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The Conformation of the Glucocorticoid Receptor AF1/tau1 Domain Induced by Osmolyte Binds Co-regulatory Proteins*

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The activation domains of many transcription factors appear to exist naturally in an unfolded or only partially folded state. This seems to be the case for AF1/tau1, the major transactivation domain of the human glucocorticoid receptor. We show here that in buffers containing the natural osmolyte trimethylamine *N*-oxide (TMAO), recombinant AF1 folds into more a compact structure, as evidenced by altered fluorescence emission, circular dichroism spectra, and ultracentrifugal analysis. This conformational transition is cooperative, a characteristic of proteins folding to natural structures. The structure resulting from incubation in TMAO causes the peptide to resist proteolysis by trypsin, chymotrypsin, endoproteinase Arg-C and endoproteinase Gluc-C. Ultracentrifugation studies indicate that AF1/tau1 exists as a monomer in aqueous solution and that the presence of TMAO does not lead to oligomerization or aggregation. It has been suggested that recombinant AF1 binds both the ubiquitous coactivator CBP and the TATA box-binding protein, TBP. Interactions with both of these are greatly enhanced in the presence of TMAO. Co-immunoadsorption experiments indicate that in TMAO each of these and the coactivator SRC-1 are found complexed with AF1. These data indicate that TMAO induces a conformation in AF1/tau1 that is important for its interaction with certain co-regulatory proteins.

Transcriptional activation by the glucocorticoid receptor (GR)[1] is mediated through the function of three regions in the protein, AF1/tau1, tau2, and AF2. Numerous mutational analyses have shown that of these, AF1/tau1 makes by far the largest quantitative contribution to transcriptional activation. Deletion or inactivating mutations of AF1/tau1 reduce the ability of the GR to activate transcription from test genes by at least 60–70% (1–3). As defined by molecular genetic analyses, tau2 and AF2 are located in the C-terminal ligand-binding domain (LBD) of the receptor, whereas AF1/tau1 lies N-terminal from the centrally located DNA-binding domain (DBD). In the human GR (hGR) AF1 is encompassed by amino acids 77–262 (3). Precisely how the AF1/tau1 transactivation region functions is unknown, in part due to lack of knowledge of its working structure; yet this structure must provide the basis for essential, specific interactions with other proteins. Studies undertaken in our laboratory and others (4, 5) have shown that when expressed independently in dilute aqueous solutions, AF1 appears to have little structure. Instead it appears to exist as a collection of conformers that overall appears as random coil. However, in the presence of the strong α-helix stabilizing agent trifluoroethanol, as many as three segments toward the C-terminal end of the AF1/tau1 region exhibit α-helical characteristics (4). Mutations in the GR tau1/AF1 transactivation region coupled with assays of function have suggested that this ability to form α-helical conformation *in vitro* correlates with the transactivation potential of the region (4). The subdomain of AF1/tau1 that subsumes the potential helices has been referred to as the AF1 core ($AF1_c$), and when this core is expressed separately while connected to an exogenous DNA-binding domain, AF1 retains considerable transactivating activity. Mutations of acidic amino acids in AF1, including the core, had little effect on such activity; however, mutations altering several hydrophobic residues of AF1/tau1 significantly diminished its transcriptional activation potential (6).

In recent years, a number of proteins that modulate GR activity, such as GRIP1, RIP140, SRC-1, and CBP/p300, have been identified (7–9). These co-regulators bind the GR and presumably act as molecular bridges to the primary transcriptional machinery. Most co-regulators have been identified through their ligand-dependent interactions with the LBD, in particular, with the AF2 domain in members of the receptor family of which the GR is part (10–14). However, it is not known whether the co-regulators function exclusively through this part of the GR or whether they can also modulate the activity of AF1/tau1. Some may well do so. CBP and several other co-regulators have been shown to bind, if not strongly, to recombinant AF1/tau1 (15–17). A recent study using $AF1_c$ or the potential helices thereof connected to a GR DNA-binding domain could interact in a yeast system with a fragment of CBP to activate appropriate promoter-reporter constructs (17). AF1 has also been shown to bind TBP (16), the critical TATA box-binding protein that forms the basis for the multiprotein transcription initiation complex. This raises the possibility that the AF1 GR domain somehow directly influences the transcription machinery. *In vitro* transcription studies indicated that the holoGR acts to stabilize the preinitiation complex (18). One possibility is that a structured conformation of AF1/tau1 is induced or stabilized during its interaction with specific target proteins. This model in fact has been supported for the activation domains of some transcription factors (19, 20). Thus, data short of actual structural proof support the idea that conditional folding of the AF1/tau1 region is an important require-

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[1] The abbreviations used are: GR, glucocorticoid receptor; hGR, human GR; TMAO, trimethylamine *N*-oxide; LBD, ligand-binding domain; DBD, DNA-binding domain; TBP, TATA box-binding protein; GST, glutathione *S*-transferase; Endo, endoproteinase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

ment for its interaction with target factors and subsequent role in gene regulation. *In vivo*, the particular conformer of AF1/tau1 required for transcriptional activation presumably is induced or stabilized by forces coming from intra- and intermolecular interactions. Knowledge of those factors and the conformation adopted by AF1/tau1 will lead to understanding of the role of this region in the transcription process, information essential to understanding how glucocorticoids affect gene regulation.

Naturally occurring solutes or organic osmolytes have been used to shift the thermodynamic balance so as to make unstructured proteins fold into native-like, functioning structures (21–26). One such osmolyte, trimethylamine *N*-oxide (TMAO), has been used very successfully to fold unstructured, inactive proteins into proteins with significant functional activity (27, 28). We recently demonstrated that TMAO induces secondary and tertiary structure in a GR fragment containing the entire N terminus plus the DBD (amino acids 1–500). The conformational transition of this GR fragment is cooperative in nature (5), a condition characteristic of proteins folding to their proper shape. Because most of the structural changes taking place in this GR fragment appear to be happening in the N-terminal domain, we hypothesize that TMAO induces an ordered conformation in the AF1/tau1 region, and this conformation is important for its interaction with target proteins. In this paper, we present studies of the TMAO-induced conformation in the AF1/tau1 region expressed alone. Our data suggest that TMAO induces secondary and tertiary structure in the AF1/tau1 region, and this induced conformation greatly enhances its interaction with certain co-regulatory proteins and TBP.

