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Effect of Geldanamycin on the Kinetics of Chaperone-Mediated Renaturation of Firefly Luciferase in Rabbit Reticulocyte Lysate†

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ABSTRACT: Renaturation of thermally denatured firefly luciferase in rabbit reticulocyte lysate (RRL) requires hsp90, hsc70, and other as yet unidentified RRL components [Schumacher, R. J., *et al.* (1994) *J. Biol. Chem. 269*, 9493−9499]. Benzoquinonoid ansamycins (BAs) have recently been shown to specifically bind hsp90 and inhibit its function. In this report, we present data that indicate BAs are specific inhibitors of hsp90 function. The effects of the BA geldanamycin (GA) on the kinetics of the luciferase renaturation in RRL were examined to gain insight into the mechanism by which GA inhibits the function of the hsp90 chaperone machinery. Chaperone-mediated renaturation of luciferase obeyed Michaelis−Menten kinetics. The GA inhibited luciferase renaturation uncompetitively with respect to ATP concentration and noncompetitively with respect to luciferase concentration, indicating that GA binds after the binding of ATP and that it binds to both the hsp90 chaperone machine/ATP complex and the hsp90 chaperone machine/ATP/luciferase complex. GA markedly decreased the K_{app} of the hsp90 chaperone machine for ATP, suggesting that GA increases the binding affinity of the hsp90 chaperone machinery for ATP or it slows the rate of ATP hydrolysis. Consistent with the notion that GA specifically binds hsp90 and inhibits its function, addition of hsp90, but not hsc70, p60, or p23, reversed GA-induced inhibition of luciferase renaturation in RRL. Hsp90, hsc70, and the hsp cohorts p60, p48, and p23 were coimmunoprecipitated with luciferase from RRL. GA increased the steady-state levels of luciferase associated with hsp90/hsp70 chaperone machine complexes that contain p60 and blocked the association of the hsp90 cohort p23 with chaperone-bound luciferase. The data suggest that the function of the hsp90 chaperone machinery is not specific to its previously described interaction with steroid hormone receptors, and that it carries out some more generalized function *in vivo*.

Protein folding and renaturation *in vivo* are facilitated by a group of proteins referred to as heat shock proteins (hsps)[1] or molecular chaperones [reviewed in Becker & Craig (1994), Craig et al. (1994), Georgopoulos (1992), Gething and Sambrook (1992), and Hartl (1995)]. Molecular chaperones appear to function in complexes referred to as chaperone machines (Georgopoulos, 1992). Chaperones primarily interact with hydrophobic regions of newly synthesized or denatured protein substrates to prevent illicit interactions between the bound protein and other cellular components. The affinity of chaperones for polypeptide substrates is modulated by associated proteins (cohorts) and conformational changes induced by the binding and hydrolysis of ATP (Becker & Craig, 1994; Georgopoulos, 1992; Hartl, 1995). Chaperone-associated cohorts modulate chaperone function by a number of mechanisms: they may interact with chaperone-bound substrates directly; they may modulate the interactions of hsps with their protein substrates; or they may modulate the rate of ATP hydrolysis or nucleotide exchange. Chaperones appear to facilitate protein folding primarily by reducing the number of molecules that follow nonproductive folding pathways that would lead to aggregation of the protein.

Hsp90 is an extremely abundant constitutively expressed heat shock protein [reviewed in Jacob and Buchner (1994)]. Genetic (Bohen & Yamamoto, 1993; Picard et al., 1990; Xu & Lindquist, 1993) and biochemical (Jacob et al., 1995; Pratt & Welsh, 1994; Schumacher et al., 1994; Wiech et al., 1992, 1993) studies indicate that members of the hsp90 gene family are involved in protein folding and renaturation under normal and adverse physiological conditions. Little is known, however, about the mechanism of action of hsp90 in the folding and renaturation of proteins, but it appears to act at later stages in protein folding pathways where extensive secondary structure has already formed (Melnick et al., 1992, 1994; Shue & Kohtz, 1994; Smith, 1993). Hsp90 functions as a chaperone machine with a number of noncovalently associated cohorts [hsc70, p60; p48; p23; and an assortment of immunophilins (Chang & Lindquist, 1994; Hutchinson et al., 1994a,b, 1995; Jacob & Buchner, 1994; Johnson & Toft, 1995; Schumacher et al., 1994; Smith, 1993; Smith et al., 1990, 1992, 1995)]. Much of what is known about the hsp90 chaperone machine comes from studies examining the factors required to reconstitute the hormone binding activity of salt-stripped glucocorticoid and progesterone steroid

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[1] Abbreviations: hsp, heat shock protein; hsc, heat shock cognate; BA, benzoquinonoid ansamycin; GA, geldanamycin; HbA, herbimycin A; RRL, rabbit reticulocyte lysate; SHR, steroid hormone receptor; GRP, glucose regulated protein; SDS, sodium dodecyl sulfate; SDS−PAGE, SDS−polyacrylamide gel electrophoresis; K_{app}, apparent K_m; V_{app}, apparent V_{max}; CP, creatine phosphate; CPK, creatine phosphokinase; GaR−agarose, goat anti-rabbit IgG cross-linked to agarose.

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hormone receptors (SHRs) in rabbit reticulocyte lysate (RRL) (Hutchinson et al., 1994a,b, 1995; Johnson & Toft, 1995; Smith, 1993; Smith et al., 1990, 1992, 1995). ATP-dependent binding of hsc70 to SHR is required for the subsequent interaction of hsp90 and its associated cohorts with SHR (Hutchinson et al., 1994a; Smith et al., 1992). Hsp90, hsc70, p60, and p48 are present in chaperone complexes at intermediate times during SHR reconstitution, while hsp90, p23, and individual immunophilins are present in later complexes formed just prior to or at the point of SHR reconstitution (Johnson & Toft, 1994, 1995; Smith, 1993; Smith et al., 1995). These studies indicate that these complexes represent dynamic, reiterative, ATP-dependent cycles of association of the SHRs with the hsp90 chaperone machine that maintain these SHRs in a conformation competent to bind hormone.

