The role of the N-terminal region in nuclear receptor function was addressed by a biochemical and biophysical analysis of the progesterone receptor A-isoform lacking only the hormone binding domain (NT-A). Sedimentation studies demonstrate that NT-A is quantitatively monomeric, with a highly asymmetric shape. Contrary to dogma, the N-terminal region is structured as demonstrated by limited proteolysis. However, N-terminal structure is strongly stabilized by the DNA binding domain, possibly explaining the lack of structure seen in isolated activation domains. Upon DNA binding, NT-A undergoes N-terminal mediated assembly, suggestive of DNA-induced allostery, and consistent with changes in protease accessibility of sites outside the DNA binding domain. Microsequencing reveals that protease-accessible regions are limited to previously identified phosphorylation motifs and to functional domain boundaries.

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. Although 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 very different functional properties. B-receptors are generally stronger transcriptional activators than A-receptors (3). Certain antiprogestins are partial agonists on B-receptors but lack transcriptional activity on A-receptors (3). Activated A-receptors are dominant inhibitors of B-receptors and other members of the steroid receptor family (6).

With the exception of estrogen receptors, PR and other steroid receptors including androgen, glucocorticoid (GR), and mineralocorticoid receptors, have highly homologous DBDs and HBDs, and bind to similar DNA response elements (7). Their N-terminal regions differ extensively, however, ranging in size from 403 to 603 amino acids, with less than 15% amino acid identity. This suggests that structural differences within these regions explain the transcriptional specificity of these receptors. Moreover, in PR, N-terminal transcriptional activity is often dependent upon the homologous DBD (4, 5); N-terminal functional domains fused to heterologous DBDs can be transcriptionally inactive. These observations suggest that the function of the N-terminal region is structurally dependent on surrounding domains, particularly the DBD. Indeed, the DBDs and the progesterone response elements (PREs) to which they bind may act as allosteric effectors, transducing information to N-terminal AFs (8). For example, DNA binding does not alter the structure of the Ets-1 DBD, but induces helical unfolding in regions distant from the DNA binding interface (9). Alternatively, N-terminal regions may acquire structure upon interaction with target proteins such as other transcription factors and/or coregulatory proteins (10). Such "induced fold" mechanisms may also explain some of the context-dependent effects of steroid receptor N-terminal regions.

We speculate that context-dependent structural differences within the N-terminal regions, generated by either induced fold or allosteric mechanisms, may explain the differences in PR isoform function. Thus, we have undertaken structural studies of the PR N-terminal regions. Here we characterize the N-terminal region of the A-receptor (NT-A) (a construct lacking only the HBD) using biochemical, biophysical, and functional assays. Our data show that highly purified NT-A is capable of transactivation, specifically recognizes a PRE in a cooperative manner, and is highly structured. Our results are the first to map the structure within the N-terminal region of a nuclear receptor, and demonstrate that NT-A has the properties of an independent functional unit.

EXPERIMENTAL PROCEDURES

Expression and Purification of Tagged and Untagged NT-A—Tagged NT-A contains a 42-amino acid N-terminal hexahistidine sequence fol-
lowed by human PR amino acids 165–688 (generously provided by D. P.
Edwards). Untagged NT-A (amino acids 165–688) was cloned into the baculovirus expression vector pVL1392 (Invitrogen, Carlsbad, CA). Both constructs were expressed in baculovirus-infected Sf9 insect cells as described (11). Whole insect cell extracts were prepared as described (12). The precipitate was centrifuged, and the resuspended pellet containing NT-A was dialyzed, and chromatographed on a Q-Sepha-
rose column. After washing and elution, NT-A was concentrated and chromatographed on a Sephacryl S-200 column. Finally, NT-A was loaded onto a SP-Sepharose column. Both tagged and untagged NT-A were judged to be greater than 90% pure by quantitation of Coomassie Blue-stained SDS-PAGE. NT-A concentration was determined using an extinction coefficient of 0.55 ml/mg/cm (13). Typical yields for both constructs were 4–6 mg/liter of culture.

