedimentation returns molecular weights in the range 41 000 ± 2000, consistent with a monomer. Furthermore, calculation of apparent molecular weight versus protein concentration shows that Cpr6 remains monomeric over a broad concentration range from sub-micromolar to sub-millimolar. In contrast, similar analyses of Sti1 sedimentation using models that assume a single species in solution return averaged molecular weights in the range 110 000–120 000, which is close to twice the value expected for monomeric Sti1. In addition, we observe a clear concentration dependence of the apparent molecular weight, consistent with oligomerization of Sti1. Best fits to the Sti1 sedimentation data are obtained with monomer–dimer equilibrium models, and these fits give dissociation constants in the low micromolar range. Thus, the gel filtration and analytical ultracentrifugation data both show that Cpr6 is a monomer and Sti1 is a dimer in solution, and these are likely to be their predominant states in their interaction with Hsp90 *in vivo*.

Sti1 binding as a dimer to the Hsp90 dimer would simultaneously occupy both TPR-binding sites and prevent any Cpr6 binding. Cpr6, binding as a monomer would nonetheless prevent binding of dimeric Sti1 even if only one TPR-binding site were occupied by Cpr6. No obvious cooperativity is evident in the ITC data for Cpr6 binding to Hsp90, suggesting that the two TPR-binding sites in the Hsp90 dimer are independent. Simultaneous binding and effective 'cross-linking' of these independent sites by the Sti1 dimer might then be the cause of the conformational change observed by CD spectroscopy and implied by the ITC data for Sti1 binding to Hsp90. No such change would be expected to accompany binding of Cpr6 monomers to independent sites.

Modulation of Hsp90 ATPase activity

Using a sensitive enzyme-coupled regenerating assay (Ali *et al*., 1993), we measured the inherent ATPase activity of yeast Hsp90 *in vitro* as previously described (Panaretou *et al*., 1998), but in the presence of the yeast co-chaperones, Cpr6 and Sti1. As the specific inherent ATPase activity of yeast Hsp90 is low (~0.2/min at 30°C), and even very pure preparations of Hsp90 are contaminated with co-purifying non-specific ATPase activities, we used the Hsp90-binding antibiotic geldanamycin, whose binding site co-localizes with the ATP-binding site in Hsp90s N-terminal domain (Prodromou *et al*., 1997a; Stebbins *et al*., 1997), to determine the Hsp90-specific ATPase in these reactions. The Hsp90 preparations, which were pure enough to grow crystals (Prodromou *et al*., 1996) nonetheless displayed a geldanamycin-resistant background activity at ~15–20% of the total ATPase activity

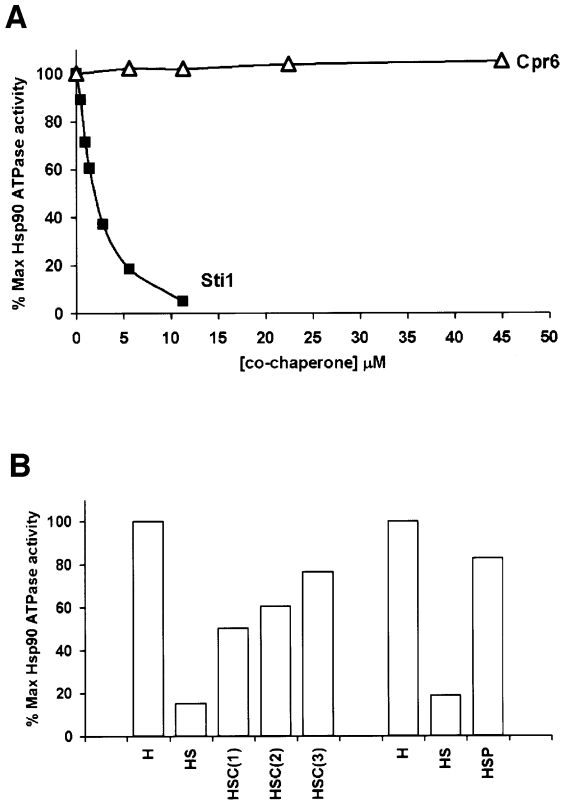

Fig. 5. ATPase activities of Hsp90 co-chaperone complexes. (**A**) Hsp90-specific ATPase activity as a function of added Cpr6 (open triangles) or Sti1 (filled squares). Hsp90 was 1.4 μM throughout. All points are averages from three independent titrations. (**B**) Relief of Hsp90 ATPase inhibition Sti1 inhibition by competing co-chaperones. Activities are shown for Hsp90 (1.4 μM) alone, H; Hsp90 (1.4 μM) after addition of Sti1 (5.6 μM), HS; Hsp90 (1.4 μM) after addition of Sti1 (5.6 μM), followed by increasing concentrations of Cpr6 (11.1, 22.2, 44.4 μM), HSC(1, 2 and 3). Activities are averages from three independent experiments. H, HS and HSP are a similar experiment with the addition of hPP5-TPR (5.6 μM) in HSP. These data are averages of two independent experiments.

detectable. Neither of the co-chaperones displayed significant ATPase activities on their own, nor in combination (data not shown).