MATERIALS AND METHODS

Expression and Purification—Construction and expression of AF1/tau1 has been described (5). The bacteria containing the recombinant vector for GST-AF1/tau1 were induced with isopropyl-β-D-thiogalactopyranoside (0.5 mM) for 3 h, lysed, and extracted. The bacterial extracts were loaded onto a glutathione-Sepharose column at 4 °C. AF1/tau1 protein was eluted from the column by thrombin digestion, followed by a Superdex-75 exclusion column as described (5). For GST adsorption experiments, GST or GST-tagged protein was purified by eluting the bound protein using 50 mM Tris, 500 mM NaCl, 50 mM reduced glutathione, pH 8.3. Protein purity was analyzed by SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue R-250 and was estimated to be greater than 95%.

Fluorescence Emission Spectroscopy—Fluorescence emission spectra of purified AF1/tau1 protein in solution were recorded at various concentrations of TMAO. The spectra were monitored using a Spex Fluoro Max spectrofluorimeter at excitation wavelengths of 278 or 295 nm as described (5). All measurements were made in 1-cm rectangular cuvettes thermostated at 22 °C, and all the data were corrected for the contribution of the solute concentrations. To prevent aggregation of protein we added proline at a constant molar ratio of TMAO:proline as 4:1 in all samples containing TMAO.

Circular Dichroism Spectroscopy—The CD spectra were recorded at 22 °C on an Aviv 62 spectropolarimeter using a 1.0-cm quartz cell, with the bandwidth of 1.0 nm and scan step of 0.5 nm. The spectra were recorded at a protein concentration of 1.0 mg/ml (in the presence and absence of TMAO) in 10 mM Tris, 10 mM NaCl, 10 mM dithiothreitol, pH 7.9, and were corrected for the contribution of solute concentrations. Each spectrum shown is a result of five spectra accumulated, averaged, and smoothed.

Limited Proteolytic Digestion—Sequencing grade trypsin, chymotrypsin, endoproteinase Gluc-C, and endoproteinase Arg-C were used for proteolytic digestions. Digestion was carried out with 5 μg of purified AF1/tau1 in 10 mM Tris, 10 mM NaCl, 2 mM dithiothreitol, pH 7.9. Except for Endo Arg-C, which was carried out at room temperature for 50 min, all other reactions were carried out at 4 °C for 20 min. Endo Gluc-C and Endo Arg-C were added at a protein:enzyme mass ratio of 50:1, whereas trypsin and chymotrypsin were added at 100:1. Reactions were terminated by adding SDS loading buffer and then placing the sample tubes in a boiling water bath. The samples were then run on SDS-PAGE gel and stained by Coomassie Blue R-250. For protein microsequencing, the proteolytic reactions were carried out in triplicate in the presence and absence of 3 M TMAO. After gel electrophoresis, the proteins were transferred onto PVDF membranes. The largest protected bands in the samples containing 3 M TMAO were pooled and sequenced by five cycles of Edman degradation (29).

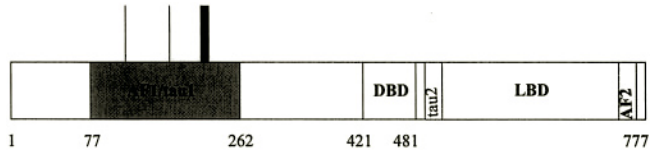

FIG. 1. **Topological diagram of human GR showing its functional regions.** The *numbers* indicate positions of amino acids. The *vertical lines* represent the positions of Trp (*bold line*), and Tyr (*thin lines*) residues in AF1/tau1.

Sedimentation Velocity—Sedimentation was carried out with a Beckman XL-A analytical ultracentrifuge, using a two-channel Epon centerpiece. Purified AF1/tau1 protein was sedimented in a buffer containing 10 mM Tris, 200 mM NaCl, 1 mM dithiothreitol, pH 7.9. Rotor speed was 60,000 rpm with scans taken at 280 nm. The apparent sedimentation coefficient distribution was determined using the time derivative of the concentration profile (30) as modified by J. Philo (DCCT+, version 1.11). Standard protocols (31) were used to correct apparent sedimentation coefficient (s^*) to 20 °C and water ($s_{20,w}$). The s^* values were corrected for viscosity, which was measured for buffer with and without 3 M TMAO, using a viscometer. For sedimentation equilibrium studies the samples were run at 20,000, 25,000, and 30,000 rpm. The samples were judged to be at equilibrium when successive scans showed no change in the distribution of protein. Data were analyzed using nonlinear least squares parameter estimation using the program NONLIN (32, 33). The buffer conditions were the same as in sedimentation velocity measurements.

GST-AF1 Adsorption Assay—Purified GST-AF1 hybrid protein in a buffer containing 10 mM Tris, 10 mM NaCl, pH 7.9, with or without TMAO was immobilized on glutathione-Sepharose beads. Nuclear extracts were prepared from HeLa cells and added (1.0 mg/ml) to the GST-AF1 bound to the beads, and the mixture was further incubated for 2 h. Any unbound protein was washed thoroughly. The appropriate concentrations of TMAO were kept throughout the assay. To the washed beads, SDS-PAGE sample buffer was added, and the sample was boiled for 5 min in a water bath. Each sample was then run on a SDS-PAGE gel and visualized by Coomassie Blue R-250 staining. To confirm CBP binding, CBP was constitutively expressed in COS-1 cells using transient transfection as described (34), and this COS-1 cell extract was used in place of HeLa nuclear extracts. All other adsorptions were carried out using HeLa nuclear extracts. After gel electrophoresis, proteins were transferred onto a PVDF membrane. The membrane was first blocked by incubation for 2 h at room temperature in PBS containing 10% nonfat milk proteins and then incubated at 4 °C with appropriate antibodies (Santa Cruz Biotechnology) overnight. The immunoreactive bands were developed using ECL method and visualized by autoradiography.

Immunoadsorption—Purified AF1/tau1 and HeLa nuclear proteins were mixed together in a buffer containing 10 mM Tris, 10 mM NaCl, pH 7.9 (with or without 3 M TMAO), to a final volume of 1 ml and incubated for 1 h at 4 °C. 5 μl of antibodies (SRC-1, CBP, or TBP; Santa Cruz) and 25 μl of protein-agarose conjugate were then added and incubated further for 2 h. Pellets were collected by centrifugation and washed thoroughly. The beads were resuspended in SDS buffer and boiled for 5 min. After SDS-PAGE gel electrophoresis, the proteins were transferred onto a PVDF membrane and immunoblotted using an antibody raised against amino acids 150–175 of the human GR as described above.