Benzoquinonoid ansamycins are antibiotics of fungal origin that revert cell transformation mediated by $p60^{v-src}$ and several other tyrosine kinases (Uehara et al., 1986, 1988). Benzoquinonoid ansamycins such as geldanamycin (GA) and herbimycin A (HbA) were traditionally thought to be direct inhibitors of tyrosine kinases (Uehara et al., 1989). However, recent work suggests that benzoquinonoid ansamycins inhibit the tyrosine kinase activity of $p60^{v-src}$ through an indirect mechanism (Whitesell et al., 1994). In extracts of cells transformed by $p60^{v-src}$, hsp90 was the predominant protein bound by immobilized GA (Whitesell et al., 1994). Binding of hsp90 to immobilized GA was specifically blocked by soluble HbA and soluble GA. Immobilized GA did not bind $p60^{v-src}$. GA treatment disrupted the interaction of hsp90 with $p60^{v-src}$ *in vivo*, concomitant with reversion of $p60^{v-src}$ transformation. Since genetic studies have previously demonstrated that hsp90 function is required for maturation or support of $p60^{v-src}$ function (Xu & Lindquist, 1993), these results strongly argue that the mechanism of GA and HbA action involves the disruption of hsp90 function. Consistent with this putative mechanism, GA induces loss of function for the *erb-B2* gene product (Chavany et al., 1996), the progesterone receptor (Johnson & Toft, 1995; Smith et al., 1995), the glucocorticoid receptor (Whitesell et al., 1995), and the *raf* kinase (Schulte et al., 1995) and disrupts the normal interaction of these proteins with hsp90 or with the hsp90 homolog GRP94. Two studies address the mechanism of GA inhibition of hsp90 chaperone machinery (Johnson & Toft, 1995; Smith et al., 1995). These studies indicate that GA decreases the recovery of progesterone receptors in mature complexes with hsp90 that contain the p23 and immunophilin cohorts. Instead, receptor accumulates in intermediate complexes containing hsp90, hsp70, and two hsp cohorts, p60 and p48.

In this report, we have utilized RRL as a high-fidelity model of the eukaryotic cytosol to identify the active principles responsible for facilitating protein renaturation. Both pharmacological and immunological approaches were used to identify the chaperones involved in the renaturation of thermally denatured firefly luciferase. We report here that: (i) GA inhibits the rate of luciferase renaturation in RRL, indicating that hsp90 plays a role in luciferase renaturation; (ii) the chaperone machinery associated with denatured luciferase is identical to that previously described for SHRs; and (iii) the association of components of hsp90 chaperone machinery with luciferase is modulated by GA in a fashion identical to that previously described for GA inhibition of reconstitution of progesterone receptor heterocomplexes. Additionally, we have investigated whether steady-state kinetics can be used to analyze the mechanism by which luciferase renaturation occurs in RRL. Steady-state kinetics indicate GA maintains the hsp90 chaperone machinery in a high-affinity ATP binding conformation and/or inhibits the subsequent ATP hydrolysis that is required for the formation of mature hsp90 chaperone machinery complexes that contain the cohort p23.

EXPERIMENTAL PROCEDURES

Materials. Bovine creatine phosphokinase (type I), serum albumin (acetylated), herbimycin A, D-luciferase, and luciferin were purchased from Sigma. Geldanamycin was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Geldampicin was provided by Dr. Kenneth L. Rinehart (University of Illinois, Urbana). The F5 anti-p60 and the JJ3 anti-p23 monoclonal antibodies were provided by Dr. David Smith (University of Nebraska, Omaha) and Dr. David Toft (Mayo Medical School, Rochester, MN), respectively. The 4322 anti-hsp70/90 and anti-hsp90 (84/86) antiserum was provided by Dr. Stephen Ullrich (NCI), and the anti-hsp40 antiserum was provided by Dr. William Welch (UCSF). Anti-luciferase affinity-purified polyclonal antibodies were purchased from Promega.

Buffers and Reagents. The buffers used were as follows: assay buffer (AB) consisting of 25 mM Tricine-HCl (pH 7.8), 8 mM $MgSO_4$, 0.1 mM EDTA, 33 mM dithiothreitol, 470 μM D-luciferin, 240 μM coenzyme A, and 0.5 mM ATP; stability buffer (SB) consisting of 25 mM Tricine-HCl (pH 7.8), 8 mM $MgSO_4$, 0.1 mM EDTA, 10 mg/mL bovine serum albumin, 10% glycerol, and 1% Triton X-100; dialysis buffer (DB) consisting of 10 mM Tris-HCl (pH 7.4), 3 mM $Mg(OAc)_2$, 100 mM KCl, and 2 mM dithiothreitol; Tris buffer (TB) consisting of 10 mM Tris-HCl (pH 7.4); Tris-buffered saline (TBS) consisting of TB and 150 mM NaCl; TB/500 consisting TB and 500 mM NaCl; and TB/50 consisting of TB and 50 mM KCl.

Luciferase Renaturation Assay. Reticulocyte lysate was prepared as described (Matts et al., 1991). Dialyzed RRL was prepared by dialysis of 2.5 mL of RRL against 500 mL of DB buffer for 6 h at 4 °C with buffer changes every 1.5 h. The endogenous ATP concentration in undialyzed lysate and the ATP and ADP concentrations remaining in the dialyzed lysate were analyzed as described (BioOrbit, 1991).

