II. LIGAND-MEDIATED ACTIVATION OF GRP94 MOLECULAR CHAPERONE AND PEPTIDE BINDING ACTIVITY

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James J. Wassenberg, Robyn C. Reed‡, and Christopher V. Nicchitta§
From the Department of Cell Biology, Box 3709, Duke University Medical Center, Durham, North Carolina 27710

The N-terminal domain of eukaryotic Hsp90 proteins contains a conserved adenosine nucleotide binding pocket that also serves as the binding site for the Hsp90 inhibitors geldanamycin and radicicol. Although this domain is essential for Hsp90 function, the molecular basis for adenosine nucleotide-dependent regulation of GRP94, the endoplasmic reticulum paralog of Hsp90, remains to be established. We report that bis-ANS (1,1-bis(4-anilino-5-napthalenesulfonic acid), an environment sensitive fluorophore known to interact with nucleotide-binding domains, binds to the adenosine nucleotide-binding domain of GRP94 and thereby activates its molecular chaperone and peptide binding activities. bis-ANS was observed to elicit a tertiary conformational change in GRP94 similar to that occurring upon heat shock, which also activates GRP94 function. bis-ANS activation of GRP94 function was efficiently blocked by radicicol, an established inhibitory ligand for the adenosine nucleotide binding pocket. Confirmation of the N-terminal nucleotide binding pocket as the bis-ANS-binding site was obtained following covalent incorporation of bis-ANS into GRP94, trypsinolysis, and sequencing of bis-ANS-labeled limit digestion products. These data identify a ligand dependent regulation of GRP94 function and suggest a model whereby GRP94 function is regulated through a ligand-dependent conversion of GRP94 from an inactive to an active conformation.

grp94,1 the endoplasmic reticulum paralog of cytosolic Hsp90, is an abundant resident endoplasmic reticulum lumenal protein that by virtue of its association with nascent polypeptides is thought to perform a chaperone function (1–6). Consistent with this role, GRP94 expression is up-regulated by stress conditions that promote protein misfolding or unfolding, such as glucose starvation and heat shock (7–9). In addition to its role in the regulation of protein folding in the endoplasmic reticulum, GRP94 can function in the intercellular trafficking of peptides from the extracellular space to the major histocompatibility complex class I antigen processing pathway of professional antigen presenting cells (10–13). The observation that GRP94 can function as a peptide-binding protein derives from the pioneering studies of Srivastava and colleagues (14) identifying GRP94 (gp96) as a tumor rejection antigen (14). Subsequent studies have demonstrated that GRP94-peptide complexes can elicit major histocompatibility complex class I restricted immune responses against a wide variety of peptide epitopes (11, 15–18). Thus, in addition to a homeostatic role in protein folding and assembly, GRP94 appears to function, in an as yet undefined manner, as a component of the major histocompatibility complex class I antigen processing and presentation pathways of mammalian cells.

The mechanism and regulation of GRP94 interactions with peptide and polypeptide substrates remains unknown. Experiments conducted with purified GRP94 and synthetic peptides have demonstrated that GRP94 binds peptides in vitro and that this peptide binding activity is markedly stimulated by heat shock (15, 19–22). The conformational and regulatory consequences of heat shock on GRP94 activity have, however, only begun to be studied. It is clear from recent data that the stimulation of peptide binding that accompanies heat shock occurs in the context of a tertiary conformational change and is accompanied by exposure of a hydrophobic domain(s) accessible to the environment sensitive fluorophore Nile Red (21).

The recent identification of a conserved adenosine nucleotide binding pocket in the N-terminal domain of eukaryotic Hsp90 proteins has focused recent investigations into the role of adenosine nucleotides in the regulation of Hsp90 and GRP94 function (23–26). It has been established that ATP is necessary for stable association of Hsp90 with the accessory factor p23 (25, 27, 28), although the identification of adenosine nucleotide-dependent regulation of Hsp90-substrate interactions has proven difficult (28, 29). Perhaps indicative of the complexity of Hsp90-adenosine nucleotide interactions, the recombinant N-terminal adenosine nucleotide-binding domain displays enzymatic activities that differ substantially from the native protein (24, 25). With respect to GRP94, evidence in support of intrinsic ATP binding and ATPase activities is controversial and a consensus regarding the molecular basis of an adenosine nucleotide-mediated regulation of GRP94-substrate interactions has yet to emerge (20, 26, 30–32).

