Partial proteolytic fragmentation of the two chick oviduct progesterone receptor subunits was used to identify structural features shared by the two proteins. Both subunits can be photoaffinity labeled at their hormone-binding sites (Birnbaumer, M., Schrader, W. T., and O’Malley, B. W. (1983) J. Biol. Chem. 258, 1637–1644) using the radioactive steroid [methyl-3H] 17α,21-dimethyl-19-nor-pregn-4,9-diene-3,20-dione. Native subunits A (M, 79,000) and B (M, 108,000) were partially purified, photoaffinity-labeled, and then subjected to various mild proteolytic digestions. Labeled fragments were analyzed by fluorography after chromatography of the digests under denaturing conditions. Digestion patterns were characteristic for each protease tested. However, fragments from both A and B were indistinguishable for all peptides of less than M, 60,000. Time course studies demonstrated the sequential production of progressively smaller discrete fragments in a manner consistent with a precursor-product relationship among them and established the existence of similar structural domains resistant to proteolysis in both proteins. Autoradiographic peptide maps were obtained by 125I-labeling of pure A and B protein isolated by two-dimensional gel electrophoresis followed by exhaustive tryptic digestion and two-dimensional separation. These studies revealed that a significant proportion of the smaller A protein differs in its primary sequence from that of the B protein which excludes the possibility of their sharing a precursor-product relationship. We conclude that B and A subunits are separate proteins with common structural features in the native state, but with considerable amino acid sequence differences. The simplest hypothesis consistent with these findings is that B and A are the products of two separate genes which have diverged to give rise to two different but related proteins that fold in such a manner as to be almost indistinguishable by proteolytic attack of their native conformation.

We have characterized two progesterone-binding subunits of the chick oviduct cytosol progesterone receptor (for review, see Ref. 1). Both proteins have been purified to apparent homogeneity (2, 3). These two proteins were termed A and B on the basis of their order of elution from DEAE-cellulose, and have M, 79,000 and 108,000, respectively. The two proteins are functionally distinct; protein A has a strong DNA-binding activity not exhibited by B, whereas B has a chromatin-binding activity that is absent on A (3–5). As assessed by reversible cross-linking experiments, they appear to exist in the cytosol as 6–7 S dimers (6).

Sherman et al. (7) found that incubation of cytosol with Ca2+ led to the appearance of two smaller progesterone-binding fragments of the receptor. One type had M, values in the range of 45,000 and was termed form IV. The other fragment had M, values close to 25,000 and was termed mero receptor. They suggested that Form IV and mero receptors were derived from proteins A and B, respectively. Subsequent studies by Vedeckis et al. (8) showed that the Ca2+-dependent reaction was due to receptor proteolysis caused by a Ca2+-activated neutral protease from chick oviduct cytosol. These workers showed that both Form IV and mero receptor fragments could be produced from either the A or B receptor proteins. The corresponding A- and B-derived fragments were indistinguishable from each other under non-denaturing conditions.

Recently, we developed methods to photoaffinity label the progesterone receptor proteins with the progesterone analogue [3H]R5020.1 This technique permitted analysis of receptor proteins in crude preparations under denaturing conditions (9, 10). By analyzing the effect of extensive proteolytic fragmentation of receptor A and B proteins by NaDodSO4-PAGE, we found that both receptor subunits yield limit digests with Staphylococcus aureus V8 protease that contain an apparently identical photoaffinity-labeled “H fragment,” of M, 9500 which has part or all of the hormone-binding site (10). The similarity in the H fragments from the A and B proteins strongly suggested that these proteins may share a common structure responsible for their reported indistinguishable progesterone-binding properties (11, 12).

This possibility raised questions as to whether subunits A and B might share other common domains, and whether the smaller A subunit might be a product of the larger B subunit. The present study examines the presence of common structural domains in the A and B proteins by analyzing in detail the size distribution of receptor fragments containing the hormone-binding site obtained under conditions of controlled proteolysis. Whether or not the B protein may bear a precursor-product relationship to A was analyzed by two-dimensional electrophoretic and chromatographic analysis of peptides obtained by exhaustive tryptic digestion of A and B proteins iodinated with 125I under denaturing conditions (13).

