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ATP sensitive tryptophans of hsp90

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Abstract

The nature of the interaction between the nucleotide ATP and hsp90 was investigated by observing fluorescence quenching of the four tryptophan residues in hsp90 as a function of quencher type and temperature. ATP and acrylamide quench the fluorescence from tryptophan free in solution principally by static and collisional mechanisms, respectively. Acrylamide quenching of tryptophan fluorescence in hsp90 is also principally collisional and identifies two classes of residues, one readily accessible to quenching the other less accessible. ATP quenching of tryptophan fluorescence in hsp90 is more complex exhibiting no overall preferred mechanism. However, ATP competitively inhibits acrylamide quenching of the readily accessible class of tryptophan residues by static quenching with the quenching constant providing an upper limit for the ATP dissociation constant. The ATP-free tryptophan dissociation constant is more than a factor of three larger than that for ATP–hsp90 suggesting that the ATP–hsp90 interaction is specific. The static quenching of tryptophan fluorescence in hsp90 by ATP implies that the nucleotide binds in close proximity to one or more of the tryptophan residues.

Keywords: Fluorescence quenching; Acrylamide; Nucleotide binding; Conformation

1. Introduction

The 90 kDa heat shock protein, hsp90, is an abundant and ubiquitous protein in both stressed and unstressed cells. Hsp90 appears to be essential to the life of eukaryotic cells, but its critical functions are still unclear. It has been characterized most extensively as a component of multiprotein complexes with steroid receptors, some additional transcription regulators, and with a variety of protein kinases [1,2]. A chaperoning activity for hsp90 has been indicated by studies on steroid receptors where hsp90 association appears to confer native conformation to the ligand binding domain of the receptor [3,4]. This association between hsp90 and the steroid receptor requires additional proteins including a 23 kDa protein of unknown function, p23 [5]. p23 is able to bind to hsp90 through a process requiring ATP and recent studies indicate that the conformation of hsp90 is altered by binding ATP to produce a structural state that can interact with p23 [6].

A second type of hsp90 chaperoning function has been indicated by studies on the refolding of the denatured proteins citrate synthatase and β-galactosidase [7,8]. In these model systems, hsp90 does not

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promote the refolding process, but it appears to maintain the denatured protein in an unaggregated state capable of refolding. This activity of hsp90 does not require ATP.

The actual binding of nucleotides to hsp90 has only recently been established. Earlier reports provided evidence that hsp90 could bind ATP [9–11], however, these reports have been challenged by other laboratories who were unable to detect ATP binding [12,13]. Conventional methods for detecting and quantitating ATP binding, including equilibrium dialysis and gel exclusion chromatography, proved inconclusive due to difficulties in detecting the apparently low affinity ATP binding at attainable hsp90 concentrations.

Two laboratories have now crystallized an amino terminal domain of hsp90 revealing a binding site for ATP [14] or its competitive inhibitor, geldanamycin [15]. The binding of this domain to an ATP affinity resin has also been demonstrated and mutations in this region block the ATP-dependent binding of hsp90 to p23 [16]. These results indicate that nucleotides act as conformational regulators of hsp90 interaction with other proteins. The identification of ATP as a ligand of hsp90 renews interest in the spectroscopic detection and characterization of the nucleotide–hsp90 interaction. We report here findings concerning the nature of this interaction using the quenching of tryptophan fluorescence.

Tryptophan accessibility to solubilized fluorescence quenchers measures the exposure of the indole group to interactions with molecules in the solvent. Two mechanisms, static and collisional quenching, describe the quencher–tryptophan interaction. Static quenching has bound quencher near the indole group of tryptophan promoting radiationless excited state relaxation. In collisional quenching, the quencher collision with indole promotes radiationless relaxation. Collisional quenching efficiency increases, while static quenching efficiency decreases, with increasing temperature because increased temperature favors the dissociated state of the static quencher while promoting the frequency of collisions of the collisional quencher [17]. These two quenching mechanisms are readily distinguishable on the basis of the temperature dependence of their efficiency.

