Trimethylamine \( N \)-Oxide-induced Cooperative Folding of an Intrinsically Unfolded Transcription-activating Fragment of Human Glucocorticoid Receptor*

(Received for publication, January 11, 1999, and in revised form, February 4, 1999)

Ilia V. Baskakov, Raj Kumar, Gainesan Srinivasan, Yan-shan Ji, D. Wayne Bolen, and E. Brad Thompson‡

From the Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, Texas 77555-0645

A number of biologically important proteins or protein domains identified recently are fully or partially unstructured (unfolded). Methods that allow studies of the propensity of such proteins to fold naturally are valuable. The traditional biophysical approaches using alcohols to drive \( \alpha \)-helix formation raise serious questions of the relevance of alcohol-induced structure to the biologically important conformations. Recently we illustrated the extraordinary capability of the naturally occurring solute, trimethylamine \( N \)-oxide (TMAO), to force two unfolded proteins to fold to native-like species with significant functional activity. In the present work we apply this technique to recombinant human glucocorticoid receptor fragments consisting of residues 1–500 and residues 77–262. CD and fluorescence spectroscopy showed that both were largely disordered in aqueous solution. TMAO induced a condensed structure in the large fragment, indicated by the substantial enhancement in intrinsic fluorescence and blue shift of fluorescent maxima. CD spectroscopy demonstrated that the TMAO-induced structure is different from the \( \alpha \)-helix-rich conformation driven by trifluoroethanol (TFE). In contrast to TFE, the conformational transition of the 1–500 fragment induced by TMAO is cooperative, a condition characteristic of proteins with unique structures.

An increasing number of biologically important proteins and protein domains have been found to be only partially structured or unstructured (unfolded) under physiological conditions (1). Notably, many of the nuclear transcription factors show disordered transactivation domains in aqueous solution (2). It is generally accepted that the structural uniqueness of proteins determines their biological function. Hence, the identification of unstructured proteins raises the question: what is the structural basis of the functional activity of such proteins/domains? Whether they act being in unfolded state (“natively unfolded” proteins) or adopt structure upon specific interaction with target molecules is a crucial question. The induced-fit and acidic blob models of the function for such transcription factor proteins represent two opposite points of view (3). Hence, methods that allow studies of the propensity of proteins to fold naturally are valuable.

Alcohols (trifluoroethanol, chloroethanol) have long been used to probe the propensity of unstructured protein/domain to form secondary structure (4–6). Their use has in part been based on the assumption that alcohols might mimic the in vivo conditions under which the disordered domain interacts with a target molecule. It has long been known, however, that alcohols favor the \( \alpha \)-helical conformation in peptides or proteins regardless of the type of the secondary structure the proteins/peptides form in the biologically relevant (native) conformation (7–9). Hence, until interacting partners of unstructured domains are identified, the current biophysical approaches using such alcohols to drive \( \alpha \)-helix formation present serious difficulties in interpreting results in the context of biology.

Recently we demonstrated the extraordinary ability of a naturally occurring solute, trimethylamine \( N \)-oxide (TMAO), to force thermodynamically unstable proteins to fold (10). Based on the two examples studied, we have shown that TMAO can increase the population of native state relative to denatured state by several orders of magnitude. These proteins regained high functional activity in the presence of TMAO. The present work addresses the question of whether TMAO can induce an unstructured region of transcription factor for which ordered conformation has never been identified to adopt a unique structure.

We studied large fragments of recombinant human glucocorticoid receptor (hGR, Fig. 1), the protein that mediates the action of glucocorticoids, hormones essential for human life. The hGR is a required intermediate in the physiological and many of pharmacological actions of the glucocorticoids, compounds often used for the treatment of lymphomas and leukemias and to inhibit the immune response. The hGR has a complex modular structure consisting of several domains: steroid-binding, DNA-binding, and two activation function domains (AF1 and AF2), which are acidic regions responsible for GR’s post-DNA-binding transactivation potential (11). The DNA-binding domains have defined secondary and tertiary structure (12, 13), and by analogy with those of other steroid receptors, so does the steroid-binding domain (14). The isolated AF1 has been found to be unstructured in aqueous solution (4). Here, we show that TMAO cooperatively induces structure in the hGR 1–500 fragment (GR 1–500) and that the TMAO-induced structure is different from the \( \alpha \)-helix-rich conformation driven by TFE. In the smaller AF1 fragment (residues 77–262) both TMAO and TFE induced similar structures.

