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Analysis of the Steroid Binding Domain of Rat Androgen-Binding Protein*

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ABSTRACT. The site-directed photoaffinity ligand [3H]17β-hydroxy-4,6-androstadien-3-one (Δ^6-testosterone) was used to label the steroid binding domain of rat androgen-binding protein (rABP). After digestion with trypsin, the major radiolabeled peptide was isolated by reverse phase chromatography. The peptide was found to have the following amino acid sequence: Ile Ala Leu Gly Gly Leu Leu Leu Pro Thr Ser. Gaps in the sequence that one would anticipate if Δ^6-testosterone formed an adduct with a single amino acid were not encountered. Several different amino acids appear to have been labeled as expected given the free radical nature of photoactivated Δ^6-testosterone. The sequence obtained corresponded to a tryptic peptide (amino acids 171–181) of the rABP precursor. The only other protein having this amino acid sequence was human sex hormone binding globulin. The binding domain lies in a hydrophobic pocket that contains a predicted β-sheet and turn secondary structure, as would be anticipated given the hydrophobic nature of the steroid molecule. A hydropathy and secondary structure analysis of rABP was performed as a basis for discussing the results of the current study in relation to previous studies on the steroid binding domain on human sex hormone binding globulin. (*Endocrinology* **129:** 690–696, 1991)

ANDROGEN-BINDING protein (ABP), a product of the Sertoli cells of the testis (1, 2), interacts with its ligands, 5α-dihydrotestosterone (5α-DHT) and testosterone, with high affinity (2, 3). ABP is primarily confined to the testis and epididymis of adult animals (4–6), but it can also be detected in the blood of immature rats from about day 15 to day 40 of postnatal life (6). ABP functions as an androgen transport protein, but it may also be involved in receptor mediated processes. The complimentary DNA (cDNA) for rat ABP (rABP) has been cloned and its primary structure has been deduced from the nucleotide sequence of the cDNA (7). The cDNA encodes a 403 amino acid precursor of ABP which contains a 30 amino acid putative signal peptide (7). Comparison of the cDNA and the deduced amino acid sequence of ABP with those of human sex hormone-binding globulin (hSHBG) has revealed that the proteins have a high degree of identity, and that they are products of the same gene (7, 8). Both proteins are composed of two subunits (9, 10); the heterogeneous appearance of the subunits is due to differential glycosylation of a single protomer (9, 11). The proteins appear to be identical except for differences in glycosylation (9, 11). A single mRNA for rABP and hSHBG has been identified (7, 8).

Relying on the use of radioactive affinity-labeling reagents to introduce a covalent tag in or near the steroid binding site, four groups have obtained data on the steroid-binding domain of hSHBG (8, 12–14). Two groups used the nucleophilic reagent bromoacetoxydihydrotestosterone to label the protein (13, 14). When analyses were performed to identify the amino acid with which the ligand had formed an adduct, Khan and Rosner (13) reported that the hystidyl residue at position 235 (this would correspond to amino acid 265 of the rABP precursor) was labeled, whereas Namkung *et al.* (14) reported that the ligand interacted with lysine 134 (amino acid 164 of the rABP precursor). The photoaffinity reagent 17β-hydroxy-4,6-androstadien-3-one (Δ^6-testosterone), has been used by Hammond *et al.* (8) and by Grenot *et al.* (12) to label hSHBG. The former group determined that the steroid binding domain on hSHBG was located between residue 296 (326 of the rABP precursor) and the carboxyl terminus of the protein, whereas the latter group interpreted their data as suggesting that methionine 139 present in the hexapeptide comprising residues 135 to 140 (residues 165 to 170 of the rABP precursor) was labeled.

Since knowledge of the molecular configuration of the binding site on steroid binding proteins is of paramount

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importance for understanding steroid specificity and structure/function relationships of the proteins, [^{3}H]Δ^6-testosterone was used to investigate the steroid binding domain of rABP. The results, presented here, indicate that the major radiolabeled peptide of rABP has the sequence Ile Ala Leu Gly Gly Leu Leu Leu Pro Thr Ser which corresponds to amino acids 171–181 of the ABP precursor molecule.

