The N-terminal Region of Human Progesterone B-receptors

BIOPHYSICAL AND BIOCHEMICAL COMPARISON TO A-RECEPTORS

Received for publication, March 22, 2001
Published, JBC Papers in Press, April 27, 2001, DOI 10.1074/jbc.M102611200

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To understand the basis for functional differences between the two human progesterone receptors (PR), we have carried out a detailed biochemical and biophysical analysis of the N-terminal region of each isoform. Extending our previous work on the A-isoform (Bain, D. L., Franden, M. A., McManaman, J. L., Takimoto, G. S., and Horwitz, K. B. (2000) J. Biol. Chem. 275, 7313–7320), here we present studies on the N-terminal region of the B-isoform (NT-B) and compare its properties to its A-receptor counterpart (NT-A). As seen previously with NT-A, NT-B is quantitatively monomeric in solution, yet undergoes N-terminal-mediated assembly upon DNA binding. Limited proteolysis, microsequencing, and sedimentation analyses indicate that the B-isoform exists in a non-globular, extended conformation very similar to that of NT-A. Additionally, the 164 amino acids unique to the B-isoform (BUS) appear to be in a more extended conformation relative to sequences common to both receptors and do not exist as an independent structural domain. However, sedimentation studies of NT-A and NT-B show differences in the ensemble distribution of their conformational states. We hypothesize that isoform-specific functional differences are not due to structural differences, per se. Rather, the transcriptional element BUS, or possibly other transcription factors, causes a redistribution of the conformational ensemble by stabilizing a more functionally active set of conformations in NT-B.

Progesterone receptors (PR) are members of the nuclear receptor family of ligand-dependent transcription factors (1). In normal and malignant tissues there are two, naturally occurring, PR isoforms, 82-kDa A-receptors and 99-kDa B-receptors (2). The proteins are identical except for a 164-amino acid extension (BUS) at the N terminus of B-receptors. Each isoform contains two well characterized autonomous units: a C-terminal hormone binding domain (HBD), and a centrally located DNA binding domain (DBD). The two domains are linked by a short nuclear localization signal and a 50-amino acid “hinge” sequence of unclear function. Though critically important to activity, the DBD and HBD together comprise only 52 and 44% of the mass of the A- and B-receptors, respectively. The remaining sequences N-terminal to the DBD are poorly characterized. The N-terminal regions of both isoforms contain a 91-amino acid transcriptional activation domain (AF1) adjacent to the DBD (3). Additionally, B-receptors contain a context-dependent activation domain (AF3) within BUS (4). The 291-amino acid region between BUS and AF1 is transcriptionally inhibitory (5). Despite their close structural similarity, the two isoforms have pronounced functional differences on artificial promoters. B-receptors are generally stronger transcriptional activators than A-receptors (8). Certain antiprogestins are partial agonists for B-receptors but lack transcriptional activity on A-receptors (5). Activated A-receptors are dominant inhibitors of B-receptors and other members of the steroid receptor family (6).

A- and B-receptors generate their isoform-specific functional effects by the presence of BUS. However, the mechanistic basis for this is unclear. Because B-receptors are generally stronger transactivators than A-receptors, BUS was thought to be a classical activation domain. However, the 164 residues unique to B-receptors do not fit the traditional definition of a transactivator, because they do not activate when linked to a heterologous DBD (7). Later work revealed that BUS is capable of direct transactivation when linked to its homologous DBD, indicating that its functional properties are influenced by its surroundings; i.e. its function is context-dependent (4). Thus BUS may indirectly influence transcriptional activity by modulating the function of B-receptors, perhaps by recruitment of coactivating proteins (7) or by directly influencing the structural properties of neighboring PR activation domains.