Addition of Cpr6 alone had no effect on the inherent ATPase activity of Hsp90 up to concentrations of 44 μM, being in ~30-fold molar excess of Hsp90. Given the affinity we have measured for the interaction of Cpr6 and Hsp90 (~0.24 μM), virtually all of the Hsp90 in this experiment would be complexed with Cpr6. In contrast, Sti1 had a very marked inhibitory effect on the inherent ATPase activity of Hsp90, reducing it to the geldanamycin-resistant background by 10 μM, with an effective IC_{50} in the assay of ~2.0 μM (Figure 5A). Inhibition of Hsp90s ATPase activity by Sti1 could be relieved by subsequent addition of Cpr6 or the isolated TPR domain of human PP5 (Figure 5B), consistent with all the co-chaperones competing for the same, or at least overlapping binding sites.

Hop, the mammalian homologue of Sti1, has been shown to interact with both Hsp70 and Hsp90 via distinct regions of the molecule, with the minimal Hsp90-interacting region being localized to the middle and C-terminus (Chen *et al*., 1996). The cSti1 construct (see Materials and methods) that lacks the regions of Sti1 implicated in interaction with Hsp70, was fully capable of inhibiting the Hsp90 ATPase activity at comparable levels to the full-length protein, and this inhibition was equally sensitive to relief by Cpr6 (data not shown). The cSti1 protein (monomer M_r = 41.7 kDa) also behaved as a dimer in gel filtration, eluting with an apparent molecular weight of 76 kDa.

Co-chaperone interaction with ATP-binding domain

The ATP-binding site of Hsp90 resides in the N-terminal domain (Grenert *et al*., 1997; Prodromou *et al*., 1997a), and this domain binds ATP with comparable affinity to the intact chaperone. However, the isolated domain displays negligible ATPase activity compared with intact Hsp90, suggesting that at least part of the catalytic apparatus for the ATP-hydrolysis reaction is delivered by domain(s) C-terminal of the ATP-binding site. The primary binding site(s) for TPR-domain co-chaperones has been localized to the C-terminal region of Hsp90 (Chen *et al*., 1998; Young *et al*., 1998). Thus, Sti1 might inhibit Hsp90 ATPase activity by interacting with the C-terminus and locking Hsp90 into a conformation in which C-terminal catalytic residues cannot interact with the γ-phosphate of ATP presented at the mouth of the N-terminal ATP-binding pocket. Although the C-terminal region of Hsp90 appears to be necessary and sufficient for TPR-domain-binding, the apparent affinity of Hop, but not immunophilins, for Hsp90 was decreased when the N-terminal domain of Hsp90 was partially deleted (Chen *et al*., 1998), suggesting a direct interaction between Hop/Sti1 and this Hsp90 N-terminal domain. ATPase inhibition by Sti1 might therefore alternatively involve direct interaction with the ATP-binding site.

To gain insight into the nature of ATPase inhibition by Sti1, we have taken advantage of a change in the CD spectrum of geldanamycin on binding to Hsp90. Difference CD spectra, obtained by subtracting the CD spectra of free geldanamycin at a range of concentrations from the CD spectra of mixtures of Hsp90 + geldanamycin at the same concentrations (Figure 6A), contain primarily the contributions of Hsp90 and the bound ligand species. Above 300 nm, the contribution from protein is negligible, and the difference CD spectrum arises entirely from the bound geldanamycin. Thus, the intensity of the difference CD spectrum in this region is indicative of the degree of saturation of the geldanamycin-binding site in the protein, and can be used to estimate the K_d for the binding interaction (Freeman *et al*., 1998). Fitting of the difference CD signal at 310 nm as geldanamycin is titrated into Hsp90 gives a K_d of ~0.25 μM, which is in the range of values previously obtained from ITC measurements (Panaretou *et al*., 1998; Roe *et al*., 1998). The CD above 300 nm can be attributed to the benzoquinone moiety of geldanamycin, which when bound to Hsp90, occupies the binding sites for the phosphate groups of ATP (Prodromou *et al*., 1997a; Roe *et al*., 1998). Geldanamycin is therefore a sensitive probe for measuring the accessibility of the nucleotide-binding pocket, and for detecting interactions with co-chaperones or client proteins close to or in the pocket.

