The presence of a nucleotide binding site on hsp90 was very controversial until x-ray structure of the hsp90 N-terminal domain, showing a nonconventional nucleotide binding site, appeared. A recent study suggested that the hsp90 C-terminal domain also binds ATP (Marcu, M. G., Chadli, A., Bouhouche, I., Catelli, M. G., and Neckers, L. M. (2000) J. Biol. Chem. 275, 37181–37186). In this paper, the interactions of ATP with native hsp90 and its recombinant N-terminal (positions 1–221) and C-terminal (positions 446–728) domains were studied by isothermal titration calorimetry, scanning differential calorimetry, and fluorescence spectroscopy. Results clearly demonstrate that hsp90 possesses a second ATP-binding site located on the C-terminal part of the protein. The association constant between this domain of hsp90 and ATP-Mg and a comparison with the binding constant obtained for the first time. Secondary structure prediction revealed motifs compatible with a Rossmann fold in the C-terminal part of hsp90. It is proposed that this potential Rossmann fold may constitute the C-terminal ATP-binding site. This work also suggests allosteric interaction between N- and C-terminal domains of hsp90.

Prokaryotic and eukaryotic cells exposed to heat and other cellular stresses synthesize several classes of highly conserved stress proteins (1). These protein families act as molecular chaperones by preventing the aggregation of nonnative polypeptides and providing the guideline for their correct folding. Heat shock protein 90 (hsp90) is one of the most abundant proteins in eukaryotic cells under heat shock and stress conditions and is also constitutively expressed, representing 1–2% of the total cellular protein in the majority of eukaryotic cells growing in unstrained conditions (2). hsp90 acts in complex with a set of partner proteins to assist target protein folding (for a review, see Ref. 3).

Sequence alignments and proteolytic digests have shown that hsp90 is composed of well conserved N-terminal and C-terminal domains linked by a charged hinge region variable in length (4). X-ray crystallographic studies of the N-terminal domain (residues 1–220) of yeast and human hsp90 allowed the identification of the ATP-Mg/ADP-Mg binding site, which can be blocked by high affinity inhibitors such as the antibiotic geldanamycin (GA) (5, 6) or radicicol (7). This site is responsible for the ATPase activity of the chaperone (8). Via the ATP-binding site, the N-terminal domain seems to regulate hsp90 conformation (9) and contains a chaperone site involved in the binding of target proteins (8). In contrast to the N terminus, the three-dimensional structure of the C-terminal domain of hsp90 is still unknown. This domain contains a second chaperone site, which has different polypeptide specificity from the N-terminal one (10). Moreover, the C-terminal region seems to be involved in both dimerization (11–13) and oligomerization (14) of hsp90. The mechanism of dimer formation has been proposed to take place through the duplicate anti-parallel interaction of fragments 542–615 and 629–731 (12). ATP binding and hydrolysis produce conformational changes that involve the entire hsp90 molecule, and the C-terminal region of hsp90 seems important for trapping the nucleotide during the ATPase cycle (15, 16). Moreover, a second ATP-binding site located in hsp90 C terminus was suggested through the use of ATP-Sepharose affinity chromatography (17); however, the association constant and stoichiometry of the complex with ATP were not determined. Thus, characterization of the C-terminal domain interaction with nucleotides is crucial to understand the hsp90 function. Therefore, we expressed C- and N-terminal domains separately and applied differential scanning calorimetry (DSC), isothermal titration calorimetry (ITC), and fluorescence spectroscopy to directly prove that hsp90 contains a second ATP-binding site located in the C-terminal part of the protein and to determine the association constant for C-hsp90-ATP-Mg complex. Then we compared this value with the binding constant obtained for the full-length protein and hypothesized the localization of the second ATP-binding site.