We used ATP and acrylamide to quench tryptophan fluorescence from free tryptophan and hsp90 in solution. ATP and acrylamide are predominantly static and collisional quenchers, respectively, of free tryptophan in solution. Acrylamide quenching of tryptophan fluorescence in hsp90 qualitatively parallels these results and also suggests heterogeneity in the tryptophan residues. We found that tryptophan fluorescence from hsp90 falls into two classes, one accessible and another relatively less accessible to collisional quenching. ATP quenching of tryptophan fluorescence in hsp90 is more complex exhibiting no overall preferred mechanism. Acrylamide quenching in the presence of ATP shows that ATP binds to hsp90 and lowers the amount of quenchable fluorescence from the readily accessible class of tryptophans by statically preempting their collisional quenching. Our findings indicate specific ATP binding to hsp90 at the site of the readily accessible class of tryptophan residues.

2. Materials and methods

2.1. Chemicals

Ultra pure acrylamide and Tris were from ICN Biomedicals (Cleveland, OH). ATP and *N*-acetyl-L-tryptophanamide (NATA) were from Sigma (St. Louis, MO). All chemicals were analytical grade.

2.2. Solutions and sample preparation

Standard buffer contained 50 mM KCl, 5 mM $MgCl_2$, and 10 mM Tris–HCl at pH 7.5. The model system for free tryptophan in solution contained 2.5 μM NATA in standard buffer that was quenched by addition of 0–14 mM ATP or 0–90 mM acrylamide.

Human hsp90β (> 98% pure) was prepared from an Sf9 cell expression system as described previously [16] and dissolved in standard buffer at 0.1 mg/ml for all of the experiments. In one kind of experiment, the tryptophan fluorescence from hsp90 was quenched by addition of 0–14 mM ATP or 0–90 mM acrylamide. In another kind of experiment, the tryptophan fluorescence from hsp90 was quenched in the presence or absence of ATP by addition of 0–450 mM acrylamide. For the latter experiments, acrylamide was added from stock solutions of 5 M acrylamide also containing 0, 5, 10, or

20 mM ATP to hold constant the ATP concentration during the acrylamide titration.

2.3. Spectroscopic measurements

We measured absorption spectra on a Beckman DU650 spectrophotometer (Beckman Instruments, Fullerton, CA) with spectral resolution of 2 nm. All absorption measurements were made at room temperature.

We measured fluorescence on an SLM 8000 spectrofluorometer (SLM Instruments, Urbana, IL). Tryptophan fluorescence intensity was recorded at an excitation wavelength of 298 nm and emission wavelength of 340 nm. Excitation and emission monochromator slits were set to 2–4 nm or in some experiments an interference filter was used with a bandwidth of 10 nm for selecting the emission wavelength. Decreases in fluorescence due to the dilution of protein or NATA by addition of quencher were corrected by the appropriate normalization of the fluorescence intensities. The NATA and hsp90 samples used in the fluorescence experiments had absorbance at 298 nm ≤ 0.05 at all quencher concentrations to avoid artifacts from inner filtering of the excitation light.

2.4. Interpreting quenching experiments

The Stern–Volmer equation relates the fluorescence intensity, F, and quencher concentration, $[Q]$, by,

$$\frac{F}{F_0} = \frac{1}{1 + K[Q]} \tag{1}$$

where F_0 is the fluorescence intensity in the absence of quencher, and K is a quenching constant representing either a collisional or static quenching mechanism, but not both. The modified Stern–Volmer equation,

$$\frac{F}{F_0} = \frac{1}{(1 + K^{c}[Q])\exp(K^{s}[Q])} \tag{2}$$

generalizes Eq. (1) for contributions from both collisional and static quenching mechanisms with constants K^{c} and K^{s} [18]. Eq. (2) is appropriate for a system with a single chromophore (or homogeneous class of chromophores) undergoing collisional and static quenching due to Q.