Materials and Methods

[^{3}H]Δ^6-testosterone (44.3 Ci/mmol), a site-specific photoaffinity ligand for androgen-binding proteins (10, 15), was obtained from Dupont/New England Nuclear (Boston, MA) who synthesized it according to our protocols (16). [^{3}H]5α-DHT (47.8 Ci/mmol) was also obtained from Dupont/New England Nuclear. Other reagents were from Fisher (Fairlawn, NJ) or Sigma (St. Louis, MO); electrophoresis supplies were from Bio-Rad (Richmond, CA).

Purification of ABP

ABP was purified from rat epididymides as previously described (11). Briefly, frozen rat epididymides were homogenized in the TE buffer [10 mM Tris-HCl (pH 7.5), 1.0 mM EDTA] and cytosol was prepared by centrifuging the homogenate at 100,000 × *g*. The ABP in the homogenate was adsorbed to diethylaminoethyl-Sephacel and eluted with KCl. The KCl extract was then chromatographed on an affinity resin consisting of 6-(5α-androstan-17β-ol-17α-yl)hexanoic acid linked to Sepharose CL-4B. The column was washed and the ABP was eluted with a solution containing 5α-DHT (11). The ABP preparation used in this study was greater than 95% pure as assessed by analysis of a silver stained (17) gel.

Photoaffinity labeling of rABP

Two-hundred and fifty micrograms (2500 pmol) of affinity-purified rABP that had been stored at −70 C was rehydrated with 3 ml 10 mM sodium phosphate buffer (pH 7.4) and added to a tube in which 250 μCi [^{3}H]Δ^6-testosterone had been evaporated to dryness. The sample was mixed on a vortex mixer and incubated on ice for 2 h during which time the sample was mixed frequently. At the conclusion of the incubation, the sample was transferred to reaction tubes and exposed to light of $\lambda > 305$ nm at 5 C for 2 h to effect photoactivation of the ligand and covalent labeling of the ABP as we have previously described (10, 15). The photolabeled sample was transferred to dialysis tubing and dialized at 4 C for 2.5 h against 4 liters deionized water and then against 2 × 4 liters dionized water at 4 C for 24 h. Aliquots (50 μl and 25 μl) of the dialized sample were saved for assessment of labeling efficiency and for analysis by polyacrylamide gel electrophoresis (18). The remainder of the sample was lyophilized and stored at −70 C until used.

A second photolysis was conducted as described above, using 200 μg (2000 pmol) rABP. In the first photolysis, 78% of the potential binding sites (assuming one binding site per ABP molecule, 2–3) were labeled. In the second experiment, 89% of the sites were labeled.

Trypsin digestion of photolabeled rABP

Lyophilized, photolabeled ABP was rehydrated in 250 μl 10 mM Tris-HCl buffer (pH 7.5); 10 mM β-mercaptoethanol were added to reduce disulfied bonds, the vial was capped, incubated at room temperature for 4 h, and the sample was lyophilized. The lyophilized sample was reconstituted in 240 μl 10 mM Tris-HCl, 50 mM $CaCl_2$; 10 μl (10 μg) *N*-Tosyl-L-Phenylalanine Chloromethyl Ketone (TPCK) treated trypsin were added to the sample and it was incubated for 15 h on a shaker at 37 C. At the conclusion of the incubation, 5 μl (5 μg) freshly prepared TPCK-trypsin were added to the sample and it was further incubated at 37 C for 6.5 h. Finally, the sample was made 1.0 mM in β-mercaptoethanol and it was stored at −20 C.

Reverse phase HPLC

Reverse-phase chromatography was performed as previously described (19) on an analytical C18 column (Brownlee, Santa Clara, CA). The column was equilibrated in 0.1% (vol/vol) trifluoroacetic acid, 0.5% (vol/vol) ethylene glycol, 1 mM triethylamine, pH 2.0. Acidified samples were loaded and eluted at 0.5 ml/min with a linear gradient of acetonitrile. Fractions were collected and when necessary stored at −70 C.

Amino acid peptide sequencing

Automated Edman degradations were done on an Applied Biosystems model 475A sequencer; phenylthiohydantoin amino acids were separated using a reverse phase C18 column and identified with a model 120A PTH analyzer and a model 900A data analysis module as previously described (20). Samples were applied to polybrene treated glass fibers.

