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Two chaperone sites in Hsp90 differing in substrate specificity and ATP dependence

(heat shock proteins/anti-tumor drugs/peptide binding/steroid receptors/src kinases)

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ABSTRACT **The abundant molecular chaperone Hsp90 is a key regulator of protein structure in the cytosol of eukaryotic cells. Although under physiological conditions a specific subset of proteins is substrate for Hsp90, under stress conditions Hsp90 seems to perform more general functions. However, the underlying mechanism of Hsp90 remained enigmatic. Here, we analyzed the function of conserved Hsp90 domains. We show that Hsp90 possesses two chaperone sites located in the N- and C-terminal fragments, respectively. The C-terminal fragment binds to partially folded proteins in an ATP-independent way potentially regulated by cochaperones. The N-terminal domain contains a peptide binding site that seems to bind preferentially peptides longer than 10 amino acids. Peptide dissociation is induced by ATP binding. Furthermore, the antitumor drug geldanamycin both inhibits the weak ATPase of Hsp90 and stimulates peptide release. We propose that the existence of two functionally different chaperone sites together with a substrate-selecting set of cochaperones allows Hsp90 to guide the folding of a subset of target proteins and, at the same time, to exhibit general chaperone functions.**

Hsp90 is one of the most abundant heat shock proteins in eukaryotic cells. Its levels amount to 1–2% of the total cellular protein even without stress (1). Members of the Hsp90 gene family are well conserved from bacteria to humans and can be found in the cytosol, in the endoplasmic reticulum and in chloroplasts. Hsp90 facilitates structural changes of key regulatory proteins of certain signal transduction pathways by chaperoning such as steroid receptors (2–4), kinases (5–7), reverse transcriptase (8), and p53 (9). In this context, Hsp90 acts in concert with a group of specific partner proteins such as prolyl isomerases, p23, Hsp70, and Hop (2–4, 8, 9).

Under heat shock conditions, when Hsp90 expression is increased severalfold, Hsp90 alone seems to perform additional functions in maintaining the structure of heat labile proteins because the expression of its partner proteins remains on similar levels. In this context, *in vitro* and *in vivo* experiments have demonstrated the general chaperone properties of Hsp90 (10–14). Although all of these experiments suggested that the chaperone properties observed *in vitro* are ATP-independent, weak ATP binding to Hsp90 was detected recently (15). Furthermore, the crystal structure of an N-terminal fragment of Hsp90 was solved in complex with ATP (16). X-ray crystallography also revealed that geldanamycin (GA), a novel anti-tumor drug that inhibits Hsp90 function, binds specifically to the ATP binding site of Hsp90 (16, 17). Despite this progress, little is known about the site of interaction with nonnative proteins on Hsp90 and the potential influence of ATP or GA on its chaperone function.

To address these issues, we have begun to analyze the chaperone function of isolated fragments of yeast Hsp90. We show here that two chaperone sites exist in Hsp90 that differ in their substrate specificity. The C-terminal fragment recognizes structured substrates, and the N-terminal fragment binds preferentially unfolded (poly)peptides and prevents unproductive side reactions such as aggregation. In addition, our results allow us to functionally link ATP binding to the chaperone site in the N-terminal domain of Hsp90.

MATERIALS AND METHODS

Protein Purifications. Yeast Hsp90 was purified as described (18). The yeast Hsp90 fragments including amino acids 1–210 (N210) and 262–709 (262C) were purified after recombinant expression in *Escherichia coli* by chromatography using DE52, hydroxyapatite, resource Q, and gel filtration using a Superdex 200 prep grade column (Pharmacia) as a final step. The purity of the fragments was >98% as measured by densitometry. The concentrations of the wild-type protein, N210 and 262C, were determined using the calculated extinction coefficients of 0.73, 0.67, and 0.85, respectively, for a 1-mg/ml solution in a 1-cm cuvette at 280 nm (19).