The context-dependent properties of BUS indicate that surrounding PR structure may influence BUS function, even as BUS may influence or modulate other regions of PR. This effect may be related to the structural stabilization that the DBD confers toward the N-terminal region of NT-A (8). Taken together, these observations make it difficult to think of PR function in terms of a simple “sum of parts.” Rather, the data suggest the presence of long-range interactions typical of multidomain regulatory proteins. Some of these effects may be explained by induced fold or allosteric mechanisms proposed to explain activation domain properties (9, 10). However, isoform-specific functional differences, by proteins with near identical primary sequences and made up of context-dependent proper
ties, suggest that intrinsic structural differences might also underlie the functional effects.

The activation properties within the N-terminal sequences of PR originally led us to hypothesize that context-dependent structural differences within these regions might explain differences in PR isoform function. Toward this end, we carried out a detailed analysis of the N-terminal region of A-receptors (NT-A), demonstrating that NT-A is a structured, functionally active construct, amenable to rigorous biophysical analysis (8). Here we extend this work, characterizing the N-terminal region of B-receptors (NT-B) using biochemical, biophysical, and functional assays. We show that the regions common to both NT-B and NT-A appear to be similarly structured, whereas the 164-amino acid BUS region appears to be in a largely open, non-globular conformation. In light of recent biochemical studies of protein stability and structure (11–14 and references therein), our data are consistent with a model in which each PR isoform can exist as an ensemble of conformational states. Accordingly, the unique functional properties of the A- and B-isoforms may reflect differential stabilization of conformations with distinct transactivation properties.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of NT-B—cDNA encoding NT-B (amino acids 1–688) was cloned into the baculovirus expression vector pVL1392 (Invitrogen, Carlsbad, CA). The construct was expressed in baculovirus-infected Sf9 insect cells as described previously (15). Whole-insect-cell extracts were prepared as described previously (16). NT-B was purified by the slow addition of an equal volume of 40% saturated ammonium sulfate to the whole-cell extract. The precipitate was centrifuged, and the resuspended pellet containing NT-B was dialyzed and chromatographed on a Q-Sepharose column. After washing and elution, NT-B was concentrated and chromatographed on a heparin-Sepharose column. Final purification of NT-B was achieved using an SP-Sepharose column. NT-B was judged to be 95% pure by quantitation of Coomassie Blue-stained SDS-PAGE (Fig. 1b). Matrix-assisted laser desorption time-of-flight mass spectrometry and N-terminal microsequencing were used to confirm the integrity of the protein. NT-B concentration was determined using an extinction coefficient of 0.56 ml/mg/cm (17). Typical yields for NT-B were 5–10 mg per liter of culture. NT-B was purified as previously described (15).

**In Vitro Transcription Assay—**The activity of purified NT-A and NT-B was assayed by *in vitro* transcription as described by Klein-Hitpass et al. (18) with minor modifications. Briefly, 1–8 μl of NT-A or NT-B were combined with 2 μl of a DNA mixture containing 100 ng of a PRE<sub>TATA</sub> and TATA template linked to a G-free reporter cassette, and 25 ng of an internal control template made up of an adenovirus major late promoter (pMLMV200) fragment linked upstream of a G-free cassette. The reaction mixture also contained 0.5 mM ATP and CTP, 1.0 mM 3'-O-methyl-GTP and 20 μM [α<sup>32</sup>P]UTP (specific activity 25 Ci/mmole). The samples were processed as described previously (18), and labeled RNA run-off fragments were separated by electrophoresis on a 6% polyacrylamide, 7% urea gel. Signals were visualized by autoradiography and quantitated by phosphorimaging. 3'-End-labeled and pMLMV200 reporter templates were kindly provided by M.-J. Tsai (Houston, TX).

**Sedimentation Equilibrium—**Sedimentation was carried out with a Beckman XL-A analytical ultracentrifuge, using a six-channel, Epon reporter templates were kindly provided by M.-J. Tsai (Houston, TX).

