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The Two-Stage Process of the Heat Shock Protein 90 Thermal Denaturation: Effect of Calcium and Magnesium

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Scanning microcalorimetry, native PAG electrophoresis, and circular dichroism were used to characterize thermal denaturation and oligomerization of heat shock protein 90 (hsp90) and the calcium and magnesium effect on these processes. The calorimetric curve of the hsp90 dimer consists of two transitions centered at 53.8 and 63.1°C. Using specific ligand geldanamycin, we have found that N-terminal domains in the hsp90 dimer are melted independently in the lower-temperature peak, while the higher-temperature one comprises unfolding of two non-interacting parts of the middle domains and dimerization region. Unfolding of the N-terminal domain gives start to oligomerization of dimers; oligomers consist of dimers not dissociating upon denaturation. Calcium and magnesium strongly decrease the hsp90 thermostability and thereby cause oligomerization at lower temperature. We suggest that calcium affects the hsp90 oligomerization, known to be important for its chaperone activity, by shifting the unfolding temperature of the hsp90 N-terminal domain close to the heat shock temperature range.

Heat shock protein 90 (hsp90) is a major stress protein highly conserved from *E. coli* to human [1,2] and thus having very similar physicochemical properties in prokaryotes and eukaryotes. Hsp90 accounts for 1-2% of the total cell protein in unstressed mammalian cells, and interacts with numerous proteins. Under stress conditions, hsp90 is overexpressed. *In vitro,* hsp90 appears to be mainly a dimer [3]. According to [2,4], hsp90 binds calcium; magnesium and manganese can favor its oligomerization [5]. Phosphorylation of hsp90 requires the presence of Ca^{2+}, whereas Mg^{2+} blocks it completely [6].

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As well as other classes of heat shock proteins, hsp90 is known to operate as a molecular chaperone, and is believed to protect cells from damage at the nonphysiologically high temperatures [5]. For this reason, it is of great interest to know the exact limit of its thermal stability. As shown in [7], the heat-induced chaperone activity of hsp90 is associated with its oligomerization. Oligomerized hsp90 keeps the substrate proteins in the folding-competent state. Some interesting data on the thermal stability of hsp90 [5] and hsp85 [8] were obtained by the fluorescence spectroscopy measurements. Unfortunately, only slight changes in the hsp90 fluorescence intensity were observed, which did not allow precise characterization of the hsp90 unfolding process. Moreover, aggregation seriously limited the accuracy of estimation of the divalent cation effect on the hsp90 heat denaturation parameters [5], and we could not find in the literature any direct data concerning the influence of calcium on the stability and conformation of this protein, although it is known to be increased under heat shock [9]. This led us to the studies of the hsp90 thermal denaturation by differential scanning calorimetry, the most convenient method for this kind of analysis, supplemented with circular dichroism (CD) and native PAG electrophoresis. We tried to characterize the thermal unfolding of hsp90 and influence of temperature on its self-oligomerization, and to assess the effect of calcium and magnesium on these processes.

MATERIALS AND METHODS

Hsp90 purification. Hsp90 was purified from porcine brain according to [1] with the following minor modification: in the first step of chromatographic procedure we used Butyl Sepharose 4 Fast Flow from Pharmacia Biotech instead of Butyl ToyoPearl 650 C. The protein was identified as hsp90 by Western immunoblotting with the SPA-830 anti-hsp90 antibody from StressGen, using the Pharmacia-LKB PhastTransfer apparatus, and its purity was checked by SDS PAGE with the Pharmacia-LKB PhastSystem. After purification hsp90 was dialysed overnight at 4°C against 20 mM sodium phos-

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phate buffer, pH 7, and hsp90 samples were then stored at −80°C. The buffers used were 20 mM sodium phosphate, pH 7, and 10 mM Tris-HCl, pH 7 for hsp90 complexes with Ca^{2+}. Protein concentration was determined from absorbance at 280 nm using an extinction coefficient of 124000 M^{-1} cm^{-1} calculated by the procedure of Gill and Von Hippel [10] considering that hsp90 is a 162-kDa dimer.

