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BINDING AFFINITY OF PROTEINS TO hsp90 CORRELATES WITH BOTH HYDROPHOBICITY AND POSITIVE CHARGES. A SURFACE PLASMON RESONANCE STUDY

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Summary

The 90 kDa heat shock protein (hsp90) is a major cytoplasmic molecular chaperone associating with numerous other proteins including steroid receptors. Here we provide the first numerical analysis of hsp90-target associations using surface plasmon resonance. Binding affinities of histones, the "native molten globule", casein and calmodulin to hsp90 decrease in the order of K_d = 70 +/- 24, 220 +/- 70 and 1800 +/- 600 nM, respectively. Analysis of the structure of binding proteins revealed that their binding affinity depends on both hydrophobicity and positive charges making the discriminative features of hsp90 similar to those of other molecular chaperones.

Key Words: molecular chaperones, hsp90, calmodulin, casein, histones, surface plasmon resonance

The 90 kDa heat shock protein (hsp90) is a major cytoplasmic molecular chaperone associating with numerous other proteins such as steroid receptors, signal transducing kinases (raf, src, wee1, sevenless, casein kinase II, cyclin dependent kinase 4) and filamentous actin (1-5). hsp90 has been shown to bind histone H1 (6), partially denatured proteins (2,7) and calmodulin (8,9) in vitro. hsp90 has been partially characterized as a molecular chaperone able to keep the target protein in a folding-competent state (10), having an enhanced chaperone activity in its oligomeric form at higher temperatures (11), and being inhibited by divalent cations (12). In spite of this information, however, relatively little is known about its binding affinity and specificity. Here we determine the binding constants of three model proteins, histones, the "native molten globule", casein (11,13) and calmodulin to hsp90 using surface plasmon resonance (SPR). Our results indicate, that the binding affinity of these proteins to hsp90 depends on both their hydrophobicity and their positive charges making the discriminative features of hsp90 similar to those of other molecular chaperones such as GroEL, dnaK, or hsp70 (14-18).

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Materials and Methods

Chemicals -- Chemicals for the coupling of hsp90 to the surface plasmon resonance sensor chips were purchased from Pharmacia Biosensor AB (Uppsala, Sweden). Hydroxyapatite was an Econo-Pac HTP column from BioRad (Richmond, CA). Chromatography media were purchased from Pharmacia LKB Biotechnology Inc. (Uppsala, Sweden). Calmodulin has been isolated from porcine brain by the method of Nishida et al (19). Histone H1 was from Boehringer Mannheim (Germany). All the other chemicals used were from Sigma Chemicals Co. (St. Louis, MO).

Isolation of hsp90 -- The 90 kDa heat shock protein was isolated from livers of 4 months old, male Sprague-Dawley rats or from the L5178Y mouse cell line using the method of Yonezawa et al (20) as described earlier (21). The purity of this preparation was higher than 95% (usually higher than 98%) as judged by densitometry of silver stained SDS slab gels (22). Protein concentrations were determined using the methods of Bradford (23).

Surface plasmon resonance (SPR) measurements -- All the solutions for the SPR measurements were passed through a 0.22 microm Millipore filter and degassed in vacuum for 5 minutes at room temperature. For coupling hsp90 to the SPR sensor chips (24,25) 50 microl rat or mouse hsp90 was dialysed overnight at 4°C against 200 ml of coupling buffer containing 5 mM Na_2HPO_4, pH 7.4 and 150 mM NaCl to remove Tris, which interferes with the coupling. Before coupling research grade carboxymethyl-dextran sensor chips (CM5, Lot 0311, Pharmacia Biosensor AB, Uppsala, Sweden) were incubated with running buffer containing 10 mM Hepes, pH 7.4, 0.15 M KCl and 0.001 % Tween-20 (supplied by Pharmacia Biosensor as 0.005 % detergent P20) to prevent the unspecific binding of proteins to the capillaries in a BIACore apparatus for 10 minutes at 25°C at a flow rate of 5 microl/min (phosphate-based buffers were omitted to prevent the precipitation of calcium- or magnesium-phosphates). The carboxymethyl-dextran coated sensor chip was activated with 200 mM of 1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide and 50 mM N-hydroxy-succinimide in Milli-Q deionized water for 5 minutes at 25°C. Rat or mouse hsp90 (protein concentration: 30 microg/ml) was coupled to the chip in a buffer containing 10 mM Na-formiate, pH 4.0, for 7 minutes at 25°C. (Increasing the pH of the coupling buffer to 5.5, the vicinity of the isoelectric point of hsp90, diminished the coupling efficiency by 95 % as expected.) The remaining active sites of the resin were inactivated by incubation with a 1 M ethanolamine.HCl solution (pH 8.5) for 7 minutes at 25°C. After coupling non-covalently bound hsp90 was removed by 2 or 3 brief (2 minutes) fluxes of 20 mM HCl. The usual change in the angle of the SPR minimum of the reflected light after coupling of hsp90 was approximately 0.9 degrees, i.e. 9,000 resonance units (RU, range: 7358-10473) corresponding to 9 ng hsp90/mm^2 sensor chip (24). Binding of various proteins to hsp90 was analysed in a running buffer. After a binding experiment has been completed, occasionally a brief (2 minutes) wash with 20 mM HCl was applied to remove the proteins, non-covalently adsorbed to the chip. With this procedure the baseline was remarkably stable (+/- 3 %) even after repeated binding experiments up to 50 times. Repeating the experiments in a different order did not result in significant differences of the binding curves, which shows that the baseline stability is a good criterium for the resorbtion of bound proteins, and also reflects that the occasional HCl-washes did not denature hsp90 significantly. With one coupling all measurements were made within 8 hours and the same sensor chip was used for maximum 3 days after opening. All the binding experiments were repeated with "mock-coupled" sensor chips, where in the coupling procedure hsp90 was omitted. Binding curves (sensorgrams) were corrected by subtracting the electrostatic