**Filter Binding Analysis—**Filter binding conditions were 20 mM Hepes, pH 8.0, 50 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mg/ml BSA (ultra-grade), and 0.002 mg/ml double-stranded salmon sperm DNA, carried out at room temperature. BSA and nonspecific DNA were included in each reaction mix to minimize protein loss, and background retention, respectively. Each reaction mix contained a 32<sup>P</sup>-end-labeled, 27-bp DNA oligomer that included 50 pM of a 15-bp palindromic tyrosine amino transferase PRE (28) and increasing concentrations of NT-B. Each sample was applied to a nitrocellulose filter, followed by a wash using the above buffer (less BSA and salmon sperm DNA). Retained protein-DNA complexes were quantified by scintillation counting. Binding was quantified as the background corrected retained counts, divided by total counts. The presence of nonspecific DNA had no effect on the measured binding transition.

**Limited Proteolysis—**EndoGluC and chymotrypsin were of sequencing grade. Bromelain was of reagent grade. Digestion was carried out with 1 μM NT-B in 20 mM Hepes, 50 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 2 mM DTT, pH 8.0, at room temperature. EndoGluC was added at an NT-A:enzyme mass ratio of 20:1. Bromelain and chymotrypsin were added at 200:1. Each reaction was allowed to proceed for 2 h with aliquots taken as a function of time. Reactions were terminated by addition of SDS loading dye and boiling of sample. Aliquots containing 1 μg of total NT-B per lane were electrophoresed on 12.5% SDS-PAGE and either stained with silver (29) or transferred to nitrocellulose for immunoblot analysis. Three antibodies were used for immunoblot analysis: AB-2, a monoclonal antibody specific to residues Val221 to Leu237 (30); and B-30, a monoclonal antibody specific for the 164 amino acid unique to B-receptors. (30). Digestion of NT-B in the presence of saturating amounts of PRE (determined by stoichiometric filter binding analysis, not shown) was carried out under identical conditions. For protein microsequencing, 30 μg of NT-B was digested per time point, electrophoresed, and transferred to a polyvinylidene difluoride membrane. Major bands were sequenced by six cycles of Edman degradation (32).

**RESULTS**

**NT-B Is an Autonomous Transcriptional Unit—**The two full-length PR isoforms are diagrammed in Fig. 1a. Each receptor contains a centrally positioned DBD and a C-terminal HBD separated by a hinge sequence (H). Both receptors contain transcriptional activation domains in the N-terminal region (AF1) and in the HBD (AF2); B-receptors have a unique AF3. NT-B and NT-A, used in the studies below, lack only the HBD. Milligram quantities of NT-B (amino acids 1–688) were purified from baculovirus-infected Sf9 insect cells. SDS-PAGE analysis showed that purified NT-B was 95% pure based on densitometric scans of Coomassie Blue-stained gels (Fig. 1b).

Microsequencing of purified NT-B resolved the second zinc finger of the DBD (31); and B-30, a monoclonal antibody specific for residues Val221 to Leu237 (30), elicited a polyclonal antibody raised against the peptide Val611 to Cys627 corresponding to the second zinc finger of the DBD (31); and B-30, a monoclonal antibody specific for the 164 amino acids unique to B-receptors. (30). Digestion of NT-B in the presence of saturating amounts of PRE (determined by stoichiometric filter binding analysis, not shown) was carried out under identical conditions. For protein microsequencing, 30 μg of NT-B was digested per time point, electrophoresed, and transferred to a polyvinylidene difluoride membrane. Major bands were sequenced by six cycles of Edman degradation (32).

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The functional activity of NT-B was tested in a PRE-dependent *in vitro* transcription assay (18). Fig. 1c compares the protein-DNA activity of purified NT-B with purified NT-A. Both NT-A and NT-B enhanced transcription above background. Although NT-B is at least ten times more transcriptionally active than NT-A in intracellular assays (5), this difference is not observed in this cell-free assay. Transcription was entirely blocked by a 100-fold excess of wild-type PRE but not by a PRE mutant (PREmut) carrying a single base pair
substitution in each half-site (data not shown, see Ref. 8). This activation was specific for the N-terminal activation domains, because highly purified DBD showed minimal transcriptional activity (8).