**EXPERIMENTAL PROCEDURES**

hsp90 Purification and Expression of N- and C-terminal Domains—The 90-kDa heat shock protein was purified from porcine brain according to the method of Ref. 18, modified by Garnier et al. (19, 20). N-hsp90 (positions 1–221) and C-hsp90 domains were obtained by PCR amplification using a chicken hsp90 cDNA-bearing plasmid pSKB3 90 (13) and...
inserted into pET 15b and pET 28a expression vectors (Novagen) in frame with the N-terminal His tag, respectively. The constructs were verified by automated sequencing and transformed into E. coli B21-Gold (DE3) pLYs Competent cells (Stratagene). Bacterial cells bearing the pET 15b and pET 28a constructs were grown overnight at 37 °C. The cultures were induced with 1 mM IPTG for 3 h at 37 °C for N-hsp90 and overnight at 20 °C for C-hsp90. Subsequently, cells were lysed by French press in extraction buffer A1 (100 mM phosphate, 100 mM NaCl, pH 7.4); the lysate was clarified by ultracentrifugation at 30,000 × g for 30 min. The recombinant proteins were purified by Hi-trap affinity chromatography (Amersham Biosciences) and eluted using a 250 mM NaCl gradient in A1 buffer. Fractions containing recombinant protein were pooled and concentrated using Amicon cells (Millipore Corp.). Concentrated proteins were desalted on a PD10 column (Amersham Biosciences).

All purified proteins were stored at −80 °C in 10 mM Tris-HCl, pH 7.5. Protein concentration was determined by UV absorbance with extinction coefficients at 280 nm of 124,000, 51,100, and 16,350 M−1 cm−1 for hsp90, C-terminal domain, and N-terminal domain, respectively. Extinction coefficients were calculated by the procedure of Gill and Von Hippel on the basis of the amino acid composition (19). All experiments were carried out in 10 mM Tris-HCl buffer, pH 7.5.

Circular Dichroism—CD spectra of 12 μM hsp90 were acquired with a Jasco J-720 spectropolarimeter using a 0.01-cm cell at 25 °C. The instrument was calibrated between 180 and 350 nm with ammonium camphorsulfonate-d50 (Katayama Chemical, Jasco) as a standard. The results were expressed as molar ellipticity, [θ] (degrees cm2 dmol−1), considering an average amino acid residue mass of 111 Da. CD spectra were analyzed by four different methods currently available in the Dicroprot program (version 2.4 by G. Deleage; available on the World Wide Web at http://www.ibp.fr/).

Fourier Transform Infrared Spectroscopy (FTIR)—FTIR spectra of 80 μl of hsp90 (100 μM) were obtained with a resolution of 4 cm−1 using a MB100 FTIR spectrometer equipped with a deuterated triglycine sulfate detector (21). Secondary structures content was calculated from Amide I and II bands using partial least-squares analysis as described in Ref. 22. This factor analysis requires a calibration set of proteins with known X-ray structures (21). The first five loading vectors were used, and three types of secondary structures were characterized: α-helix, β-sheet, and undefined structures.

hsp90 Cross-linking Experiments—Chemical cross-linking of hsp90 was performed using N-[3-dimethylaminopropyl]-N′-ethylcarbodiimide hydrochloride (EDC) (Sigma) as cross-linking reagent. The optimal EDC concentration determined by titration was equal to 1.5 mM. hsp90 (6 μM) was incubated 10 min at room temperature in the presence or absence of various concentrations of Mg2+, and the presence of ligands (3.5 × 10−4 M GA and 5 mM ATP-Mg complex) on hsp90 cross-linking was tested. For cross-linking, 32 mM EDC stock solution was added (5% of hsp90 sample volume), and samples were incubated for 30 min at room temperature. To stop the cross-linking process, samples were diluted 10-fold with buffer (a 20-fold excess of amine containing buffer is enough to quench the reaction). Then samples were submitted to 4–15% gradient native PAGE using the PhastSystem apparatus (Amersham Biosciences). Gels were stained with Coomassie Brilliant Blue.

Differential Scanning Calorimetry—Measurements of hsp90 and its N- and C-terminal domain heat denaturation and influence of ATP-Mg or ATP alone on this process were carried out on a MicroCal VP-DSC instrument in 0.51 ml cells at a heating rate of 1 K/min. Protein concentration varied from 2.3 to 6 μM. The heating curves were corrected for the instrumental base line obtained by heating the solvent alone at 20 °C and equilibrium constant (Kc) was obtained using the following procedure. 5–10-μl aliquots of ligands (2–4 × 10−3 μM) were injected from a 250-μl microsyringe into the 1.34-ml calorimeter cell containing protein solution (1.0–2.5 × 10−3 μM) to achieve a complete binding isotherm. The heat of dilution was measured by injecting the ligand into the buffer solution or by additional injections of ligand after saturation; the value obtained was subtracted from the heat of reaction to obtain effective heat of binding. Titration curves were fitted using the MicroCal Origin software, assuming a single class of site.