To further investigate the structural basis for the activation of GRP94 peptide binding activity that accompanies heat shock, we examined the interaction of GRP94 with the environment-sensitive fluorophores, Prodan, 8-ANS, and bis-ANS. Because of its unique characteristics, we focused attention on bis-ANS. Bis-ANS has been used as a probe of the conformational state of a wide range of proteins including the molecular...
chaperones GroEL and DnaK (33, 34). bis-ANS is known to bind to hydrophobic sites on proteins and, unlike Prodan and 8-ANS, bis-ANS also interacts with nucleotide-binding sites (35–37). In this study we report that bis-ANS elicits a conformational change in GRP94 that is accompanied by a substantial increase in chaperone and peptide binding activity. The activation of GRP94 function by bis-ANS is blocked by radi- cal, an established ligand for the conserved, N-terminal nucle- otide-binding pocket of the eukaryotic Hsp90s (38, 39). On the basis of these data, we propose a model in which ligand binding to the N-terminal nucleotide-binding domain of GRP94 elicits a conformational change that converts GRP94 from an inactive to an active conformation, wherein the chaperone and peptide binding activities of GRP94 are markedly stimulated.

**EXPERIMENTAL PROCEDURES**

**Materials—**Fluorescent probes were obtained from Molecular Probes (Eugene, OR). bis-ANS concentration was determined by absorbance at 385 nm (εex = 16, 790 cm−1 M−1 in water). Citrate synthase (EC 4.1.3.7) was purchased from Roche Molecular Biochemicals (Mannheim, Ger- many). Radical was obtained from Dr. Leo Neukers, Beckman Institute, Frederick, MD. Peptide VS8 (RGYVYQGL) was synthesized by the University of North Carolina at Chapel Hill Peptide Synthesis Facility (Chapel Hill, NC). Na0.125M] was purchased from Amersham Pharmacia Biotech. All other reagents were obtained from Sigma unless otherwise indicated. GRP94 was purified from porcine pancreas as described previously (40). The concentration of GRP94 was determined by absorbance at 280 nm (1A280 = 1.18 mg/ml).

**Fluorophore Binding Reactions—**All binding reactions, with the exception of the indicated circular dichroism and citrate synthase aggre- gation experiments, were conducted in buffer A (110 mM KOAc, 20 mM NaCl, 2 mM Mg(OAc)2, 25 mM K-HEPES, pH 7.2, 100 μM CaCl2). Fluorescent probe and radiolig stocks were prepared in dimethyl form- amide at 5 mM final concentration. For all assays, control reactions at identical conditions were performed to correct for any solvent effects. Where indicated, GRP94 was heat shocked by incubation in a 50 °C water bath for 15 min followed by cooling to 37 °C.

**Fluorescence Measurements—**Emission spectra were obtained in a FluorMax spectrofluorometer (SPEX Industries Inc., Edison, NJ) operat- ing in photon counting mode. Spectra were recorded and processed with the SPEX 38000 operating software, version 2.1 (SPEX Industries Inc., Edison, NJ). For emission scans, slit width was set at 1 nm. Excitation wavelengths were as follows: Prodan, 360 nm; ANS, 372 nm; bis-ANS, 393 nm; trypophan, 295 nm. All spectra were background corrected.

**Circular Dichroism Measurements—**Far-UV CD spectrometry was performed on an AVIV Associates 62DS circular dichroism spectrome- ter. Samples were prepared in 0.1% dimethylformamide (DMF) which is transparent to far-UV CD measurements and binds weakly to proteins. Circular dichroism samples were analyzed in 1-mm path length quartz cuvette at 37 °C. GRP94 samples (1 μM) were prepared in standard phosphate-buffered saline solution as buffer A produced unacceptable dynode voltages in the relevant region of the spectrum. GRP94 was incubated with 10 μM bis-ANS for 2 h at 37 °C prior to obtaining spectra. Spectra were recorded from 300 to 195 nm.