binding of proteins (which was significant only in the case of histones) and the occasional changes in the refractive index after various additions. Special care was taken to reduce the latter phenomenon to the minimum throughout the experiments. Repeating some of the experiments with certified sensor chips (CM5, Lot 02103) did not show any significant difference.

Analysis of the binding data -- The portion of the sensorgrams which corresponds to the dissociation of various proteins from hsp90 was analyzed by the BIA evaluation program 2.1 to obtain k_d. The experimental data fit well the single exponential kinetic model used. Some experiments have been repeated at a flow rate of 10 microl/min to check the effect of possible rebinding. Rebinding and mass transfer limitation was also checked by the repetition of the experiments at lower levels of immobilized hsp90. The identical results showed that these phenomena did not occur under the conditions we used. Using the corresponding k_d values nonlinear curve fitting of the association phase of the corresponding sensorgram was carried out with the BIA evaluation program 2.1 to obtain k_a. K_d was calculated as k_d/k_a.

Measurement of the tryptophane fluorescence of hsp90 -- Fluorescence measurements were carried out in a Hitachi 2500 spectrofluorimeter at 37°C as described before (26).

Results and Discussion

Figure 1 shows the binding and dissociation of histones (lysine-rich, Type III-S, Sigma), alpha-casein (Sigma C 6780) and calmodulin to immobilized hsp90 measured by surface plasmon resonance (SPR) in the presence of 1 mM $CaCl_2$. Binding has been corrected to the electrostatic binding of the proteins to the carboxymethyl-dextran coated SPR sensor chips. This correction was significant in the case of positively charged histones, but was almost negligible (< 3 % of total binding) in case of alpha-casein and calmodulin. Binding was analysed in the absence of divalent cations and in the presence of 1 mM $MgCl_2$ which gave identical results in the binding of histones and casein (data not shown). Binding of calmodulin to hsp90, however, was a strictly calcium-dependent process, which is in agreement with earlier observations (8,9). Examining the binding of dephosphorylated-casein (Sigma C 8032) did not show significant differences when compared to the binding of casein (data not shown) which indicates, that the binding was mainly not due to electrostatic interactions between the negative phosphates of casein and lysines/arginines of hsp90. Addition of globular actin, native alpha-lactalbumin and bovine IgG, proteins known (5) or supposed not to bind to hsp90, did not show any increase in the SPR signal (data not shown).

Numerical analysis of the binding curves indicates that histones, casein and calmodulin bind to hsp90 with an approximate K_d of 70, 220 and 1800 nM, respectively (Table I.). Dissociation of histones from hsp90 occurs very slowly, while in case of calmodulin the relatively slow association is accompanied with a quite fast dissociation which results in a small overall binding affinity.