Expression and Purification of DBD—GST-DBD was constructed by polymerase chain reaction amplification of the human PR DBD (amino acids 556–645), and subcloned into a pGEX-5x-3 bacterial expression vector (Amersham Pharmacia Biotech). Escherichia coli BL-21 cells transformed with the recombinant plasmid were grown in LB medium, and induced with 0.1 mM isopropyl-thiogalactoside for 20 h at 25°C. The supernatant was centrifuged, and the resuspended pellet containing NT-A was dialyzed, and chromatographed on a Sephacryl S-200 column. After washing and elution, NT-A was concentrated and chromatographed on a Sephacryl S-200 column. Finally, NT-A was loaded onto a SP-Sepharose column. Both tagged and untagged NT-A were judged to be greater than 90% pure by quantitation of Coomassie Blue-stained SDS-PAGE. NT-A concentration was determined using an extinction coefficient of 0.55 ml/mg/cm (13). Typical yields for both constructs were 4–6 mg/liter of culture.

In Vitro Transcription Assay—The activity of purified receptors was assessed by in vitro transcription as described by Klein-Hitpaas et al. (14) with minor modifications. Briefly, 1–8 µg of NT-A or DBD was combined with 2 µl of a DNA mixture containing 100 ng of a PRE, TATA template linked upstream of a G-free cassette. The reaction mixture also contained 0.5 mM rATP and rCTP, 1.0 mM [32P]UTP (specific activity 25 Ci/mmol). The reactions were allowed to proceed for 2 h at 25°C. The products were separated by electrophoresis on a 12.5% SDS-PAGE, and either stained with silver (24) or transferred to nitrocellulose for immuno blot analysis. Two antibodies were used for immunoblot analysis: AB-52, a monoclonal antibody specific to residues Val-221 to Leu-237 (25); and α268, a polyclonal antibody raised against the peptide Val-611 to Cys-627 corresponding to the second zinc finger of the DBD (26). Digestion of NT-A 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-A was digested per time point, electrophoresed, and transferred to a polyvinylidene difluoride membrane. Major bands were sequenced by six cycles of Edman degradation (27).

RESULTS

NT-A Is an Autonomous Transcriptional Unit—The two 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 (AP1), and in the HBD (AP2); B-receptors have a unique AP3.

Milligram quantities of hexa-His-tagged and untagged NT-A (amino acids 165–688) were purified from baculovirus-infected Sf9 insect cells, and judged to be greater than 90% pure by densitometric scans of Coomassie Blue-stained SDS-polyacryl amide gels (Fig. 1b). The PR-DBD (amino acids 556–645) was expressed in E. coli as a GST fusion protein. The DBD was cleaved from GST by factor Xa digestion, and purified to homogeneity (Fig. 1b).

The functional activities of DBD and NT-A were tested in a PRE-dependent in vitro transcription assay (14). Fig. 1c compares the transcriptional activity of purified hexa-His-tagged NT-A with purified DBD. At increased DBD levels (5.6–56 pmol), there is weak transcriptional activation (3-fold over basal) of the reporter gene from the PRE-containing promoter. At similar levels, hexa-His-tagged NT-A enhanced transcription 30-fold. This transcription was entirely blocked by a 100-fold excess of wild-type PRE but not by PRE mutant PREmut carrying a single base pair substitution in each half-site. Untagged NT-A behaved identically to tagged NT-A (data not shown), indicating that the hexa-His tag has no discernible effect on the transactivation function of NT-A. All further studies employed the tagged protein.

Sedimentation Equilibrium Demonstrates That NT-A Is a Monomer in Solution—Accurate interpretation of structural influences on protein function requires knowledge of its assembly state, size, and hydrodynamic shape. Analytical ultracen-
Experimental Procedures.

**HBD** NT-A and the PR DBD. A–C, hormone binding domain; H, hinge; AF, activation function; BUS, B-upstream segment. NT-A includes amino acids 165–688. **b**, baculovirus-expressed NT-A was purified as described under "Experimental Procedures." 50 μg of NT-A and DBD were resolved by 15% SDS-PAGE and stained with Coomassie Blue. Lane 1, DBD; lane 2, tagged NT-A; lane 3, untagged NT-A. M, markers are shown on the right. NT-A and DBD were judged to be 90% and 98% pure, respectively. c, in vitro transcription of NT-A compared with DBD. Increasing concentrations of either purified NT-A or DBD were titrated into a transcription reaction mix containing a PRE2-TATA, a positive control AdML promoter linked to a G-free cassette, and 32P-labeled UTP, either in the presence or absence of wild-type or mutant PRE. Labeled RNA run-off fragments were resolved on a sequencing gel, visualized by autoradiography, and quantitated by phosphorimaging.