### Experimental Procedures

**Materials**

[3H]R5020 (85 Ci/mmol) was obtained from New England Nuclear. 125I (carrier-free in 0.1 M NaOH) was purchased from Iso-Tex Diag.

1 The abbreviations used are: [3H]R5020, [17α-methyl-3H]17α,21-dimethyl-19-nor-pregn-4,9-diene-3,20-dione; NaDodSO4, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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Dialyzed affinity-labeled partially purified receptor proteins were used in the determination of their spectrophotometric properties. The proteins used throughout as molecular markers were: β-galactosidase, phosphorylase b, β-galactosidase, bovine serum albumin, ovalbumin, and α-chymotrypsin. The proteins were radioiodinated with [125I] to determine their molecular weights. The proteins were heated for 5 min at 100 °C, centrifuged, and the supernatants were removed and lyophilized. The samples were then analyzed on cellulose-coated TLC plates (10 x 10 cm) (EM Laboratories, Pomona, NY). The samples were redissolved in 10 μl of Buffer I (acetic acid/formic acid:water; 15:5:80) and 2-5 μl were spotted onto each plate (~50,000 cpm). Electrophoresis was carried out at 15 °C on a Desaga thin layer electrophoresis apparatus in Buffer I for 1 h at 500 V. To ensure that the peptides of each sample migrated a consistent distance, progress of electrophoresis was monitored by using a mixture of 2% orange G (w/v) and 1% acid fuchsin (w/v) in Buffer I. The dye was spotted at the opposite end from each sample and each plate was removed when the lead dye component reached a preset mark. The plates were dried and the peptides were chromographed in the second dimension in Buffer II (butanol/pyridine/acetic acid:water; 32:5:25:20). The plates were again dried and then analyzed by autoradiography at room temperature using Kodak X-Omat AR film. Exposure times were 6 and 18 h.

Peptide maps were obtained four times. In assessing the amount of receptor protein present in the gel slices by the intensity of the Coomassie blue stain we notice a 10-fold variation in the amount of protein used. Thus, at the time of digestion the tryptic/protease ratio varied up to 10-fold between experiments. However, this variation had no effect either on the number or the relative intensities of the spots seen in the tryptic peptide maps, indicating both that these maps are reproducible and that digestions with trypsin are complete under the conditions used.

**RESULTS**

Receptor A and B subunits labeled with [3H]R5020 by the methods recently described (10) were analyzed by NaDodSO4-PAGE in 7.5% gels as shown in the outer lanes of Fig. 1 (top). Single intense fluorographic signals were seen in both cases at the molecular weights expected for undegraded receptor peptides (2, 3, 10). The six inner lanes show results of partial proteolysis with S. aureus V8 protease, as analyzed on 12.5% gels. After as little as 1 min of digestion using 0.1 μg/ml of enzyme, detectable proteolysis had occurred. The figure shows that the pattern of partial proteolytic fragmentation of the A and B proteins is complex. When different times of incubation as well as different concentrations of the protease were used, the proteolytic degradation proceeded in a sequential manner, with accumulation of intermediate sized fragments. These fragments ranged in size from 50,000-60,000, then from 40,000-45,000 followed by the accumulation of fragments 26,000-29,000. The 40,000-45,000 fragments and the 26,000-29,000 fragment groups are in the size range that upon analysis under nondenaturing conditions would be classified as Form IV and mero receptors, respectively (7, 8). More striking than the patterns of proteolytic degradation themselves, was that below Mr = ~60,000, the proteolytic patterns obtained were indistinguishable for the A and B proteins.

Thus, the overall structural arrangements of A and B are strikingly similar, when analyzed by S. aureus V8 protease digestion under mild conditions. The appearance of distinct bands of [3H]R5020 site shows that certain domains are highly resistant to the enzyme, whereas other regions are highly sensitive. If general hydrolysis were occurring, the bands would not be distinct, due to size heterogeneity. Furthermore, these results cannot be due to [3H]R5020 labeling at multiple loci on the proteins, since we have shown (10) that exhaustive digestion with this enzyme yields single labeled peptides from both A and B, as analyzed by two-dimensional gel analysis by the method of O'Farrell (22).