For a protein system with two classes of tryptophans that are inequivalently accessible to quenching, Eq. (2) further generalizes to,

$$\frac{F}{F_0} = \frac{f_a}{(1 + K_a^{c}[Q])\exp(K_a^{s}[Q])} + \frac{(1 - f_a)}{(1 + K_b^{c}[Q])\exp(K_b^{s}[Q])} \tag{3}$$

where f_a is the fraction of the emission intensity belonging to fluorophores in class a that are quenched by Q with collisional and static quenching constants of K_a^{c} and K_a^{s}. K_b^{c} and K_b^{s} are the quenching constants for the other class of fluorophores. We also define the accessible class quenching curve, A/A_0, using Eq. (3) such that,

$$\frac{A}{A_0} \equiv \frac{f_a}{1 + K_a^{c}[Q]} = \frac{F\exp(K_a^{s}[Q])}{F_0} - \frac{(1 - f_a)\exp(K_a^{s}[Q])}{(1 + K_b^{c}[Q])\exp(K_b^{s}[Q])} \tag{4}$$

Similarly, the less accessible class quenching curve, B/B_0, is given by,

$$\frac{B}{B_0} \equiv \frac{1 - f_a}{1 + K_b^{c}[Q]} = \frac{F\exp(K_b^{s}[Q])}{F_0} - \frac{(f_a)\exp(K_b^{s}[Q])}{(1 + K_a^{c}[Q])\exp(K_a^{s}[Q])} \tag{5}$$

2.5. Curve fitting

Quenching curves were fitted using a least squares protocol with inequality constraints to locate the best fitting linear parameters. The best nonlinear parameters (when using Eq. (3)) were located by grid search. All error estimates are standard error of the mean.

3. Results

3.1. The quenching of NATA in solution

The free tryptophan model system of NATA (*N*-acetyl-L-tryptophanamide) characterizes ATP and acrylamide quenching of a highly accessible chromophore. Fig. 1 shows the ATP and acrylamide quenching of NATA in standard buffer at two temperatures. The plots of F_0/F vs. $[Q]$ are approximately linear for both quenchers and at both temperatures indicating a homogeneous chromophore undergoing collisional or static quenching, as expected. Eq. (1) inverted to give F_0/F, adequately describes this system with the slopes of the curves giving the quenching constants. We found $K = 0.088 \pm 0.003$ or 0.060 ± 0.01 mM^{-1} for ATP at 4 or 37°C, and, 0.0173 ± 0.0004 or 0.022 ± 0.0005 mM^{-1} for acrylamide at 4 or 37°C.

In the foregoing experiments, temperature had opposite effects on the quenching constants for the two kinds of quenchers. Increasing temperature increased the acrylamide, but decreased the ATP quenching constants. These data demonstrate that over the quencher concentrations studied, acrylamide and ATP are predominantly collisional and static quenchers of tryptophan, respectively.

3.2. The quenching of tryptophan fluorescence from hsp90

Fig. 2A shows the ATP quenching of tryptophan fluorescence from hsp90 at two temperatures. Changing temperature causes no statistically significant change in the quenching constants given by $K = 0.018 \pm 0.001$ and 0.017 ± 0.002 mM^{-1} for 4 and 37°C. ATP is a static or dynamic quencher of tryptophan fluorescence when directly interacting with these residues, or, it binds to and alters hsp90 conformation to quench tryptophan fluorescence without

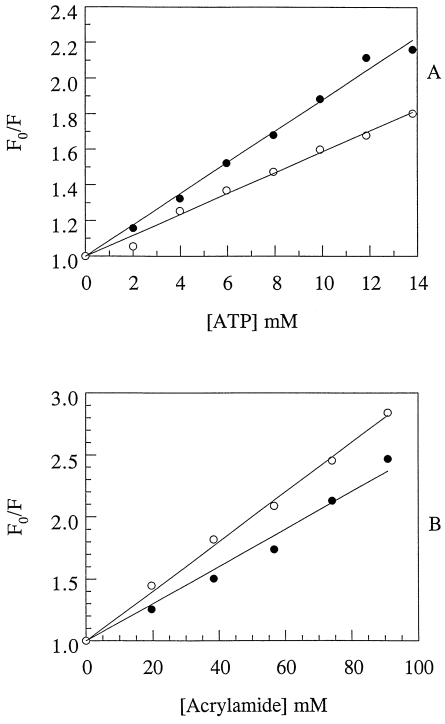

Fig. 1. The Stern–Volmer plot for quenching of NATA at different temperatures. NATA at 2.5 μM concentration was quenched by ATP (panel A) or by acrylamide (panel B) at 4°C (closed symbols) and 37°C (open symbols).