Fluorescence—Relative fluorescence intensity was measured on a Perkin-Elmer LS 50 with slit widths of 5 and 10 nm. Fluorescence data were obtained using 0.2 cm (excitation direction) × 1 cm (emission direction) cells at 25 °C. The excitation was done at 295 nm to specifically excite the hsp90 tryptophan residues and to minimize the ATP absorption. The measurements were carried out in the presence of 2 mM magnesium. Equilibrium difference fluorescence measurements were conducted on constant hsp90 concentration and increasing ATP concentrations. The difference in fluorescence (ΔF) at 340 nm between hsp90 fluorescence and hsp90-ATP complex fluorescence (ΔF = Fhsp90 − Fcomplex) was plotted versus ATP concentrations. The inner filter was corrected, and the curve was fitted to the saturation curve equation by nonlinear least-squares regression analysis (24).

Sequence Analysis and Predictive Methods—The α- and β-hsp90 sequences available in SWISS-PROT data base were used for the multiple-sequence alignments. Location of sequence variation within aligned family members was determined by the PHD program as previously described (25). Predictions of secondary structure and solvent accessibility for pig and human hsp90α (HS9A_PIG, HS9A_HUMAN) and hsp90β (HS9B_HUMAN) were obtained from the PHD server (26). The Predictive and Inferential Function using the inter-residue sequence alignments as input to a neural network trained with a nonredundant protein structure data base. It has been reported to have the best predictive accuracy when only sequence information is available (31). Predictions of the secondary structure through the amino acid sequences of HS9A_PIG, HS9A_HUMAN, and HS9B_HUMAN were also performed with the npredict program (32, 33).

Sedimentation Velocity—Sedimentation velocity experiments were performed at 55,000 rpm in a Beckman Optima XL-A analytical ultracentrifuge equipped with an AnTi 55 rotor and 12-mm aluminum double-sector centripieces. Data were acquired in continuous mode at 280 or 230 nm depending on protein concentrations. The data were analyzed using the Svedberg (34) and dcdt (35) computer programs.

Sedimentation Equilibrium—Sedimentation equilibrium runs were performed at 20 °C at three loading concentrations and at three speeds in a six-channel centerpiece of charcoal-filled Epon. The protein gradients were detected at 280 nm. High speed sedimentation was conducted afterward for base-line correction. Data analysis was performed by global analysis of several data sets obtained at different loading concentrations at one speed using the program MULTIEQ3B (36).

RESULTS

Characterization of hsp90 and Its Recombinant Domains—Two HSP90 fragments were expressed in Escherichia coli and purified. The amino-terminal domain (N-hsp90) was designed to end at residue 221, which includes the ATP-binding site (5). The carboxyl-terminal domain (C-hsp90) started at position 446 to the end of the sequence. It included the recently suggested second ATP-binding site (17). The two proteins showed electrophoretic homogeneity in SDS-PAGE, with molecular masses of about 30 and 40 kDa, respectively. N-hsp90 eluted from size exclusion chromatography-HPLC with an apparent molecular mass of 28 kDa and a Stokes radius of 19.2 Å. Sedimentation equilibrium experiments at three loading concentrations and three speeds confirmed that N-hsp90 was monomeric. Global analysis of three concentrations at one speed using a single ideal species model converged to the value of 28.1 kDa and a Stokes radius of 19.2 Å. Velocity sedimentation experiments allowed the determination of a sedimentation coefficient at infinite dilution at 20 °C in water, s20,w,0 of 2.45 ± 0.02 S. Analysis of C-hsp90 by more PAGE and size-exclusion chromatography by demonstrated that the C-hsp90 dimer contained 40–45% dimers with an apparent molecular mass of 100 kDa and a Stokes radius of 41.7 Å and 60–70% of oligomers with apparent molecular masses ranging from 170 to 400 kDa and Stokes radii ranging from 41.7 to 60 Å. Analytical ultracentrifugation experiments confirmed heterogeneity of C-hsp90 quaternary structures and indicated a polydispersity sample.
ATP Binds to hsp90 C-terminal Domain