Temperature dependence of hsp90 oligomerization. The temperature dependence of hsp90 oligomerization was studied by heating 0.3 ml of the hsp90 solution (1 or 2 mg/ml) at 1 K/min (Haake PG20 apparatus). The hsp90-geldanamycin complex was prepared with the saturating geldanamycin (Drug synthesis and chemistry, NCI) concentration in the presence of 0.14% dimethyl sulfoxide (DMSO, Sigma) used to dissolve geldanamycin. The presence of DMSO did not influence the experiments. Aliquots were taken in every three degrees, immediately cooled to 4°C, and then resolved at 15°C by native PAGE on 7.5% homogeneous gels with the Pharmacia LKB PhastSystem in order to follow the disappearance of hsp90 dimers with temperature. Gels were stained with Coomassie blue and the intensity of the dimer bands was determined by scanning the gels with the Image Master System of Pharmacia. The dimer quantity present at 20°C was taken as 100%. The temperature corresponding to disappearance of 50% of hsp90 dimers ($T_{50\%}$) was taken as a reference.

Differential scanning microcalorimetry. Microcalorimetric measurements were carried out on DASM-4 instrument (NPO Biopribor, Pushchino, Russia) in 0.48-ml cells at the heating rates of 1 and 2 K/min and on MicroCal VP-DSC instrument (U.S.A.) in 0.51-ml cells at a heating rate of 1 K/min. Protein concentration varied from 0.8 to 2.1 mg/ml. The partial molar heat capacity of the protein (C_p) was determined as described elsewhere [11], taking 0.73 cm^3 g^{-1} for the partial specific volume. To analyze functions of the excess heat capacity, we used the MicroCal Origin (4.1) software package. The calorimetric denaturation enthalpy values were accurate to ±8% and the temperatures for the transition peaks were accurate to ±0.3°C.

Circular dichroism. CD spectra were recorded on Jasco J-715 spectropolarimeter (Japan) equipped with the thermostated water-jacketed cells (Hellma Cells, U.S.A.) and the Neslab RTE-111 programmable circulating water bath (Neslab Instruments, U.S.A.). The cells had the light path of 1.0 cm, protein concentration was 2.0-2.2 mg/ml (near-UV). For continuous melting of the samples, the temperature was increased at a rate of 1 K/min and the transition temperatures were determined from the peaks on the first temperature derivatives of the normalized melting profiles. The results were expressed as molar ellipticity, [Θ] (deg cm^2 $dmol^{-1}$), based on the mean amino acid residue weight of 111 for hsp90.

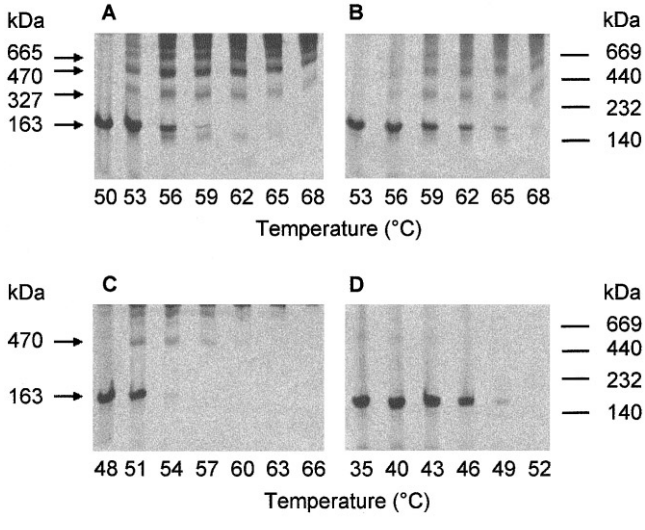

FIG. 1. Temperature dependence of oligomerization of hsp90 (A), hsp90 complex with geldanamycin (13 μM) (B), hsp90 with 5 mM $MgCl_2$ (C), and hsp90 with 5 mM $CaCl_2$ (D) at pH 7. The concentration of hsp90 was 2 mg/ml (A, C, D) or 1 mg/ml (B). The migration of molecular mass markers is indicated on the right of the gels. The apparent molecular masses produced by hsp90 oligomerization are indicated on the left of the gels. Temperature was increased at a rate of 1 K/min.

TABLE 1

The Influence of Geldanamycin, Mg^{2+}, and Ca^{2+} on the Temperature Corresponding to Disappearance of 50% of hsp90 Dimers ($T_{50\%}$), the Temperatures of the First (T^1) and Second (T^2) Transitions on the hsp90 Calorimetric Curve and the Denaturation Enthalpy (ΔH_{cal})

Added compound	$T_{50\%}$[a] (°C)	T^1 (°C)	T^2 (°C)	ΔH_{cal} (kcal/mol)
none	56	53.8 (53.3[b])	63.1	490
none[c]		53.6[c]	64.6[c]	460[c]
geldanamycin	60	57.4	63.1	590
5 mM Mg^{2+}	52	49.9 (50.1[b])	60.9	400
5 mM Ca^{2+}	46/47	45.7 (45.1[b])	56.3	360

[a] PAGE data.
[b] Denaturation temperature obtained by CD.
[c] Heating rate 2 K/min.