RESULTS

TMAO Causes the hGR AF1/tau1 Transactivation Region to Fold into a Native-like Conformation—As with other related receptors, the GR contains several major functional domains. These are shown diagrammatically for the human GR in Fig. 1. The AF1/tau1 transactivation region, amino acids 77–262 is *highlighted*, with *vertical lines above the bar* indicating the location of the two Tyr and one Trp residues within AF1/tau1. This domain was expressed in a bacterial system as a hybrid protein tagged at the N-terminal residue with glutathione *S*-

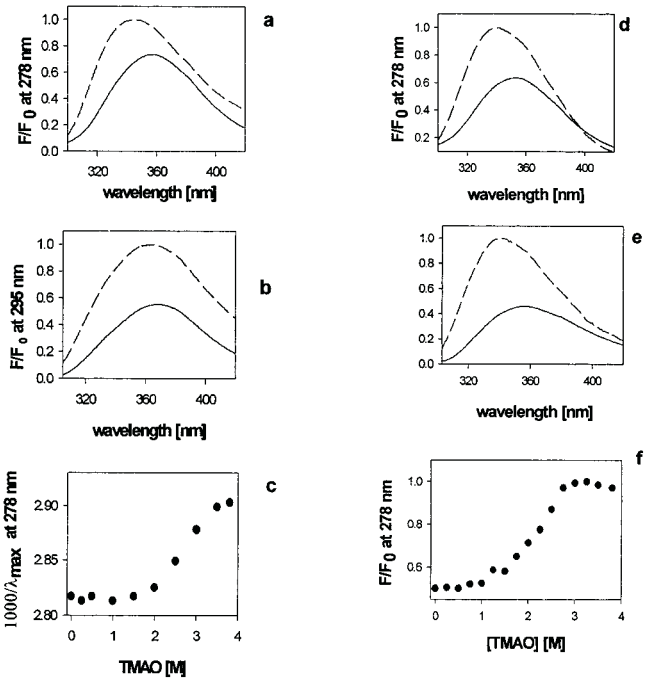

FIG. 2. **Fluorescence emission spectra of AF1/tau1 in the absence and presence TMAO.** The *curves a, b, d and e* show the spectra in the absence (—) and presence (- - -) of 3.5 M TMAO. Experiments *a–c* were carried out in low salt; *d–f* were carried out in high salt. *a* and *d*, excitation at 278 nm; *b* and *e*, excitation at 295 nm. *c* and *f* show the reversible conformational transition of AF1/tau1 induced by TMAO, as monitored by change in emission maxima upon excitation at 278 nm with respect to increasing concentrations of TMAO. The spectra in low salt were recorded in buffer containing 10 mM Tris, 10 mM NaCl, 10 mM dithiothreitol, pH 7.9. Those of *d–f* were recorded in the same buffer except with 200 mM NaCl. The linear least squares best fit of experimental data to the two-state model of protein folding/denaturation using linear extrapolation methods (42) gives apparent thermodynamic parameters of TMAO-induced folding: $\Delta G = -2.8 \pm 1.1$ kcal/mol, $m = 1.1 \pm 0.6$ in 10 mM NaCl, and $\Delta G = -3.9 \pm 0.7$ kcal/mol, $m = 1.6 \pm 0.3$ in 200 mM NaCl.

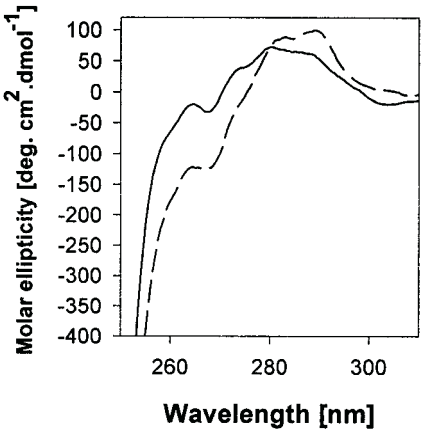

FIG. 3. **Near UV-CD spectra of AF1/tau1 in the absence (—) and presence (- - -) of 3 M TMAO.** Each spectrum is the result of five spectra accumulated, averaged, and smoothed.

transferase (GST). After removing the GST moiety, we studied the folding of the AF1/tau1 protein. Fluorescence emission was used to monitor the environment around the Trp and Tyr residues. The fluorescence emission spectra of >95% pure recombinant AF1/tau1 are presented in Fig. 2. Fig. 2*a* shows the spectra in the absence and presence of 3.5 M TMAO after excitation at 278 nm, which reflect the changes coming both from Tyr and Trp residues. Fig. 2*b* indicates the fluorescence emission changes after excitation at 295 nm, which specifically follows changes in the environment of the single Trp residue located between the first two potential helices of the AF1 core. In both sets of spectra, the quantum yield of the fluorescence is increased in the presence of TMAO, with an increase of about 2-fold at 3.5 M TMAO. There are blue shifts in the emission maxima in the presence of TMAO. These fluorescence changes are typical of those accompanying the removal of aromatic residues from polar, aqueous solution into a more hydrophobic environment within the protein. Both the increase in quantum yield and the blue shift in fluorescence maxima indicate the formation of compact structure in the presence of TMAO. Because the three amino acids excited are located well apart in AF1/tau1, the conformational changes reflected in the fluorescence emission changes may be happening throughout the peptide. TMAO induces this conformational transition in AF1/tau1 in a cooperative manner, as shown by monitoring the shift in fluorescence emission maximum after excitation at 278 nm as a function of TMAO concentration (Fig. 2*c*). These observations strongly suggest that TMAO folds AF1/tau1 region into a more compact structure, and the cooperativity of the process is a hallmark of native protein folding (35, 36).

Because the ultracentrifugation studies (discussed below) required a buffer containing 200 mM NaCl, we repeated the fluorescence emission spectra in this buffer to find out whether the structural changes we observed in AF1/tau1 following TMAO exposure occurred at this salt concentration. In Fig. 2, (*d* and *e*) the quantum yield of the fluorescence is increased significantly following TMAO exposure, as was seen in Fig. 2 (*a* and *b*). Interestingly, the blue shift in emission maxima at 295 nm is significantly increased compared with that at low salt (Fig. 2, *b versus e*), indicating that higher salt concentration favors the conformational transition of folding of AF1/tau1 by TMAO. The TMAO-induced conformational transition in AF1/tau1 in 200 mM salt also is cooperative (Fig. 2*f*) and reaches saturation at 3 M compared with that of 3.5 M in 10 mM salt (Fig. 2*c*). These fluorescence emission studies thus indicate that the TMAO-induced cooperative conformational transition in AF1/tau1 is more favored at 200 mM.