ATPase Activity. ATPase assays were performed as described (20). Hsp90 and both fragments were incubated at 30°C with 2 mM (final concentration) unlabeled ATP and 3 μCi of [α-^{32}P]ATP in a total volume of 20 μl, containing 40 mM Hepes·KOH (pH 7.5) and 2 mM $MgCl_2$. Protein concentrations varied between 1 and 3 μg/μl. GA was obtained from the Experimental Drugs Division of the National Institutes of Health (Bethesda, MD).

Citrate Synthase (CS) Assay. CS was denatured from pig heart mitochondria (Boehringer Mannheim) either chemically in 6 M guanidinium chloride, 50 mM Tris·HCl (pH 8.0) and 20 mM dithioerythritol for 1 h at 25°C or thermally by incubation at 43°C in 40 mM Hepes (pH 7.5).

Aggregation of nonnative CS was measured by light scattering in stirred quartz cuvettes in a Perkin—Elmer MPF 44A luminescence spectrophotometer with a thermostated cell holder. The excitation and emission wavelengths were set to 500 nm with a spectral bandwidth of 2 nm for the thermally denatured protein; in the case of the chemically denatured protein, excitation and emission wavelength were set to 360 nm with a spectral bandwidth of 2.5 nm. All assays were performed according to Buchner *et al*. (21) except that, in the case of the chemically denatured CS, the buffer contained 50 mM Tris·HCl (pH 8.0), 50 mM NaCl, and 20 mM dithioerythritol.

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Abbreviations: GA, geldanamycin; GR, glucocorticoid receptor; CS, citrate synthase.

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Insulin Assay. The insulin aggregation assay was performed in 10 mM sodium phosphate buffer (pH 7.0) and 100 mM NaCl. Turbidity was monitored at 650 nm in a Pharmacia Ultrospec 4060 UV-Vis spectrophotometer equipped with a temperature control unit using micro-cuvettes (100 μl) with a path length of 10 mm. Insulin (45 μM) was preincubated at 30°C with varying concentrations of Hsp90 and fragments. The aggregation reaction was started by the addition of dithioerythritol to a final concentration of 20 mM (22). Peptides (see Table 1) were synthesized as described (20). The HD peptides derived from the antibody 3E6 are described in Knarr *et al.* (20).

RESULTS AND DISCUSSION

Influence of Hsp90 Fragments on Insulin Aggregation. Analysis of all available Hsp90 sequences together with data from limited proteolysis and mass spectrometry revealed the existence of two highly conserved domains in Hsp90 separated by a charged region of variable length (23). To determine their function, the respective domains of yeast Hsp90 were cloned, expressed in *E. coli*, and purified to homogeneity. The monomeric N-terminal domain consists of residues 1–210 (N210), and the dimeric C-terminal domain consists of residues 262–709 (262C).

To analyze whether any of the stably folded recombinant Hsp90 fragments exhibit chaperone function, we studied the interaction of full length Hsp90s and the Hsp90 fragments with reduced insulin, an established chaperone model substrate. Upon reduction of the disulfide bonds, the B-chain of insulin, a 30-residue polypeptide, readily aggregates (22). We found that the Hsp90 fragments and wild-type Hsp90 from *E. coli*, yeast, and humans effectively suppressed insulin aggregation in a concentration-dependent way (Fig. 1 *A*–*C*). A 1:1 ratio (Hsp90-to-insulin) already was sufficient to suppress aggregation whereas for CS, another chaperone model substrate, an excess of Hsp90 was required (refs. 10 and 11 and see below). This result reflects the differences in the kinetics of aggregation that are slow in the case of insulin and much faster especially in the case of chemically denatured CS (see below). The mode of interaction of Hsp90 fragments with insulin seems to mimic the interaction of Hsp90 with peptides because a set of peptides, including a 19-aa fragment of the glucocorticoid receptor (GR1) that had been shown previously to inhibit Hsp90 function in receptor activation (24), effectively competed for insulin binding (*cf.* Fig. 3 and see below). In summary, the functional analysis of the Hsp90 fragments suggested the existence of two different chaperone sites in Hsp90.