RESULTS AND DISCUSSION

Native PAGE analysis of the hsp90 oligomerization temperature dependence. Native gel electrophoresis showed that at pH 7 hsp90 is in a dimer form which is confirmed by our sedimentation experiments (data not shown). There is no change in this state in the temperature range 20-50°C (Fig. 1A). Further increase of temperature induced a decrease in the hsp90 dimer quantity (Fig. 1A) associated with the emergence of higher molecular bands corresponding to protein oligomerization: 50% of hsp90 was oligomerized at 56°C (Table 1). Estimation of the oligomer molecular weight clearly demonstrate that upon heating, self-association of hsp90 dimers occurred, leading to the formation of tetramers, hexamers, octamers, and higher molecular weight polymers (Fig. 1A), i.e., the hsp90 dimer does not dissociate upon heating. Moreover, the oligomers formed were still present up to 68°C (Fig. 1A). No difference was observed when protein concentration changed from 1 to 2 mg/ml.

The influence of geldanamycin, an antibiotic known to bind hsp90 [12], on the hsp90 thermal oligomerization is shown in Figure 1B. Geldanamycin shifts the oligomerization process towards higher temperatures: indeed, 50% of hsp90 dimers were oligomerized at the temperature of 60°C instead of 56°C for hsp90 alone (Table 1). Therefore, geldanamycin protects hsp90 from temperature oligomerization.

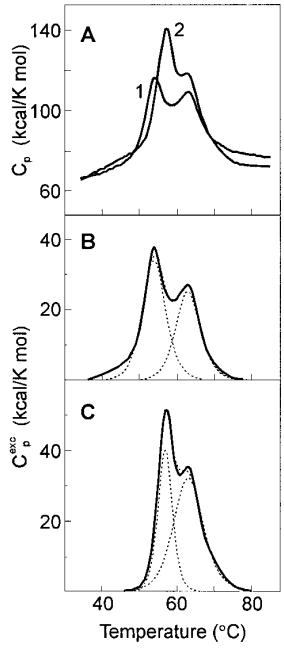

FIG. 2. (A) Temperature dependence of the partial molar heat capacity of hsp90 (1) and its complex with geldanamycin (2) at pH 7. Computer deconvolution of the transition excess heat capacity of hsp 90 (B) and its complex with geldanamycin (C): experimental results (solid line), deconvoluted peaks, and their sum (dotted line). Temperature was increased at a rate of 1 K/min.

In the presence of magnesium and calcium, we observed a significant decrease of the temperature at which the disappearance of hsp90 dimers began (Fig. 1C, D). With 5 mM magnesium or 5 mM calcium the temperatures corresponding to 50% of the hsp90 dimer disappearance were 52°C and 46/47°C, respectively (Table 1), thus by 4 and 10°C lower than in the absence of these ions. Moreover, in the presence of divalent cations, when the hsp90 dimer band disappeared, we did not observe emergence of oligomers as for hsp90 or the hsp90-geldanamycin complex. In the presence of magnesium only few high-molecular-weight oligomers were seen on the gel and they rapidly disappeared as temperature increased (Fig. 1C); in the presence of calcium no oligomer appeared following the disappearance of dimers, and the hsp90 dimers aggregated immediately (Fig. 1D). The formed aggregates cannot penetrate in the gel and stay at the sample deposition in the staking gels. At the same time, lowering the calcium concentration to 1-2 mM allowed us to observe hsp90 oligomerization. Thus, Mg^{2+} and much more strongly Ca^{2+} favour the hsp90 self-association.

Scanning calorimetry of hsp90. Figure 2A (curve 1) shows the temperature dependence of the hsp90 partial molar heat capacity at pH 7. This value at 20°C (0.35 cal K^{-1} g^{-1}) is close to the average value for small globular proteins [11], indicating a comparably tight packing of polypeptide chains within the dimer. The calorimetric curve consists of two clearly visible peaks centered at 53.8 and 63.1°C. The existence of two peaks indicates that the denaturation process does not conform to a two-state mechanism and involves the presence of significantly populated partially folded states. Melting of hsp90 was irreversible. However, calorimetric curve shows no sign of detectable aggregation and there was no concentration dependence of denaturation parameters. In addition, the first peak temperature and overall denaturation enthalpy did not change and the second peak temperature slightly raised upon the heating rate increase from 1 to 2 K/min (Table 1). This shows that the process, which is responsible for the irreversibility, is slower than the denaturation process and thus cannot influence its parameters.