Analytical procedures

Proteins were electrophoretically analyzed on sodium dodecyl sulfate 10% polyacrylamide gradient slab gels under reducing conditions with the Laemmli buffer system (18). When required, the gels were fluorographed with diphenyloxazole in acetic acid (21) or silver stained (17). ABP was also detected immunochemically (22) after its electrophoretic transfer to nitrocellulose (23).

Results

Assessment of rABP preparations

Aliquots of the affinity-purified rABP used in this study were subjected to polyacrylamide gel electrophoresis under reducing and denaturing conditions (18). As can be seen (Fig. 1, lane 1) two major bands (10, 11) that constitute the heavy (M_r 48,000) and light (M_r 42,000) protomers of rABP are detectable by silver staining (17). These proteins constitute greater than 95% of the total protein on the gel. Only one contaminant of band M_r approximately 65,000 could be detected which may represent serum albumin. A monoclonal antibody raised against rABP (22) (Fig. 1, lane 2) detected both protomers of rABP and indicated that no cross-reacting material was present. The image shown in Fig. 1, lane 3,

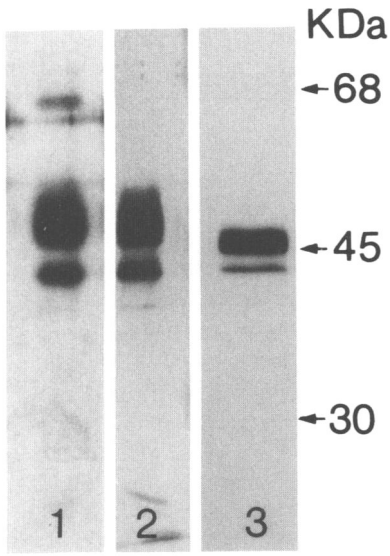

FIG. 1. Characterization of the affinity purified rABP preparation. ABP was subjected to electrophoresis under denaturing conditions (18). One lane of the gel (Fig. 1, lane 1) was silver stained (17), the other lane (Fig. 1, lane 2) was transferred to nitrocellulose (23) and the rABP was detected using a monoclonal antibody raised against rABP (22). ABP was photolabeled with [^{3}H]Δ^6-testosterone (10, 15) and electrophoresed as above; the gel was processed for fluorography (21), dried, and exposed to Kodak X-Omat film (Fig. 1, lane 3).

indicates that only the heavy and light protomers of rABP were covalently labeled with [^{3}H]Δ^6-testosterone; there was no evidence of contaminating radiolabeled proteins at this exposure time (~24 h). This assessment indicated that the rABP preparation was suitable for conducting the binding site studies.

Fractionation of rABP tryptic peptides

When the TPCK-trypsin-treated photolabeled rABP was fractionated by reverse phase HPLC, more than 50 peptides were detected by their absorbance at 214 nm (Fig. 2A). There are 34 predicted tryptic fragments of the rABP precursor (GenBank), the additional fragments detected here are presumably derived from trypsin. In contrast, there were only 8 radioactive peptides (Fig. 2B). The largest labeled peak, with a retention time of 80–90 min, constituted about 34% of the total radioactivity present in the sample (Fig. 2B). The next largest peak (retention time of 64–70 min) represented about 13% of the total radioactivity. The fractions containing the major peak were pooled and rechromatographed. The absorbance (214 nm) profile obtained indicated the presence of three minor peaks and one asymmetrical major peak (Fig. 3A); the latter peak was coincident with the major peak of radioactivity (Fig. 3B).

To determine if the minor radioactive peaks seen in Fig. 2B represented an incomplete digest of the photolabeled rABP, the fractions (retention time 40–79 min) were pooled and digested with trypsin again. When the