Sedimentation Velocity Demonstrates That NT-A Is Structurally Asymmetric in Solution—Sedimentation velocity analysis was used to establish the hydrodynamic shape of NT-A monomers. Fig. 3 shows the distribution of the apparent sedimentation coefficient determined under identical conditions as the sedimentation equilibrium studies. The distribution is well described by a gaussian curve, consistent with the presence of a single species. The peak s value corresponds to s0*, the sedimentation coefficient under these conditions. The temperature and buffer corrected sedimentation coefficient (s0,20) was calculated to be 2.75 s, which together with the experimentally determined molecular weight yielded a frictional coefficient (f) of 9.7e–8 g/s. Comparison of this value to the predicted frictional coefficient for a sphere of the same molecular weight (f0) indicated that NT-A is highly asymmetric with a frictional ratio (ff0) of 1.74. Modeling of NT-A as a prolate ellipsoid yielded a ratio of major to minor axes of approximately 9:1, with a Stokes radius of 52 Å.

**NT-A Binds to a PRE Cooperatively**—The DNA binding of NT-A and DBD to a PRE were compared (Fig. 4) using the nitrocellulose filter binding assay (28). Filter binding of NT-A under equilibrium conditions generated a steep binding transition at 70 nM, suggestive of cooperative binding. (The curve is also sigmoidal in linear units.) In contrast, equilibrium binding of the DBD to the PRE shows a broad binding curve with a binding transition at 700 nM, 10-fold weaker than the NT-A binding. This difference indicates that the N-terminal region of the A-receptor enhances DNA binding affinity. Additionally, these interactions are specific for a PRE, since binding studies using the mutated PRE described in the in vitro transcription assay resulted in no detectable signal (data not shown).

**NT-A Has an Ordered Structure That Is Stabilized by the DBD**—The above data demonstrate that the N-terminal region contributes to transactivation and influences the DNA binding properties of the A-receptor. To determine whether definable structural features explain these properties, we carried out a detailed peptide mapping analysis of NT-A, using the highly specific proteases EndoGluC and EndoArgC, the semispecific protease chymotrypsin, and the nonspecific protease bromelain. 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. All four proteases generated discrete digestion patterns, some of which developed immediately as brief intermediates, while others appeared only after extended exposure to protease (Figs. 5–7). The identities and cleavage sites of all major bands were directly established by protein microsequencing. The presence of N-terminal and/or DBD-containing sequences was determined by immunoblotting with AB-52, a monoclonal antibody specific to residues Val-221 to Leu-237 (25); and α266, a polyclonal antibody raised against the peptide Val-611 to Cys-627 in the second zinc finger of the DBD (26). These data, together with molecular weight estimates, were used to define the N terminus and estimate the C terminus of each major band. To verify that the digestion patterns of NT-A reflected meaningful structure, proteolysis was also carried out with denatured NT-A.
In contrast to the definitive structure observed with native NT-A, denatured NT-A was rapidly digested to small peptides that ran at the dye front (data not shown).

**EndoArgC Cleaves NT-A at a Limited Number of Sites**—Proteolysis of NT-A with EndoArgC in the absence of a PRE generates 10 major bands that are detectable by silver staining (Fig. 5a, left). Immunoblotting with a266 shows that each band contains the DBD (Fig. 5b), and immunoblotting with AB-52 identifies bands that retain N-terminal sequences (Fig. 5c). Microsequencing analysis (Fig. 5d) demonstrates that the bands fall into four sets. (i) One set of bands (bands 1–3) is cleaved at Arg-187. All three react with AB-52 and a DBD; therefore, the size variations must be due to variable cleavage within the hinge region. (ii) Another set of bands (bands 4 and 6) is cleaved at Arg-293. The bands have lost the AB-52 epitope but retain the DBD, and thus also vary in the length of the hinge. (iii) Two bands (bands 5 and 7) are cleaved at Arg-319 and retain the DBD; their variable size is again due to cleavage within the hinge. (iv) The last set of bands (bands 8–10) retains the DBD, but by molecular weight estimate, has lost the hinge. Band 8 is cleaved at Arg-402. It is slowly cleaved to band 9 by secondary cleavage at Arg-428, N-terminal to the currently defined border of AF1 (29). Upon extended exposure to EndoArgC, band 9 is eventually reduced to the limit band 10 by cleavage at Lys-481, 26 amino acids within the N-terminal border of AF1, and indicative of considerable structure within AF1.