Firefly luciferase (2 mg/mL) in SB was denatured by incubation at 41 °C for 10 min (Schumacher et al., 1994) unless otherwise specified. After being cooled on ice, aliquots were diluted 20-fold into heme-deficient protein synthesis mixes (Matts et al., 1991) containing 10 μM edeine or dialyzed reticulocyte lysate containing 10 mM creatine phosphate, 20 units/mL creatine phosphokinase, and varying concentrations of ATP. Samples were incubated at 28 °C, and at various time points (usually 10 min), the luciferase activity present in 2 μL aliquots (unless otherwise stated) was determined by dilution into 50 μL of AB. Light production was measured for 10 s in a Lumac (3M) Bioluminometer. The rate of luciferase renaturation was determined to be linear over the time period for all assays, and the rate of luciferase renaturation is expressed in the figures and table as light-producing units renatured per 10 min interval (light units/10 min). For the kinetic analysis,

less than 1% of the denatured luciferase was renatured over the time course of the assay, so the concentration of denatured luciferase effectively remained constant. Curve fitting was done with the nonlinear least-squares program STEPT (Chandler, 1988) using weighting factors equal to the reciprocal of the variances of each data point. As described previously (Schumacher et al., 1994), (i) luciferase renaturation was ATP-dependent; (ii) no significant spontaneous (chaperone-independent) renaturation of the luciferase was observed when the denatured luciferase stock was incubated at 28 °C in the absence of RRL; and (iii) at very limiting luciferase concentrations (0.03−0.3 nM) 60−100% of the denatured luciferase could be renatured upon prolonged incubation (75−90 min) in RRL.

Several factors were found to affect the rate of luciferase renaturation in the assay. As previously reported, different lysate preparations renatured luciferase at different rates because the preparations contain different quantities of hsps (Matts & Hurst, 1992; Schumacher et al., 1994). Because of the expense of the enzyme, stock solutions of native luciferase were stored at 4 °C and used over the period of a week. Aliquots of the stock solution were clarified by centrifugation prior to use. The rate of luciferase renaturation was observed to decrease with time of storage of the stock solution. This correlated with the accumulation of some insoluble material in the stock solution over the storage period. Ongoing protein synthesis in RRL was also observed to inhibit luciferase renaturation.[2] Undialyzed lysate was thawed rapidly at 30 °C, followed by the addition of ediene to ensure that translation was fully inhibited. All data points reported in the figures represent measurements determined in a single experiment using a single lysate preparation and luciferase stock. All experiments were carried out at least 3 times.

Purification of Hsps and Hsp Cohorts. Hsc70 was purified to apparent homogeneity from postribosomal supernatant of rabbit reticulocyte lysate as described for the purification of supernatant factor (Gross, 1976; Gross et al., 1994). The initial steps in the purification of the hsp90 were similar to the steps for the purification of supernatant factor: precipitation at pH 5.4; reprecipitation at 40−80% saturated ammonium sulfate; and chromatography on DEAE-cellulose. These steps were followed by chromatography on Superose 12/HR and monoQ columns as previously described (Denis, 1988). p60 copurified with hsc70 up to the ATP−agarose affinity chromatography step, where p60 was present in the fraction of proteins that did not bind to the column in buffer containing 0.1 M KCl (Gross et al., 1994). The p60 was approximately 67% pure at this stage, with hsc70 being the primary contaminating protein (p60:70 ∼5:1). Chicken p23 (Johnson & Toft, 1994) was generously provided by Dr. David Toft (Mayo Medical School). Western blotting with an anti-hsp40 antiserum indicated that none of these preparations were contaminated with hsp40.

Immunoadsorptions of Proteins. Goat anti-rabbit IgG was cross-linked to agarose (GaR−agarose) as previously described (Matts et al., 1992). Affinity-pure anti-luciferase antibody (10 μL) was adsorbed to 20 μL of GaR−agarose (diluted 1:1 in TBS) in the presence or absence (control) of luciferase (40 μg) for 2 h on ice. In the absence of stability buffer, purified luciferase spontaneously denatures. The GaR−agarose was then washed with 500 μL each of TBS, TB/500, TBS, and TB/50. RRLs (35 μL), diluted 1:1 to contain 10 mM creatine phosphate, 20 units/mL creatine phosphokinase, 75 mM KCl, and either DMSO or GM (1 μg/mL), were then added to GaR−agarose. The mixtures were incubated for 5 min at 30 °C with periodic mixing to prevent settling of the agarose. The unadsorbed proteins were separated from adsorbed proteins (pellets) by centrifugation. Pellets were washed 4 times with TB/50.

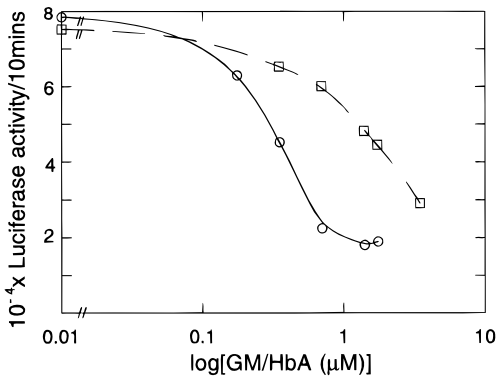

FIGURE 1: Concentration dependence for benzoquinonoid ansamycin-induced inhibition of luciferase renaturation in RRL. Heme-deficient protein synthesis mixes (100 μL) containing 10 μM edeine were preincubated for 20 min at 30 °C in the presence of 0.1 μL of DMSO (vehicle control), or 0.1 μL of DMSO containing varying concentrations of GA (○) or HbA (□). Five microliters of denatured luciferase (10 μg/mL) was then diluted into 100 μL of lysate. After 10 min, the amount of luciferase activity present in a 20 μL aliquot of each reaction was measured as described under Experimental Procedures. Light-forming units renatured per 10 min of incubation is plotted versus the final concentration of GA or HbA.

Western Blot Analysis. Samples were prepared for SDS−PAGE, separated in 10% gels, and transferred to PVDF membrane as previously described (Matts & Hurst, 1992; Matts et al., 1992). Hsp70 and hsp 90 were detected by Western blotting with the 4322 anti-hsp70/hsp90 antiserum (Ehrhart et al., 1988) or an anti-hsp90 (anti-mouse hsp84/86 antipeptide) antiserum provided by Dr. Stephen Ullrich (NCI) as previously described (Matts & Hurst, 1992; Matts et al., 1992). p60, p48, and p23 were detected by Western blotting with the F5 anti-p60 (Smith et al., 1993), the 3G6 anti-p48 (Smith et al., 1995), and the JJ3 anti-p23 (Johnson et al., 1994) mAb, respectively.