**Sedimentation Equilibrium Demonstrates That NT-B Is a Monomer in Solution**—Fig. 2 shows the results of a series of sedimentation equilibrium studies carried out at pH 8.0, 100 mM NaCl, and 4 °C. These data consist of three different initial loading concentrations of NT-B covering a 10-fold concentration range (17, 5.1, and 1.7 μM, Fig. 2, A, B, and C, respectively). Each sample was allowed to reach equilibrium at three different rotor speeds (16,000, 24,000, and 32,000 rpm). Simultaneous analysis of all nine data sets resolved a weight-average molecular mass for NT-B of 68,556 ± 2,779 Da, in close agreement with the predicted monomer molecular mass of 70,848 Da. The square root of the variance was 0.0087 absorption units. Analysis of any individual data set to a monomer model resulted in a comparable molecular weight and quality of fit. Attempts to fit the data to any other model (e.g. monomer-dimer equilibrium) yielded poorer fits or failed to converge. Over a wide range of conditions we could find no evidence for NT-B solution self-assembly.

**NT-B Binds to a PRE Cooperatively**—The DNA binding of NT-B to a PRE was analyzed using the nitrocellulose filter binding assay (Fig. 3, squares) (33). Filter binding of NT-B under equilibrium conditions generated a steep binding transition at 40 nM, indicative of cooperative binding. (The curve is also sigmoidal in linear units.) Comparison to NT-A binding (Fig. 3, triangles) demonstrates that the data are essentially identical, indicating that BUS does not play a role in cooperative assembly to the PRE. In contrast, equilibrium binding of the isolated PR DBD to the PRE is ~10-fold weaker (8), indicating that N-terminal regions common to NT-A and NT-B play a role in cooperative assembly on the PRE.

**NT-B, Like NT-A, Has an Ordered Structure**—The above data demonstrate that NT-A and NT-B have very similar functional, self-association, and DNA binding properties. To determine whether the two isoforms have similar structural features, we carried out a detailed peptide mapping analysis of NT-B, using the highly specific protease EndoGluC, the semi-specific protease chymotrypsin, and the nonspecific protease bromelain, under identical conditions as used in our previous work on NT-A (8). Briefly, proteolysis was carried out in the absence or presence of a PRE, under conditions similar to those used in the DNA binding and sedimentation assays. Specific peptide fragments, generated under conditions of limited proteolysis, were visualized by silver staining (Figs. 4 and 5) and immunoblotting (not shown). The identities and cleavage sites of all major bands were directly established by protein microsequencing.

Proteolysis of NT-A and NT-B with EndoGluC in the absence (Fig. 4a) or presence (Fig. 4b) of a PRE generates a series of discrete fragments that are detectable by silver staining (shown) and by immunoblotting (not shown). EndoGluC generates a similar digestion pattern for NT-A and NT-B fragments of less than ~40 kDa. Indeed, microsequencing of fragments common to both NT-A and NT-B (fragments 3–8) resolved identical N-terminal sequences. The identity of each of these fragments, including the high molecular weight NT-A fragments, is summarized in Fig. 4c.

With regard to the higher molecular weight fragments of NT-B, immunoblot analysis of the digest using N-terminal antibodies suggested that the different patterns at high molecular weight were due to simultaneous cleavage of BUS at multiple sites (data not shown). Microsequencing of these fragments revealed multiple cleavage sites within BUS, suggestive of an extended, non-globular structure. This interpretation was confirmed by sedimentation velocity analysis (see below). Two major cleavage sites mapped to Glu-135 and Asp-150. We were unsuccessful in sequencing the remaining high molecular weight bands due to their low abundance and rapid degradation.

When NT-B is bound to a PRE (Fig. 4b), there are subtle changes in the proteolysis patterns characterized by the appearance of several new bands, and a change in stability of a subset of other bands (bands 4 and 6–8; Fig. 4b). As determined previously with NT-A, these effects are due to changes in stability of regions within the hinge and AF1. Overall, the presence of the PRE strongly stabilizes the hinge and subtly stabilizes AF1. Finally, the presence of BUS appears to have no detectable influence on the proteolytic profile either in the absence or presence of the PRE.