**Conformational Analysis by Proteolysis—**The conformational state of GRP94 was assessed by tryptic digestion of the protein and subsequent SDS-PAGE analysis. For simple proteolysis experiments, 10 μM of a 0.5 mg/ml GRP94 stock, with or without prior heat shock, was combined with 1 μl of bis-ANS and/or radiolig stock solutions and incubated for the indicated times at 37 °C. Samples were then digested with 0.1% trypsin and digested for 30 min at 37 °C. An equal volume of SDS-PAGE sample buffer was added and the samples were snap frozen in liquid nitrogen. Immediately before gel analysis, samples were thawed and boiled for 5 min. Samples were then separated on 12.5% SDS-polyacrylamide gels. Gels were fixed and stained with Coomassie Blue. For time course experiments, excess free bis-ANS was removed immedi- ately prior to trypsinization by gel filtration on 0.5-ml G-25 Sephadex spin columns.

**Identification of the bis-ANS-binding Site—**The bis-ANS-binding region of GRP94 was identified by covalent incorporation of bis-ANS into GRP94 following the bis-ANS photolysis procedures described in Refs. 42 and 43. Briefly, 50 μg of GRP94 was combined with 50 μM bis-ANS in a final volume of 100 μl and photocross-linked for 15 min on ice with a 366-nm hand-held UV lamp (Ultra-violet Products, Inc., San Gabriel, CA). Following photocross-linking, GRP94-bis-ANS complexes were di- gested with trypsin for 1 h at 37 °C. The trypsin derived limit digestion products were then separated by C-18 reverse phase high performance liquid chromatography using a continuous acetone/water gradient in 20 mM ammonium bicarbonate, with sequential detection by UV absorbance (220 nm) and fluorescence emission (excitation 418 nm; emission 498 nm). The major resultant fluorescent peak was collected and the corresponding peptide sequenced by Edman degradation on an Applied Biosystems Procise model 492 automated protein sequencer.

**Native Blue Electrophoresis—**The oligomeric state of GRP94 was assayed by blue native-PAGE as described previously (44). GRP94 was lyophilized or shock frozen to a 10-fold molar excess of bis-ANS for the indicated times. Samples were then dissolved in 15% glycerol and loaded onto 5–18% gradient gels with 0.02% Coomassie Brilliant Blue in the cathode buffer. Gels were run at 4 °C, stained with Coomassie Blue, destained, and dried.

**Peptide Synthesis Aggregation Assays—**The effects of GRP94 on the thermal aggregation of citrate synthase were assayed by the methods of Buchner and colleagues (45). Samples containing no protein, or GRP94 (1 μM), were incubated in 40 mM HEPES, pH 7.5, for 2 h at 37 °C with either 0.2% N,N-dimethylformamide or 10 μM bis-ANS. The samples were then warmed to 43 °C for 5 min and placed in a spectrofluorometer thermostatted at 43 °C. Citrate synthase was then added to 0.15 μM final concentration and the thermal aggregation of the enzyme followed by its emission scattering. Excitation and emission wavelengths were both 500 nm with 2 nm slit width. The time course of citrate synthase aggregation was followed for 100 s.

**RESULTS**

**Binding of Polarity-sensitive Fluorescent Probes to GRP94—** Recent studies on the conformational regulation of GRP94 have identified a tertiary structural change that occurs in response to heat shock and is associated with an activation of peptide binding activity (21, 22). Coincident with the heat shock-elicited conformational change, GRP94 displays enhanced binding of environment sensitive fluorescent probes such as Nile Red, which preferentially bind to hydrophobic domains (21). GRP94 contains two domains of significant hydrophobicity, a C-termi- nal assembly domain and a highly conserved N-terminal region, which corresponds to the Hsp90 geldanamycin and adenosine nucleotide-binding site (24, 46). To characterize the structural basis for the heat shock-dependent activation of GRP94 activity, the interaction of polarity-sensitive fluoro- phores with native and heat-shocked GRP94 was examined. The three probes tested, Prodan (6-propionyl-2-(dimethylami- no)napththalene), 8-ANS, and bis-ANS are structurally related probes which bind to hydrophobic sites on proteins and undergo substantial fluorescence spectrum changes upon introduction into nonpolar environments (34–36, 47). The following experimental protocol was utilized. GRP94 was warmed to 37 °C and either maintained at 37 °C or heat shocked for 15 min at 50 °C, followed by incubation at 37 °C. Subsequently, probe was added to the GRP94 solution and emission spectra were recorded after 30 min at 37 °C. As depicted in Fig. 1A, the emission maxima of Prodan in the presence of native or heat-shocked GRP94 were essentially identical, indicating that Prodan does not interact with the hydrophobic binding pocket(s) displayed by heat-shocked GRP94. In contrast, the structurally related probe, 8-ANS, displays weak interactions with native GRP94,
yet binds avidly following heat shock (Fig. 1B). At present, it is not known if 8-ANS binding to native GRP94 represents the binding of 8-ANS to a weakly hydrophobic site present on the entire population of molecules or whether 8-ANS binds to a strongly hydrophobic site present on a small fraction of native GRP94 molecules, but uniformly represented on the heat shocked population of GRP94.