The EndoArgC data are consistent with the presence of considerable structure in the A-receptor N-terminal region. Of the 16 arginine residues, only 4 are immediately accessible to EndoArgC (Arg-187, Arg-293, Arg-319, and Arg-402). However, only DBD-containing fragments retain stability; fragments lacking the DBD are immediately degraded (Fig. 5, compare b and c). This strongly suggests that the DBD plays a significant role in stabilizing the structure of the N-terminal region.

When NT-A is bound to a PRE, there are subtle changes in the EndoArgC proteolysis pattern characterized by an increase in the apparent molecular mass of bands above 30 kDa (compare PRE in Fig. 5a and b). Bands 8a, 8b, 8c on the right, like band 8 on the left, are cleaved at Arg-402. Thus, the higher molecular mass of bands 8a–8c is due to persistence of the hinge sequence. Stabilization of the hinge also explains the

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**FIG. 2.** Sedimentation equilibrium analysis of NT-A, plotted as absorbance versus radius. Panels represent each initial loading concentration: 22 μM (a), 6.6 μM (b), and 2.2 μM (c). Symbols represent NT-A absorbance at each rotor speed (squares, 18,000 rpm; circles, 27,000 rpm; triangles, 36,000 rpm). NT-A at 22 and 6.6 μM was scanned at 280 nm, and NT-A at 2.2 μM was scanned at 230 nm. Solid lines represent the best fit model (monomer) from simultaneous analysis of all nine data sets. Square root of the variance for the analysis was 0.0087 OD units. For clarity, only every fifth data point is shown. Conditions were 20 mM Hepes, pH 8.0, 100 mM NaCl, 2.5 mM MgCl₂, 1 mM DTT, 4 °C.

**FIG. 3.** Sedimentation coefficient distribution of NT-A as determined from sedimentation velocity. Distribution was determined by analysis of 25 successive scans taken 5 min apart at 50,000 rpm. The peak s* corresponds to the apparent sedimentation coefficient. Initial loading concentration of NT-A was 5.0 μM. Solution conditions were identical to sedimentation equilibrium studies.

**FIG. 4.** Filter binding analysis of NT-A and DBD under equilibrium binding conditions. Equilibrium binding titration of 32P-labeled PRE oligomer with either NT-A (triangles) or DBD (squares). [32P]PRE concentration in both experiments was estimated to be 50 pM. Data were not analyzed explicitly, due to the complexity of binding, but were simply fit to sigmoidal curves.
increased molecular mass of the bands analogous to bands 1–7; microsequencing of these bands revealed the same set of cleavage sites as seen in the absence of the PRE. One additional subtle change occurs in the presence of the PRE; proteolysis at Lys-481 (generating band 10) is retarded, concomitant with stabilization of band 9. Overall, the presence of the PRE strongly stabilizes the hinge and subtly stabilizes AF1.

Digestion with Low Specificity Enzymes Confirms That NT-A Is Structured—To rule out that the limited number of bands generated by EndoArgC were due to the limited number of cleavage sites for this highly specific enzyme, NT-A was also analyzed with the nonspecific protease, bromelain, and the semispecific protease, chymotrypsin (Fig. 6). Peptide fragments were analyzed by silver staining, immunoblotting, and microsequencing. Only the silver-stained bands are shown. Bromelain cleaves at any accessible peptide bond, yet only three bands appear at 5 min (Fig. 6a), suggesting that few sites are initially accessible to the protease. Band 1 is generated by cleavage at Ser-345, band 2 at Ala-407, and band 3 at Ala-429. Note that bands 2 and 3 are cleaved at sites near the EndoArgC-scissible residues, Arg-402 and Arg-428 (Fig. 5, bands 8 and 9). All three bands contain the DBD (data not shown), with the AB-52 monoclonal antibody. Numbered arrows refer to bands sequenced by Edman degradation and described in the text. Molecular weight markers are indicated. d, schematic of NT-A showing defined N-terminal borders and estimated C-terminal borders of EndoArgC fragments.
also consistent with the EndoArgC results. As with EndoArgC, no AB-52-positive bands were stable for more than 5 min. Thus, the nonspecific enzyme generated discrete cutting patterns analogous to those generated by the highly specific enzyme, and provides further evidence of significant structure within the N-terminal region. Finally, it is of interest that, in the absence of a PRE, bromelain will eventually digest the entire NT-A molecule, including the DBD (Fig. 6a), suggesting that the DBD is not entirely conformationally stable in solution.