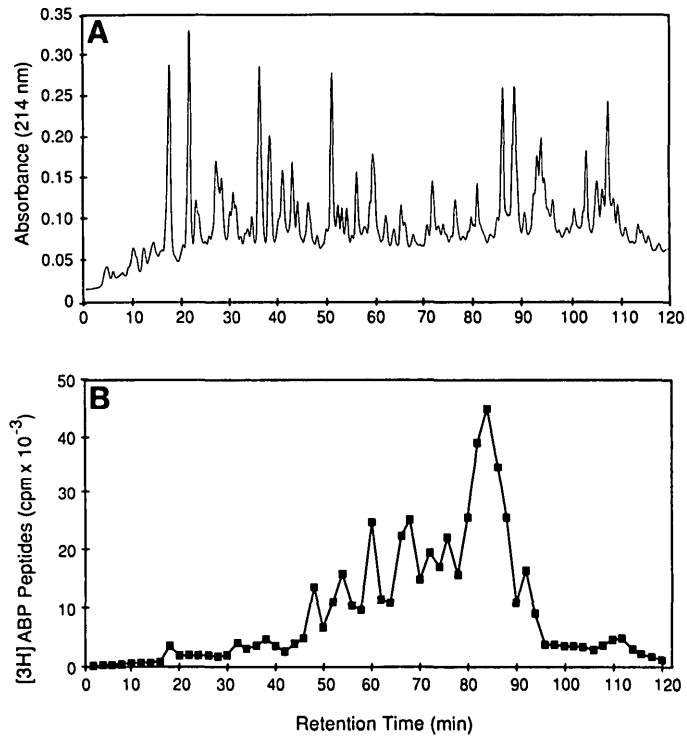

FIG. 2. Fractionation of rABP tryptic peptides by reverse phase HPLC. Peptides were eluted from a C18 reverse-phase column with a 120 min, 10–35% linear gradient of acetonitrile. Peptide elution was monitored at 214 nm (A) and retention time is expressed in minutes. The radioactivity in a 50-μl aliquot of each 1 ml fraction was determined on a liquid scintillation counter and is expressed as CPM $\times 10^{-3}$ (B). The profile presented is representative of three different experiments.

redigested material was analyzed many peptides absorbing at 214 nm (Fig. 4A) were detected. However, the same minor peaks of radioactivity seen in Fig. 2B were present (Fig. 4B); indicating that the original digest was complete and that these labeled peptides are distinct from those present in the major peak of Fig. 2B. No radioactivity or absorbance at 214 nm was detected beyond the 80-min period.

Sequencing of the major radioactive tryptic peptide

The major radioactive peptide (Fig. 2B) was then sequenced. For the sequencing procedure, the radioactive peak was subdivided into two portions: one representing the sharp portion of the absorbance peak, and one representing the shoulder region. The consensus sequence obtained from sequencing four peptides, two from each of two photolabelings of rABP is:

Ile Ala Leu Gly Gly Leu Leu Leu Pro Thr Ser

No gaps (missing amino acids) were encountered in the sequence indicating that Δ^6-testosterone did not form an adduct with a single amino acid. Radioactivity was associated with amino acids eluted in each cycle of the sequence (data not shown).

When the above sequence was entered into a protein

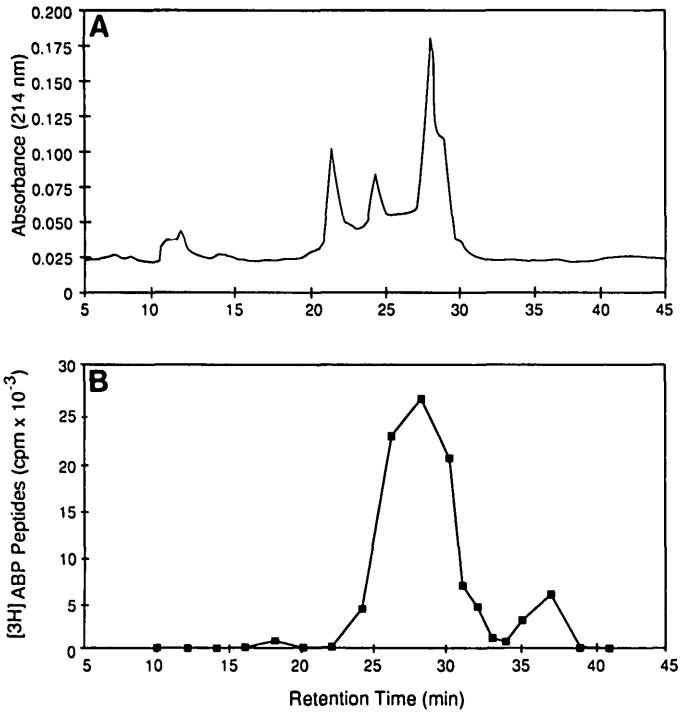

FIG. 3. Purification of the major [^{3}H]ABP tryptic peptide. The major radioactive peak shown in Fig. 2 was rechromotographed on a C18 reverse-phase HPLC column and eluted with a 25–30% linear gradient of acetonitrile. Peptide elution was monitored at 214 nm absorbance (A) and radioactivity (B) in the 0.5 ml fractions collected was determined. The profile presented is representative of two different experiments.