The interaction of bis-ANS with GRP94 was complex, and displayed clear time and concentration dependence. As depicted in Fig. 1, C and D, the initial bis-ANS binding to native GRP94 was biphasic and following extended incubations in the presence of bis-ANS, a level of fluorophore binding similar to that seen with heat-shocked GRP94 was observed. Maximal bis-ANS binding to heat-shocked GRP94 occurred very rapidly and under the described experimental setting, could not be time resolved. At 10-fold higher probe and protein concentrations (as used in later experiments) bis-ANS binding to native GRP94 was much more rapid, with 85% maximal binding occurring in 1 h (data not shown). These data suggest that maximal bis-ANS binding to GRP94 required a slow structural transition. This transition may represent a bis-ANS elicited conformational change in GRP94 and/or the bis-ANS dependent stabilization of a distinct conformational state accessed by the native protein.

Analysis of bis-ANS Binding to Heat-shocked GRP94—To determine the affinity of bis-ANS for GRP94, bis-ANS was added to increasing concentrations of heat-shocked GRP94, the fluorescence spectrum was determined, and the emission intensity at 475 nm plotted as a function of GRP94 concentration (Fig. 2, A and B). Under the described experimental conditions, bis-ANS binding to GRP94 was near-maximal at a 20-fold molar excess of GRP94 monomer over bis-ANS. From the Klotz plot (59, 60), a $K_d$ of 110 nM for GRP94-bis-ANS interactions was determined. In this experimental format, where the ligand concentration is constant and the receptor concentration is varied, the $K_d$ value more accurately indicates the concentration of unbound receptor (GRP94) at which the ligand (bis-ANS) is 50% saturated (Fig. 2B). From a mathematical standpoint, however, the determined $K_d$ value is identical to that viewed from a more traditional perspective, where the $K_d$ represents that concentration of ligand at which the receptor is 50% occupied. Importantly, these data indicate that bis-ANS binds in a saturable manner to heat-shocked GRP94.

As mentioned previously, bis-ANS serves both as a probe of GRP94 conformation, in that it preferentially binds to the conformation accessed by GRP94 during heat shock, and as a ligand to elicit such a conformational change. Regarding the former, additional experiments were performed to further characterize the temperature dependence of the heat shock-elicited conformational change. Fig. 2C depicts an experiment in which GRP94 (50 µg/ml) was incubated for 15 h at temperatures ranging from 4 to 50 °C, and the relative efficiency of conversion to the heat shock conformation determined following addition of 5 µM bis-ANS. As depicted in Fig. 2C, a small but...
reproducible increase in the GRP94 fraction present in the heat shock conformation was observed following extended incubation at 37 °C (as compared with paired incubation at 4 °C). Under the described experimental conditions, the temperature-dependent structural transition is half-maximal at 42 °C, and maximal at 45 °C (Fig. 2C). Because swine, the source organism for the GRP94 used in this study, commonly achieve febrile body temperatures of 41–42 °C during periods of bacterial or viral infection, the conformational change identified in these studies may represent a physiologically relevant mechanism for regulating GRP94 activity.