Table 1. Influence of peptides on the chaperone activity of Hsp90 fragments

Peptide	Sequence	Turbidity N210	Turbidity 262C
No peptide	—	30	30
HD 25	SGFTFND	35	n.d.
HD 86	LRAEDMA	39	n.d.
HD 131	GPSVFPL	53	58
HD 131 L	GPSVFPLAPS SKC	95	56
HD 132	PSVFPLA	38	60
HD 133	SVFPLAPS	55	58
HD 131 + HD 133	GPSVFPL + SVFPLAPS	51	n.d.
HD 167	WNSGALT	34	n.d.
GR 1	AKAILGLRNL HLDDQMTLL	92	60
GR 3	AKAILGLRN	76	55
GR 4	LRNLHLDDQ	65	62
GR 5	LHLDDQMTLL	46	54
GR 3 + GR 5	AKAILGLRN + LHLDDQMTLL	80	n.d.
VSV 8	RGYVYQGL	71	53

Aggregation of insulin was measured as described above. The numbers represent the normalized turbidity in arbitrary units determined 35 min after reduction of insulin.
n.d., not determined.

Binding of Insulin to N210 Is Influenced by ATP. The *in vitro* chaperone function of Hsp90 has been reported to be ATP-independent (10, 14, 25), although Hsp90 binds ATP weakly (15). Furthermore, x-ray crystallography of an N-terminal fragment of Hsp90 revealed an ATP binding site (16). This site previously had been identified as the binding pocket for the anti-tumor drug GA (17), which is a highly specific inhibitor of Hsp90 (26). Therefore, we investigated the influence of adenosine nucleotides on the interaction of Hsp90 with insulin (Fig. 2). ATP or GA had no effect on the chaperone function of 262C (Fig. 2*A*). However, ATP and GA decreased the affinity of N210 in the aggregation assay (Fig. 2*B*). Addition of ATP or GA to a preformed N210·insulin complex resulted in the slow release and aggregation of insulin (Fig. 2 *C* and *D*) whereas ADP had no effect on the chaperone function of both 262C and N210 (Fig. 2 *A* and *B*).

The chaperone function of wild-type Hsp90 was also partially inhibited by ATP or GA. As for the fragments, no influence of ADP could be detected (Fig. 2*E*). Furthermore, preformed complexes between insulin and Hsp90 could be disrupted to some extent by the addition of ATP or GA (Fig. 2*E* and data not shown). To determine whether the partial inhibition observed for the wild-type protein by ATP was due to the rendering the N-terminal chaperone site inactive, we performed experiments in which ATP was added to assays containing both the N- and C-terminal domains (Fig. 2*F*). The observed inhibition of their chaperone activity corresponds well to that of the wild-type protein (Fig. 2*E*) and for the isolated N-terminal fragment (Fig. 2*C*), suggesting that the properties of the intact protein are reflected in the isolated domains.

Taken together, our data suggest that ATP or GA binding to Hsp90 and N210 induces conformational changes that decrease the affinity for nonnative protein and favor dissociation of the complex. ADP showed no influence in the assays used. Additionally, we found that ATP is hydrolyzed by Hsp90 and N210 using radiolabeled ATP. The ATPase activity of both proteins could be inhibited by equimolar amounts of GA. The turnover number was determined to be 0.02 min^{-1} for Hsp90 and 0.008 min^{-1} for N210. Thus, the catalytic activity of Hsp90 is $\approx$15 times lower than that of BiP (data not shown) and $\approx$300 times lower than that of GroEL. The ATPase activity of neither the intact protein nor the N-terminal fragment could be stimulated by the addition of peptides used in our chaperone assays (see Table 1). Given the low ATPase activity, it is reasonable to assume that, in the assays used, ATP hydrolysis is not required for efficient discharge of N-terminally bound proteins.