RESULTS

GA and HbA Inhibit Luciferase Refolding. Recent studies suggest that benzoquinonoid ansamycins bind hsp90 and inhibit its function (Whitesell et al., 1994). Since we have previously demonstrated that purified fractions enriched in hsp90 stimulated renaturation of denatured luciferase in RRL (Schumacher et al., 1994), we examined the effect of GA and HbA on the renaturation of luciferase. While neither GA nor HbA had any effect on the activity of native luciferase (not shown), they inhibited luciferase renaturation in a concentration-dependent manner (Figure 1). The concentrations of GA and HbA that inhibited luciferase renaturation by 50% of the maximally induced inhibition (IC_{50}) were estimated to be 0.2 and 1.3 μM, respectively. Maximum inhibition of luciferase renaturation was observed at a GA concentration of 0.7 μM. Addition of the DMSO vehicle at concentrations up to 0.2% (v/v) had no effect on the rate of luciferase renaturation. The pharmacologically inactive BA geldampicin (Whitesell et al., 1994) did not

[2] S. D. Hartson, V. Thulasiraman, and R. L. Matts, manuscript in preparation.

Table 1: Degree of GA-Induced Inhibition of Luciferase Renaturation Depends on the Temperature of Denaturation[a]

temp of denaturation (°C)	light units renatured/10 min upon addition of		ratio (DMSO/GA)
	DMSO	GA	
38	40176	20505	1.96
41	39357	9883	3.98

[a] Luciferase (0.7 μM), denatured at 38 or 41 °C for 10 min, was diluted 20-fold into heme-deficient protein synthesis mixes supplemented with edeine and containing either GA (1.8 μM) or DMSO (1 μL/mL). The amount of luciferase renatured after incubation for 10 min at 28 °C was measured as described under Experimental Procedures. The rate of luciferase renaturation is reported as light units renatured/10 min. The experiment was repeated 2 times with similar results.

inhibit luciferase renaturation when added at concentrations up to 15 μM (not shown).

The extent of GA-induced inhibition of the rate of luciferase renaturation was observed to be dependent on the temperature at which the luciferase was denatured (Table 1). GA decreased the initial velocity of luciferase renaturation by 50% when the luciferase was denatured at 38 °C and by 80% when the luciferase was denatured at 41 °C. These observations suggest that the more severely luciferase was denatured, the more dependent luciferase became on a GA-inhibitable event for rapid renaturation.

Kinetic Analysis of Chaperone-Mediated Luciferase Renaturation. We next examined the kinetics of luciferase renaturation in reticulocyte to determine whether the mechanism by which BAs inhibit the renaturation of luciferase could be analyzed using steady-state kinetics. At saturating ATP concentrations, the rate of luciferase renaturation was saturable with respect to the concentration of denatured luciferase present, and followed a rectangular hyperbolic curve that is characteristic of enzymes following Michaelis−Menten kinetics (not shown). The K_{app} and V_{app} for the reaction were calculated to be 1.0 ± 0.17 μM and (1.0 ± 0.097) × 10^5 light units/10 min, respectively. The K_{app} was calculated assuming that the luciferase preparation was homogeneous, and that all the denatured luciferase (MW = 65 000) present represented soluble monomeric substrate with a single chaperone binding site. Therefore, this estimated K_{app} represented a maximum value, since some insoluble material was noted to be present in the luciferase stock.

The renaturation of luciferase in RRL requires the presence of an optimum concentration of K^+, Mg^{2+}, and ATP (Schumacher et al., 1994). To allow the kinetic analysis of the effects of varying ATP concentrations on the rate of luciferase renaturation, RRL was dialyzed to remove endogenous ATP. The ATP remaining in the lysate was quantified and was found to be 5 and 20 μM in the absence and presence of a CP/CPK ATP regenerating system, respectively. The rate of luciferase renaturation was determined at a near-saturating concentration of denatured luciferase and varying ATP concentrations. The K_{app} and V_{app} for ATP in the reaction were estimated to be 61 ± 8.0 μM and (4.2 ± 0.25) × 10^4 light units/10 min, respectively. Renaturation rate was next determined while varying both ATP and luciferase concentrations. An Eadie−Hofstee plot of the data generated a series of nearly parallel lines that is characteristic of sequential reaction mechanisms (Figure 2).

Kinetic Analysis of GA-Induced Inhibition of Luciferase Renaturation. We next examined the kinetics of luciferase renaturation in the presence of GA to gain insight into the mechanism by which GA inhibited chaperone-mediated luciferase renaturation. Luciferase renaturation rates were determined at saturating ATP and varying luciferase and GA concentrations. Eadie−Hofstee plots of the data indicated that GA inhibited luciferase renaturation noncompetitively, affecting both the V_{app} and the V_{app}/K_{app} (Figure 3A). Replots of V_{app}/K_{app} *versus* [GA] (Figure 3B) and $1/V_{app}$ *versus* [GA] were hyperbolic (Figure 3C), indicating that luciferase renaturation could not be completely inhibited even at saturating concentrations of GA. Concentrations of GA up to 3.5 μM inhibited luciferase renaturation no more than that observed with 0.7 μM GA.

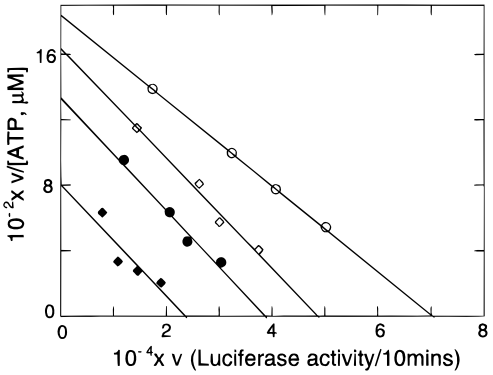

FIGURE 2: Eadie−Hofstee plot of kinetics of luciferase renaturation. The rate of luciferase renaturation was measured in dialyzed RRL in the presence of varying ATP and luciferase concentrations as described under Experimental Procedures. Concentration of luciferase present in the renaturation assay was 1.03 μM (○), 0.77 μM (◇), 0.51 μM (●), and 0.26 μM (◆), respectively. ATP concentrations were varied between 0.02 and 0.1 mM. The experiment was repeated 3 times with similar results. Lines were analyzed as described under Experimental Procedures. As noted under Experimental Procedures, in repeats of the experiment the rate of luciferase renaturation varied between different lysate preparations and the age of the luciferase stock. The K_{app} for ATP varied from 40 to 80 μM, and the K_{app} for luciferase varied from 0.8 to 1.5 μM. The V_{max} for the reaction varied from 40 000 to 200 000 light units/10 min.