Titration of geldanamycin into a pre-formed Hsp90–Cpr6 complex, gives essentially the same difference CD

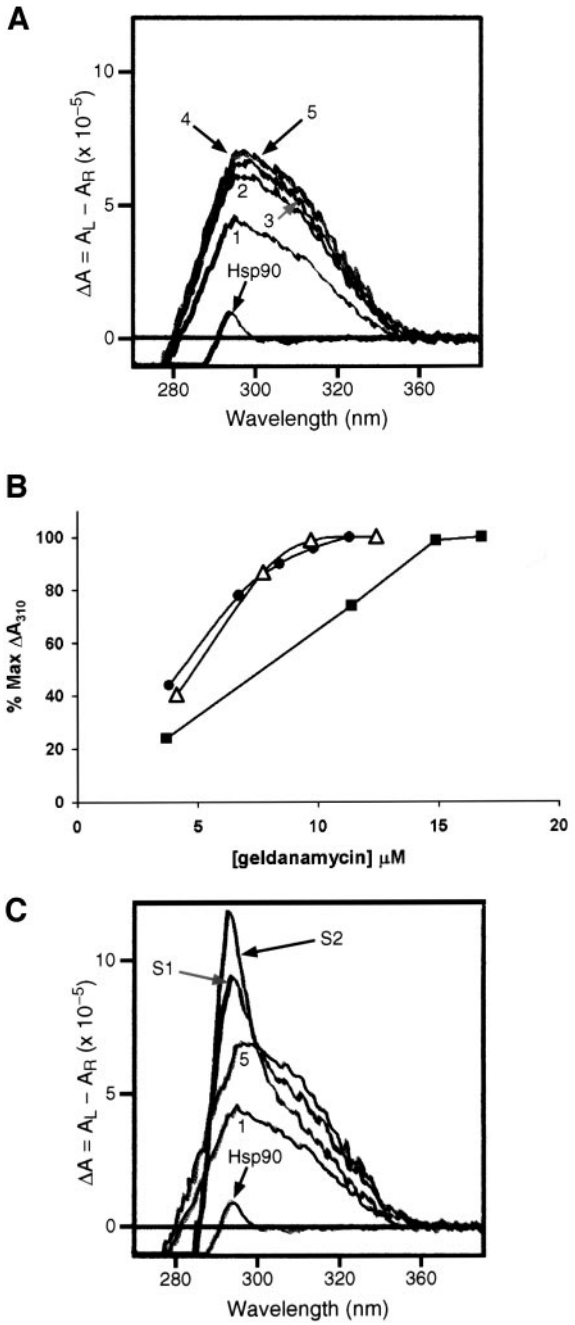

Fig. 6. CD titration of geldanamycin binding. (**A**) Near-UV difference spectra for the titration of geldanamycin into Hsp90, obtained by subtracting spectra for free geldanamycin from spectra for Hsp90/geldanamycin at the same geldanamycin concentrations. The spectrum for Hsp90 alone is indicated; 1–5 are spectra for geldanamycin at 2.4, 4.8, 7.2, 9.5 and 13.9 μM, respectively. (**B**) Change in difference CD at 310 nm as a function of geldanamycin concentration added to Hsp90 alone (filled circles), Hsp90+Cpr6 (open triangles) and Hsp90+Sti1 (filled squares). (**C**) Displacement of geldanamycin from Hsp90 by Sti1. Spectra 1 and 5 are as in (A); S1 and S2 are difference CD spectra obtained by subtraction of spectra for free geldanamycin and for free Sti1 from the spectra obtained by addition of Sti1 (2.6 and 5.2 molar excess over Hsp90), to a saturated Hsp90/geldanamycin complex. The increase in ΔA_{295} due to binding of Sti1 to Hsp90 is accompanied by a decrease in the ΔA_{310} due to displacement of bound geldanamycin.

at 310 nm as a function of added geldanamycin as with Hsp90 alone (Figure 6B). Thus, consistent with its lack of effect on Hsp90 ATPase activity, bound Cpr6 does not block access to the nucleotide-binding pocket, either directly, or by altering the conformation of Hsp90. In contrast, when geldanamycin is titrated into a pre-formed Hsp90–Sti1 complex, the difference CD as a function of geldanamycin concentration is considerably reduced (Figure 6B) showing that access to the nucleotide-binding pocket is substantially hindered in that complex. Furthermore, in the reverse experiment, titration of Sti1 into Hsp90 pre-saturated with geldanamycin causes a progressive decrease in the difference CD at 310 nm, accompanied by an increase at ~295 nm, consistent with displacement of Hsp90-bound geldanamycin and formation of an Hsp90–Sti1 complex (Figure 6C).