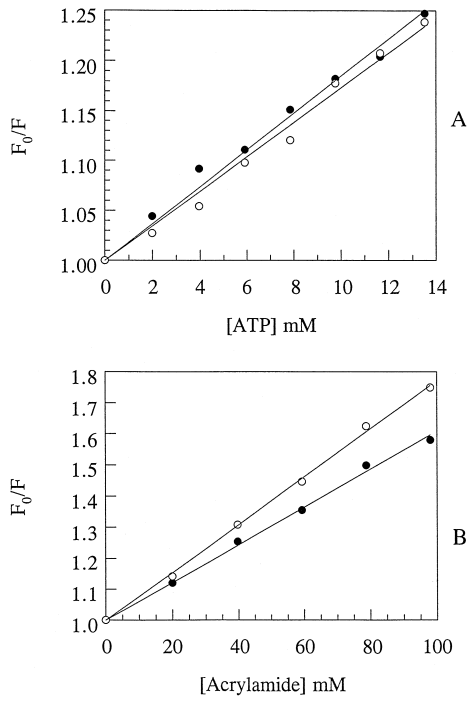

Fig. 2. The quenching of tryptophan fluorescence from hsp90 at different temperatures. Hsp90 at 0.1 mg/ml (1.18 μM) was quenched with ATP (panel A) or acrylamide (panel B) at 4°C (closed symbols) and 37°C (open symbols).

direct interaction with tryptophan. The latter case is unlikely since the data in Fig. 2A would then imply a large ATP dissociation constant, $K_d = 1/K \sim 56$ mM, that is inconsistent with subsequent observations showing an ATP effect on hsp90 that saturates at [ATP] < 5 mM. More likely, ATP directly interacts with several tryptophan residues in hsp90 by either static or collisional quenching mechanisms nullifying the effect of temperature on the quenching efficiency. The presence of any static quenching also implies ATP binding to hsp90. Fig. 2B shows data similar to that in Fig. 2A but with acrylamide quenching. Rising temperature significantly increases the quenching constants given by $K = 0.0061 \pm 0.0001$ and 0.0077 ± 0.0003 mM^{-1} for 4 and 37°C.

Fig. 3 shows the acrylamide quenching of tryptophan fluorescence in hsp90 in the absence and presence of ATP at 4°C and over a large range of acrylamide concentrations. The plots of F_0/F vs. $[Q]$ are bimodal, with the apparent slope of the curves changing at $[Q] \approx 100$ mM, and characteristic to the collisional acrylamide quenching of tryptophan fluorescence in a protein with inhomogeneously accessible tryptophan residues [18]. The curves are also concave upward at the high $[Q]$ end indicative of contributions from static acrylamide quenching of tryptophan, as observed in other tryptophan containing protein systems [18]. The curves in Fig. 3 are averages from 12 quenching curves, 3 at each ATP concentration of 0, 5, 10, and 20 mM. The 12 curves, sharing the same quenching constants but differing in their fraction of accessible chromophores due to the presence of ATP, were fitted simultaneously with Eq. (3) and the inequality constraint $f_a \geq 0$ such that each curve had the identical quenching constants K_a^c, K_a^s, K_b^c, and K_b^s, but differing f_a's. The common best values for the nonlinear parameters K_a^c, K_a^s, K_b^c, and K_b^s, showed that the static quenching term for the readily accessible class of tryptophans does not affect the goodness of fit permitting elimination of K_a^s from further consideration. The f_a's so determined were averaged and their standard error calculated at each ATP concentration. Values for all of the fitting parameters are summarized in Table 1. Errors shown in Table 1 for the quenching constants were estimated in a separate calculation where we fitted each of the 12 quenching curves individually.

The results summarized in Table 1 show that the best choices for the quenching constants K_a^c, K_b^s, and K_b^s indicate, as expected, that the acrylamide quenches predominantly by the collisional mechanism and with a small but significant static quenching component in the less accessible class of trypto-