Structural Consequences of bis-ANS Binding to GRP94—Following an extended incubation period, the emission spectra of bis-ANS bound to native GRP94 bears substantial similarity to that emission spectra of bis-ANS bound to heat shocked GRP94 (Fig. 1C). Because heat shock is known to elicit a stable tertiary conformational change in GRP94 (21), these data suggest that the binding of bis-ANS to GRP94 induces, or stabilizes, a conformational change similar to that occurring in response to heat shock. To determine whether the GRP94 conformation seen upon addition of bis-ANS is similar to that observed following heat shock, a series of structural studies on the bis-ANS-GRP94 complex was performed. In one series of experiments, the proteolysis patterns of native, heat-shocked, and bis-ANS-treated GRP94 were examined. As shown in Fig. 3A, lanes 2 and 3, incubation of native GRP94 with low levels of trypsin yields two prominent proteolysis products, representing known structural domains of the protein (24, 46, 48). In contrast, proteolysis of either bis-ANS-treated or heat-shocked GRP94 yields a substantially reduced recovery of these prominent proteolysis products, with the concomitant appearance of a diverse array of proteolytic fragments of higher SDS-PAGE mobility. Essentially identical proteolysis patterns were observed following either heat shock or bis-ANS treatment of Hsp90 (data not shown). These data provide evidence that bis-ANS binding to GRP94 elicits or stabilizes GRP94 in a conformational state that can be readily accessed and/or stabilized by either heat shock or ligand (bis-ANS) binding.

Effects of bis-ANS Binding on GRP94 Quaternary and Secondary Structure—When purified from tissue, GRP94 exists as a homodimer (40, 49). Following heat shock, however, GRP94 forms higher molecular weight complexes (21). To further characterize the effects of bis-ANS on GRP94 quaternary structure, the oligomerization states of native, heat-shocked, and bis-ANS-treated GRP94 were assayed by the blue native-PAGE

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2 M. Morrow, personal communication.
technique (44). In these experiments, GRP94 was incubated with bis-ANS or briefly heat shocked and subsequently incubated at 37 °C. The samples were then analyzed by gradient blue native-gel electrophoresis (44). In this procedure, nondenaturing Coomassie dye binds to the protein and acts as the dominant charge carrying moiety, thus allowing assignment of molecular weights with an accuracy comparable to that of SDS-PAGE (44). As is apparent in Fig. 4, in the absence of heat shock or bis-ANS treatment the majority of GRP94 exists as a dimer with an apparent molecular mass of approximately 200 kDa. However, exposure to heat shock causes a relatively rapid formation of tetramers, hexamers, and octamers (Fig. 4, lanes 2–4). Incubation of GRP94 with a 10-fold molar excess of bis-ANS induces changes in the quaternary structure of GRP94 that mimic those seen upon heat shock (Fig. 4, lanes 4 and 5). These data lend further support to the hypothesis that bis-ANS induces or stabilizes a structural transition in GRP94 that is similar to that occurring in response to heat shock.

To gain additional insights into the GRP94 conformational occurring in response to bis-ANS treatment, native and bis-ANS-treated GRP94 were analyzed by circular dichroism (CD) spectroscopy. By providing a structure-averaged estimate of α-helix and β-sheet content, CD spectroscopy can identify conformational changes occurring at the secondary structure level. As shown in Fig. 5, the CD spectra for native, heat-shocked, and bis-ANS-treated GRP94 were identical, indicating that the conformational change occurring in the presence of bis-ANS is primarily, if not entirely, limited to a tertiary structural change.

**Radicicol Inhibits Temperature and bis-ANS-induced GRP94 Conformational Changes**—Radicicol, a macrocyclic antibiotic, binds to the highly conserved N-terminal nucleotide binding pocket of Hsp90 and thereby blocks Hsp90 function (38, 50). To determine if radicicol binding also influenced the structural dynamics of GRP94, the following experiments were performed. GRP94 was incubated with increasing concentrations of radicicol, subsequently heat-shocked, cooled, and digested with trypsin. SDS-PAGE analysis of GRP94 treated in this fashion demonstrated that radicicol inhibited the heat-induced structural transition, as assayed by the similarities in proteolysis patterns between native GRP94 and radicicol-treated, heat-shocked GRP94 (data not shown). Similar inhibition of the heat shock-induced structural transition of Hsp90 by radicicol was also observed (data not shown). To determine if radicicol could also inhibit the bis-ANS-dependent GRP94 structural transition, GRP94 was incubated with increasing concentrations of radicicol, bis-ANS was then added, and the samples were incubated for 1 h. Samples were subsequently digested with trypsin and the proteolysis patterns determined by SDS-PAGE. As is depicted in Fig. 6A, radicicol, when present at a 10-fold molar excess over bis-ANS, efficiently blocked the bis-ANS-dependent GRP94 conformation change.