The far-UV CD spectrum of the full-length hsp90 was analyzed by four different methods (see “Experimental Procedures”), and results are shown in Table I. The average values were as follows: 38% α-helix, 15% β-sheet, and 47% other structures. The component assignment of the deconvoluted spectra of the amide I and II infrared absorption bands of hsp90 by FTIR showed that hsp90 contained 44% α-helix, 17% β-sheet and 39% undefined structure. The PHD algorithm and the nnpredict program were used to create a picture of human hsp90 secondary structure content (Fig. 1, line PHDsec and line nnp, respectively). Prediction methods did not show significant differences between hsp90α and hsp90β (data not shown). In addition, the secondary structures content of the N-terminal subdomain (residues 12–223) obtained by predictive methods (PHDsec and nnpredict) and the secondary structure content deduced from x-ray three-dimensional structure (6) (Fig. 2), were highly similar with the exception of the first β-strand (residues 16–21) and the sixth α-helix (residues 129–135); all remaining structural elements predicted are present in the crystallographic structure. This indicates that the PHD method gave a good estimation of secondary structure, and it may be used for the entire protein (Fig. 1).

Influence of ATP on Mg2+-dependent Oligomerization Process of hsp90—As demonstrated earlier, hsp90 dimer undergoes a Mg2+-dependent oligomerization process implicating the C-terminal domain (19). GA did not influence the cross-linkage profile observed in the presence or absence of Mg2+ (Fig. 3, lines A4 and B4). Contrary to GA, ATP inhibited the Mg2+-dependent oligomerization process (Fig. 3, line C2). Considering a binding constant of $2.2 \times 10^4$ M$^{-1}$ (37), in our experimental conditions about 90% of Mg2+ was bound to ATP, and only 0.5 mM Mg2+ was free (Fig. 3, gel C). To further raise the free Mg2+, the total Mg2+ concentration was increased to 10 mM, maintaining the same ATP concentration (5 mM) (Fig. 3, gel D). In these conditions and despite the presence of more than 5 mM free Mg2+, no hsp90 oligomerization process was observed (Fig. 3, lane D2). Thus, ATP inhibited hsp90 oligomerization induced by Mg2+ ions. Since GA does not influence the Mg2+-dependent oligomerization and ATP is known to compete with GA in our experimental conditions about 90% of Mg2+ was bound to ATP, and only 0.5 mM Mg2+ was free (Fig. 3, gel C). To further raise the free Mg2+, the total Mg2+ concentration was increased to 10 mM, maintaining the same ATP concentration (5 mM) (Fig. 3, gel D). In these conditions and despite the presence of more than 5 mM free Mg2+, no hsp90 oligomerization process was observed (Fig. 3, lane D2). Thus, ATP inhibited hsp90 oligomerization induced by Mg2+ ions. Since GA does not influence the Mg2+-dependent oligomerization and ATP is known to compete with GA in our experimental conditions about 90% of Mg2+ was bound to ATP, and only 0.5 mM Mg2+ was free (Fig. 3, gel C). To further raise the free Mg2+, the total Mg2+ concentration was increased to 10 mM, maintaining the same ATP concentration (5 mM) (Fig. 3, gel D).

Influence of ATP on Thermal Stability of hsp90, N-hsp90, and C-hsp90—To obtain additional proof that ATP binds to the C-terminal part of hsp90, heat denaturation of the three proteins (hsp90, N-hsp90, and C-hsp90) and effects of ATP-Mg and ATP alone were studied by DSC. In a previous study, we showed that the melting curve of hsp90 consists of two transitions; on the basis of the specific GA binding and stabilization, the lower temperature peak corresponded to the melting of the N-terminal domain, while the higher temperature one comprised denaturation of the C-terminal domain (19). As already reported (5), the N-terminal domain of hsp90 does not contain a divalent cation-binding site but binds ATP-Mg. Fig. 4A and Table II show that ATP-Mg increased by 2.6 °C the denaturation temperature of N-hsp90 and increased by 2.0 °C the denaturation temperature of C-hsp90.

| Method of analysis | α-Helix (%) | β-Strand (%) | Other structures (%) |
|-------------------|-------------|--------------|---------------------|
| CD$^a$            | 0.38 ± 0.05 | 0.15 ± 0.06  | 0.47 ± 0.03         |
| FTIR$^b$          | 0.44 ± 0.02 | 0.17 ± 0.03  | 0.39 ± 0.03         |
| PHDsec$^c$        | 0.45        | 0.15         | 0.35 (0.38)$^d$     |
| nnpredict$^e$     | 0.39        | 0.09         | 0.53                |

$^a$ Average of results obtained by the four methods.
$^b$ See Refs. 27–30.
$^c$ The content of secondary structure expected to have an accuracy better than 81.2%.
$^d$ See Refs. 27 and 33.