The fluorescence emission spectra of AF1/tau1 in the presence and absence of TMAO suggest that TMAO causes tertiary structure to form in this protein. To acquire further evidence for tertiary structure occurring in AF1/tau1, we recorded the near-UV CD spectra of this protein in the presence and absence of TMAO (Fig. 3). Comparison of the spectra shows that in the presence of TMAO, the maximum at around 290 nm is significantly increased, reflecting perturbation of the Trp residue. These data support the conclusion that TMAO causes three-dimensional structure to occur in the domain.

The Structure Induced in AF1/tau1 by TMAO Resists Proteolysis—As another way of evaluating the changes in the tertiary structure of the AF1/tau1 region brought about by TMAO, we carried out limited proteolytic digestions of the protein with four different proteases in the presence and absence of the osmolyte. To be sure that the compound was not interfering with enzyme activity, we first tested trypsin activity on the artificial substrate, *N*α-benzoyl-L-arginine *p*-nitroanilide. Tryptic activity was not blocked by TMAO. In fact, proteolysis of the test substrate was somewhat enhanced, perhaps due to the osmolyte preserving the natural active conformation of the enzyme (data not shown). The patterns of proteolytic products of AF1/tau1 after digestion by trypsin and chymotrypsin are shown in Fig. 4*a*. It is evident that the partial cleavage patterns vary as the concentration of TMAO increases. At 0 and 0.5 M TMAO, the protein is nearly completely digested by both proteases (*lanes 3* and *4,* and *9* and *10*), whereas it is partially protected at 1 and 1.5 M TMAO (*lanes 5* and *6* and *lanes 11* and

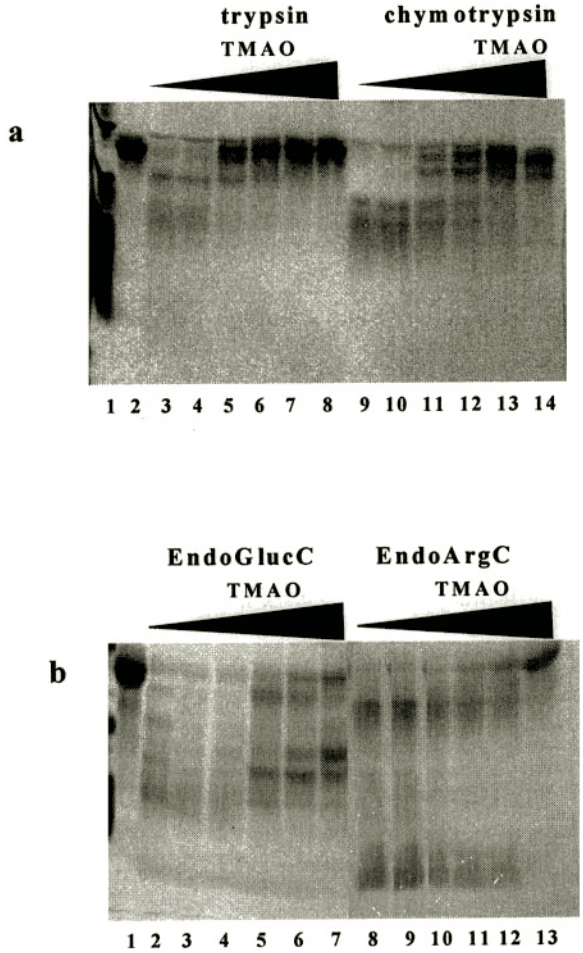

FIG. 4. **TMAO-induced folding protects AF1 against proteolysis by trypsin (*lanes 3–8*) and chymotrypsin (*lanes 9–14*) (*a*) and endoproteinase Gluc-C (*lanes 2–7*) and endoproteinase Arg-C (*lanes 8–13*) (*b*).** The lanes show the products of digestion resolved by SDS-PAGE and stained with Coomassie Blue. *a, lane 1,* molecular weight markers; *a*, *lane 2,* and *b, lane 1,* purified undigested AF1. Each set of 6 lanes shows the results from digestion in the presence of 0, 0.5, 1.0, 1.5, 2.0, or 3.0 M TMAO.

12). At 2 M or higher TMAO concentrations, AF1/tau1 appears to be mostly protected, suggesting that it has folded into a tertiary structure that moves the residues attacked by these enzymes to positions not easily reached by them. Similar results were obtained when AF1/tau1 was digested with the proteases Endo Gluc-C and Endo Arg-C (Fig. 4*b*), although in the case of Endo Gluc-C, the protection was not as complete. In the case of Endo Arg-C, full protection was seen only at 3 M TMAO.

We compared the peptide patterns resulting from digestion of heat-denatured and non-denatured AF1/tau1, to see whether the non-denatured form contained folded regions resistant to peptidases. The data (not shown) indicated no difference in patterns, consistent with lack of significant structure in native recombinant AF1/tau1. We then compared the peptide patterns of the folded forms induced by TMAO. Upon exposure to each of the four proteases, both non-denatured and denatured AF1/tau1 show closely similar protected digestion patterns after incubation in the presence of 3 M TMAO for 15 min up to 16 h (data not shown). The similar patterns of protected peptides at every time point in the presence of 3 M TMAO demonstrate that TMAO can induce similar structures in AF1/tau1 whether it is initially non-denatured or denatured. Acute (15 min) TMAO exposure is enough to fold the recombinant protein domain into a tertiary structure not distinguishable, in this test, from that

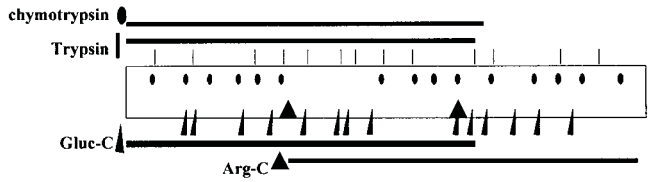

FIG. 5. **Location of some major proteolytic fragments from AF1/tau1, protected by TMAO.** Proteolytic digestion data are combined with protein microsequencing. The *horizontal solid bar* diagrams AF1/tau1 (amino acid 77–262). The symbols indicate the amino acid positions which trypsin, *thin vertical line*; chymotrypsin, *oval*; endoproteinase Gluc-C, *thin black triangle*; or endoproteinase Arg-C, *wide triangle*; can cut. The *horizontal lines* indicate the largest protected peptide after digestion in each enzyme in the presence of 3 M TMAO. Partial sequencing provided identification of the N termini of these peptides. Sizes estimated by electrophoresis on denaturing gels.

produced by the longest exposure. These observations suggest that once in a sufficient concentration of TMAO, AF1/tau1 folds rapidly to a protease-resistant shape and that it remains in this conformation, irrespective of the duration of TMAO exposure.