The Two Chaperone Sites of Hsp90 Differ in Substrate Specificity. Having established that the two chaperone sites differ in their nucleotide dependence, we further analyzed their substrate range. As mentioned above, we tested the specificity of the interaction of Hsp90 with insulin in a competition assay with various peptides including the steroid receptor fragment GR1 (Table 1; Fig. 3). The 19-aa peptide GR1 was unfolded completely in solution (data not shown). It can therefore be viewed as a model for sequence-dependent peptide interaction with Hsp90. When GR1 was added at an equimolar ratio to N210 before the addition of reduced insulin, the influence of N210 on insulin aggregation could no longer be detected (Fig. 3*A*). Addition of GR1 to preformed complexes between N210 and insulin resulted in dissociation and the subsequent aggregation of insulin. In the case of 262C, only a partial inhibition of its chaperone function could be achieved by the addition of GR1, supporting the assumption that the two

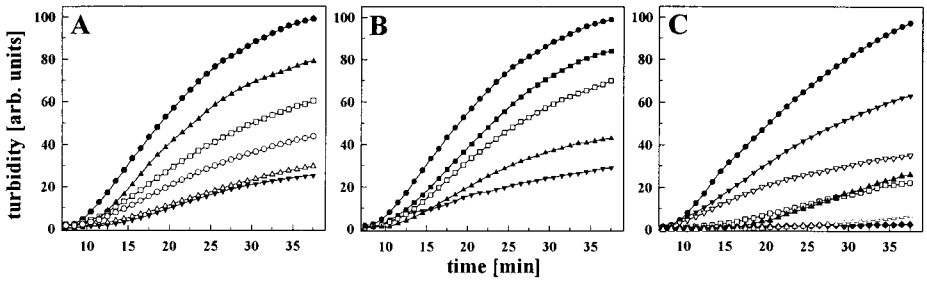

FIG. 1. Influence of Hsp90 on the aggregation of insulin B-chain. Insulin aggregation (45 μM) was monitored in the absence (●) or presence of Hsp90 fragments or the wild-type proteins from yeast, humans, and *E. coli*. Hsp90 or domains of Hsp90 exhibited no detectable influence on the turbidity of the solution. (*A*) Influence of increasing concentrations of N210 on insulin aggregation: 4.5 μM N210 (▲), 9 μM N210 (□), 22.5 μM N210 (○), 45 μM N210 (△), and 225 μM N210 (▼). (*B*) Influence of increasing concentrations of 262C on insulin aggregation: 4.5 μM 262C (■), 9 μM 262C (□), 22.5 μM 262C (▲), and 45 μM 262C (▼). (*C*) Influence of increasing concentrations of yeast Hsp90, human Hsp90, or *E. coli* Hsp90 on insulin aggregation; yeast Hsp90: 2 μM (▼), 5 μM (▽), 18 μM (□), 45 μM (◆), *E. coli* Hsp90 18 μM (▲), and human Hsp90 18 μM (△).

binding sites differ in their substrate specificity (Fig. 3*B*). To further analyze the binding properties of the N-terminal chaperone site, we investigated the interaction of N210 with a number of peptides differing in sequence and length. Some of these peptides previously had been used in studying the peptide binding site of BiP (20), the ER-specific member of the Hsp70 family. In addition, we used VSV8, an 8-residue long peptide known to interact with Grp94 (27, 28), an ER member of the Hsp90 family and peptides derived from GR1 (Fig. 3*C* and Table 1). Of all peptides tested, GR1 and HD131L bound most efficiently to N210 as determined by competition with insulin. GR subfragments of 9 or 10 residue lengths were significantly less efficient than full length GR1. Furthermore, combinations of the different subfragments did not restore the high affinity binding of GR1 (Table 1 and data not shown). VSV8 was about as efficient as the GR1 subfragments in binding to Hsp90. For the heptapeptides previously analyzed for BiP binding, no correlation could be detected between binding to BiP (20) and Hsp90. Of interest, HD131L, a longer peptide consisting of a fusion of two BiP binding heptapeptides, was found to bind to Hsp90 as efficiently as GR1 whereas the respective heptapeptides showed only a partial effect on insulin aggregation (Table 1). Thus, the competition experiments clearly showed a correlation between peptide length and binding to N210. This specificity could not be detected for 262C, for which all peptides tested were able to inhibit the chaperone function to some extent.