Digestion of NT-A with chymotrypsin generates seven bands (Fig. 6b). Bands 1–3 correspond to cleavage at Leu-316, in close proximity to the cleavage site at Arg-319 seen with EndoArgC (bands 5 and 7). All three bands contain the DBD (data not shown), and thus vary in the length of the hinge. Band 4 contains two peptides: a DBD-positive sequence corresponding to cleavage at Tyr-463, and the hexa-His tag sequence. Band 5 also contains two peptides with the same sequences seen in band 4. Since the (Tyr-463-cleaved) bands 4 and 5 are also DBD-positive, the entire hinge region (−6 kDa) is likely removed from band 4 to generate band 5. The remaining (hexa-His-tagged) bands 4 and 5 likely correspond to the N-terminal fragments generated by cleavage at Tyr-463 and Leu-316, respectively. After 60 min, band 5 is cleaved to generate the limit bands 6 and 7, cleaved at Leu-530 and Tyr-544, respectively. Their molecular weights are consistent with cleavage at the C terminus of the DBD.

As with EndoArgC, the chymotryptic and bromelain digestion patterns of NT-A do not change dramatically in the presence of the PRE (Fig. 6, a and b, right). However, the PRE protects the DBD from bromelain cleavage; note the appearance of a stable 14–15-kDa, DBD-positive, band present only in the PRE-containing samples. Upon binding to the PRE, the DBD must become resistant to cleavage by bromelain, due either to steric occlusion by DNA, or to structural stabilization of the PRE-bound DBD. In the chymotryptic digest, appearance of the isolated DBD is accelerated by the presence of the PRE (Fig. 6b, bands 6 and 7). This is due to the accelerated proteolysis of band 5, suggesting that the region surrounding Leu-530 and Tyr-544, within AF1, becomes more solvent-accessible upon DNA binding. Finally, digestion with bromelain and chymotrypsin does not reveal a PRE-dependent stabilization of the hinge region, suggesting that any PRE-induced stability is only local in origin.

EndoGluC Digestion Demonstrates Independent Structure of the Entire N-Terminal Region—Digestion of NT-A with EndoGluC generated eight major bands by silver staining (Fig. 7, a and b). Band 1 begins at the hexa-His tag. Its size (−45 kDa) is consistent with a fragment terminating at Glu-560 (44.5 kDa), i.e. the entire N-terminal region lacking the DBD and hinge. The corresponding C-terminal fragment, Ser-561 through the hinge, is found in band 7. Band 7 is slowly cleaved to band 8 by release of the hinge, since its N terminus also begins at Ser-561. Band 8, the isolated DBD, is highly stable. Additionally, the persistence of band 1 suggests that the N-terminal region, when intact, maintains significant independent stability.

Band 1 is, however, slowly cleaved to generate bands 2 and 4. Band 2 (−40 kDa) begins at the hexa-His tag. Its molecular weight predicts that there must be an additional cleavage site within the N-terminal region. Indeed, sequencing of the faint band 6 (−17 kDa) revealed a cleavage at Glu-532. Thus band 2 is consistent with a fragment from the hexa-His tag to Glu-532 (41.3 kDa), while band 6 is likely to extend from Gly-533 through the hinge (17.4 kDa). Band 4 (−28 kDa) contains two peptides; one has the hexa-His tag, and the other is the product of cleavage at Glu-459. The molecular weights of the two peptides in band 4 are roughly consistent with one peptide extending from Cys-460 through the hinge (24.4 kDa), and a second peptide from the N terminus to Glu-459 (34.2 kDa). The rapid disappearance of the DBD-containing bands 4 and 6 is presumably due to rapid cleavage at Glu-560 to generate band 7.

The remaining bands, 3 and 5, are products of cleavage at Glu-231. The mobility of band 3 (−32 kDa) is consistent with a fragment from Ser-232 to Glu-560 (33.1 kDa). Band 5 (−23 kDa) is faint, and appears after 90 min (Fig. 7a). Its size is consistent with a Ser-232 to Glu-459 fragment (22.8 kDa). Taken together, these data indicate that within the N-terminal region of NT-A, only 3 of 23 glutamate residues are immediately accessible for cleavage by EndoGluC: Glu-231, Glu-459, and Glu-560.