We next examined the effect of GA on the kinetics of luciferase renaturation at near-saturating luciferase and varying ATP concentrations in a dialyzed RRL as described above (Figure 4A). Eadie−Hofstee plots of the data generated a series of straight lines that intersected at a single point, a pattern which is characteristic of uncompetitive inhibition. However, the lines intersected to the right of the *y*-axis, indicating that GA should theoretically stimulate luciferase renaturation in a competitive manner with respect to ATP, at very low ATP concentrations. The replots of K_{app}/V_{app} *versus* [GA] (Figure 4B) and $1/V_{app}$ *versus* [GA] (Figure 4C) appeared nonlinear. The best-fit of the $1/V_{app}$ *versus* [GA] replot was parabolic, but a rigorous exclusion of a linear fit would require more data. While the stimulatory effect of GA on luciferase renaturation that is predicted to occur at very low ATP concentrations is curious, it is physiologically irrelevant, since the parabolic shape of the [GA] *versus* $1/V_{app}$ replot indicates that this phenomenon does not occur at higher ATP concentrations where GA inhibits luciferase renaturation.

Effects of GA on the Interaction of Components of the Hsp90 Chaperone Machine with Denatured Luciferase. To further elucidate the mechanism by which GA inhibits luciferase renaturation, the components of the hsp90 chaperone machine that were coimmunoprecipitable with dena-

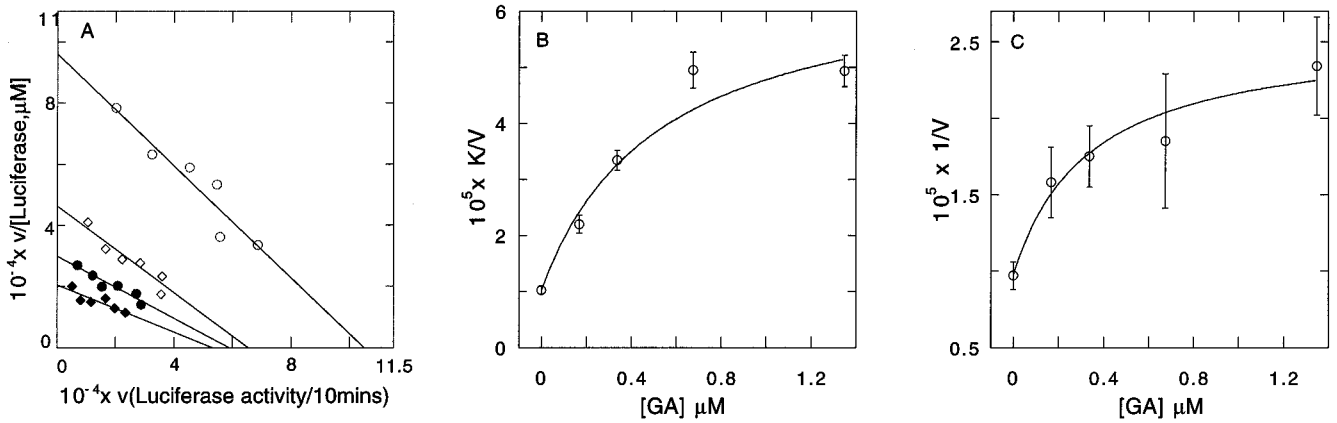

FIGURE 3: Effect of GA on the rate of luciferase renaturation in the presence of saturating ATP and varying luciferase concentrations. (A) Eadie−Hofstee plot of the kinetics of luciferase renaturation measured at saturating ATP (1.7 mM endogenous) and varying luciferase and GA concentrations. The rate of luciferase renaturation was measured in 100 μL of heme-deficient protein synthesis mixes containing 10 μM edeine and 0.1 μL of DMSO (○) or 0.17 μM (◇), 0.34 μM (●), and 0.67 μM (◆) GA as described under Experimental Procedures. (B) Replot of K_{app}/V_{app} versus [GA]. (C) Replot of $1/V_{app}$ versus [GA]. The experiment was repeated 5 times with similar results. The data were analyzed as described under Experimental Procedures. The smooth curves (B and C) represent the best-fit hyperbola. A linear fit can be excluded by a χ^2 probability >0.9995 for the K_{app}/V_{app} curve, and ~0.90 for the $1/V_{app}$ curve.