* This work was supported by NCI Grant CA41407 (to E. B. T.) and NIGMS Grant GM 49760 (to D. W. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Human Biological Chemistry and Genetics, University of Texas Medical Branch, 301 University Blvd., 605 Basic Science Bldg., Galveston, TX 77555-0645. Tel.: 409-772-2271; Fax: 409-772-5159; E-mail: bthompson@utmb.edu.

1 The abbreviations used are: TMAO, trimethylamine \( N \)-oxide; TFE, trifluoroethanol; hGR, human glucocorticoid receptor; DBD, DNA-binding domain; GR 1–500, recombinant hGR fragment containing residues 1–500; AF1, recombinant hGR transactivation domain 1 containing residues 77–262; AAD, acidic transactivation domain.
All Trp (bold vertical lines) and Tyr (thin vertical lines) residues are shown.

EXPERIMENTAL PROCEDURES

Solutions of TMAO (Sigma) were prepared as described by Baskakov and Bolen (15).

Construction and expression of GR 1–500 has been described (16, 17).

The expression vector contained a frameshift mutant of hGR coding for amino acids 1–500 plus codons for a unique 5-amino acid sequence followed by a stop codon. Cytosolic fractions were prepared from the cell pellet (18). The AF1 domain fragment was extracted from hGR cDNA digested with BglII and inserted into an expression vector pGEX-4T-1 (Amersham Pharmacia Biotech). The recombinant expression plasmid pGEX-4T(AF1) was selected and transformed into Escherichia coli BL21. The bacteria containing the recombinant vector for GST-AF1 were induced with isopropyl-β-D-thiogalactopyranoside (0.5 mM) for 3 h, lysed, and extracted. Insect cell cytosol containing the expressed protein (GST-GR 1–500) or bacterial extracts containing (GST-AF1) were loaded onto a glutathione-Sepharose column at 4 °C. After thorough washing of column, the GST-GR 1–500 or GST-AF1 bound to the resin was incubated with alkaline phosphatase for 30 min at room temperature to dephosphorylate the peptide. The hGR fragments were then cleaved from GST by digesting with thrombin at 4 °C for 4 h, collected, and concentrated using Amicon Centriprep units. Protein purity was analyzed by SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue R-250 and estimated to be greater than 90%.

Analyzed by SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue R-250 and estimated to be greater than 90%.

RESULTS

TMAO Forces the GR 1–500 Fragment to Fold in a Cooperative Manner—Fig. 1 diagrams the regions of the hGR studied, with the location of the Tyr and Trp residues therein. Fig. 2 presents the fluorescence emission spectra of GR 1–500 measured either upon excitation at 295 nm, to follow changes in the environment of Trp residues specifically, or upon excitation at 278 nm, in which emission arises from Tyr and Trp residues as well as being a result of energy transfer from Tyr to Trp residues. Thus, the latter protocol links Tyr probes distributed throughout the protein (Fig. 1) with fluorescence emission from the two Trp residues. Because a substantial fraction of the fluorescence probes of GR 1–500 located outside of the DBD sequence (both Trp residues, Trp214 and Trp280, and 7 out of 10 Tyr residues, Fig. 1), the intrinsic fluorescence reflects mainly changes involving the AF1 and adjacent regions. The quantum yield of the emission spectra obtained in aqueous solution and in a strongly denaturant conditions (7 M guanidine HCl) are similar, illustrating that the extra DBD portion of GR 1–500 is largely unfolded in this low-salt buffer solution. GR 1–500 is a 2.5-fold increase in quantum yield and a shift in emission maximum from 350 nm in the absence of TMAO to 331 nm in the presence of 3.5 mM TMAO. The fluorescence changes are typical of those accompanying the removal of aromatic residues from polar aqueous solution into a more hydrophobic environment. Both the increase in quantum yield and the blue shift in fluorescence maximum indicate the formation of compact structure in the presence of TMAO. TMAO induces the conformational transition in GR 1–500 in a cooperative manner, as shown by monitoring the level of fluorescence at 329 nm (upon excitation at 278 and 295 nm) and the shift in emission maximum, as a...
function of TMAO concentration (Fig. 2C).