Hsp90 melting curve can be deconvoluted into two overlapping transitions (model for independent non-two-state transitions of MicroCal Origin), Figure 2B. As was shown in [12,13], hsp90 contains three structural domains: N-terminal (residues 1-400), middle (401-615), and C-terminal (621-732). Keeping this in mind, we conclude that two transitions on the hsp90 denaturation curve reflect melting of two different regions of the protein. Indeed, heating the protein to the first peak top does not affect the second peak upon rescanning, whereas the first peak is significantly decreased. In order to assign denaturation transitions to specific regions of hsp90, we studied melting of the hsp90 complex with geldanamycin (Fig. 2A, curve 2). Geldanamycin binds exclusively to the N-terminal domain of hsp 90 (residues 9-232) and inhibits the chaperone functions of hsp 90 [12,14]. In the presence of geldanamycin the melting curve is also deconvoluted into two transitions (Fig. 2C) but the low temperature peak is shifted to higher temperatures by 3.6°C, whereas the high temperature peak remains unchanged (Table 1). Consequently, the first transition corresponds to melting of the hsp90 N-terminal domain, whereas the second transition relates to denaturation of the rest of the protein. The lower thermostability of the N-terminal domain was found also for hsp70 [15]. The temperature of disappearance of 50% of hsp90 dimers is shifted to higher temperatures in comparison with the temperature of the N-terminal domain melting (Table 1). Hence, the unfolding of the N-terminal domain precedes oligomerization of the protein.

The ratio between calorimetric and effective or van't Hoff enthalpies, which determines the number of cooperative units in the molecule [11], is equal to two for the first denaturation transition of hsp90 alone and that complexed with geldanamycin. It should be noted that the calorimetric enthalpy was calculated for the molecular weight of the dimer. Thus, N-terminal domains of the two monomers in hsp90 dimer melt relatively independently and do not interact with each

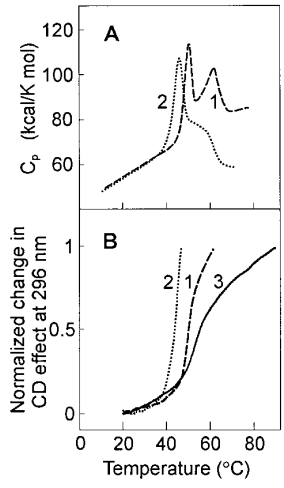

FIG. 3. Temperature dependence of the partial molar heat capacity (A) and normalized change in CD intensity at 296 nm (B) for hsp90 at pH 7: (1) 5 mM $MgCl_2$, (2) 5 mM $CaCl_2$, (3) without divalent cations. Temperature was increased at a rate of 1 K/min.

other. This is in good agreement with the fact that N-terminal domain is not involved in dimerization [16].

The mechanism of the hsp90 dimer formation is realised through the duplicate interactions of residues 542-615 of the middle domain and C-terminal domain (629-732) of each monomer molecule oriented in the opposite direction [16]. Our data show that the oligomers consist of hsp90 dimers in the whole range of temperatures. It follows that segments of two monomers involved in dimerization remain bound and do not dissociate upon denaturation. The ratio between calorimetric and van't Hoff enthalpies for the second calorimetric peak is about three both for hsp90 alone and complexed with geldanamycin. Owing to this, three cooperative units of the dimer may melt in this peak: two non-interacting parts of the middle domains (401-541) and the dimerization region.

The results of the hsp90 heat denaturation in the presence of 5 mM $MgCl_2$ and 5 mM $CaCl_2$ are demonstrated in Fig. 3A. The recording of curve 2 was disturbed by a high exothermal aggregation heat effect at high temperatures. The partial molar heat capacity at 20°C and the slope of the heat capacity vs. temperature dependence before denaturation in the presence of divalent cations were similar to those for hsp90 alone. The data prove that Mg^{2+} and Ca^{2+} did not alter the compactness and the dimer form of hsp90 before the denaturation. This agrees with our PAGE analysis of hsp90 oligomerization (Fig. 1C,D). Contrary to the free hsp90, heating the protein to the first peak top leads to complete disappearance of both peaks upon rescanning. In the presence of cations, temperature association between partially-unfolded (molecules with unfolded N-terminal part) and native molecules also takes place. The total melting heat effect decreased by 18% and 26% upon adding Mg^{2+} and Ca^{2+}, respectively (Table 1). The temperatures for the first and second peaks were lower by 3.9°C and 2.2°C in the presence of Mg^{2+}, and by 8.1°C and 6.8°C in the presence of Ca^{2+} (Table 1). Thus, divalent cations lead to a decrease in the hsp90 thermostability, and self-association started at lower temperatures (Table 1). The effect of calcium on the hsp90 melting was much stronger than that of magnesium.