Discussion

Previous studies have shown that a variety of proteins associated with Hsp90 complexes, interact with Hsp90 via TPR domains (Radanyi *et al*., 1994; Owens-Grillo *et al*., 1996; Ratajczak and Carrello, 1996; Silverstein *et al*., 1997). The binding affinity for Hsp90 we observe for two such proteins, Cpr6 and Sti1, is in the sub-micromolar range, sufficient to ensure that *in vivo*, these co-chaperones would predominantly exist in complexes with Hsp90. A variety of studies have demonstrated competition between TPR-domain co-chaperones (Owens-Grillo *et al*., 1995, 1996; Ratajczak and Carrello, 1996; Silverstein *et al*., 1997), suggesting that these proteins bind to a common, and presumably single TPR-domain-binding site on the Hsp90 dimer. However, our ITC data for the binding of Cpr6 and Sti1 to Hsp90 clearly demonstrate the presence of two TPR-binding sites in the Hsp90 dimer. Furthermore, gel-filtration chromatography and sedimentation-equilibrium data both indicate that while Cpr6 is monomeric, Sti1 itself exists as a dimer and would therefore associate with Hsp90 in a dimer–dimer interaction. Mutual exclusion of Sti1 and Cpr6 (and other immunophilins) from Hsp90 complexes (Owens-Grillo *et al*., 1996; Chang *et al*., 1997) would then result from the requirement of the Sti1 dimer to simultaneously occupy both TPR-domain-binding sites.

Sti1/Hop is able to bind simultaneously to Hsp90 and Hsp70 (Smith *et al*., 1993) and appears to act as a scaffold protein, physically coupling these two chaperones (Chen *et al*., 1996; Johnson *et al*., 1998). Given that Sti1 is a dimer in free solution and interacts with Hsp90 as a dimer, it seems reasonable to suppose that Sti1 can recruit two molecules of Hsp70 into the Hsp70–Sti1–Hsp90 ternary complex. However, the affinity and stoichiometry of the Sti1/Hop–Hsp70 interaction remain to be experimentally determined.

Structural and biochemical studies have unambiguously identified an ATP-binding site in the N-terminal domain (Grenert *et al*., 1997; Prodromou *et al*., 1997a). More recently, we have demonstrated the presence of an inherent ATPase activity in Hsp90, dependent on this binding site, which is essential to the function of this chaperone *in vivo* (Panaretou *et al*., 1998). The turnover rate we observe for this inherent ATPase activity in yeast Hsp90 is comparable with the unstimulated ATPase activities of Hsp70s (O'Brien and McKay, 1995; Bukau and Horwich, 1998). The inherent ATPase activity of Hsp70/DnaK is significantly stimulated by interactions with the co-chaperone

DnaJ (or its eukaryotic homologues) (Jordan and McMacken, 1995; Nagata *et al*., 1998). In marked contrast, the inherent ATPase activity of Hsp90 is more or less abolished by interaction with Sti1. As Cpr6 neither stimulates nor inhibits Hsp90s ATPase activity, binding to the C-terminal TPR-binding sites on Hsp90 appears to have no direct influence on the ATPase activity *per se*. However, as the TPR-domain co-chaperones compete for binding to Hsp90, addition of Cpr6 or the hPP5-TPR domain can restore the Hsp90 ATPase activity by displacing bound Sti1.

Mutagenesis studies of human Hsp90 (Chen *et al*., 1998) indicated a weakening of Hop binding to Hsp90 when part of the N-terminal ATP-binding domain of Hsp90 was deleted. Genetic studies in yeast showed synthetic effects between Sti1 expression levels and *ts* mutations of residues in the N-terminal domain of Hsp90 (Chang *et al*., 1997). Here we have shown that binding of Sti1 to Hsp90 *in vitro* inhibits Hsp90s ATPase activity, and displaces geldanamycin bound in the ATP-binding site in the N-terminal domain. Taken together these results suggest a direct interaction between Hop/Sti1 and the ATP-binding site in the N-terminal domain.