In our earlier studies, hsp90 was shown to bind ATP, possess an autokinase activity and undergo a conformational change after ATP addition. The effects of ATP were more pronounced in the presence of Ca-ATP, while Mg-ATP showed only a weak affinity for hsp90 (21,26). Recently, the ATP-binding of hsp90 has been challenged demonstrating that Mg-ATP shows a very weak,

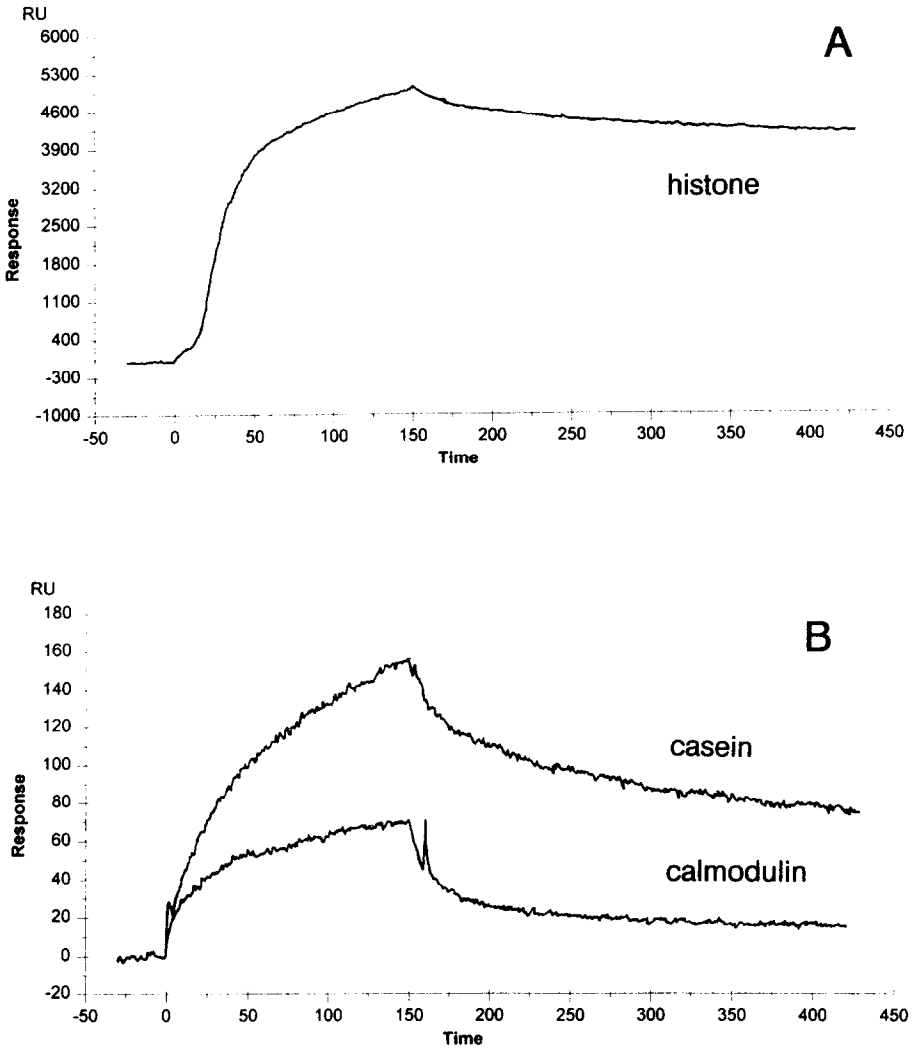

Fig. 1

Surface plasmon resonance sensorgram of the binding of histones (panel A), alpha-casein and calmodulin (panel B) to hsp90. Histones (Sigma, Type III-S, lysine-rich), alpha-casein (Sigma C 6780) and procine brain calmodulin were added to immobilized mouse hsp90 at a final concentration of 50 microg/ml in the presence of 1 mM $CaCl_2$. Coupling of hsp90 to the sensor chip and measurement of surface plasmon resonance was performed as described in Materials and Methods. After allowing the binding of the respective protein for 2 minutes, the sensor chip was washed with the running buffer to induce the dissociation of the protein. Sensorgrams have been corrected to the eletrostatic binding of proteins to the carboxymethyl-dextran matrix using "mock-coupled" sensor chips. Sensorgrams are representatives of minimum 3 experiments.

TABLE I

Numerical Analysis of the Binding of Histones, Casein and Calmodulin to hsp90

		- ATP	+ ATP
histone	$k_d \times 10^{-3}$ (s^{-1})	0.4 ± 0.1	0.5 ± 0.2
	$k_a \times 10^{3}$ ($M^{-1}s^{-1}$)	5.3 ± 1.2	6.6 ± 1.9
	K_d (nM)	70 ± 24	80 ± 19
casein	$k_d \times 10^{-3}$ (s^{-1})	1.4 ± 0.3	1.7 ± 0.7
	$k_a \times 10^{3}$ ($M^{-1}s^{-1}$)	8.6 ± 3.2	6.8 ± 3.4
	K_d (nM)	220 ± 70	280 ± 110
calmodulin	$k_d \times 10^{-3}$ (s^{-1})	2.4 ± 0.6	2.2 ± 0.7
	$k_a \times 10^{3}$ ($M^{-1}s^{-1}$)	1.4 ± 0.5	1.5 ± 0.6
	K_d (nM)	1800 ± 600	2000 ± 700