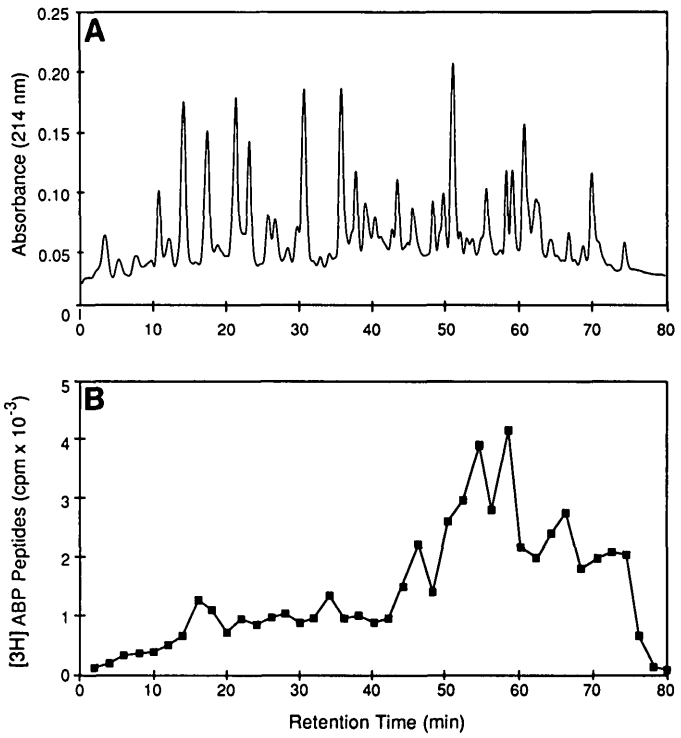

FIG. 4. Redigestion of the minor [^{3}H]ABP tryptic peptides. The radioactive tryptic peptides eluting from 40–79 min retention time from the profile shown in figure 2 were pooled, redigested, and rechromotographed on a C18 reverse-phase HPLC. The peptides eluted were monitored at 214 nm absorbance (A) and the radioactivity (B) present was determined. The profile presented is representative of two different experiments.

sequence data bank (GenBank), it was found to be present in both the rABP precursor and hSHBG. No other proteins were found to contain this sequence. The sequence we have determined corresponds to amino acids 171 to 181 of the rABP precursor (7). These amino acids correspond exactly to an anticipated tryptic fragment of the rABP precursor. The published amino acid sequence of rABP (7) is presented in Fig. 5; the peptide that we have sequenced is enclosed. The peptide with a retention time of 21 min was also sequenced and yielded Lys Ser Ala Try Pro Gly Gln Ile Thr Ser Asn Met Phe which corresponds to a tryptic peptide fragment of trypsin.

Structural analysis

Figure 6 presents a hydropathy plot of the rABP precursor as determined using window values obtained from Kyte and Doolittle (24). Window averaging at residue i is calculated across six residues. *Arrow A* indicates the region on the rABP precursor that corresponds to the region on rABP that we have labeled; it also indicates the region on the rABP precursor that corresponds to the region on hSHBG labeled by others (12, 14). *Arrow B* indicates the region on the rABP precursor that corresponds to the region on hSHBG labeled by Khan and Rosner (13) while the *bar* indicates the region corresponding to that on hSHBG that was labeled by Hammond *et al.* (8).