Four of the most prominent protected bands seen after partial digestion of AF1 in the presence of 3 M TMAO were identified by sequencing their N termini. In Fig. 5 the positions of these bands in the AF1/tau1 domain are shown diagrammatically. A long region starting at the N terminus of AF1/tau1 was protected from digestion by trypsin, chymotrypsin, and Endo Gluc-C. Many potential substrate sites for these enzymes exist within that region. All three enzymes cut in a relatively short region lying approximately beyond amino acid 217. Although we did not identify them by sequencing as yet, the sizes of smaller peptides protected against these three enzymes suggest that in TMAO a relatively short segment of AF1/tau1 beyond amino acid 217 is open to attack. Peptide 4 was produced by Endo Arg-C, which has only two potential substrate sites in AF1/tau1. The protected site is Arg-214. This result is consistent with the interpretation that in the TMAO-folded portion, a short region beyond amino acid 217 is open to proteolysis. It is evident from these results that TMAO-induced structure in AF1/tau1 is not confined to only one part of the molecule.

AF1/tau1 Exists as a Monomer Both in the Absence and Presence of TMAO—It is evident from our data that TMAO exposure apparently leads to the formation of a significant amount of secondary and tertiary structure in the AF1/tau1. The observed structural changes in the presence of 3 M TMAO might originate from an oligomerization of the protein induced by the presence of the co-solvent. Analytical sedimentation studies were conducted to monitor the effects of TMAO on the quaternary structure of the protein. Data from sedimentation velocity studies of AF1/tau1 in the absence and presence of 3 M TMAO were found to fit a single species model, as shown in Fig. 6. No additional species were detected, and the total amount of protein loaded in the cell was accounted for in that single species. The s^* ($s_{20,w}$) value for AF1/tau1 obtained in the presence of 3 M TMAO is ≈2.4, which corresponds to the expected value for a globular protein of ~20–25 kDa (37). However, the s^* value for AF1/tau1 in buffer without TMAO is ≈1.6, consistent with that of a protein of the same molecular weight but one that assumes either an asymmetric shape or adopts an unstructured conformation. Sedimentation equilibrium experiments were also run under similar conditions at three different speeds (20,000, 25,000, and 30,000 rpm). The results (not shown) indicated the presence of a protein of a molecular mass of 21 kDa, which corresponds to the monomeric molecular weight for AF1/tau1. Thus these ultracentrifugation studies clearly indicate that up to 0.5 mg/ml AF1/tau1 exists as a monomer in the aqueous solutions employed in this study and

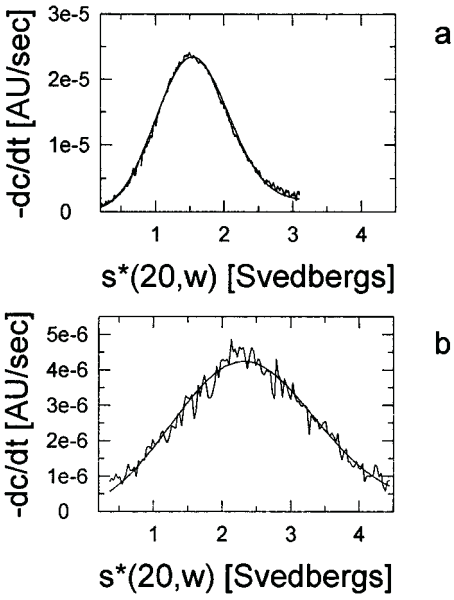

FIG. 6. **Sedimentation coefficient distribution of AF1/tau1 as determined from sedimentation velocity.** The peak *s** corresponds to the apparent sedimentation coefficient. *a,* in buffer; *b,* in 3 M TMAO.

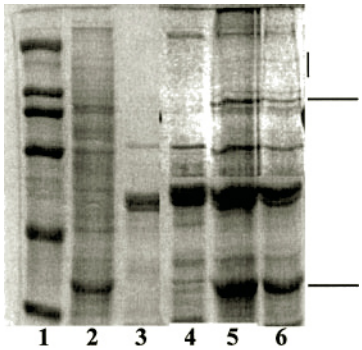

FIG. 7. **TMAO enhances GST-AF1/tau1 interactions with several proteins from HeLa cell nuclear extract in a GST pull-down assay.** Proteins were identified by SDS-PAGE and Coomassie Blue staining. *Lane 1,* molecular weight markers; *lane 2*, nuclear extract; *lane 3*, GST-AF1/tau1 with no nuclear extract; *lane 4*, GST-AF1/tau1 + nuclear extract; *lanes 5* and *6*, GST-AF1/tau1 + nuclear extract + 3.0 M TMAO. The beads used in *lane 5* were washed with a buffer with 3 M TMAO, whereas in *lane 6*, beads were washed with buffer only. *Horizontal lines* to the *right* indicate two of the 5 or 6 proteins retained in TMAO.

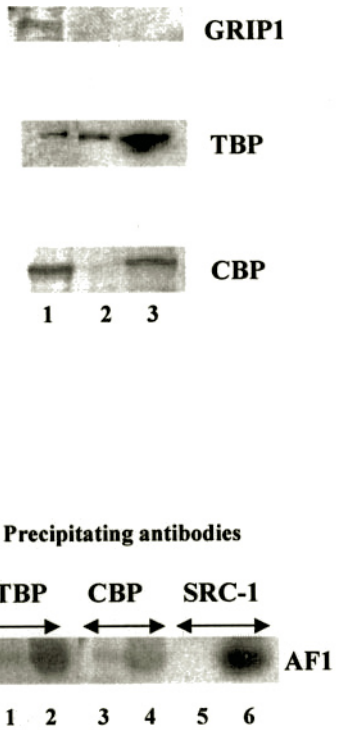

FIG. 8. **TMAO enhances the association of TBP, CBP, and SRC-1, but not all known steroid receptor cofactors, with AF1/tau1.** In the *upper three panels*, GST-AF1/tau1 linked to glutathione-Sepharose beads were used to adsorb proteins from HeLa cell nuclear extracts (see "Materials and Methods"). *Lanes 1*, nuclear extract without adsorption to beads. *Lanes 2,* adsorption without TMAO. *Lanes 3,* adsorption in the presence of 3 M TMAO. The data shown are immunoreactions of the proteins resolved by denaturing gel electrophoresis, using antibodies to GRIP1, TBP, or CBP. *Lower panel* shows the immunoreaction to antibodies against AF1 (resolved by gel electrophoresis), after immunoprecipitation with the indicated antibodies. *Lanes 1, 3,* and *5*, immunoadsorption in the absence of TMAO. *Lanes 2, 4,* and *6*, immunoadsorption in 3 M TMAO.

that exposure to 3 M TMAO does not induce protein aggregation.