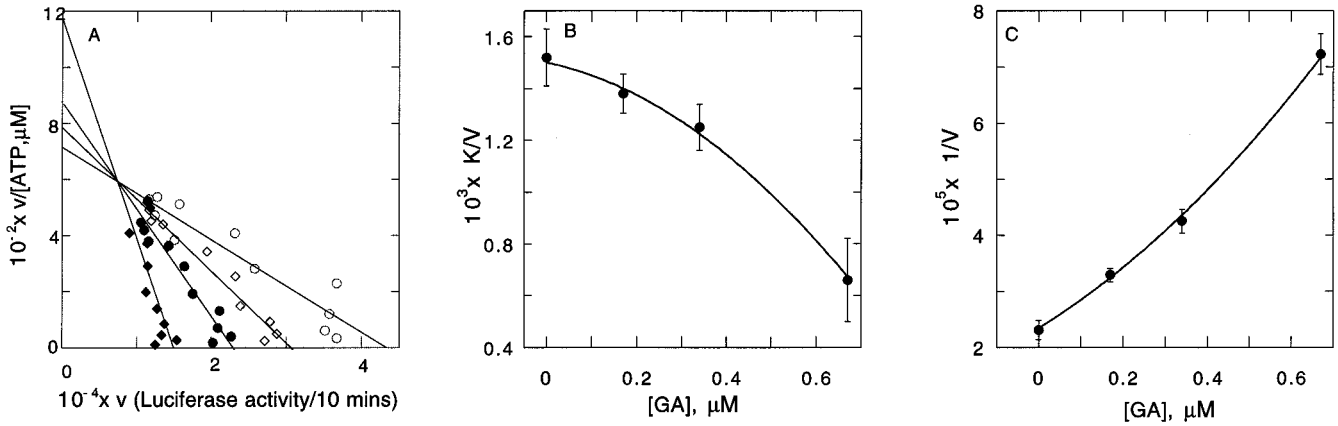

FIGURE 4: Effect of GA on the rate of luciferase renaturation in the presence of saturating luciferase and varying ATP concentrations. (A) Eadie−Hofstee plot of the kinetics of luciferase renaturation measured at saturating luciferase and varying ATP and GA concentrations. The rate of luciferase renaturation was measured in 100 μL of dialyzed lysate containing 2.1 μM luciferase and varying ATP concentrations in the presence of 0.1 μL of DMSO (○), or 0.17 μM (◇), 0.34 μM (●), and 0.67 μM (◆) GA as described under Experimental Procedures. (B) Replot of K_{app}/V_{app} versus [GA]. (C) Replot of $1/V_{app}$ versus [GA]. The experiment was repeated 3 times with similar results. The data were analyzed as described under Experimental Procedures. The smooth curves (B and C) represent the best-fit parabola.

tured luciferase in RRL were characterized. Hsp90, hsc70, and the cohorts p60, p48, and p23 were observed to interact with luciferase (Figure 5). The interaction of hsc70 with luciferase was indicated by the increase in the amount of hsc70 present in the immune pellet relative to the nonspecific binding apparent in the minus luciferase control (Figure 5, lane 1 *vs* lanes 2 and 3). GA treatment was found to increase the steady-state level of p60, while it decreased the steady-state level of p23 interacting with luciferase relative to the control (Figure 5, lane 2 *vs* lane 3).

Hsp90 Specifically Reverses Inhibition of Luciferase Renaturation Induced by Geldanamycin. To further test the hypothesis that GA binds hsp90 and inhibits its function, we examined the effect of purified hsps and hsp cohorts on luciferase renaturation in reticulocyte lysate (Figure 6). In the absence of GA, addition of purified hsp90, p60, or p23 to control RRL had no effect on luciferase renaturation (Figure 6, closed bars: con *vs* 90, 23, or 60). Luciferase renaturation was stimulated by 70% upon the addition of purified hsc70 (Figure 6: 70). Addition of both hsp90 and hsc70 stimulated luciferase renaturation by 80% (Figure 6: 90+70). A small (an additional 5%) but reproducible stimulation of luciferase renaturation was observed when p23

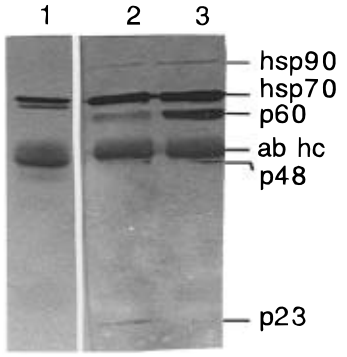

FIGURE 5: Effect of GA on the interaction of denatured luciferase with heat shock proteins. Luciferase was adsorbed to affinity-purified anti-luciferase antibody bound to GaR−agarose as described under Experimental Procedures. The immobilized luciferase was incubated for 5 min at 30 °C in heme-deficient protein synthesis mixes in the presence (lane 3) or absence (lane 2) of GA (1 μg/mL). A control incubation containing anti-luciferase antibody adsorbed to GaR−agarose, but no luciferase, is shown in lane 1. The agarose pellets were quickly washed, and analyzed by SDS−PAGE and Western blotting as described under Experimental Procedures. The experiment was repeated 4 times with similar results.

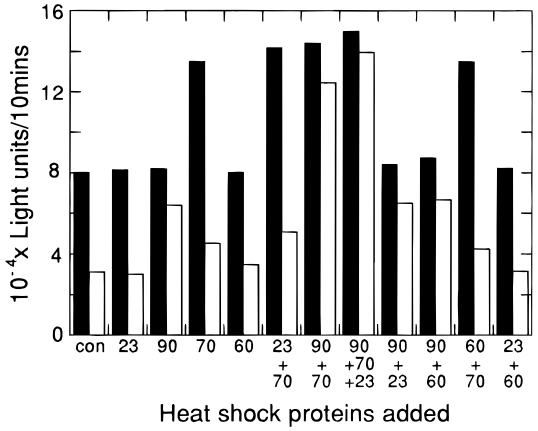

FIGURE 6: Effects of purified hsps and hsp cohorts on GA-induced inhibition of luciferase renaturation in RRL. Rabbit reticulocyte lysate containing 10 mM creatine phosphate, 20 units/mL creatine phosphokinase, 75 mM KCl, 1 mM $Mg(OAc)_2$, and 1 μL/mL DMSO (closed bars) or 0.7 μM GA (open bars) was supplemented with: buffer (con); 20 μg/mL p23 (23); 160 μg/mL hsp90 (90); 80 μg/mL hsc70 (70); 25 μg/mL p60 (60); or combinations of these quantities of proteins. Denatured luciferase was diluted 20-fold into the reaction mixes, and the amount of luciferase renatured after incubation for 10 min at 28 °C was measured as described under Experimental Procedures. The experiment was repeated 3 times with similar results.