No obvious effect of the PRE on NT-A structure was revealed by EndoGluC digestion (Fig. 7a). Note, however, that the first cleavage reaction (band 1) results in release of the DBD, which eliminates the DNA binding activity of NT-A. However, proteolysis at the hinge is clearly reduced when the isolated DBD is bound to the PRE (compare the rate of appearance of band 8, the isolated DBD, in the presence and absence of PRE). Additionally, the appearance of band 7 (DBD and the hinge) is slowed 15–30 min by the presence of the PRE. This is likely to be due to the reduced accessibility of Glu-560, and is consistent with the apparent increased stability of bands 4 and 6.

**DISCUSSION**

The Size and Shape of NT-A—Due to the thermodynamic linkage between protein self-assembly and DNA binding, any quantitative analysis of binding data necessitates a quantitative determination of solution assembly state (33). Using sedimentation equilibrium, we demonstrate that NT-A is exclusively monomeric in solution. This is completely consistent with the long held notion that the HBD serves as the primary mediator of solution dimerization in full-length receptors (1), and demonstrates that interactions between the DBD and the
N-terminal region must be intramolecular in nature. In contrast, Tetel et al. (12) recently reported that unpurified PR N-terminal regions are capable of dimeric interactions under solution conditions similar to those described here, based on a qualitative protein-protein interaction “pull-down” assay. However, this assay does not measure stoichiometry directly. Rather, it measures interactions, possibly due to polydisperse aggregation, as might be found in a cellular extract. In contrast, the defined conditions of sedimentation equilibrium used here provide a quantitative determination of assembly stoichiometry. Further, our data also show that highly purified NT-A is amenable to rigorous thermodynamic analysis, lending credence to the interpretation of the structural and hydrodynamic analyses.

Sedimentation velocity analysis shows that the sedimentation coefficient of NT-A (2.75 s) is smaller than that expected for a sphere of equivalent mass. This s value translates into a frictional ratio of 1.74, indicative of significant structural asymmetry. Such asymmetry increases the surface to volume ratio, creating extensive surfaces for the potential recruitment of coregulatory proteins, and consistent with our observations of multiple proteolytic cleavage sites for NT-A. More precise information about the structure of NT-A will require crystallographic analysis. However, if our solution data are modeled as a prolate ellipsoid, the major to minor axis ratio of NT-A is approximately 9:1, and corresponds to a major axis length of 260 Å. In such a model, NT-A would be capable of spanning 76 base pairs of linear, B-form DNA, comparable to the distance from a PRE binding element to the TATA box (4).

**DNA Binding of NT-A**—Previous studies have demonstrated cooperative binding of the isolated PR and GR DBD to a PRE (34, 35). However, our study demonstrates that NT-A has an approximately 10-fold greater DNA binding affinity relative to the DBD. Further, the shapes of the two binding curves indicate an apparent cooperative transition occurring with NT-A, which is absent with the DBD (due to both specific and non-specific binding, not shown). This phenomenon may not be limited to PR. N-terminal GR (GR lacking its HBD) also has an increased binding affinity to a GRE, relative to its DBD (36). The presence of the GR N-terminal region also results in greater discrimination between specific and nonspecific DNA (37). The GR results were interpreted as being due to steric effects (38), to additional protein-protein interactions occurring outside the DBD (35), or possibly to induction by the GRE (36). The PR and GR data could also be explained by a pseudo-cooperativity due to GR association in solution, because we have determined explicitly that NT-A does not associate in solution, we can conclude that for PR, the apparent cooperativity is due to *bona fide* allostery, and implicate the N-terminal region in this effect. This is also likely to explain the GR data since, relative to a non-cooperative system, cooperative interactions will increase the apparent DNA binding affinity. Finally, N-terminal GR has recently been reported to be monomeric in solution (39), consistent with our conclusion. The PR cooperativity presumably arises through interactions driven by the PRE, and transduced by the DBD. This predicts a conformational switch in the N-terminal region, resulting in formation of a more favorable N-terminal oligomerization interface. This interpretation is consistent with our proteolysis data, which indicate a PRE-induced change in stability of a subset of sites within AF1 (Fig. 8), and also with recent spectroscopic studies of GR demonstrating a PRE-dependent signal between the DBD and the N-terminal region (39). Thus our results may provide a molecular explanation for both the GR and PR data, and further, suggest that DNA-driven, N-terminal conformational switching may be common to the steroid receptor family.