The mouth of the ATP-binding pocket in Hsp90, opens into an extended channel, traversing one face of the N-terminal domain, whose dimensions suggest it could provide a binding site for a segment of polypeptide chain. The lining and side walls or the channel are formed by residues which are highly conserved in all Hsp90 sequences (Prodromou *et al*., 1997a), and one end of this channel is adjacent to an exposed hydrophobic patch on the N-terminal domain which has the characteristics of a protein–protein interaction site (Prodromou *et al*., 1997b). Two temperature-sensitive mutations of yeast Hsp90 (Thr22Ile, Thr101Ile) (Nathan and Lindquist, 1995) lie in this putative 'interaction patch', and both of these mutants show synthetic effects in the presence of Sti1 overexpression. Thus, this surface of the isolated N-terminal domain of Hsp90 containing the mouth of the nucleotide-binding pocket is a reasonable candidate for the putative secondary site of interaction of Sti1 with intact Hsp90. The observed displacement of geldanamycin on binding of Sti1 is fully consistent with bound Sti1 making direct interactions in or very close to the mouth of the ATP-binding pocket. Blockade of the ATP-binding pocket by Sti1 would prevent binding of ATP, and block access of catalytic residues from C-terminal regions, inhibiting the ATPase activity as observed.

Binding of denatured polypeptides and hydrophobic peptides to the isolated N-terminal domain of Hsp90 has been reported (Young *et al*., 1997; Scheibel *et al*., 1998) and interpreted as indicating a second 'chaperone' site in Hsp90, distinct from the main binding site for client proteins, which is localized in the C-terminal regions of Hsp90. In one study, peptide binding to the isolated N-terminal domain was found to be strongly inhibited by geldanamycin, but not by ATP, while the other study showed strong competition for peptide binding by ATP but less so with geldanamycin. Competition for binding by ATP and/or geldanamycin would suggest that these peptides probably bind in the vicinity of the ATP-binding pocket close to the site of Sti1 interaction we identify here, and may even be mimicking Sti1 interactions. As neither study provides any quantitation of the affinity of the isolated N-terminal domain for their different peptide and denatured polypeptide reagents, it is difficult to ascertain the significance of these observations, which may just represent a non-specific 'detergent' effect provided by the hydrophobic patch exposed on the surface of the isolated N-terminal domain.

Cell-free assembly studies (principally of steroid hormone receptors) have shown an ordered pathway of chaperone complexes associated with folding client proteins (Smith *et al*., 1995). Consistent with the sub-micromolar affinity we observe *in vitro*, Hsp90 and Hop/Sti1 exist in mammalian and yeast cells as an adundant and stable complex which accounts for the majority of the cellular Hop/Sti1 (Smith *et al*., 1993; Chang *et al*., 1997). Our results suggest that the ATPase activity of Hsp90 in this Hsp90–Hop/Sti1 complex will be substantially inhibited and the Hsp90 dimer will be conformationally locked by its stable bidentate interaction with the Sti1 dimer. Hsp90 only becomes associated with the folding client protein in the intermediate complex, which is probably formed by binding of an 'early' Hsp70–client complex, to the pre-existing Hsp90–Hop/Sti1 complex. As the ATPase inhibitory properties of Sti1 are retained in the truncated version (cSti1) lacking the Hsp70-binding regions, it is unlikely that binding of Hsp70 to the Hsp90–Hop/Sti1 complex would relieve this inhibition, so that the ATPase activity of Hsp90 in the intermediate complex, would also be silent. Hop does not appear to influence the ATPase activity of Hsp70 (Johnson *et al*., 1998), so that unlike Hsp90, the ATPase of Hsp70 in the intermediate complex is likely to be active.