To address the question of whether the two chaperone sites cooperate in the wild-type protein, we performed the insulin/peptide competition assay in the presence of Hsp90 or N210 and 262C. The previous experiments had shown that the chaperone function of N210 can be suppressed completely by addition of GR1 and that, in the case of 262C, GR1 causes only half maximum inhibition (Fig. 3 *A* and *B*). The chaperone activity of full length Hsp90 was inhibited by GR1 to an extent corresponding to a total inhibition of the N-terminal site and a partial inhibition of the C-terminal site as observed in the isolated fragments (Fig. 3*D*). Therefore, in the case of the insulin/peptide

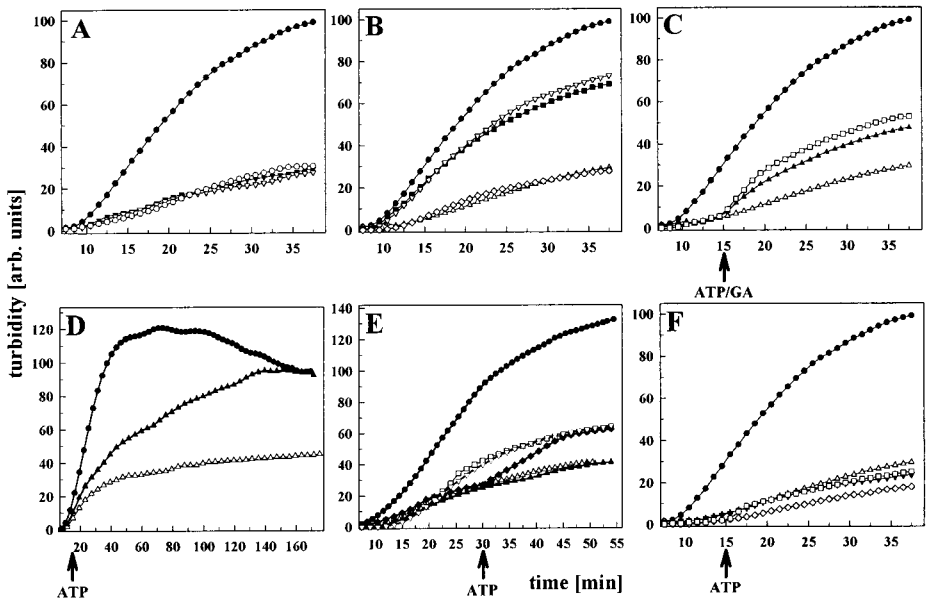

FIG. 2. ATP dependence of the interaction between Hsp90 and nonnative protein. The effects of Hsp90 and fragments thereof (45 μM each) on insulin B-chain aggregation (45 μM) were monitored in the absence (●) and presence of 1 mM of nucleotides or 100 μM GA (see below). (*A*) Influence of ATP (○) or GA (▽) on the chaperone function of 262C (▼). (*B*) Influence of ATP (▽), ADP (◇), or GA (■) on the chaperone function of N210 (△). (*C*) Influence of N210 (△) on insulin aggregation and the effect of ATP (▲) or GA (□) on preformed N210·insulin complexes. The arrow indicates the time of addition of ATP or GA. (*D*) Influence of N210 (△) on insulin aggregation and the effect of ATP (▲) on preformed N210·insulin complexes in a long time kinetic. The arrow indicates the time of addition of ATP. (*E*) Influence of ATP (▽), ADP (△), or GA (□) on the chaperone function of yeast Hsp90 (18 μM) (▲). Addition of ATP to preformed Hsp90·insulin·complexes (◆) is indicated by an arrow. (*F*) Influence of 262C (▼), N210 (△), and 262C+N210 without ATP (◇) and with ATP (□) on insulin aggregation. ATP addition to a preformed N210·insulin complex is indicated by an arrow.