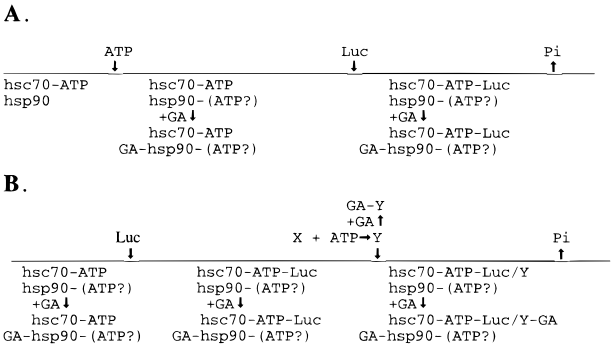

FIGURE 7: Possible schemes for GA-induced inhibition of luciferase renaturation. (A) A possible reaction sequence for the hsp90 chaperone machinery. The hsp90 chaperone machinery is assumed to be a large complex (foldasome) containing p60, p48, p23, and the immunophilins. Only hsp90 and hsc70 are shown. (B) An alternate scheme in which there is an ATP-dependent assembly of chaperones X (hsp90, p23, and immunophilins) to form complex Y which then adds to the hsp90/hsc70 complex containing p60, p48, and bound luciferase. The ? after ATP associated with hsp90 denotes the fact that the binding of ATP to hsp90 has recently been questioned (Jacob et al., 1996).

was added in conjunction with hsc70 alone, or with hsc70 and hsp90 (Figure 6: 23+70 and 90+70+23). Addition of other combinations of components had no effect on luciferase renaturation.

The effect of the purified hsps and hsp cohorts on GA-induced inhibition of luciferase renaturation was then examined. Luciferase renaturation was inhibited by 60% in RRL in the presence of 0.7 μM GA (Figure 6, closed *vs* open bars: con). GA inhibited luciferase renaturation to a similar degree (60−70%) in RRL supplemented with purified p23, p60, or hsc70 or any combination of these components (Figure 6) compared to RRL that was supplemented with the purified components and incubated in the absence of GA.

In contrast to the other components, hsp90 was observed to specifically reverse GA-induced inhibition of the rate of luciferase renaturation. In the presence of GA, addition of hsp90 to RRL stimulated luciferase renaturation by 100% relative to the control containing GA but lacking hsp90 (Figure 6, open bars: con *vs* 90). In hsp90-supplemented RRL containing GA, the rate of luciferase renaturation was restored to within 80% of the rate observed in hsp90-supplemented RRL incubated in the absence of GA (Figure 6, closed *vs* open bars: 90). In RRL supplemented with hsp90 in combination with the other purified components, GA-induced inhibition of the rate of luciferase renaturation ranged from 7 to 20%. In addition, the extent to which hsp90 reversed the inhibitory effect of GA was proportional to the amount of hsp90 added to the lysate (data not shown). The ability of hsp90 to protect against GA-induced inhibition of the rate of luciferase renaturation was not simply an effect of adding protein to the lysate, since addition of BSA showed no protection against GA-induced inhibition of luciferase renaturation.

DISCUSSION

Kinetics of Chaperone-Facilitated Protein Renaturation in RRL. While the mechanism by which protein renaturation occurs in RRL is undoubtedly complex, the data presented here indicate that the problem is at least partially tractable to analysis using steady-state kinetics. Luciferase renaturation showed substrate saturation with respect to both ATP and luciferase concentrations and followed Michaelis−Menten kinetics. Eadie−Hofstee plots of the initial velocity data (Figure 2) were a family of nearly parallel lines characteristic of sequential mechanisms. The parallel pattern observed for the chaperone-mediated luciferase renaturation of this study could be explained by either of the abbreviated alternatives presented in Figure 7. Specifically, addition of denatured luciferase could immediately follow addition of ATP to the chaperone machine (Figure 7A). In this scheme, the hsp90 chaperone machine would be a single large complex of proteins [e.g., a foldasome (Hutchinson et al., 1994b, 1995)]. The data are also consistent with models proposed by the laboratories of Smith and Toft (Johnson & Toft, 1995; Prapapanich et al., 1996; Smith et al., 1995) in which there is an ATP-dependent assembly of an hsp90/p23/immunophilin complex which subsequently binds to SHR/hsp90/hsc70/p60/p48 complexes and from which the previously bound chaperones dissociate (Figure 7B).

The association of hsp90, hsc70, and cohorts p60, p48, and p23 with denatured luciferase suggests that renaturation of luciferase occurs by a mechanism similar to that involved in restoring the hormone binding activity of SHRs (Hutchinson et al., 1994a,b, 1995; Johnson & Toft, 1995; Smith, 1993; Smith et al., 1995). This observation implies that the function of the hsp90 chaperone machine is not specific to its previously described interactions with signal transduction proteins, and that it is capable of carrying out a more generalized protein folding function *in vivo*. This folding activity appears to target proteins containing partially unfolded structure. This notion is consistent with previous reports that indicate hsp90 interacts with late folding intermediates of proteins prior to their conversion to the native state (Hartson & Matts, 1994; Jacob et al., 1995; Melnick et al., 1992, 1994; Shue & Kohtz, 1994; Smith, 1993).

An Alternate Pathway for Luciferase Renaturation Exists in RRL. Kinetic analyses of GA-induced inhibition of luciferase renaturation at varying luciferase concentrations indicate that luciferase can renature by more than one

pathway. GA inhibited luciferase renaturation noncompetitively with respect to luciferase concentration, indicating that at saturating ATP concentrations GA binds to both the hsp90 chaperone machine/ATP complex and the hsp90 chaperone machine/ATP/luciferase complex. The hyperbolic replots of GA concentration *versus* $1/V_{app}$ and K_{app}/V_{app} indicate that GA cannot completely inhibit luciferase renaturation even at saturation. This indicates that luciferase can renature by a slower alternate reaction pathway in the presence of GA. The existence of an alternate pathway is consistent with the observations that: (i) folding of newly synthesized luciferase in RRL requires hsc70, hsp40, and additional lysate chaperone components (e.g., TRiC), but does not appear to involve hsp90 (Frydman et al., 1994); and (ii) refolding of luciferase that has been denatured by treatment with guanidine hydrochloride can be chaperoned by hsc70, hsp40, and Hip (p48) *in vitro* (Freeman et al., 1995; Hohfeld et al., 1995).

The observations that the extent to which GA inhibits the rate of luciferase renaturation depends on the age of the luciferase stock and the temperature at which it was denatured also suggest that denatured luciferase is a mixed population of molecules whose rate of renaturation varies in their sensitivity to inhibition by GA. Evidence for the existence of multiple unfolding intermediates of thermally denatured citrate synthase that renatured at different rates in the presence of hsp90 *in vitro* has been previously reported (Jacob et al., 1995).