GR 1–500 Adopts Different Secondary Structure in TMAO and TFE Solutions—GR 1–500 in the absence of solute shows considerable unfolded secondary structure in aqueous solution as measured by CD (Fig. 3A). It is reasonable to believe that the small amount of secondary structure observed is due to DBD, which is known to be an independently folding domain (12, 13). However, addition of TMAO caused significant changes in the CD spectra, consistent with reduction of random coil conformation and formation of secondary structure with a large contribution of α-helical structure. The absence of an isodichroic point (the single point of intersection of spectra) demonstrates that the conformational transition in TMAO cannot be described in terms of a simple two-state model. The broad peak observed at 218–222 nm in the presence of 2.37 M TMAO may arise from the contribution of conformation other than α-helix (perhaps β-strand). Unfortunately, we cannot estimate the contribution of β-strand conformation in the presence of TMAO by this method, due to high adsorption of the solvent in the far UV region. In the presence of TFE the CD spectra displays clear α-helical character with characteristic maxima at 190 nm and minima at 208 (Fig. 3B). As a first approximation, the conformational transition of GR 1–500 in TFE can be described in terms of a “random coil” to “α-helix” transition with the intersections of six spectra forming an approximate isodichroic point. In contrast to the TMAO-induced transition, which is cooperative, the TFE-induced conformational change is noncooperative (Fig. 3C), typical for the helix induction curves described for peptides in TFE/H₂O mixtures (19).

TFE and TMAO Induce Similar Conformational Change in the Isolated AF1—When the AF1, which lacks DBD, was expressed, both solutes, TFE and TMAO, induce similar CD changes (Fig. 4). With this peptide, addition of either TFE or TMAO decreases the amount of random coil character while promoting formation of α-helical structure. The only data suggesting that the TMAO-induced structure differs from the TFE-induced structure is that the two solvents produce different isodichroic points (202 nm in TFE and >205 nm in TMAO, Fig. 4). If we assume that both solutes induce only α-helical structure in hGR AAD, the efficacy of α-helix formation by TMAO and TFE is approximately equal (compare spectrum obtained in 3 M TMAO with spectrum measured in 20% TFE (2.8 M TFE)). Comparison of the differing CD spectra obtained with GR 1–500 and the AF1 in the presence of TMAO (Fig. 3A and Fig. 4) suggests that the DBD and/or regions adjacent to AF1 may be important for the conformational transition of AF1.

**DISCUSSION**

Our fluorescence and CD data support the earlier observation that the transactivation domain AF1 of the hGR is largely unstructured (4). Because the hGR with AF1 deleted has only 25–30% transactivation activity of the holo-hGR, the AF1 region is clearly important for determination of the level of transcription of genes that are under glucocorticoid regulation (20). The AF1 of the hGR is a member of a large family of activation domains (AAD) defined by their richness in acidic residues and little sequence similarity (2). Since many such domains are rich in acidic amino acids, previous work led to the idea that acidic residues are crucial for the function of AADs (21). According to the “acidic blob” concept (22), AADs do not adopt a defined structure in vivo, rather it functions by general ionic interactions with target proteins. However, mutagenesis studies of hGR AF1 demonstrate that acidity is not the most important determinant of activity, and negative charges per se are not sufficient for activation (20, 23). Rather, key hydrophobic amino acids appear to be crucial for activity (24). That the hGR
Folding of Unstructured Transactivation Region

AF1 might be structured in vivo is suggested by the pattern of AF1 degradation in cell-free extracts, which show defined degradation products that are inconsistent with the indiscriminate proteolysis expected for an unfolded peptide (20). Structural studies have shown that AADs have the propensity to form α-helical structure in the presence of alcohols, and several proline substitution mutants reduce both helix-forming potential and transactivation activity (4–6). Keeping in mind the ability of AADs to form α-helix in the presence of alcohols, one could hypothesize that acidic activation domains are unstructured in vivo until they adopt a more ordered conformation when directly involved in transcriptional activation, according to the induced-fit model of folding (3).

The major concern over using alcohols (TFE, chloroethanol) to probe secondary structures of peptides and proteins is the question of the relevance of alcohol-induced structure to the biologically important conformations of the proteins. These alcohols are such potent inducers of α-helices that helices are forced to occur in peptides/proteins, whereas such structures may be unlikely to exist in vivo (7–9). As an example, the work of Hoy et al. (25) with AADs from yeast GAL4 and GCN4 transcription factors demonstrated the ability of AADs to adopt a β-sheet conformation under slightly acidic pH condition, a conformation that is biologically important for interaction with target molecules (26). TFE, on the other hand, induces only α-helical conformation in AADs of GAL4 and GCN4 (25). This result emphasizes the importance of the question about the applicability of TFE in probing biologically relevant structures and illustrates the intrinsic plasticity of the transactivation region, i.e. the ability to adopt different conformations depending upon solution conditions.