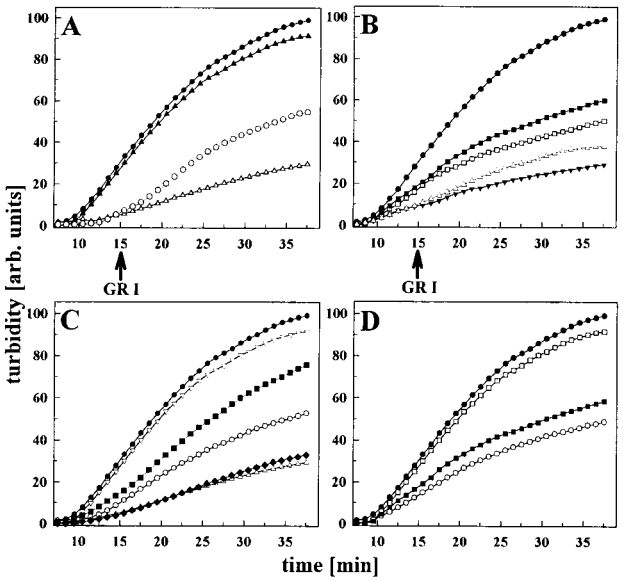

FIG. 3. Substrate specificity of the Hsp90 chaperone sites. The effects of Hsp90 and fragments thereof (45 μM each) on insulin aggregation (45 μM) were monitored in the presence of various peptides. (*A*) Influence of GR1 peptide (45 μM) on insulin binding to N210. Aggregation of insulin in the absence (●) and in the presence of N210 (△), N210 + GR1 (▲), and addition of GR1 (indicated by the arrow) to a preformed N210 insulin complex (○). (*B*) Influence of GR1 peptide on insulin binding to 262C. Aggregation of insulin in the absence of Hsp90 (●), in the presence of 262C (▼), 262C + 45 μM GR1 (□), and 262C + 225 μM GR1 (■) and addition of GR1 to a preformed 262C·insulin complex (△). (*C*) Influence of various peptides on insulin binding to N210. Aggregation of insulin in the absence of Hsp90 (●) and in the presence of N210 (△), N210 + GR1 (▽), N210 + VSV8 (■), N210 + HD 131 (○), and N210 + HD25 (◆). (*D*) Comparison of the influence of peptide on the chaperone activity of wild-type Hsp90 and fragments thereof. Aggregation of insulin in the absence of Hsp90 (●) and in the presence of Hsp90 + GR1 (■), 262C + GR1 (○), and N210 + GR1 (□). Concentrations of the fragments were 45 μM, and concentration of wild-type Hsp90 was 18 μM (based on the respective monomers). GR1 was added at a 1:1 ratio to insulin.

assay and in their ATP-dependence, the two chaperone sites seem to act independently in a similar way in the fragments and in the wild-type protein.

Influence of Hsp90 Fragments on Aggregation of CS. To further characterize the substrate specificity of the Hsp90 fragments, we examined the influence of the Hsp90 fragments on preventing citrate synthase (CS) aggregation during refolding and unfolding (Fig. 4), a well established model substrate protein (21), which had been used to analyze the chaperone function of Hsp90 from different species (11). When monitoring aggregation of CS during refolding from the chemically denatured state, both the N- and the C-terminal fragments suppressed aggregation (Fig. 4 *A* and *B*), implying that the N-terminal fragment is capable of interacting with nonnative proteins—albeit with more unfolded conformations. This effect was specific because it could be suppressed by GA (Fig. 4*A*) whereas this inhibitor of Hsp90 had no effect on the chaperone function of the C-terminal fragment (Fig. 4*B*).