**NT-B Structure was also analyzed with the nonspecific protease bromelain, and the semispecific protease chymotrypsin (Fig. 5, a and b). Silver staining revealed that both proteases generated discrete fragments, indicative of structure within**
much of the N-terminal region of NT-B. Sequencing of these fragments resolved the same series of cleavage sites as seen previously with NT-A. Cleavage sites were mapped to Leu-316, Tyr-463, Leu-530, and Tyr-544 for chymotryptic cleavage, and Ser-344, Ala-407, and Ala-429 for cleavage by bromelain. Finally, as seen with the EndoGluC digest, we could detect no independent structure of BUS, suggesting that it is rapidly cleaved to small fragments.

Sedimentation Velocity Demonstrates That NT-B Is Homogeneous and Structurally Asymmetric in Solution—Sedimentation velocity analysis was used to establish the hydrodynamic shape and distribution of NT-B monomers. Fig. 6 shows the integral distribution plot of the data determined under identical conditions as the sedimentation equilibrium studies. NT-B migrates as a single hydrodynamic species as judged by the near vertical distribution of $S$ (solid circles). The change in $S$ over the range of the boundary is typically only 4%, indicative of a homogenous species (homogeneity being defined as a change of less than 5% (24)). The temperature- and buffer-corrected sedimentation coefficient ($S_{20,w}$) for NT-B was determined to be 2.70 S, which together with the experimentally determined molecular weight yielded a frictional coefficient ($f$) of 1.18$-\cdot 7$ $g/s$. Comparison of this value to the predicted frictional coefficient for a sphere of the same molecular weight ($f_s$) indicated that NT-B is extremely asymmetric with a frictional ratio ($f/f_s$) of 1.99. Modeling of NT-B as a prolate ellipsoid yielded a ratio of major to minor axes of ~20:1, with a Stokes radius of 63 Å. These results were not significantly affected even by assuming a maximal hydration value based upon the amino acid sequence of NT-B (0.41 g of H$_2$O/g of protein) (19).

NT-A sediments at essentially the same $S_{20,w}$ as NT-B (Fig. 6, open squares), although typically we see a rightward drift in the distribution from 2.5 to 2.8 S, indicative of a heterogeneous system (12% change in $S_{20,w}$). Because we previously demonstrated that NT-A is also strictly monomeric under these conditions (8), this result is not due to heterogeneity in molecular weight. Rather, this reflects heterogeneity in the frictional coefficient, $f$. Thus, under identical conditions, NT-A is structurally heterogeneous in contrast to NT-B.

**DISCUSSION**

Structural Properties of NT-B—The accessible cleavage sites of NT-B, mapped with proteases of widely different specificities, reflect the tertiary structure of the monomer. As seen previously for NT-A (8), these data map the structural boundaries of the AF1 (minimally, residues 463–530) and DBD functional domains (residues 560–638) within PR (3, 34), the proteolytic accessibility of previously identified phosphorylation motifs (Ser-190, Ser-294, Ser-345, Ser-400) (35–37), and to two residues that are chemically competent to be phosphorylated (Ser-232, Thr-320). The microsequencing data indicate that the enzymes make single, highly localized cuts. Obvious protease recognition sequences, even a few amino acids away, are protected from cleavage. Thus although the cleavage sites must be solvent-accessible, nearby residues appear to be in an ordered conformation.

Proteolysis in the presence of a PRE suggests that there are only subtle DNA-induced changes in NT-B structure (Fig. 4b). As described for NT-A (8), the appearance of more stable products as seen with NT-B is due to a PRE-dependent stabilization of the hinge region (bands 7 and 8) and of regions within and around AF1 (bands 4 and 6). Residues outside of AF1 and the hinge appear to be unaffected by DNA binding, indicating that the structural changes imposed by PRE binding are both subtle and localized.