Fig. 1. Secondary structure and solvent accessibility predictions of pig hsp90α. The residue number and the sequence of amino acid residues are indicated. The secondary structure prediction by the PHDsec method is graphically displayed with helical symbols, arrows, and ribbons for α-helices, extended β-sheet strands, and undefined, respectively. The predictions with an expected accuracy under and over 81.2% are in light yellow and red, respectively. Secondary structures predicted by the nnpredict method (nnp) (32, 33) are shown on the secondary structure scheme (PHDsec) (27–30); residues enclosed in an α-helix and a β-strand are indicated by a and β, respectively. The three-state solvent accessibility prediction by the PHDsec method is shown below. e, exposed; b, buried; blank, intermediate solvent accessibility.

Table I

| Method of analysis | α-Helix (%) | β-Strand (%) | Other structures (%) |
|-------------------|-------------|--------------|---------------------|
| CD$^a$            | 0.38 ± 0.05 | 0.15 ± 0.06  | 0.47 ± 0.03         |
| FTIR$^b$          | 0.44 ± 0.02 | 0.17 ± 0.03  | 0.39 ± 0.03         |
| PHDsec$^c$        | 0.45        | 0.15         | 0.35 (0.38)$^d$     |
| nnpredict$^e$     | 0.39        | 0.09         | 0.53                |

$^a$ Average of results obtained by the four methods.
$^b$ See Refs. 27–30.
$^c$ The content of secondary structure expected to have an accuracy better than 81.2%.
$^d$ See Refs. 27 and 33.
temperature of the N-terminal domain unfolding in the entire protein. Unfortunately, strong aggregation, accompanying thermal denaturation of C-hsp90 in the presence of magnesium, did not allow us to register a reliable heat absorption peak, and the addition of ATP shifted the thermogram to higher temperatures without decreasing the aggregation (Fig. 4B). The same was true for the second peak of the hsp90 melting curve (Table II). The aggregation was also observed after completion of heat denaturation of N-hsp90 in the presence of ATP-Mg (Fig. 4A).

To avoid aggregation, the influence of ATP on thermal denaturation of hsp90 and its recombinant domains was studied in the absence of added Mg²⁺ (but possibly with residual Mg²⁺ on the protein), thus allowing us to register a reliable heat absorption peak, and the addition of ATP shifted the thermogram to higher temperatures without decreasing the aggregation (Fig. 4B). The same was true for the second peak of the hsp90 melting curve (Table II). The aggregation was also observed after completion of heat denaturation of N-hsp90 in the presence of ATP-Mg (Fig. 4A).

Fig. 2. Comparison between the N-terminal domain secondary structure of HSP90 obtained through crystal three-dimensional structure (upper line) and the predicted secondary structure obtained by the PHDsec (27–30) and nnpredict (32,33) methods. α-Helices and extended β-sheet strands are represented by cylinders and arrows, respectively. For the PHDsec method, only predictions corresponding to an expected accuracy over 81.2% are represented.

Fig. 3. Effect of GA and ATP on the hsp90 Mg²⁺-dependent oligomerization process. The presence and the absence of reagents is indicated by + and −, respectively. Concentrations of Mg²⁺ and ligands are indicated on the left. In all experiments, the last component added was EDC. Samples were analyzed on 4–15% gradient native PAGE with the PhastSystem apparatus. D and O indicate migration of dimeric and oligomeric species, respectively.

Fig. 4. Raw DSC data for N-hsp90 (A) and C-hsp90 (B) at pH 7.5 with (dashed line) and without (solid line) 5 mM ATP in the presence of 5 mM magnesium.

pearmance of protein aggregation occurring immediately after completion of its melting. According to size exclusion chromatography-HPLC data, increasing temperature until 61 °C (near the beginning of the C-hsp90 heat absorption peak) led to the complete aggregation of C-hsp90 oligomers without modification of dimer quantity (data not shown). For this reason, (i) the melting peak for C-hsp90 (Fig. 5B, 2) represented melting of only dimers, and (ii) the low value of denaturation enthalpy was due to the fact that the calculation was based on total C-hsp90 concentration, without any consideration of the more complex C-hsp90 quaternary structure. Fig. 5 and Table II show that free hsp90 domains and those in the intact protein exhibited different melting sensitivity. Noteworthy, the N-hsp90 stability decreased by 5.7 °C as compared with that of...
Hsp90 and without (H11002) C-Hsp90. N-Hsp90 (H11001) and Hsp90 (H11002) N-hsp90 (H11001) and the stoichiometric equilibrium constant was in the milli-molar range ($K_a = 5 \times 10^8$ M$^{-1}$), and the binding was enthalpy-driven with an unfavorable entropy.