Of interest, the N-terminal fragment was completely inactive in preventing the thermal aggregation of CS (Fig. 4*C*) whereas the C-terminal fragment of Hsp90 inhibited aggregation similar to wild-type Hsp90 in this assay (Fig. 4*D* and data not shown). Because it had been shown that Hsp90 interacts with highly structured unfolding intermediates of CS in an ATP-independent manner (11), these results suggested that the chaperone site recognizing these intermediates is located exclusively in the C-terminal domain of Hsp90.

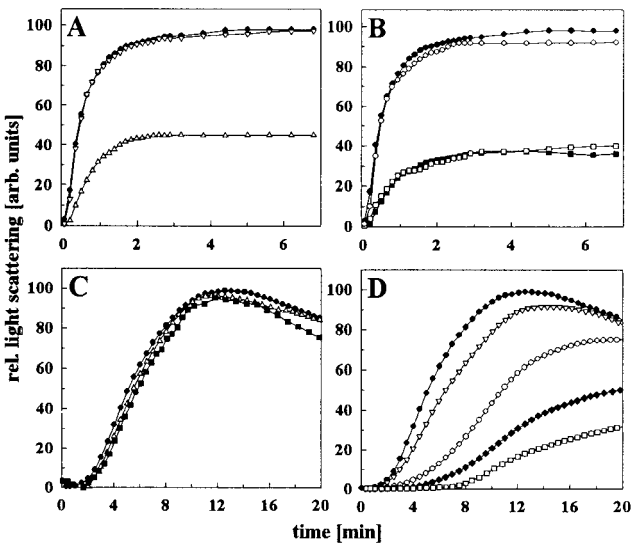

FIG. 4. Influence of Hsp90 fragments on the aggregation of CS. (*A*) Aggregation of chemically denatured CS (150 nM) in the absence (●) or in the presence of 3 μM N210 (△) and 3 μM N210 + 100 μM GA (▽). (*B*) Aggregation of chemically denatured CS (150 nM) in the absence (●) or in the presence of BSA (1.5 μM) (○), 1.5 μM 262C (■), and 1.5 μM 262C + 100 μM GA (□). (*C*) Aggregation of thermally denatured CS (150 nM) in the absence (●) or in the presence of 150 nM N210 (△) or 3 μM N210 (■). (*D*) Aggregation of thermally denatured CS (150 nM) in the absence (●) or in the presence of increasing amounts of 262C: 150 nM (▽), 750 nM (○), 1.5 μM (◆), and 3 μM (□).

Model for the ATP-Dependent Reaction Cycle of N210. Taken together, our results suggest that the N-terminal chaperone site of Hsp90 binds both peptides and unfolded proteins. This property is reminiscent of the Hsp70 family of molecular chaperones. However, although for different members of the Hsp70 family it is established that the peptide binding site occupies seven residues, Hsp90 seems to bind larger peptides more efficiently. A precise dissection of the properties of the N-terminal binding site with respect to a preferred binding motif cannot be given with the set of peptides used in this study. However, a functional cycle for the ATP-dependent substrate interaction of the N-terminal domain is now becoming evident (Fig. 5). Interesting to note, although the N-terminal chaperone site appears to be selective concerning substrate specificity and although the interaction with nonnative polypeptide is regulated tightly by ATP binding, the C-terminal site seems to be more promiscuous in binding, with a preference for partially folded structures occurring under stress conditions and during *de novo* folding. However, no specific regulation of this interaction has been discovered yet. *In vivo*, Hsp90 performs part of its function in the context of specific complexes with so-called partner proteins or cochaperones (2–4, 8, 9, 29, 30). It thus is reasonable to assume that the partner proteins may influence predominantly the domain to which they bind. Therefore, we investigated the binding of partner proteins to the isolated domains by chemical crosslinking. We found that, of all of the partner proteins tested, Hop, Hop-Hsp70, and FKBP52 bind specifically to 262C and not to N210 (data not shown). Therefore, it is tempting to speculate that these proteins modulate the binding properties of the C-terminal chaperone site of Hsp90. For p23, another partner protein of Hsp90, ATP is required for the efficient association with Hsp90 (31). It thus may well be that this cochaperone affects both the nucleotide binding properties and chaperone activity of Hsp90. The importance of ATP binding (and hydrolysis) by Hsp90 *in vivo* is highlighted by mutations in Hsp90, which were