We were able to obtain sequence from only two sites within BUS (Glu-135 and Asp-150) due to its instability. This region is rapidly attacked by all the proteases, and shows no evidence of independent structure (Figs. 4 and 5), suggesting that, even within the intact B-receptors, BUS appears to be in a non-globular conformation. Of the cleavage sites successfully sequenced in BUS, none were found to correlate with known phosphorylation sites, as seen with NT-A (8). Finally, we see no influence of the DBD or PRE on the structure of BUS.

Size and Shape of NT-B—Using sedimentation equilibrium,
we demonstrate that NT-B is exclusively monomeric in solution. This is in agreement with previous studies indicating that the HBD serves as the primary mediator of solution dimerization in full-length receptors (3) and is identical to our results with NT-A, demonstrating that BUS plays no role in solution assembly. The results further demonstrate that NT-B, like NT-A, is amenable to rigorous thermodynamic analysis, lending credence to our interpretations below.

Sedimentation velocity analysis (Fig. 6) shows that the sedimentation coefficient of NT-B (molecular mass of 71 kDa) is essentially identical to our previously determined value for NT-A (molecular mass of 59 kDa). The Svedberg equation (see “Experimental Procedures”) demonstrates that, with an increase in molecular weight, the frictional coefficient must also increase to maintain a constant S. This result translates into a frictional ratio of 1.99 for NT-B, indicative of enormous structural asymmetry. If our results are modeled as a prolate ellipsoid, the major to minor axis ratio of NT-B is 20:1. By comparison, the axial ratio of the A-receptor was estimated to be 9:1 (8). In conjunction with our proteolytic studies (Figs. 4 and 5) the hydrodynamic results suggest that BUS is in a very
extended, non-globular conformation that has no obvious influence on the remaining structure of NT-B.²

The observed deviation from symmetry as seen in the frictional ratio suggests that both NT-A and NT-B can be modeled as highly elongated, rod-like structures (prolate ellipsoids). Highly elongated molecules typically exhibit a strong concentration dependence to their sedimentation coefficients (24). However, our studies show that, over a 20-fold range of concentration, only minimal changes in S are detectable (data not shown). Moreover, analysis of sedimentation data by the method of Van Holde-Weischet (23) shows that structural elongation would generally be detected by a negatively sloping change in the distribution of S. Again, we see no such effect (Fig. 6). Taken together, these data suggest that the observed deviation from symmetry is due to a highly extended (i.e. non-compact) conformation rather than due to a true elliptical shape. This extended conformation translates into a larger effective volume due to the lower packing density of the NT-B polymer chain and thus leads to an increased frictional coefficient. This interpretation is perfectly consistent with our proteolysis data, the kinetics of each cleavage reaction suggest that NT-A and NT-B exist in conformations that, although structured, are highly protease accessible. Finally, a more open, possibly flexible structure is likely to have more conformational degrees of freedom and is consistent with the structural heterogeneity seen especially for NT-A (Fig. 6).

Possible Mechanisms of Action—We have postulated that the different functional effects of A- and B-receptors are due to different structural properties. However, much of our data suggest that NT-A and NT-B share very similar if not identical structures and features: Both are quantitatively monomeric in solution (Fig. 2), both bind a PRE with identical apparent affinity (Fig. 3), and each displays similar structural features (Figs. 4 and 5). Perhaps not surprisingly, both isoforms also appear to transcriptionally activate a reporter to a similar extent in vitro (Fig. 1c), although the transcription results, by their qualitative nature, are of limited utility for making mechanistic interpretations.

If indeed isolated NT-A and NT-B are structurally identical, then either coactivating proteins or possibly the HBD are necessary to induce structural differences in PR N-terminal regions. A role for the HBD is less likely, because NT-A and NT-B are functionally different in cellular transcription assays (5). However, this hypothesis cannot be tested until the putative coactivating proteins are identified, purified, and characterized as to their interactions with PR. Furthermore, this explanation begs the question as to how PR structural transitions occur, and how, mechanistically, they translate into isofor-specific functional effects. Nor does this hypothesis explain the differences seen in the structural homogeneity plots of NT-A and NT-B (Fig. 6).