TMAO Facilitates the Interaction of AF1/tau1 with Important Target Proteins—It is presumed that AF1/tau1 makes physical interactions with other factors in order to transactivate gene(s) (16) and that conditional folding is important for these interactions (6). We therefore evaluated whether the conformation induced in AF1/tau1 by TMAO is important for specific protein-protein interactions. By using a GST "pull-down" assay with GST-AF1/tau1 attached to solid beads as the adsorptive reagent, we observed that in the presence of TMAO, several proteins from HeLa cell nuclear extracts were adsorbed. Fig. 7 shows an example of the results. It is evident from the Coomassie-stained gel that when sufficient TMAO is present, GST-tau1 binds only certain proteins from HeLa nuclear extracts. Several higher molecular weight bands that are not prominent in the extract become so in the adsorbed proteins, and one low molecular weight band that is prominent in the crude extract is strongly retained on the GST-AF1/tau1 column. GST alone or GST-AF1 in low concentrations of TMAO did not retain these proteins. Several similar experiments using both unlabeled and metabolically labeled proteins confirmed the fact that in TMAO certain proteins have high affinity for AF1/tau1. In the experiment shown, after binding the proteins to the GST-AF1 on beads in the presence of 3 M TMAO, the column with bound proteins was washed extensively with a buffer containing no TMAO. Subsequent elution and electrophoresis of the retained proteins showed that removal of TMAO from the washes had not completely released these proteins from the GST-AF1, suggesting that once the AF1/tau1 complexes were formed in the presence of TMAO, they were relatively stable (Fig. 7, compare *lanes 5* and *6*).

To begin to identify specific AF1/tau1 target factors, we screened the bound proteins by immunoreacting these with antisera against a selected set of known co-regulators. RIP140, CBP, NcoR, TBP, GRIP, p/Cip, SRC-1 have been shown previously to interact with nuclear receptors and/or to affect receptor-specific transcriptional activation (7–14). No immunoreactions were observed with the sera we employed for N-CoR, GRIP1, RIP140, or p/Cip, either in the presence or absence of 3 M TMAO. (One such experiment is shown in Fig. 8.) Consistent with previous reports (6), in the absence of TMAO we detected a weak interaction with TBP and CBP. This was increased dramatically in the presence of 3 M TMAO (Fig. 8). In the absence of TMAO the antiserum did not detect a p250/300 band in the adsorbed HeLa nuclear proteins but did show a slight reaction with a protein of 160 kDa. But in 3 M TMAO, a reaction at 250/300 kDa and a very strong reaction at 160 kDa were seen. To determine whether TMAO truly enhanced an interac-

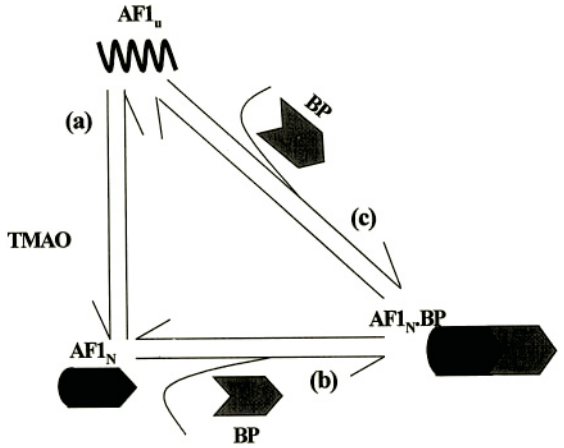

FIG. 9. **Model for folding of the AF1 domain of the GR.** $AF1_U$ represents the assembly of unfolded conformers of AF1 in the absence of its proper high affinity protein binding partner (*BP*). In this circumstance, AF1 could exist only in its unfolded state(s) or in equilibrium with a properly folded state, $AF1_N$. *BP* could induce folding directly to $AF1_N$ by binding, shifting AF1 directly to the heterodimer $AF1_N$·BP. Alternatively, $AF1_U$ could be in equilibrium with its native folded state $AF1_N$. Without BP, $[AF1_N]$ is very small relative to $[AF1_U]$; however, BP could bind $AF1_N$ and eventually shift AF1 to the complex, by the law of mass action. Agents such as the osmolyte, TMAO, by shifting the equilibrium toward $AF1_N$, enhance the quantity of AF1 available immediately for binding to BP.

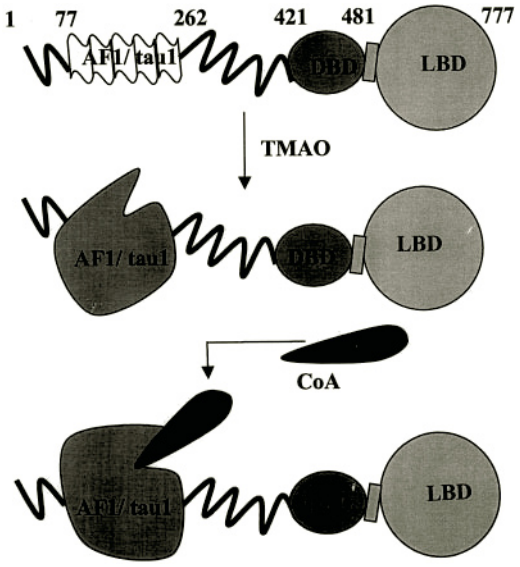

FIG. 10. **Model applying Fig. 9 to the GR.** The *top schematic* shows the GR with its globular DBD and LBD but a nonspecifically conformed major transactivation domain, AF1/tau1. The osmolyte TMAO gives structure to AF1 such that specific binding to co-regulators can occur.

tion between CBP and AF1/tau1, we transfected COS-1 cells with a CBP plasmid designed to express CBP constitutively. In extracts from these cells we found that in the presence of 3 M TMAO a protein of 250/300 kDa, reactive to the anti-CBP antiserum, was trapped by the GST-AF1 column (Fig. 8). Among the other HeLa proteins trapped in the presence of 3 M TMAO on the GST-AF1 column was TBP. Only a weak reaction was seen unless TMAO was present.