Our work shows that: 1) the naturally occurring solute TMAO induces compact structure in the GR 1–500 as indicated by the substantial enhancement in intrinsic fluorescence and radiation products that are inconsistent with the indiscriminate denaturation products of Hoy helical conformation in AADs of GAL4 and GCN4 (25). This action of both solutes, TMAO and TFE, focus on the peptide backbone, and this ensures that the effect of both solutes are general in scope, because the backbone is the most prevalent structural element of the protein fabric. In opposition to TFE solution, the propensities of hydrophobic groups to interact with solvent are essentially the same in water as they are in TMAO solution (30). Thus, due to weakening the hydrophobic interactions the dominant effect of TFE on protein is denaturation with preferential formation of α-helices as a result of strengthening peptide hydrogen bonds, whereas TMAO forces unfolded proteins to fold by providing an additional force for folding that has no preference for any particular secondary structure. Based on the molecular origin of TMAO-driven protein folding, if biologically relevant structure can be induced into the transactivation region of hGR (or any other intrinsically unstructured AAD) without its target molecule, it is more likely to be induced by solutes (like TMAO) that have been selected in nature for their ability to fold and stabilize proteins than it is by alcohols.

REFERENCES

1. Weinreb, P. H., Zhen, W., Poon, A. W., Conway, K. A., and Lansbury, P. T. (1996) Biochemistry 35, 13709–13715
2. Hahn, S. (1995) Cell 72, 481–483
3. Frankel, A. D., and Kim, P. S. (1991) Cell 65, 717–719
4. Dahlman-Wright, K., Baumann, 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. Schmitz, M. L., Silva, M. A. S., Altmann, H., Czisch, M., Holak, T. A., and Baurele, P. A. (1994) J. Biol. Chem. 269, 25613–25620
6. Donaldson, L., and Capone, J. P. (1992) J. Biol. Chem. 267, 1411–1414
7. Buck, M., Schwabhe, H., and Dobson, C. M. (1995) Biochemistry 34, 13219–13223
8. Fan, P., Bracken, C., and Baum, J. (1999) Biochemistry 38, 1573–1582
9. Sönnichsen, F. D., Van Eyk, J. E., Hodges, R. S., and Sykes, B. D. (1992) Biochemistry 31, 8790–8798
10. Baskakov, I. V., and Bolen, D. W. (1998) J. Biol. Chem. 273, 4831–4834
11. Beato, M. (1989) Cell 56, 335–344
12. Hard, T., Kellenbach, E., Boehles, R., Maler, B. A., Dahlman, K., Friedman, L. P., Carlstedt-Duke, J., Yamamoto, K. R., Gustafsson, J.-A., and Kaptein, R. (1990) Science 249, 357–360
13. Luisi, B. F., Xu, W. X., Owinskiowski, Z., Friedman, L. P., Yamamoto, K. R., and Sigler, P. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1699–1703
14. Wurtz, J.-M., Bourguet, W., Renaud, J.-P., Vivat, V., Champon, P., Moras, D., and Gronemeyer, H. (1993) Nat. Struct. Biol. 3, 97–95
15. Baskakov, I. V., and Bolen, D. W. (1998) Biophys. J. 74, 2666–2673
16. Chen, H., Srinivasan, G., and Thompson, E., B. (1993) J. Biol. Chem. 267, 25873–25880
17. Graham, F. L., and Van der Eb, A. J. (1973) Virology 52, 456–467
18. Srinivasan, G., and Thompson, E. B. (1990) Mol. Endocrinol. 4, 209–216
19. Luo, P., and Baldwin, R. L. (1997) Biochemistry 36, 8413–8421
20. Almlof, T., Wright, A. P. H., and Gustafsson, J.-A. (1996) J. Biol. Chem. 271, 17535–17540
21. Mitchell, P. J., and Tjian, R. (1989) Science 245, 371–378
22. Sigler, P. B. (1988) Nature 333, 210–212
23. Mason, S. A., and Housley, P. R. (1993) Biochemistry 32, 5879–5894
24. Hoy, M. V., Leuther, K. K., Kodadek, T., and Johnston, S. A. (1993) Cell 72, 587–594
25. Leuther, K. K., Salmeron, J. M., and Johnston, S. A. (1993) Cell 72, 575–585
26. Yancey, P. H., Clark, M. E., Hand, S. C., Bowls, R. D., and Sonner, G. N. (1982) Science 217, 1214–1222
27. Cammers-Grabowin, A., Allen, T. J., Oslick, S. L., McClure, K. F., Lee, J. H., and Kemp, D. S. (1996) J. Am. Chem. Soc. 118, 3082–3096
28. Luo, P., and Baldwin, R. L. (1998) J. Biol. Chem. 273, 49–57
29. Wang, A., and Bolen, D. W. (1997) Biochemistry 36, 9101–9108
30. Santoro, M. M., and Bolen, D. W. (1988) Biochemistry 27, 8063–8068