Subsequent to formation of the intermediate complex, Hsp70 (and associated Hip and Hsp40s) and Hop/Sti1 are replaced by one of several large immunophilins (FKBP51, FKBP52 or Cyp40/Cpr6/Cpr7) or by PP5, all of which contain TPR domains by which their interaction with Hsp90 is mediated. Our data suggest that replacement of Hop/Sti1 by an immunophilin or PP5, would unblock the N-terminal ATP-binding site on Hsp90, so that the Hsp90 in mature complexes would be able to bind and hydrolyse ATP. As the displacement of Hsp90-bound Hop/Sti1 by immunophilins is somewhat diminished in the presence of geldanamycin (Smith, 1995), conversion from the intermediate to the mature complex, may require the concomittant binding of a TPR-domain co-chaperone and ATP. Mature steroid receptor folding complexes also contain an acidic protein, p23/Sba1, whose association with Hsp90 is dependent on the presence of ATP or ATP analogues (Johnson and Toft, 1994, 1995; Fang *et al*., 1998), and of an intact ATP-binding site in Hsp90 (Grenert *et al*., 1997), but does not bind to the isolated N-terminal domain. Consistent with its requirement for ATP loaded-Hsp90, binding of p23 is inhibited by Hop/Sti1 (Johnson *et al*., 1998).

It is now clear that Hsp90 both binds and hydrolyses ATP, and that this ability is essential to its function *in vivo*. Here we have shown that this inherent ATPase activity is regulated by interaction with TPR-domain co-chaperones, so that it is inhibited in the early phases of Hsp90-mediated protein folding, when Hop/Sti1 is present in the 'intermediate' folding complex, and this inhibition is relieved in the later phases when Hop/Sti1 is replaced by

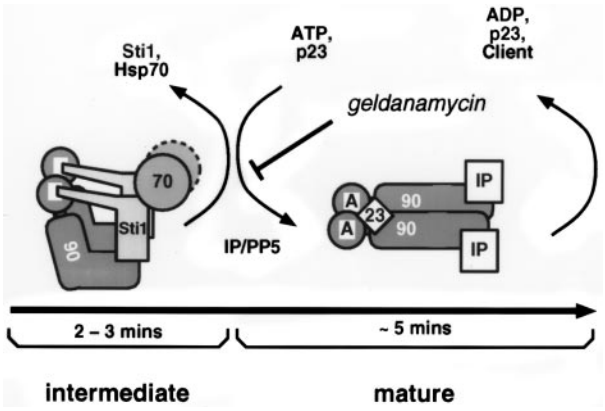

Fig. 7. Composition and interchange of Hsp90-based chaperone complexes. Schematic diagram showing the composition of, and transition between the 'intermediate' and 'mature' Hsp90 chaperone complexes, incorporating the current literature plus the new data presented here. The client protein is not shown. The 'intermediate' complex, which lasts around 2–3 min post-translation of the client protein (Smith, 1993; Smith *et al*., 1995), contains an Hsp90 dimer–Sti1 dimer complex, with access to the ATP-binding sites in the N-terminal domains of the Hsp90, blocked by Sti1. The number of Hsp70 (plus associated Hsp40 and Hips) in this complex remains to be determined. Displacement of Sti1 and Hsp70 etc. unblocks the ATP-binding site and allows binding of other TPR-domain co-chaperones and p23/Sba1, forming 'mature' complexes whose lifetime is ~5 min. The number of p23/Sba1 molecules bound per 'mature' complex has not been determined. Geldanamycin blocks ATP binding and prevents the conversion into the 'mature' complex. Hydrolysis of bound ATP by Hsp90 releases p23/Sba1 and the client protein. Subsequent re-binding of Sti1 would promote release of ADP and immunophilins, priming the Hsp90 complex for binding of a new client protein molecule delivered into the 'intermediate' complex by Hsp70 (plus Hsp40 and Hip).

other TPR-domain co-chaperones in the 'mature' complex (Figure 7). Consistent with this, client protein binding is not prevented by geldanamycin (Smith *et al*., 1995; Dittmar and Pratt, 1997) a competitive inhibitor of the ATPase activity of Hsp90. Thus, the ATP-dependent step in Hsp90-mediated protein folding occurs after the client protein has bound to the Hsp90-based chaperone complex, as we have previously suggested (Panaretou *et al*., 1998).

The nature of the ATP-bound 'mature' complex is still obscure, but is the state of Hsp90 complexes in which productive folding and/or activation appears to take place, as blocking its formation with geldanamycin inhibits production of folded client proteins (Blagosklonny *et al*., 1995; Chavany *et al*., 1996; Schulte *et al*., 1996; An *et al*., 1997; Bamberger *et al*., 1997; Chen *et al*., 1997; Dasgupta and Momand, 1997; Hartmann *et al*., 1997; Segnitz and Gehring, 1997; Uma *et al*., 1997; Whitesell *et al*., 1997; Garcia-Cardena *et al*., 1998). Hsp90 locked into a pseudo-ATP-bound state by the presence of molybdate ions displays a lowered affinity for hydrophobic resins (Sullivan *et al*., 1997), which could indicate a decreased affinity for client proteins analogous to the ATP-bound states of Hsp60/GroEL or Hsp70/DnaK (Bukau and Horwich, 1998), but how this would promote folding in the case of Hsp90 remains unclear. The role of immunophilins, PP5 and p23/Sba1, which are recruited to ATP-bound Hsp90 complexes *in vivo*, is also unclear, as these proteins appear to be non-essential for refolding of glucocorticoid receptor in *in vitro* reconstructions (Dittmar and Pratt, 1997).