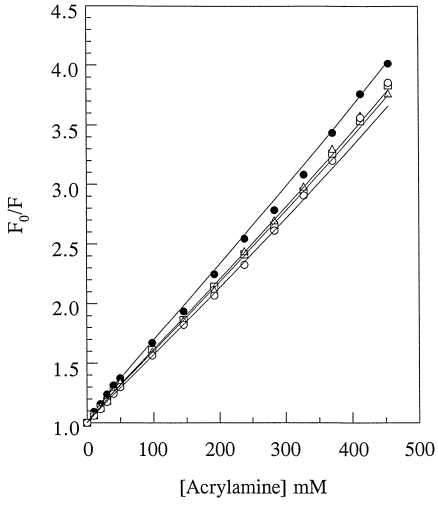

Fig. 3. The acrylamide quenching of tryptophan fluorescence from hsp90 at 4°C in the absence (●) and presence of 5 mM (□), 10 mM (△), or 20 mM (○) ATP. The curves are averages of several experimental curves and are nonlinear indicating inhomogeneous quenching of the four tryptophan residues in hsp90. The solid lines are constructed from Eq. (3) and the values of f_a, K_a^c, K_b^c, and K_b^s, indicated in Table 1.

Table 1
Quenching constants and fractional intensities for tryptophan fluorescence from hsp90 in the presence of ATP[a]

Quenching constants $(mM)^{-1}$		[ATP] (mM)	f^a
K_a^c	0.03 ± 0.01 (12)	0	0.21 ± 0.003 (3)
K_a^s	–	5	0.12 ± 0.010 (3)
K_b^c	0.005 ± 0.001 (12)	10	0.13 ± 0.040 (3)
K_b^s	0.0002 ± 0.0001 (12)	20	0.09 ± 0.020 (3)

[a] Quenching constants and the dimensionless fractional intensities are defined in Eq. (3). Parameter K_a^s was not included in the analysis because no static quenching of the readily accessible class of tryptophans was detected. The average of f_a in the presence of ATP is 0.11. Errors are standard error of the mean for (n) observations.

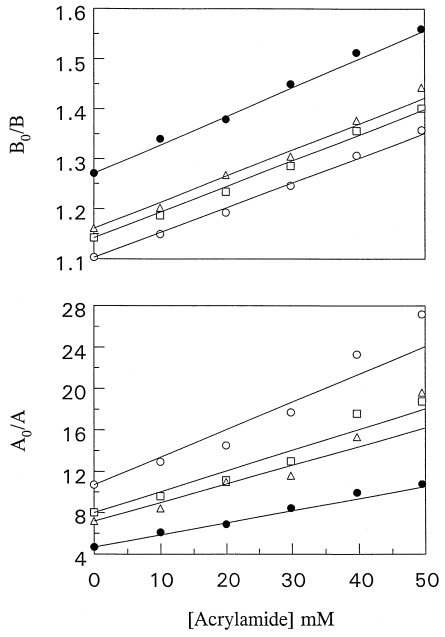

Fig. 4. The acrylamide quenching of the readily accessible (bottom) and less accessible (top) classes of tryptophan fluorescence from hsp90 at 4°C in the absence (●) and presence of 5 mM (□), 10 mM (△), or 20 mM (○) ATP. The solid lines in the bottom (top) panel are from the middle term, and the data points from the right hand side term, in Eqs. (4) and (5). The *y*-intercept indicates $1/f_a$ (bottom) or $(1-f_a)^{-1}$ (top) for the various concentrations of ATP.

phans. The findings for the readily accessible fraction, f_a, show that it decreases by approximately a factor of 2 upon addition of 5 mM ATP and that this effect saturates at [ATP] < 5 mM.

Fig. 4 shows the data from Fig. 3 rearranged to indicate the individual components of the quenching curves. Quenching curves for the less accessible (top) and readily accessible (bottom) component are shown at each ATP concentration. The linear fits indicate the contrast observed in the values for f_a vs. [ATP] by differences in their *y*-intercept.

4. Discussion

The ATP quenching of tryptophan fluorescence suggested earlier that ATP interacts with the tryptophan residues of hsp90 [10]. However, a subsequent report by Jakob et al. [12] argued that this ATP effect was caused mainly by an artifactual inner filtering effect. Our results show that ATP quenches hsp90 tryptophan fluorescence by a complex mechanism. ATP quenching of tryptophan occurs by ATP binding very near tryptophan in the static quenching mechanism, by direct collision of ATP and tryptophan in the dynamic quenching mechanism, or by ATP binding to hsp90 and affecting conformation to quench tryptophan but without direct interaction with the residue (the indirect mechanism). Static and indirect ATP quenching of tryptophan fluorescence from hsp90 are consistent with the recent findings that the protein contains an ATP binding site [14,16,19]. ATP collisional quenching of tryptophan fluorescence from hsp90 has no implication of a binding site for ATP. Consequently, the mechanism is critical for determining the physiological significance of the quenching results.