The C-hsp90/ATP-Mg binding curves obtained by ITC were also fitted (Fig. 6) assuming a single class of site. According to the thermodynamic parameters reported in Table III, the binding was enthalpy-driven. The variation of enthalpy ($\Delta H$ = $-79 \pm 20$ kJ mol$^{-1}$) and the affinity constant ($K_a = 5.5 \times 10^4$ M$^{-1}$) were stronger than those obtained for the entire protein ($K_a = 5 \times 10^3$ M$^{-1}$) (Table III). Noteworthy, the peaks corresponding to the heat signal associated with each injection were wider than for the protein alone, showing that a slow mechanism, probably a structural change, also took place during the binding of ATP-Mg (Fig. 6B).

C-hsp90 possesses one tryptophan residue. During complex formation with ATP, the fluorescence emission spectrum can either shift in the wavelength of maximum fluorescence emission or shift in fluorescence intensity. These shifts can therefore be used to evaluate the association constant. In the presence of Mg$^{2+}$, when ATP-Mg was added, the maximum of fluorescence emission spectrum at 340 nm of C-hsp90 decreased, confirming the formation of a C-hsp90-ATP-Mg complex. The interaction did not induce any wavelength shift (data not shown). Fluorescence changes were recorded as successive ATP aliquots were added to a solution of 5 $\mu$M C-hsp90. The intensity changes at 340 nm (mean of 10 values) were plotted.
of N-hsp90 from yeast and human are almost identical (5, 6, 38). Vertebrates hsp90α and hsp90β are related by 85% amino acid identity in the same species, and all hsp90αs or hsp90βs show more than 95% identity between them (96% for chicken and human). Thus, results obtained for native pig hsp90, composed of 83% α-isoform and 17% β-isoform (20), recombinant α-chicken domains, and α- or β-human (predictions) are highly comparable and could be applied to α- or β-hsp90 from other eukaryotic species. This was confirmed by investigation of hsp90 secondary structure by spectral (CD and FTIR) and by predictive methods (PHD and nnpredict).

Using hydrophobic cluster analysis, we proposed that hsp90 contains a potential Rossmann fold between amino acids 490 and 630, suggesting that the protein could have an NADH reductase activity (45). However, using ITC, we did not detect the binding of NADH to hsp90. Using deletion/mutation analysis, a binding site for novobiocin/ATP was localized in a region overlapping the carboxyl-terminal dimerization domain of the chaperone (mutant Δ4, amino acids 538–728), where the two ligands compete (17). Thus, it seems reasonable to propose that the C-terminal ATP-binding site could be localized between amino acids 538 and 630, because the secondary structure analysis showed that this region is highly structured in a succession of α-helices and β-sheets (α555–581 β586–591 β598–601 α597–613) compatible with a Rossmann fold motif known to bind ATP. Since γ-phosphate-linked ATP-Sepharose bound the mutant Δ4, amino acids 538–630 seems sufficient for purine base binding as confirmed by competition with GTP and not with CTP (17). The peptide 657–677 seems to be crucial for nucleotide and novobiocin binding, because the corresponding deletion mutant was unable to bind ATP Sepharose, and an excess of the same peptide (residues 657–677) inhibited the binding of mutant Δ4 to ATP and novobiocin-Sepharose (17). As the mechanism of dimer formation occurs through the duplicate antiparallel interaction of fragments 542–615 and 629–731 (12), the 657–677 peptide from one monomer could participate in the formation of the ATP-binding site of the other monomer, and vice versa. Moreover, deletion of this peptide induced hsp90 monomerization (13). The C-terminal ATP-binding site thus overlaps with the dimerization domain, explaining why ATP binding, dimerization, and magnesium-dependent oligomerization processes are closely linked.

To sum up, we characterized the second ATP-binding site located on the C terminus of hsp90. The binding may occur on a Rossmann fold region previously suggested (45). The comparison of binding constants for ATP-Mg between this domain of hsp90 and the full-length protein and the nucleotide obtained by ITC and the DSC results demonstrate interdomain interactions. Whether this second site contributes to hsp90 ATP activity and how it communicates with the N-terminal one remains to be determined.

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