The hydropathy plot indicates approximately 20 major hydrophobic pockets, with the most prominant one corresponding to the signal peptide (amino acid residues 1–30). One would expect that the binding site for the steroid molecule would lie in one of these hydrophobic regions. Figure 7 presents an expanded view of the region on Fig. 6 indicated by *arrow A*. The *arrow A* in Fig. 7 indicates the position corresponding to Lys 134 on hSHBG (His 164 of the rABP precursor) labeled by Petra's group (14). This is one of the amino acids not conserved between rABP and hSHBG. The second arrow (B) corresponds to the Met 139 of hSHBG (Met 169 of the rABP precursor) labeled by Grenot *et al.* (12). The *bar* indicates the peptide we have labeled and sequenced that corresponds to amino acids 171 to 181 of the rABP precursor and to amino acids 141 to 151 on the mature form of rABP. Also indicated in Fig. 7 are conformational data for this region of the rABP molecule. The region contains α-helix (A), β-sheets (B), and two turns (T) which make up the proposed pocket. As can be noted, the rABP region labeled is a hydrophobic pocket that contains the predicted secondary structure of a β-sheet and turn. The

FIG. 5. The amino acid sequence of rABP. The amino acid sequence derived from the cDNA of rABP (7) is presented. The first 30 amino acids form a putative signal peptide. Amino acid differences between rABP and hSHBG (8) at points of interest are indicated beneath those for ABP and are indicated in bold type. The region we have sequenced is enclosed. The amino acids identified by others (12–14) as being involved in the steroid binding-site domain are indicated by *asterisks* and *solid lines*. Potential sites of Asn-glycosylation are underlined with a *dashed line*.

region labeled by the other investigators lies near this pocket in a less hydrophobic region predicted to have α-helix structure. The single letter designation for the amino acids of this region is indicated in the figure. Putative steroid binding domains on hSHBG reported by Khan and Rosner (13) and Hammond *et al* (8) lie at some distance from this region (see Figs. 5 and 6) in regions of lesser hydrophobicity.

Discussion

Previously Δ^6-testosterone was shown to be a site-specific photoaffinity label for rabbit (25) and rABP (10, 15) and for human (9) and rabbit (26) SHBG. In this paper [^{3}H]Δ^6-testosterone was used to covalently label and identify the steroid-binding-site peptide of rat ABP. The major radiolabeled tryptic peptide was selected for sequencing. This peptide is a complete tryptic fragment of rABP since cleavage occurred after an arginine residue on its amino terminus and before a lysine residue on its carboxy terminus (see Fig. 5). Failure of the minor labeled peptides to coelute with the major peptide upon redigestion and rechromatography confirms that the original digest was complete and indicates that they are distinct from the major radiolabeled peptide. We concluded, therefore, that the major peak most likely contains the steroid binding site domain.

When the major peptide was sequenced, all of the amino acids in the peptide could be detected. It is clear, therefore, that the Δ^6-testosterone did not form an adduct with a single amino acid on the ABP molecules. The resultant effect is that a mixture of peptides were present containing complexed and noncomplexed amino acids. The putative interaction of Δ^6-testosterone with more than one amino acid is not unexpected given the free radical nature of the photoactivated molecule (15). During the sequencing only the noncomplexed amino acids would be detected. It is unlikely that homogeneous labeling of all the amino acids in the peptide occurred; however, the limited availability of ABP constrains us from performing sufficient sequence analyses to pinpoint the one(s) most frequently labeled. The most frequently labeled amino acids would most likely be those in proximity to the A ring of the steroid nucleus, which is the portion of the molecule that is activated by UV light (15).

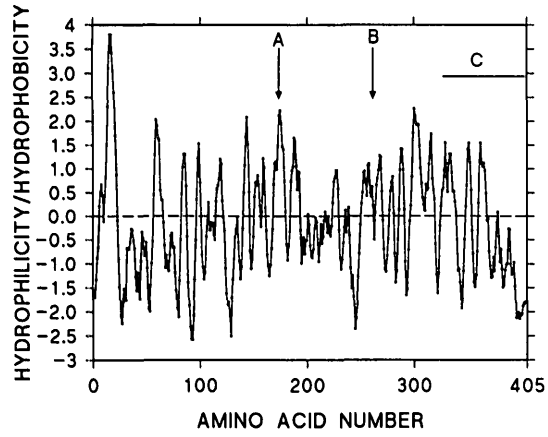

FIG. 6. Hydropathy plot of ABP. Data on the hydrophobicity and hydrophilicity of the amino acid sequence of the ABP precursor were obtained from the Genbank data base and analyzed with an Intelegenetics software package (Mountain View, CA) by the method of Kyte and Doolittle (24). The data are plotted versus the amino acid number beginning at the N-terminal of the protein. The *letters, arrows* and *bar point* out areas putatively containing the steroid binding site domain. A indicates the region labeled by us and (11, 14). B indicates the region labeled by Khan and Rosner (13). C indicates the region labeled by Hammond *et al.* (8).