To confirm further these AF1/tau1 interactions, we carried out immunoadsorption experiments, using CBP and TBP primary antibodies in HeLa nuclear extracts that had been supplemented with recombinant AF1/tau1. We also probed for adsorbed proteins with an antibody to the 160-kDa protein SRC-1. After allowing the extracts to react with each antiserum, the protein complexes were trapped by adding secondary antibodies linked to inert beads. The beads were washed extensively, and the bound proteins were released and resolved by electrophoresis in denaturing conditions on polyacrylamide gels. An antiserum to amino acids 150–175 of the hGR was used to identify AF1/tau1. In the absence of TMAO, a small amount of AF1 was found to have been retained on the beads precipitated with anti-CBP and anti-TBP. No SRC-1/AF1 interaction was seen without TMAO. In the presence of 3 M TMAO, a very strong interaction of AF1 with each of the other proteins has been shown to have occurred. Taken together, these protein-protein interaction data indicate that TMAO-induced folding in AF1/tau1 is important for its interaction with target factors. Among these may be CBP, TBP, and SRC-1.

DISCUSSION

Deletion studies have indicated that hGR lacking the AF1/tau1 transactivation region retains only a small portion of its transactivation activity (1, 2); hence, it has been concluded that this region plays a major role in gene regulation by GR. It has been reported that AF1/tau1 makes physical contact with certain other proteins to activate genes (6, 15–17). It has also been shown that a C-terminal portion of AF1/tau1 is indispensable for its transactivation activity. This "core" region has a propensity to form α-helix, and in the presence of trifluoroethanol, it can form three helical segments (4). These potential helices seem important for functional interactions with a fragment of CBP in a yeast system (17). The induced fit model of folding hypothesizes that the AF1/tau1 region is unstructured *in vivo* until it adopts a more ordered conformation (38). In this paper we have studied the conditional folding of recombinant AF1/tau1 in the presence of a naturally occurring osmolyte, TMAO, which has been shown to fold proteins into native-like structures (27). Our results clearly demonstrate that TMAO causes a significant amount of secondary/tertiary structure to form in the AF1/tau1 region. The fluorescence emission data indicate a shift of hydrophobic amino acids into a more non-polar environment, just the type of change seen as natural hydrophobic forces drive protein folding. In both low salt and near physiological salt concentrations, the conformational transition is cooperative, a hallmark of naturally folding proteins (35, 36), and the free energy shift is similar to that seen during spontaneous folding of globular proteins. Osmolyte-driven stabilization of protein folding is in fact a process used often in nature (23). Due to its solvophobic effect on the peptide backbone (25), TMAO forces thermodynamically unstable proteins to fold to active conformations without altering the rules for folding (25). Osmolytes, including TMAO, serve this purpose in a wide range of organisms. Based on these facts and on our observations, it is highly likely that TMAO enhances folding to a natural structure in AF1/tau1. The near-UV CD and the fluorescence emission data both point to this conclusion. These data monitor signals indicating that the 2 Tyr and 1 Trp residues move to more hydrophobic locations, *e.g.* to the interior of the protein. The TMAO-induced increased s^* value without a change in molecular weight suggests that the protein is more tightly packed and assumes a globular structure in the presence of this co-solvent. Since the breadth of the sedimentation profiles shown in Fig. 6 is proportional to the diffusion coefficient of the protein, the larger breadth in the profile for AF1/tau1 in the presence of 3 M TMAO indicates a higher diffusion coefficient. A higher diffusion coefficient for the same molecular weight protein implies that the hydrodynamic shape of the protein is more compact or symmetric. Thus, the hydrodynamic data are in complete agreement with the conclusion that the presence of 3 M TMAO induces AF1/tau1 to assume a more structured state without inducing formation of large aggre-

gates. The data from limited protease digestions combined with microsequencing analysis of peptides in the digests indicate that extensive regions of AF1 are protected, implying that much of the recombinant peptide is involved.

Based on these observations we hypothesize that an induced conformation or limited set of conformations occurs in AF1/tau1 in order for it to carry out its transcription function. This could happen either by an induced fit mechanism, in which AF1/tau1 would fold as the direct result of interaction with its target factor(s), or by a shift in the equilibrium between a large proportion of the unstructured form and a small proportion of properly structured form (Fig. 9). In the latter case, the small proportion of structured molecules can make physical interactions with target molecules. By shifting the equilibrium to favor structured molecules, TMAO would enhance the interaction of AF1/tau1 with its partners. Of course, in the holoGR, AF1/tau1 may be partially structured. Our data on two domain fragments of the GR (39) and the data of others (40) on the progesterone receptor and its N-terminal region are consistent with this. But no data available suggest even in the holoreceptor that AF1/tau1 is a fully structured globular domain.

If TMAO causes AF1 to assume a native, functional structure, then the interaction of the domain with partner proteins should be enhanced. It is very likely that AF1/tau1 makes a physical contact with cofactors or other proteins to activate gene transcription. It has been shown that the AF1/tau1 core can bind to several proteins important for transcription, including CBP and TBP (6, 15–17). These proteins have also been reported to interact with the LBD of the GR, presumably at AF2. A careful examination of the data for the observed AF1/tau1 interactions with these proteins shows that although specific, only a limited amount of protein-protein binding could be demonstrated. This is consistent with the aspect of our model (Fig. 9) in which a small portion of AF1/tau1 is in the folded state, capable of such interactions, whereas most of AF1/tau1 remains unfolded due to the unfavorable equilibrium of Fig. 9*a*. Our protein-protein interaction experiments show that in TMAO, the binding of AF1/tau1 to a finite number of other cellular proteins is greatly enhanced. Specifically, the interactions of AF1 with CBP, TBP, and SRC-1 are enhanced in the presence of TMAO.