Other observations reported here suggest that the GA-sensitive folding pathway is normally preferred. GA inhibited the rate of luciferase renaturation by 70% in the RRL lysate supplemented with hsc70. However, a 35% inhibition would have been expected if hsc70 was stimulating luciferase renaturation solely through a GA-insensitive pathway. In addition, hsp90 reversed GA-induced inhibition of luciferase renaturation in hsc70 supplemented lysate nearly completely. Thus, the exogenous hsc70 appeared to be preferentially stimulating luciferase renaturation through the GA-sensitive pathway in RRL.

Effect of Geldanamycin on the Interactions of Hsps and Associated Cohorts with Denatured Luciferase. The data presented here support the hypothesis that GA is a specific inhibitor of hsp90 function. GA inhibited luciferase renaturation in RRL, but had no direct effect on luciferase activity *in vitro* or in RRL after its renaturation. The difference observed between the IC_{50} values for GA and HbA-induced inhibition of luciferase renaturation is similar to differences observed between the potency of the two drugs in *in vivo* assays of their tumoricidal activity (Whitesell et al., 1992). GA-induced inhibition of luciferase renaturation was specifically reversed by the addition of hsp90 to RRL, but not by the addition of hsc70, p60, or p23.

The binding of GA to hsp90 appears to inhibit the normal sequential interaction of components of the hsp90 chaperone machinery with substrate. When progesterone receptor/hsp90 complexes are reconstituted in RRL in the presence of GA, the progesterone receptor accumulates in intermediate hsp90 complexes containing hsc70, p60, and p48 (Johnson & Toft, 1995; Smith et al., 1995). GA blocks the formation of mature complexes of the progesterone receptor with hsp90 complexes that contain p23 and immunophilins. However, GA does not inhibit the dynamic cycling of the progesterone hormone receptor through the hsp90/hsc70/p60/p48 intermediate complexes (Smith et al., 1995). Similarly, during luciferase renaturation in GA-treated RRL, GA increased the amount of p60 and decreased the amount of p23 associated with the hsp90 chaperone machinery containing bound luciferase. Thus, as for the progesterone receptor, GA appears to prevent the transition of hsp90/hsc70/p60/p48 complexes that contain bound luciferase to hsp90 complexes containing p23 and bound luciferase. We speculate that the intermediate chaperone machinery may represent, in part, the chaperone machinery responsible for the alternate folding pathway based on two observations. First, the chaperones which interact with denatured luciferase in GA-treated lysate are characteristic of the intermediate chaperone machinery. Second, GA does not appear to inhibit the dynamic interaction of this machinery with substrates (Smith et al., 1995). However, the identities and roles of the individual chaperones that are responsible for luciferase renaturation in RRL remain to be elucidated. We have not yet examined whether immunophilins interact with hsp90−p23-bound luciferase or whether GA blocks this interaction as has been reported for the progesterone receptor, but recent results indicate that hsp40 also binds to denatured luciferase present in the intermediate complexes.[3]

Mechanism of Action of Geldanamycin. Kinetic analysis of GA-induced inhibition of luciferase renaturation with respect to varying ATP concentration suggests a possible mechanism by which the binding of GA to hsp90 inhibits hsp90 function. Inhibition of luciferase renaturation by GA was uncompetitive with respect to ATP (Figure 4A). This shows either that the inhibition by GA is not reversibly connected with the addition of ATP or that GA inhibits an enzyme form downstream from that with which ATP combines. These possibilities are consistent with the schemes presented in Figure 7. The best-fit curve of the replot of [GA] *versus* $1/V_{app}$ was parabolic, suggesting that two molecules of GA are binding to each hsp90 chaperone machine complex. This is consistent with the notion that hsp90 is present in the chaperone complex as a dimer (Jacob & Buchner, 1994), if the conformation of each hsp90 present in the dimer is not equivelent. Alternatively, two GA binding sites may be present in each hsp90 monomer. Since no amount of ATP relieves the inhibitory effect of GA, GA and ATP are clearly binding to different sites.

The data suggest that GA acts by inducing changes in the binding or hydrolysis of ATP by the hsp90 chaperone machinery. GA caused a significant reduction in the K_{app} of the hsp90 chaperone machine for ATP from ~60 to ~12 μM. This observation indicates that the binding of GA to the hsp90 chaperone machine complex either decreases the rate of ATP dissociation (e.g., increases the affinity of the hsp90 chaperone machine for ATP) or slows the rate at which ATP is converted to product (e.g., inhibits the rate of ATP hydrolysis). The effects of GA on the K_{app} for ATP and the interaction of p23 with the hsp90 chaperone machine containing bound luciferase are consistent with previous reports that (a) p23 interacts with hsp90 in the presence of either ATP or nonhydrolyzable ATP analogs; but (b) the presence of hydrolyzable ATP is required for the stable association of p23 with hsp90/progesterone receptor complexes (Johnson & Toft, 1995); and (c) the hsp90 cohort p23 does not interact with the hsp90 chaperone machinery containing bound substrate (SHR) in the presence of GA (Johnson & Toft, 1995; Smith et al., 1995; and Figure 5).

[3] V. Thulasiraman and R. L. Matts, unpublished observations.

Based on the correlation between GA's effects on p23 binding to the hsp90 chaperone machinery and GA's effects on ATP affinity or hydrolysis, we speculate that p23 plays a role in regulating either ATP hydrolysis or nucleotide exchange during the action of the hsp90 chaperone machine. The binding of GA to hsp90 could be directly affecting the ATP binding site that has been proposed to be present on hsp90 (Figure 7A) (Csermely et al., 1993; Johnson & Toft, 1995; Nadeau et al., 1993). However, since the existence of the hsp90 ATP binding site has recently been questioned (Jacob et al., 1996), the binding of GA to hsp90 may affect the ATP binding site present on some other component of the hsp90 chaperone machinery, such as hsc70, through allosteric interactions.

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