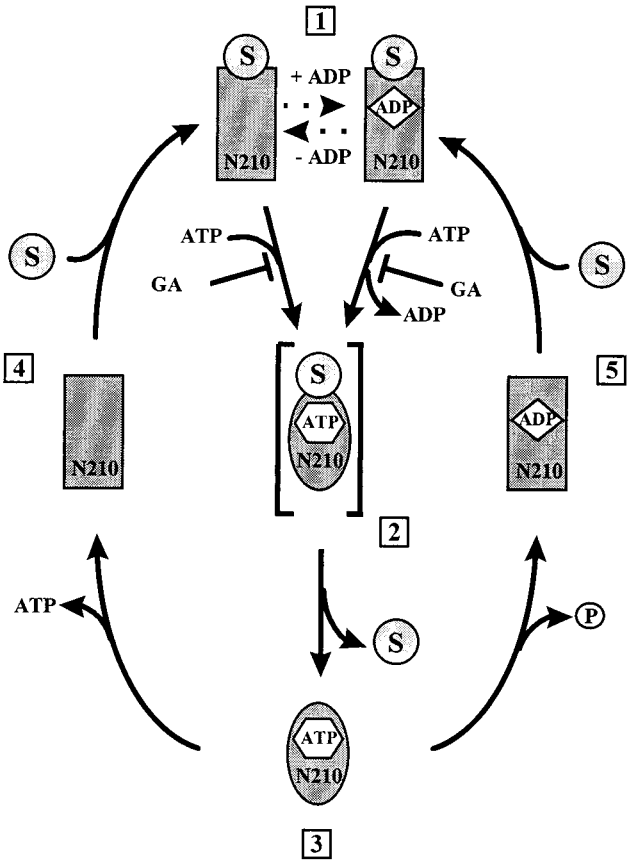

FIG. 5. Model for the ATP-regulated chaperone activity of Hsp90. The model describes the substrate binding cycle of the N-terminal domain of Hsp90. In the absence of ATP or in the presence of ADP, N210 has a high affinity binding site for substrate. Addition of nonnative proteins results in efficient complex formation. Functional differences between the ADP form and the nucleotide-free form could not be detected so far (1). Binding of ATP to the nucleotide-free or ADP-bound complexes between N210 and nonnative protein results in a conformational change that decreases the affinity of N210 for nonnative proteins (2). This complex seems to be short-lived (indicated by brackets), and, as a consequence, immediate dissociation of the substrate is observed (3). Alternatively, GA can bind to N210 and, similar to ATP, promote the release of bound substrates. To restore the high affinity binding site, ATP can either be released (4) or hydrolyzed (5). It remains to be seen which of the two mechanisms for reset to the high affinity state is used *in vivo*. S, substrate.

found to be defective in the folding of specific Hsp90 substrates in yeast (32) and which might affect the ATP binding site (16).

In vivo, the functional interplay of the two distinct chaperone sites of Hsp90 and their selective modulation by partner proteins seems to allow the conformational regulation of specific target proteins in addition to preventing the deleterious effects of stress on protein structure in general.

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