ATP is predominantly a static quencher of the NATA model system. Fig. 1A shows that increased temperature lowers the quenching constant of ATP indicative of static quenching. We investigated the effect of temperature on ATP quenching of hsp90. Fig. 2A shows that the ATP–hsp90 system gives no significant change in quenching efficiency over the temperature range of 4°C–37°C. These data are consistent with all of the above mentioned ATP quenching mechanisms of tryptophan fluorescence in hsp90, however, they also imply a $K_d \sim 56$ mM if the indirect quenching mechanism predominates. Additional experiments investigating ATP inhibition of acrylamide tryptophan quenching provided the information needed to decide the mechanism of ATP interaction with hsp90.

Fig. 2B shows that acrylamide is a collisional quencher of tryptophan in hsp90 while Fig. 3 shows that acrylamide quenching of hsp90 is sensitive to the local environment surrounding the tryptophan residues in the protein. F_0/F vs. $[Q]$ in Fig. 3 in the absence of ATP shows heterogeneity in the accessibility of tryptophan residues to acrylamide quenching suggesting that at least two tryptophan environments exist, one that is readily accessible to collisional quenchers in the solvent and another less accessible. We found that the readily accessible residue or residues accounts for ~ 0.21 of the total fractional emission from the native protein at 4°C.

We probed the nature of the ATP–tryptophan interaction by examining how ATP inhibits acrylamide quenching of the two classes of tryptophan residues in hsp90.

Table 1 shows that the fractional emitted light intensity from the readily accessible tryptophans decreases from 0.21 to 0.11 by introduction of ATP. This decrease saturates at [ATP] < 5 mM ruling out indirect quenching as a significant mechanism of ATP action in these experiments. Saturation at [ATP] < 5 mM also rules out the collisional quenching mechanism since it would imply a quenching constant $> 0.2\ \mathrm{mM}^{-1}$, a value of an order of magnitude higher than that observed for any collisional quenching of hsp90 or NATA. Evidentially, ATP statically quenches the readily accessible tryptophans in hsp90. The model proposed in Appendix A for tryptophan quenching in hsp90 indicates that the saturation of the ATP quenching implies an ATP $K_d < 4$ mM and that the readily accessible tryptophans have an ATP static quenching constant of $> 0.25\ \mathrm{mM}^{-1}$. This K_d is significantly smaller than the $K_d = 11$ mM observed for NATA in solution under similar conditions (see Fig. 1). Since the NATA–ATP binding constant is an appropriate model for nonspecific ATP binding on a protein we conclude that hsp90 specifically binds ATP. Furthermore, the observation that about half of the fluorescence from the readily accessible tryptophans cannot be statically quenched by ATP (since $f_a \rightarrow 0.1$ when [ATP] $\rightarrow \infty$) indicates heterogeneity (of a different sort than that discerned by acrylamide) *within* this class of residues.

$K_d < 4$ mM agrees with previous estimates of this constant [14,19]. Scheibel et al. [19] observed binding of a spin-labelled ATP analog to human hsp90 with a $K_d \sim 0.4$ mM and a value of 0.13 mM was obtained by calorimetry for the binding of ATP to yeast hsp90 [14]. Several conventional methods for measuring ATP binding have not been successful because of the low affinity of this binding [19]. This lack of success was the main motivation for the present study. The reason for this low affinity is not known. Since hsp90 is thought to function with the cooperation of several chaperones or co-chaperones [1,2], it is quite possible that interaction with these factors or with unfolded protein substrates generates a more effective binding site on hsp90 with higher affinity for ATP.