Although the experiment depicted in Fig. 6A indicated that radicicol was able to inhibit the appearance of the bis-ANS-dependent conformational state, it was necessary to determine if bis-ANS binding to GRP94 was blocked by radicicol treatment. To this end, the following experiment was performed. GRP94 was incubated in the presence of increasing concentrations of radicicol, subsequently heat treated under conditions sufficient to elicit efficient bis-ANS binding, and bis-ANS binding assayed. As shown in Fig. 6B, radicicol, in a dose-dependent manner, inhibited bis-ANS binding to heat-treated GRP94. Because radicicol itself blocks the heat shock-induced conformation change, these data present two models of bis-ANS action. In one model, bis-ANS binds to the nucleotide-binding domain and directly elicits the observed conformational change. Radicicol, by binding to the adenosine nucleotide binding pocket, would then be predicted to inhibit the bis-ANS-dependent conformational change. In an alternative model, GRP94 interconverts, in a temperature-sensitive manner, between two conformational states, arbitrarily referred to as the open or the closed state. In the open state, bis-ANS would bind and thereby stabilize the open conformation whereas radicicol binding would stabilize the closed conformation. For both models, bis-ANS binding to the N-terminal adenosine nucleotide-binding domain was predicted and was subsequently examined.
binding sites (42, 43). As described under “Experimental Procedures.” GRP94 was combined with an excess of bis-ANS and photocross-linked on ice for 15 min. GRP94 was subsequently digested with trypsin, the fluorescent peptides purified by high performance liquid chromatography, and the sequence of the labeled peptides determined by Edman sequencing. The major resultant fluorescent peptide yielded the sequence YSQFINFPIYV, which mapped to residues 271–281 of the N-terminal domain of GRP94. This segment is homologous to the human HSP90 sequence HSQFIGYPITLFV from amino acids 210 to 222, and overlaps with the C-terminal region of the adenosine nucleotide/geldanamycin/radicicol-binding domain (24, 46).

bis-ANS Activates GRP94 Chaperone Activity—To determine if the bis-ANS-dependent conformational changes in GRP94 were of functional significance, the molecular chaperone activities of native, heat-shocked, and bis-ANS-treated GRP94 were evaluated in a thermal aggregation assay (29, 45). In these experiments, citrate synthase aggregation was assayed in the presence of buffer, native GRP94, heat-shocked GRP94, or GRP94 that had been previously exposed to bis-ANS for 2 h. Following experimental treatment of the GRP94, reactions were equilibrated at 43 °C, citrate synthase then added and aggregation, as represented by light scattering, was measured. In the absence of GRP94, citrate synthase undergoes rapid thermal aggregation and under the experimental conditions depicted in Fig. 7A, reaches a plateau level within 15 min. In the presence of native GRP94, the degree of aggregation is reduced, suggesting that at least a fraction of the population of native GRP94 molecules are in an active conformation. Under these experimental conditions, approximately 50% of the citrate synthase aggregated. At the concentration of GRP94 used in these experiments, and assuming a stoichiometric interaction, these results indicate that roughly 8% of the native GRP94 is in the active conformation. In the presence of heat-shocked or bis-ANS-treated GRP94, no thermal aggregation of citrate synthase was detectable (Fig. 7A). These data indicate that the ability of GRP94 to bind to substrate proteins is greatly enhanced by prior heat shock or bis-ANS treatment and suggest that the GRP94 conformation elicited by heat shock or bis-ANS binding represents an active state of the molecule.

bis-ANS Activates Peptide Binding Activity to GRP94—To assess the effects of bis-ANS treatment on the peptide binding
activity of GRP94, GRP94 was either treated with bis-ANS, or briefly heat shocked. A 10-fold molar excess of I25I-VSV8 was then added and the mixture incubated for 30 min at 37 °C. Free peptide was separated from bound peptide by Sephadex G-75 spin column chromatography and the bound peptide was quantitated by γ-counting. As shown in Fig. 7B, treatment of GRP94 with bis-ANS significantly enhanced the peptide binding activity of GRP94, yielding approximately a 4–5-fold stimulation over native protein. Under similar conditions, heat-shocked GRP94 displayed approximately a 10-fold stimulation of binding. From the data presented in Fig. 7A and B, it is apparent that bis-ANS elicits or stabilizes a GRP94 conformation that displays markedly enhanced molecular chaperone and peptide binding activities.