The alternative explanation is that the native structures of the two isoforms are in fact different, yet the techniques that sample the macroscopic average of the system are incapable of detecting microscopic differences. We favor this interpretation for the following reason: Recent biophysical studies on protein stability and structure (for reviews, see Refs. 38–40) suggest that native protein structure should be considered as a statistical ensemble of conformational states rather than as a unique, singular conformation. Within the native state, even globular proteins undergo local folding and unfolding reactions, each representing a different structure. Taken as a collection, these structural states represent the conformational ensemble. Furthermore, the distribution of this ensemble reflects the functional properties of the protein: Of the many conformations present, some may be functionally active while others are not. For allosteric systems such as PR, where ligand or protein binding is linked to transcriptional activation, such binding will stabilize the subset of binding-competent structures. This will cause a shift in the distribution of the ensemble, enriching the population of transcriptionally active conformations. This viewpoint is a departure from traditional two-state models of structural transitions (e.g. “active versus inactive” and “folded versus unfolded”) that predict ligand binding induces a structural transition within a macromolecule.

Thus, the ensemble perspective may be applicable to understanding the properties of transactivation domains such as BUS. Isolated transactivation domains typically have minimal structure, existing as disordered, or unfolded conformational ensembles (9, and references therein). They are thought to function by acquiring structure either upon interaction with coactivating proteins or by allosteric effects induced by DNA binding (9, 10). From a statistical viewpoint, we might imagine instead that coactivating proteins, DNA response elements, or surrounding intramolecular protein structure stabilize a subset of folded conformations and thus shift the equilibrium to favor transcriptionally active (i.e. folded) structures.

Our proteolysis results can be interpreted within the framework of an ensemble hypothesis. Because limited proteolysis measures an average conformation over the population of structures, it may not resolve only subtle shifts in the ensemble distribution. Also, cleavage at structurally important residues may release conformational constraints, causing the protein structure to relax to a “low energy” state conformation, thus masking any initial structural differences in the ensemble distribution. In contrast, under equilibrium conditions, the ensemble hypothesis predicts that we should observe subtle differences in apparent structure between A and B due to different distributions of the conformational ensemble. We are currently investigating this possibility by hydroxyl-radical footprinting.

This view leads us to propose the following mechanism for A- versus B-isofrom functional differences. The greater degree of structural heterogeneity seen for NT- A (Fig. 6) indicates that it can take on a larger variety of conformations than NT-B. If some conformations within regions common to NT-A and NT-B are more transcriptionally active than others, for example, by presenting an activation domain surface in a more accessible state, a role for BUS may be to selectively stabilize and enrich those functionally active states. This mechanism is consistent with our observation that NT-B is homogeneous compared with NT-A. Furthermore, this provides a compelling rationale, based on biophysical theory and experiment, as to why the B-isofrom is generally a stronger activator than the A-isofrom. Importantly, this hypothesis also reconciles apparent contradictory data of BUS function. Namely, that BUS acts to modulate the A-receptor (7) by stabilizing one or more active states, yet it also acts as a transactivator (4) by taking on an active conformation due to the stabilizing influence of surrounding PR sequence. In summary, this scenario may describe only a restricted case of a larger theme; it is the sum of many small contributions from ligand binding, DNA binding, coactivator interactions, and intramolecular interactions that are necessary to stabilize the active conformer sufficiently enough to cause a shift in the conformational ensemble.

Acknowledgments—We thank Drs. Dean Edwards and Ming Tsai for constructs and Dr. James Goodrich for helpful discussions.

² Preliminary data employing hydroxyl radical footprint analysis of NT-B indicates that BUS is structured, yet highly solvent-accessible, as evidenced by the appearance of discrete footprints throughout the BUS region. Thus BUS does not maintain a random coil conformation within NT-B.
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