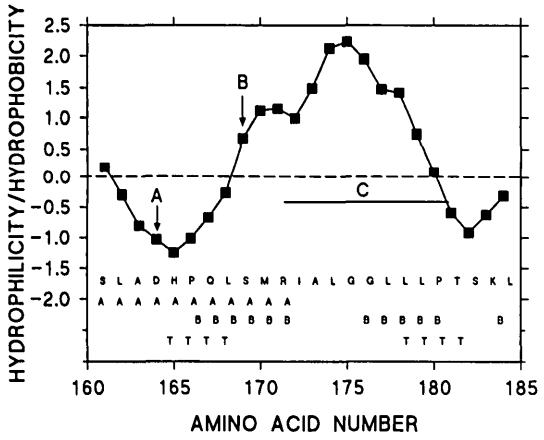

FIG. 7. Data on binding site domain A. The hydrophobicity/hydrophilicity data on region A indicated in Fig. 6 are plotted. The *bar* indicates amino acids 171 to 181 of the ABP precursor that we have determined is the binding site domain of rABP. *Arrow A* indicates amino acid His 164 of the rABP precursor (Lys 134 of hSHBG) labeled by Petra's group (14). Arrow B indicates Met 169 of the rABP precursor (Met 139 of hSHBG) labeled by Grenot *et al.* (12). The amino acid sequence and secondary structure configuration are indicated below the graph and are off-set slightly from the graph. A, α-helix; B, β-sheet; T, turn.

The radiolabeled rABP peptide that we have sequenced (corresponding to amino acids 171 to 181 of the rABP precursor) is immediately adjacent to the region containing amino acids Lys 134 of hSHBG (164 of the rABP precursor) and Met 139 of hSHBG (169 of the rABP precursor) that have been previously reported to be involved in the steroid binding domain of hSHBG (12, 14). Therefore, three laboratories have independently identified a restricted portion of the rABP/hSHBG molecule as a site for interaction with androgens. The sites labeled by Namkung *et al.* (14) and Grenot *et al.* (12) are just outside of the hydrophobic pocket that we contend is the steroid binding site. Therefore, their data may represent covalent attachment of the affinity ligands to hSHBG before the ligand had entered the binding site or before binding equilibrium had been reached. The fact that two different ligands, bromoacetoxydihydrotestosterone (14) and Δ^6-testosterone (12), can label this region of ABP/SHBG provides evidence that the labeling is not a function of the ligand used. The regions identified by Khan and Rosner (13) and Hammond *et al.* (8) as putative androgen binding sites are at some distance in the linear array of hSHBG from this concensus binding site domain. Furthermore, the sites they have proposed are in less hydrophobic regions of the protein thus diminishing the theoretical likelihood that they are the primary androgen binding sites. Nevertheless, it is possible that secondary folding could bring all of the identified regions into close proximity resulting in sites other than the primary steroid binding site being labeled.

Photo-affinity-labeling data presented here and elsewhere (10, 25, 26) show that both protomers of androgen-binding proteins bind [^{3}H]Δ^6-testosterone. This observation may indicate that there is a discrete steroid binding site on each protomer, *i.e.* that there are two binding sites per dimer. The fact that both protomers have an identical primary structure and the fact that only one major radiolabeled peptide was detected upon photolysis and trypsin digestion would be consistent with this possibility. If two binding sites are present on the rABP dimer, they would appear to be equivalent since Scatchard analysis (27) of steroid binding data yield a straight line (2, 3). It is also possible that both protomers contribute to the formation of a single binding site. When the dimer is dissociated under denaturing conditions, the protomers would be found labeled according to the statistical proability of the photoprobe reacting with each of them. In this case, two (or more) distinct radiolabeled peptides would be detected, one being derived from each of the protomers. In this study one major radiolabeled tryptic peptide of rABP was detected, this is likely to be the primary site of interaction of the steroid with the protein. We cannot exclude the possibility that some of the minor labeled peptides in our study, or peptides labeled by others, may constitute a portion of the other protomer that is involved in formation of the binding site. The elucidation of whether there is a steroid binding site on each protomer or whether the two protomers form a single binding site awaits further investigation.

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