Human hsp90β contains four tryptophans at positions 156, 288, 311, and 597. Only one of these, Trp156, is in the amino terminal domain known to bind ATP [14–16]. Thus, it is likely that the static quenching by ATP observed in the present study relates primarily to Trp156. Trp156 does not contact ATP in the crystal structure, but lies in a β structure ~ 9 Å away [14]. Static quenching by ATP requires the close proximity, probably within ~ 4 Å, of the ATP-sensitive tryptophan. The discrepancy in the ATP–Trp distance between our data and the crystallography is potentially due to the different systems studies. Our native system is in solution, intact, and much larger (724 vs. 214 residues) than the crystallized fragment. An interesting speculation is that the ATP–Trp distance discrepancy is the ATP-induced conformation change in the native hsp90 [6,10,16]. If so, then the heterogeneity in the ATP quenching of the readily accessible class of chromophores suggests that at 4°C half of the ATP-hsp90's maintain the 9 Å ATP–Trp distance conformation and the rest maintain the 4 Å conformation. Additional studies are needed to further characterize the binding of nucleotides to hsp90 and to establish the relationship of this binding to hsp90 structure and function.

In summary, we have identified an ATP sensitive class of tryptophans in hsp90 that are readily accessible to collisional quenching and that are statically quenched by specific nucleotide binding to the protein. Static quenching is produced by the close proximity of quencher and chromophore so that an ATP binding site in hsp90 must lie in close proximity, probably within ~ 4 Å, of the ATP sensitive tryptophan residues. The saturation of the ATP binding in hsp90 indicates that hsp90 specifically binds ATP with $K_d < 4$ mM.

Acknowledgements

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Appendix A. Model for tryptophan quenching in hsp90

The four tryptophan residues in human hsp90 [10] are inhomogeneously accessible to collisional quenching by acrylamide such that each residue is assigned to one of four classes. Class A tryptophans are readily available for collisional quenching by acrylamide and are statically quenched by specific interaction with ATP. When class A tryptophans interact with ATP they are quenched and become class C tryptophans. Class B tryptophans are relatively less accessible to quenching by either mechanism and class D tryptophans are readily accessible to acrylamide quenching but are not affected by ATP. The reaction and conservation equations for this model are,

$$A + ATP \rightleftharpoons C \tag{A1}$$

$$\begin{array}{ll} [A] + [C] = \alpha[H] & [B] = \beta[H] \\ [D] = \delta[H] & \alpha + \beta + \delta = 4 \end{array} \tag{A2}$$

where [H] is the total protein concentration and α, β, and δ are the integer number of tryptophan residues in class A + C, B, and D tryptophans. For $x \equiv [A]/[H]$ the K_d of Eq. (A1) is,

$$K_d^{-1}[ATP] = (\alpha - x)/x \tag{A3}$$

We estimate x from the fluorescence quenching data using,

$$f_a = \frac{q_A[A] + q_C[C] + q_D[D]}{q_A[A] + q_B[B] + q_C[C] + q_D[D]} \tag{A4}$$

where q_i is the quantum efficiency for the i-th tryptophan class. We may assume $q_C = 0$ because class C tryptophans are quenched statically by ATP. Substituting from Eq. (A2) gives,

$$f_a = \frac{x + \delta(q_D/q_A)}{x + \beta(q_B/q_A) + \delta(q_D/q_A)} \tag{A5}$$

We solve for the two independent constants in Eq. (A5), $\delta(q_D/q_A)$ and $\beta(q_B/q_A)$, using limiting cases for f_a such that, $f_a \rightarrow f_a' = 0.09$ when $[ATP] \rightarrow \infty$ or $x \rightarrow 0$, and, $f_a \rightarrow f_a'' = 0.21$ when $[ATP] = 0$ or $x = 1$. Solving for x and substituting this value into Eq. (A3) we find,

$$K_d^{-1}[ATP] = \frac{(1-\alpha)(f_a' + f_a f_a') + f_a''(\alpha - f_a') - f_a(1 - \alpha f_a')}{(1 - f_a'')(f_a - f_a')} \tag{A6}$$

for any [ATP]. When one tryptophan residue occupies the readily accessible class of chromophores ($\alpha = 1$) we estimate an upper limit for K_d by substituting $[ATP]_{1/2} < 5$ mM, where $[ATP]_{1/2}$ is the ATP concentration when $f_a = 1/2(f_a' + f_a'')$, giving $K_d < 4$ mM. For larger α, the upper limit on K_d decreases implying the possibility of higher affinity ATP–hsp90 binding.

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