The ATP-bound 'mature' complex will be converted into an ADP-bound complex by the inherent ATPase activity of Hsp90 (Panaretou *et al*., 1998). The turnover rate we have measured for this activity at 30°C in free Hsp90 and in an Hsp90–Cpr6 complex, is ~0.2/min. As the affinity of Hsp90 for ADP is relatively low (unlike Hsp70), nucleotide exchange will be rapid, so that the lifetime of the ATP-bound state of an Hsp90–Cpr6 complex would be ~5 min. This is very similar to the lifetime reported for the association of progesterone receptor with immunophilin–Hsp90–p23 'mature' complexes at 30°C, in reticulocyte lysates (Smith, 1993; Smith *et al*., 1995). The similarity of these lifetimes suggests that ATP hydrolysis provides the trigger for release of bound client protein from the mature complex.

Materials and methods

Expression and purification of Hsp90, Sti1 and Cpr6

An overnight culture of *Escherichia coli* BL21(DE3)pLysS carrying a His-tagged version of Hsp90, Sti1, cSti1 (C-terminal domain of Sti1, residues 237–589) or Cpr6 cloned into pRSETA was used to inoculate 4–8 l of LB containing 100 μg/ml ampicillin and 10 μg/ml chloramphenicol. The culture was grown to an OD_{650} of 0.7, induced with 1 mM IPTG and grown for 3 h prior to harvesting by centrifugation. Cells containing Sti1, cSti1 or Cpr6 were re-suspended in 20 mM Tris–HCl pH 8.0 containing 100 mM NaCl (buffer A), while those containing Hsp90 were re-suspended in 20 mM Tris–HCl pH 7.4, 10 mM EDTA, 1 mM dithiothreitol and 15% glycerol. Cells were disrupted by sonication in the presence of protease inhibitors (Boeringer Mannheim), and the cell lysate spun at 18 000 r.p.m. at 4°C for 60 min. Hsp90 cell lysate was loaded onto a Q-Sepharose column which was subsequently developed with a NaCl gradient of 0–2 M. The other His-tagged proteins were loaded onto a Talon column equilibrated in buffer A. The column was washed extensively with buffer A, followed by buffer A containing 10 mM imidazole. The bound His-tagged protein was subsequently eluted with buffer A (pH 7.0) containing 100–300 mM imidazole. His-tagged proteins were then concentrated in an Amicon stirred cell equipped with a XM50, YM30 or YM10 membrane or by ammonium sulfate precipitation (60 or 80% saturation). The concentrated protein was subsequently loaded onto a Sephacryl 400 HR (Hsp90), a Superdex 200 PG (Sti1 and cSti1) or a Superdex 75 PG (Cpr6) gel-filtration column equilibrated in 20 mM Tris–HCl pH 8.0, 500 mM NaCl, 5 mM EDTA and protease inhibitors. With His-tagged Hsp90 gel filtration was performed in the same buffer except that EDTA was omitted. Fractions containing His-tagged protein were pooled. His-tagged Hsp90 was diluted 5-fold with 20 mM Tris–HCl (pH 8.0) and subjected to Talon affinity chromatography as described above. Finally all His-tagged proteins were dialysed against 3×5 l of 20 mM Tris–HCl pH 7.4 containing 1 mM EDTA and concentrated using Amicon Centriplus and Centricon concentrators with appropriate molecular weight retention. Proteins were concentrated to the following levels: Hsp90, 30 mg/ml; Sti1, 31.6 mg/ml; cSti1, 11.7 mg/ml; and Cpr6, 82 mg/ml. The TPR domain of human protein phosphatase 5 (hPP5-TPR; residues 16–181) (2 mg/ml) was a kind gift from David Barford (University of Oxford, UK).