**Structural Properties of NT-A**—Based on previous proteolytic studies of PR and GR (40, 41) and spectroscopic studies of N-terminal AFs lacking the DBD (42), it is generally thought that the N-terminal regions are unfolded. This conclusion, however, may have been premature, since these studies used techniques that would not have detected context-dependent structure within N-terminal sequences. Additionally, earlier proteolytic studies used stoichiometric or near-stoichiometric ratios of target protein to enzyme (43), which select for only the most stable of domains. Under conditions commonly used to probe protein structure, like the 20:1 or 200:1 protein to enzyme ratios used in this study, we find only a small subset of potential sites in NT-A available for attack, indicative of considerable structure. This conclusion is supported by the observation that urea-denatured NT-A was rapidly and completely degraded to small peptide fragments at even the lowest protease concentrations.

The accessible cleavage sites, mapped with proteases of widely different specificities, reflect the tertiary structure of monomeric NT-A (summarized in Fig. 8). These data provide direct evidence for the structural basis of the AF1 and DBD functional domains within PR (7, 29). Specifically, residues between 428 and 463 likely define the structural N-terminal boundary of AF1, and residues between 530 and 560 define its C-terminal boundary, and are consistent with the N-terminal boundary of the DBD (7). Further, the hinge region is readily removed when NT-A is in solution, consistent with the previously defined C-terminal boundary of the DBD (7). Importantly, there are many obvious additional sites for enzymatic attack very close to the mapped sites surrounding, and internal to, AF1, but we find no evidence for proteolysis at those sites. This suggests that AF1 is tightly folded, at least when linked to the DBD. We also note that EndoArgC preferentially cleaves at Lys-481 (band 10), despite the presence of a nearby arginine (Arg-492). EndoArgC does not typically cleave at lysines, suggesting that Lys-481 is in a non-canonical conformation.

The remaining cleavage sites occur within the poorly understood region N-terminal to AF1 (Fig. 8). Six clustered sites show a remarkable correlation to the location of all four previously identified phosphorylated residues (Ser-190, Ser-294, Ser-345, and Ser-400) (30–32), and to two residues that are chemically competent to be phosphorylated (Ser-232 and Thr-320). Indeed, it is thought that there are at least two additional phosphorylation sites in the PR N-terminal region (30). The microsequencing data indicate that the enzymes make single, highly specific cuts. Once again, obvious protease recognition sequences, even a few amino acids away, are protected from cleavage. Thus, while the cleavage sites must be solvent-accessible, nearby residues must be in an ordered conformation. Comparison of limited proteolysis data with high resolution structural studies (44, 45) indicates that the ability of proteases to cleave peptide bonds is dependent upon local segmental motion of the peptide substrate, as well as solvent accessibility.
This local mobility within an ordered structure is apparently due to transient unfolding, allowing the substrate to momentarily conform to the active site of the enzyme, thus generating scission. It may not be surprising, then, that these sites are susceptible to attack; kinases as well as proteases are likely to use local unfolding to optimize enzymatic reactivity and specificity to take advantage of a induced fit mechanism (46).

The precision of cleavage and the persistence of EndoGluC band 1 (Fig. 7) indicate a structured N-terminal region. However, the kinetics of each cleavage reaction suggest that the conformation of this region is highly accessible to proteolytic attack. Additionally, we see no evidence of highly stable, independent subdomains of the globular N-terminal region, suggesting that it is not composed of globular domains. Classical domains, when attacked by multiple proteases, are resistant to prolonged enzymatic attack (47). In this study, only the DBD approximates this definition. (The DBD is degraded in solution by bromelain.) Rather, the data suggest a non-globular, or extended conformation consistent with this definition. (The DBD is degraded in solution by bromelain.) This is consistent with recent NMR and structural analysis of hPR which showed, for example, that the isolated GR 889–895 demonstrated a highly asymmetric structure. The local mobility within an ordered structure is apparently due to transient unfolding, allowing the substrate to momentarily conform to the active site of the enzyme, thus generating scission. It may not be surprising, then, that these sites are susceptible to attack; kinases as well as proteases are likely to use local unfolding to optimize enzymatic reactivity and specificity to take advantage of a induced fit mechanism (46).

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