**DISCUSSION**

In this article, we present evidence demonstrating that bis-ANS binds to the conserved, N-terminal adenosine nucleotide-binding domain of GRP94 and elicits a tertiary conformational change yielding markedly enhanced molecular chaperone and peptide binding activities. The binding of bis-ANS to GRP94 is biphasic, with an initial rapid binding phase followed by a slow, extended binding phase. In accord with these data, we hypothesize that bis-ANS binds to and stabilizes a low abundance GRP94 conformation, referred to as the open state. In this model, GRP94 molecular chaperone and peptide binding activity is intimately coupled to such a conformation change. In the absence of regulatory ligands, access to this conformation is proposed to occur in a time and temperature-dependent manner through intrinsic structural fluctuations. Inhibitory ligands, such as geldanamycin and radicicol, are proposed to function by binding to and stabilizing GRP94 in a closed, or inactive, conformation (46).

The molecular basis for the regulation of GRP94 function is currently under investigation. By analogy to its cytosolic paralog, Hsp90, current views favor regulation of GRP94 activity through cycles of ATP binding and hydrolysis (24, 25, 51, 52). Arguments favoring an adenosine nucleotide-based regulatory mechanism for the Hsp90 family of chaperones extends from crystallographic data demonstrating that the conserved N-terminal structural domain of the Hsp90 family of chaperones contains an ATP/ADP-binding site (24, 25). Furthermore, the amino acid residues that function in adenosine nucleotide binding and ATP hydrolysis, as identified by analysis of crystallographic data, have been demonstrated to be essential in yeast (25, 52). Although the structural and molecular genetic data implicating ATP and ADP in the regulation of Hsp90 function are robust, biochemical analyses of ATP and ADP effects on Hsp90 activity in vitro have not identified the expected mode of regulation. For example, recent studies of Hsp90 domain function using an insulin B-chain aggregation assay, indicated that ATP, in a manner similar to the inhibitory ligand geldanamycin, inhibited the chaperone activity of the Hsp90 N-terminal domain (51). Thus, although ATP and ADP can be demonstrated to bind to and influence Hsp90 conformation (27, 28, 53), it is not yet clear how cyclic ATP binding and hydrolysis are mechanistically coupled to a cycle of interaction between Hsp90 and unfolded polypeptide substrates.

Consistent throughout studies of the molecular regulation of Hsp90 function has been a strong correlation between conformational state and activity. For example, heat shock has been demonstrated to elicit a conformational change accompanied by the activation of molecular chaperone activity (54) (current paper), peptide binding activity (19, 20), and oligomerization state (21, 55). Interestingly, the heat shock sensitive increase

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**FIG. 8. Model for the ligand-dependent conformational regulation of GRP94 activity.** The nucleotide-binding domains of GRP94 are depicted as residing in either of two conformations that differ in their capacity to bind the adenosine derivative N-ethylcarboxamidoadenosine (NECA). Following heat shock, or in the presence of activating ligands, such as bis-ANS, GRP94 undergoes a tertiary conformational change accompanied by a marked elevation of chaperone and peptide binding activity. In this conformation, GRP94 can undergo, in a concentration-dependent manner, homotypic oligomerization. Inhibitors of GRP94 activity, such as geldanamycin and radicicol are proposed to bind the nucleotide-binding domain of both subunits and block the conformational acquisition of the active state.
in Hsp90 oligomerization state can be blocked by ATP and geldanamycin (55), again suggesting that ATP and geldanamycin influence Hsp90 conformation in a similar, if not identical manner. Are such conformational changes relevant to Hsp90 or GRP94 function? In the following, we provide a rationale for the physiological significance of such conformational changes and argue for a ligand-mediated regulation of GRP94 conformation.