Molecular weight determination by gel filtration

One hundred microlitre aliquots of protein (1 mg/ml) were loaded onto a Superdex 200 PG 16/60 column equilibrated in 10 mM Tris–HCl pH 7.4, 1 mM EDTA and 250 mM NaCl. The column was run at 1 ml/min and was calibrated using gel-filtration standards from Bio-Rad (bovine thyroglobulin, M_r = 670 000; bovine γ-globulin, M_r = 158 000; chicken ovalbumin, M_r = 44 000; horse myoglobin, M_r =17 000; and vitamin B12, M_r = 1350). Molecular weights of Sti1, cSti1 and Cpr6 were determined by logarithmic interpolation.

Molecular weight determination by analytical ultracentrifugation

Sedimentation equilibrium experiments were conducted at 16°C in a Beckman-Optima XL-I analytical ultracentrifuge. Starting concentrations of ~15 and 45 μM were used for Sti1 and Cpr6, respectively, in order to give initial A_{280} values of ~1 in 1.2 cm path length cells. For each protein, ~100 μl samples were spun at 5000, 10 000, 20 000 and 40 000 r.p.m. in an An-60 Ti rotor, and allowed to reach equilibrium, which

took ~24 h in each case. Sedimentation curves were measured by Rayleigh Interference and by absorbance at 280 nm. For each sample, the various data sets were fitted both individually and simultaneously using routines in the Beckman–Optima XL-A/XL-I data analysis software (v4.0), which employ a non-linear least-squares method (Johnson *et al*., 1981). Two fitting models were used; the first assumed solutions of ideal single species whilst the second assumed monomer–dimer equilibria and fixed monomer molecular weights of 67 647 and 43 541 for Sti1 and Cpr6, respectively, which were calculated from their amino acid sequences. The viscosity of the buffer (20 mM Tris pH 7.4, 1 mM EDTA) at 16°C was calculated to be 1 mg/ml and the partial specific volumes for Sti1 and Cpr6 were calculated on the basis of amino acid composition as 0.724 and 0.733 ml/mg, respectively.

Hsp90 ATPase activity assay

Proteins destined for ATPase assays were passed through a 2 ml column of C8-coupled ATP–agarose (Sigma A2767) equilibrated in 20 mM Tris–HCl pH 7.4, 10 mM MgCl and 100 mM NaCl, to minimize contamination with non-specific ATPase activities. None of the His-tagged proteins bound to the resin and were therefore collected as a flow-through fraction. Proteins were subsequently concentrated and the buffer exchanged to 20 mM Tris–HCl pH 7.4 containing 1 mM EDTA using Centricon 10 micro-concentrators (Amicon). Hsp90 was used at 1.4 μM throughout. ATPase activity was assayed at 37°C using a regenerating enzyme-coupled system (Ali *et al*., 1993) and followed as a decrease in absorbance at 340 nm. Hsp90-specific ATPase activity was determined using geldanamycin as previously described (Panaretou *et al*., 1998). Sti1, cSti1, Cpr6 and hPP5-TPR were added at various concentrations and the change in the specific ATPase activity of Hsp90 observed. Geldanamycin was kindly provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda MD, USA.

Isothermal titration calorimetry

Titration experiments were performed using the MSC system (MicroCal Inc., MA). In each experiment, 16 aliquots of 15 μl were injected into 1.3 ml of the other protein component at 25°C. Protein concentrations (injected component first) used were as follows: 105 μM Hsp90 and 7 μM Sti1; 88 μM Sti1 and 5.8 μM Hsp90; 224 μM Hsp90 and 25 μM Cpr6; 63 μM Cpr6 and 7.3 μM Hsp90; 224 μM Hsp90 and 25 μM TPR-PP5. The resulting data were fitted after subtracting the heats of dilution as described previously (Prodromou *et al*., 1997a). Heats of dilution were determined in separate experiments by diluting protein into buffer and buffer into protein. Titration data were fit using a non-linear least squares curve-fitting algorithm with three floating variables: stoichiometry, binding constant ($K_B = 1/K_D$) and change of enthalpy of interaction ($\Delta H°$).

Circular dichroism spectroscopy

CD spectra were recorded with nitrogen-flushed JASCO spectropolarimeters J600 and J720 using 4 s time constant, 10 nm/min scan speed and a spectral bandwidth of 2 nm. One or two cm path-length cells were used for measurements in the near-UV region (240–320 nm) and 0.02 cm or 0.05 cm cells were used for measurements in the far UV region (185–250 nm). CD spectra are reported in $\Delta A = A_L - A_R$ based upon an average molecular weight per amino acid of 113. Both spectropolarimeters were calibrated with ammonium D-champor-10-sulfonate.

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