Calcium-binding proteins with EF-hand are known to be stabilized by binding of Ca^{2+} ions [17]. The destabilization of hsp90 in the presence of Ca^{2+} indicates that hsp90 is unlikely to be a Ca-binding protein with EF-hand as asserted in [4]. Moreover, PROSITE (ExPASy-SWISS-PROT tools) examination did not reveal an EF-hand motif or other known calcium-binding patterns in the primary structure of hsp90 sequences available in the SWISS-PROT database.

Hsp90 melting by CD. Near-UV CD of proteins is a highly sensitive criterion for the native state of a protein, and can thus be used as a "fingerprint" of the tertiary structure. Hsp90 contains four tryptophan, 25 tyrosine, and 25 phenylalanine residues. The spectrum was not modified by 5mM $MgCl_2$, whereas calcium ions changed the CD sign in the 280 nm region (where Tyr and Trp contribute) from negative to positive (Fig. 4). This indicates that calcium influences the local structure and environment of the side groups of some Tyr and Trp residues, i.e., the tertiary structure of the protein. The fact that this CD effect in the 280 nm region varies in sign is not surprising, since slight differences in the orientation of side groups can reverse the sign of the Cotton effect [18]. In the near-UV CD spectra of hsp90 there is a positive peak at 296 nm due to Trp, which was not disturbed by cations (Fig. 4). We followed protein denaturation by the changes in the amplitude of this band. Hsp90 undergoes a sharp denaturation transition with a midpoint (T_d) at 53.3°C (Fig. 3B, curve 3). The presence of divalent cations lowers T_d to 50.1°C (Mg^{2+}) and 45.1°C (Ca^{2+}). These values coincide with the corresponding values obtained from scanning calorimetry for the first transition (Table 1), i.e., CD reflects the unfolding of hsp90 N-terminal do-

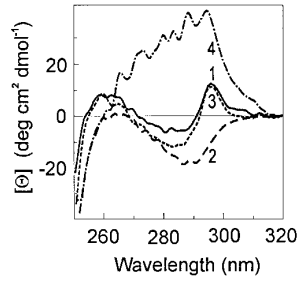

FIG. 4. CD spectra of hsp90 in the near-UV region at pH 7: (1) 20°C, (2) 90°C, (3) 20°C, 5 mM $MgCl_2$, (4) 20°C, 5 mM $CaCl_2$.

main. This is in agreement with the localization of tryptophans in the hsp90 molecule sine three of four residues belong to the N-terminal domain: Trp161, 296, and 319 (hs9a_human, SWISS-PROT database). The fourth residue (Trp 605) is in the C-terminal part dimerization region [16] melting in the second calorimetric peak. Absence of the second transition on the CD melting curve is indicative of the invariability of the asymmetry of this residue microenvironment confirming that hsp90 dimer does not dissociate upon denaturation. As we have observed, hsp90 tends to oligomerization as soon as temperature increased, and cations strongly influence this process, lowering the temperature threshold of oligomer formation. This tendency is confirmed by the marked increase in hsp90 absorption upon heating in the presence of cations, followed by the colloid-type structure formation and precipitation at 62°C (Mg^{2+}) and 47°C (Ca^{2+}) (Fig. 3B, curves 1 and 2).

To sum up, thermal denaturation of hsp90 exhibits two well defined transitions: N-terminal domains of the two monomers in hsp90 dimer melt independently in the lower-temperature peak, while the higher-temperature one comprises unfolding of the middle and C-terminal domains. Unfolding of the N-terminal domain is an important stage in the start of oligomerization; the oligomers consist of dimers not dissociating upon denaturation. As it is known, the hsp90 oligomerization is responsible for its ability to bind substrate proteins and prevent their irreversible aggregation [7]. Magnesium and to a much greater extent calcium proved to decrease the hsp90 thermostability and thereby to cause oligomerization at lower temperature. Calcium appeared to alter the protein tertiary structure. It should be noted that calcium shifts the unfolding temperature of the hsp90 N-terminal domain close to the heat shock temperature range (42-45°C [8]). Hence, the unfolding of N-terminal domains in the dimer is an important step in the initiation of functional activity of hsp90. The regulation of hsp90 oligomerization by calcium is in good agreement with the phenomena occurring under heat stress. Indeed, the rise in intracellular calcium observed in such conditions [9] can account for that hsp90 oligomerizes easier and becomes fully functional as a molecular chaperone.

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