Binding of proteins to hsp90 was measured and analysed as described in Materials and Methods. Data are means +/- SDs of minimum 3 independent experiments.

if any binding to yeast and bovine hsp90 (27). On the other hand we have observed, that higher (millimolar) concentrations of ATP induce the dissociation of filamentous actin from nitrocellulose-coated microscope coverslip-bound hsp90 even in the presence of magnesium (28). To address this question in this system we examined the effect of ATP to the binding of histones, casein and calmodulin to hsp90.

Data of Table I. show, that the binding of proteins examined was not affected by ATP. We have tested the binding of casein in the presence of other nucleotides or nucleotide-analogues, such as AMP, ADP, GTP, ATP-gamma-S, and vanadate which induced no significant difference in the binding affinity of casein either in the presence of Mg^{2+} or Ca^{2+} (data not shown). These observations fit to the previous data showing that ATP-related effects of hsp90 may be restricted to a subset of hsp90-binding proteins and that for some ATP-effects (such as hsp90-associated ATP-ase activity) the presence of an additional protein might be required (2,10,28,29).

TABLE II

Comparison of the Net Charges of Histones, Casein, Calmodulin and hsp90 with their Binding Affinities

	Net charge at neutral pH	Binding affinity to hsp90 (K_d, nM)
hsp90	-38	
C-terminal	-13	
middle	-13	
N-terminal	-12	
histone H1	+59	70
H2A	+15	
H2B	+19	
H3	+20	
H4	+17	
casein	-14	220
calmodulin	-25	1800

The net charges at neutral pH were calculated using the primary structure of murine hsp90 (30), histones (31,32), casein (33) and calmodulin (34), respectively. Binding affinities are from Table I. The C-terminal, middle and N-terminal domains of hsp90 represent the amino acid sequences of 1-190, 191-284 and 285-733 of murine hsp90 (30), respectively. The middle, highly charged domain is characteristic to the eukaryotic hsp90 homologues.

The dissociation constant of calmodulin (approximate K_d of 2 microM) shows a good agreement with earlier estimates of 1-10 microM using equilibrium gel filtration (7). Similarly, the binding affinity of casein (200 nM) is in the range, where the protein shows a half-maximal inhibition of binding of denatured dihydrofolate-reductase to hsp90 (11). Since our earlier studies (6) did not result a direct estimation of the binding affinity of histones to hsp90 we wanted to address this question utilizing the fact that histone H1 does not contain tryptophanes. Tryptophane fluorescence of hsp90 showed a small increase and blue-shift after the addition of histone H1 indicating that one or both of the two tryptophanes in hsp90 has been shielded from water after histone H1-binding. Unfortunately the numerical analysis of this phenomenon was not possible, since the signal-to-noise ratio was to low for a "histone H1-titration" of hsp90 tryptophane fluorescence (data not shown).

Speculating on the reasons, why these three proteins have so different binding affinities to hsp90 one might compare their net charges at neutral pH (Table II) and hydrophobicity. hsp90 has 37 negative charges at neutral pH. This might explain the tight association of positively charged histones to the protein and partially explain, why the negatively charged casein and (especially) calmodulin bind to hsp90 at a lower affinity. Electrostatic repulsion between hsp90 and calmodulin is partially reduced by the positively charged calmodulin binding-site of hsp90 (9). This effect, however, seems not to be enough to induce a tight binding of calmodulin to hsp90. hsp90 is a hydrophobic protein (26) which suggests, that similarly to other molecular chaperones, hydrophobic interactions might also contribute to the binding of other proteins, such as the hydrophobic casein (11,13) to hsp90. Studies with other molecular chaperones, such as with GroEL, dnaK, or with hsp70 indicated, that similarly to hsp90, both hydrophobic and electrostatic interactions play a role in determining their binding affinities (14-18). However, one should be cautious with direct comparisons, since histones, casein and calmodulin are not direct targets of the hsp90-chaperone, and from the data available, the overlap between their binding site and the presumed peptide binding site of hsp90 can not be assessed at the moment. Discrimination between some hsp90-binding proteins and direct targets of the hsp90-chaperone may be important all the more since our other experiments indicate that binding of guanidine-HCl denatured lactalbumine to sensor surface-immobilized hsp90 displays some ATP-dependent changes in the presence of divalent cations (P. Csermely, Y. Miyata and I. Yahara, unpublished results).

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