Taken together, our observations suggest that TMAO folds the intrinsically unstructured conformers of AF1/tau1 into a conformation which is well suited for its interaction with other cofactors. Once AF1/tau1 reaches this proper shape, co-regulator or basal TATA box-binding protein(s) can interact efficiently with this part of the receptor and stabilize its structure. We have noticed during our GST pull-down assays that once the target factors interact, removal of TMAO does not alter this interaction, suggesting relatively tight binding, not dependent on constant presence of TMAO. Therefore, it can be speculated that TMAO thermodynamically favors a well structured conformation of AF1/tau1, and co-regulator proteins stabilize this structure. We have also presented data that binding an hGR fragment containing the DBD to its cognate DNA-binding site causes AF1 to fold (39). Fig. 10 presents a model in which the available data for AF1/tau1 are accommodated in the context of holo-receptor. This model leaves open the possibility that additional interactions occur between the proteins binding at AF1 and other parts of the GR, *e.g.* AF2. Some reports suggest that GR AF1 and AF2 work in conjunction with each other through the DRIP complex (41). Conformational changes from other factors such as cross-domain communication, binding to glucocorticoid response elements, or even steroid binding could also play a significant role.

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REFERENCES

1. Dahlman-Wright, K., Almlof, T., McEwan, I. J., Gustafsson, J. A., and Wright, A. P. H. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1619–1923
2. Giguere, V., Hollenberg, S. M., Rosenfeld, M. G., and Evans, R. M. (1986) *Cell* **46,** 645–652
3. Hollenberg, S. M., and Evans, R. M. (1988) *Cell* **55,** 899–906
4. Dahlman-Wright, K., Baumannm, H., McEwan, I. J., Almlof, T., Wright, A. P. H., Gustafsson, J. A., and Hard, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 1699–1703
5. Baskakov, I. V., Kumar, R., Srinivasan, G., Ji, Y., Bolen, D. W., and Thompson, E. B. (1999) *J. Biol. Chem.* **274,** 10693–10696
6. Almlof, T., Gustafsson, J. A., and Wright, A. P. H. (1998) *Biochemistry* **37,** 9586–9594
7. McKenna, J., Lanz, B., and O'Malley, B. W. (1999) *Endocr. Rev.* **20,** 321–344
8. Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (1997) *Curr. Opin. Cell Biol.* **9,** 222–232
9. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996) *Mol. Endocrinol.* **10,** 1167–1177
10. McEwan, I. J., Wright, A. P. H., and Gustafsson, J. A. (1997) *BioEssays* **19,** 153–160
11. Hong, H., Kohli, K., TrivediA., Johnson, D. L., and Stallcup, M. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 4948–4952
12. Cavailles, V., Dauvois, S. S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. (1995) *EMBO J.* **14,** 3741–3751
13. Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1995) *Science* **270,** 1354–1357
14. Chrivia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) *Nature* **365,** 855–859
15. Henriksson, A., Almlof, T., Ford, J., McEwan, I. J., Gustafsson, J. A., and Wright, A. P. H. (1997) *Mol. Cell. Biol.* **17,** 3065–3073
16. Ford, J., McEwan, I. J., Wright, A. P., and Gustafsson, J. A. (1997) *Mol. Endocrinol.* **11,** 1467–1175
17. Warnmark, A., Gustafsson, J. A., and Wright, A. P. H. (2000) *J. Biol. Chem.* **275,** 15014–15018
18. Tsai, S. Y., Srinivasan, G., Allan, G. F., Thompson, E. B., and O'Malley, B. W. (1990) *J. Biol. Chem.* **265,** 17055–17061
19. Mc Ewan, I. J., Dahlman-Wright, K., Ford, J., and Wright, A. P. H. (1996) *Biochemistry* **35,** 9584–9593
20. Shen, F., Triezenberg, S. J., Hensley, P., Porter, D., and Knutson, J. R. (1996) *J. Biol. Chem.* **271,** 4827–4837
21. Back, J. F., Oakenfull, D., and Smith, M. B. (1979) *Biochemistry* **18,** 5191–5199
22. Yancey, P. H., Clarke, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. (1982) *Science* **217,** 1214–1222
23. Burg, M. D. (1995) *Am. J. Physiol.* **268,** F983–F996
24. Lee, J. C., and Timasheff, S. N. (1981) *J. Biol. Chem.* **256,** 7193–7201
25. Liu, Y., and Bolen, D. W. (1995) *Biochemistry* **34,** 12884–12891
26. Qu, Y., Bolen, C. L., Bolen, D. W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 9268–9273
27. Baskakov, I. V., and Bolen, D. W. (1998) *J. Biol. Chem.* **273,** 4831–4834
28. Baskakov, I. V., and Bolen, D. W. (1998) *Biophys. J.* **74,** 2666–2673
29. Matsudaria, P. (1987) *J. Biol. Chem.* **262,** 10035–10038
30. Stafford, W. F. (1992) *Anal. Biochem.* **203,** 295–301
31. Van Holde, K. E. (1971) *Physical Biochemistry*, pp. 98–121, Prentice-Hall, Englewood Cliffs, NJ
32. Johnson, M. L., and Fraiser, S. C. (1985) *Methods Enzymol.* **117,** 301–342
33. Johnson, M. L., Correia, J. J., Yphantis, D. A., and Holvorson, H. R. (1981) *Biophys. J.* **36,** 575–588
34. Chen, H., Srinivasan, G., and Thompson, E. B. (1997) *J. Biol. Chem.* **272,** 25873–25880
35. Baldwin, R. L., and Rose, D. G. (1999) *Trends Biochem. Sci.* **24,** 26–33
36. Dalby, P. A., Oliveberg, M., and Fersht, A. R. (1998) *J. Mol. Biol.* **276,** 625–646
37. Chervenka, C. H. (1969) *A Manual of Methods for the Analytical Ultracentrifuge,* p. 45, Spinco Division, Beckman Instruments, Palo Alto
38. Tjian, R., and Maniatis, T. (1994) *Cell* **77,** 5–8
39. Kumar, R., Baskakov, I. V., Srinivasan, G., Bolen, D. W., Lee, J. C., and Thompson, E. B. (1999) *J. Biol. Chem.* **274,** 24737–24741
40. Bain, D. L., Franden, M. A., McManaman, J. L., Takimoto, G. S., and Horwitz, K. B. (2000) *J. Biol. Chem.* **275,** 7313–7320
41. Hittelman, A. B., Burakov, D., Iniguez-Lluhi, J. A., Freedman, L. P., and Gorabedian, M. J. (1999) *EMBO J.* **18,** 5380–5388
42. Santro, M. M., and Bolen, D. W. (1988) *Biochemistry* **27,** 8063–8068