In previous studies, it was observed that native GRP94, as purified from tissue, was present in two conformational states. These conformational states could be distinguished by the environment-sensitive fluorophore Nile Red and can be referred to as the open (Nile Red accessible) and closed (Nile Red inaccessible) conformations (21). The majority of native GRP94 is found in the closed conformation, although the fraction present in the open conformation can be rapidly and dramatically increased by heat shock. Thus, in a minimal model GRP94 exists in two conformational states, with the interconversion between the two states reflecting the structural micro-unfolding fluctuations that are characteristic of protein structural dynamics in their native thermal environments (56–58). That the interconversion between the two conformational states could be regulated by ligand binding to the N-terminal adenosine nucleotide-binding domain is suggested by the following observations. One, heat shock elicits a conformational change in Hsp90 and GRP94 that is accompanied by enhanced molecular chaperone activity (54) (current report). Two, geldanamycin and ATP block the acquisition of the heat shock-dependent conformation (55), and three, in the present study, it was observed that bis-ANS binds to the N-terminal adenosine nucleotide-binding domain of GRP94 and elicits a conformational change accompanied by enhanced molecular chaperone and peptide binding activities. These observations highlight the importance of the conformational state of GRP94 in the regulation of its molecular chaperone and peptide binding activities and demonstrate that ligand binding to the conserved, N-terminal adenosine nucleotide-binding domain can regulate the conformation and activity state of GRP94.

Previous studies on bis-ANS binding to the Hsp70 chaperone, DnaK support a model in which bis-ANS binds to and stabilizes an open DnaK conformational state that is spontaneously accessed by the protein during steady state, thermally driven, conformational fluctuations (34). By analogy, we hypothesize that GRP94 spontaneously interconverts between two conformational states, inactive, or closed, and active or open. In the inactive, or closed state, GRP94 is unable to stably interact with substrate (poly)peptides, whereas in the open state a pronounced molecular chaperone activity is displayed. Although such conformational dynamics are readily apparent in the native protein and can be rapidly elicited upon elevation of the temperature above 37 °C, such conformational changes, if regulated only by temperature, would limit the activation of GRP94 chaperone function to the heat shock state. However, the fact that such conformational changes can be modulated by ligand binding to the N-terminal adenosine nucleotide-binding domain suggests that ligands of the appropriate structure can elicit a conformational change identical or similar to that occurring during heat shock, even in the absence of thermal stress. A ligand-mediated regulation of GRP94 conformation would allow the activation of GRP94 chaperone activity under conditions other than heat shock that disrupt protein folding, such as nutrient deprivation and oxidative stress. In this and the accompanying manuscript (61), data are presented in support of an adenosine-based ligand, other than ATP/ADP, that would regulate GRP94 function. At present, the existence of such a native ligand(s) is entirely speculative.

A model summarizing the primary observations of this and the preceding manuscript (61) is depicted in Fig. 8. In this model, we postulate that the nucleotide-binding domains present in each of the two identical subunits of GRP94 exist in either of two conformational states. Operationally, the two conformations can be distinguished by their ability to bind the adenosine derivative N-ethylcarboxamidoadenosine. In this conformational state, GRP94 displays low chaperone activity. During heat shock, or in the presence of a suitable ligand (i.e. bis-ANS), GRP94 undergoes a tertiary conformational change that is accompanied by the activation of chaperone activity. In this conformation, and at sufficiently high GRP94 concentrations, GRP94 undergoes homotypic oligomerization. In contrast, radiolabelled geldanamycin, established inhibitors of GRP94, are proposed to bind to the nucleotide-binding domains of both subunits and thereby elicit a conformation in which GRP94 chaperone activity is dramatically reduced or eliminated. In the radiolabelled or geldanamycin bound state, GRP94 is unable to access an active conformation, as would occur in response to heat shock.

In conclusion, we report the identification of a ligand elicited conformational change in GRP94 that is accompanied by a marked activation of molecular chaperone and peptide binding activities. The similarities between the conformations of GRP94 following heat shock activation and bis-ANS binding support the conclusion that GRP94 conformation and activity can be regulated by ligand binding to the N-terminal adenosine nucleotide-binding domain and that the conformation of the protein in the bis-ANS liganded state is physiologically relevant. Future studies will focus on the relationships between GRP94 conformation and function, with an emphasis on the identification of adenosine-based native ligands that stimulate GRP94 molecular chaperone activity.

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Ligand Regulation of GRP94 Function