More extensive digestions were then carried out to continue...
FIG. 1. **S. aureus** V8 protease treatment. **Top**, proteolytic fragmentation of partially purified A and B progesterone receptor proteins (left and right, respectively) covalently labeled with $[^{3}H]R5020$. The figure shows the fluorography of the polyacrylamide slab gels after electrophoresis in the presence of NaDodSO, of the untreated A and B proteins (left-most and right-most lanes) as well as of the A and B proteins treated with 0.1 and 0.5 $\mu$g/ml of **S. aureus** V8 protease for 1 and 30 min as shown. Untreated receptor proteins were electrophoresed in 7.5% acrylamide slabs, while treated proteins were analyzed in 12.5% polyacrylamide slab gels. Molecular weights of bands obtained were calculated on the basis of mobilities of standard proteins in parallel lanes (see "Experimental Procedures"). All lanes depicted after proteolytic treatment stem from a single polyacrylamide slab. **Bottom**, proteolytic fragmentation by **S. aureus** V8 protease of partially purified A and B progesterone receptor proteins covalently labeled with $[^{3}H]R5020$ and comparison of fragment sizes obtained. The electrophoretic analysis was carried out in 12.5% polyacrylamide slab gels. A, treated A protein; B, treated B protein; A + B, mixture of the amount of treated A protein electrophoresed in lane A plus the amount of treated B protein electrophoresed in lane B. Other details were as in the top. **BPB**, bromphenol blue.

degradation to produce the 9500 peptide limit fragments. These results are shown in the Fig. 1, bottom. The two panels show digestions using two different concentrations of **S. aureus** V8 protease. The outer lanes of each show results obtained when either A or B protein was digested alone; the middle lanes are mixtures of the two digests. The extent of digestion of the mixture applied to the left panel, obtained using 0.1 $\mu$g/ml of protease is similar to that seen in Fig. 1, top. Under these conditions the Form IV fragments predominate. Electrophoresis of the mixture of A and B fragments failed to resolve the partial bands from each; thus, the fragment sizes are indeed indistinguishable by this method of analysis. The protease digestion was carried out in 12.5% polyacrylamide slab gels. A, treated A protein; B, treated B protein; A + B, mixture of the amount of treated A protein electrophoresed in lane A plus the amount of treated B protein electrophoresed in lane B. Other details were as in the top. **BPB**, bromphenol blue.

FIG. 2. **Tryptic proteolysis.** Tryptic proteolysis under mild conditions of partially purified A and B progesterone receptor proteins (not denatured) covalently labeled with $[^{3}H]R5020$ and comparison of fragment sizes obtained. The electrophoretic analysis was carried out in 12.5% polyacrylamide gel slabs. For the rest of details see the figure, the legend to Fig. 1 and "Experimental Procedures."

and B, and a different set of other digestion products from those obtained in Fig. 1. The noticeable feature in this experiment was again the striking similarity of fragments produced from both subunits. These intermediate fragments are indistinguishable from each other in the mixture analysis (middle
Proteolytic Analysis of Progesterone Receptor Proteins

Fig. 3. α-Chymotryptic proteolysis. α-Chymotryptic proteolysis of the partially purified A and B progesterone receptor proteins covalently labeled with [3H]R5020 and comparison of fragment sizes obtained. The electrophoretic analysis was carried out in 12.5% polyacrylamide slab gels. Nondenatured A and B proteins were treated with addition of 0.1 μg/ml of α-chymotrypsin and electrophoresed alone or in combination as shown in the figure. For the rest of details see the figure, the legend to Fig. 1 and “Experimental Procedures.”

Fig. 4. Two-dimensional gel electrophoresis of covalently labeled partially purified A and B receptor proteins. The units of pH are indicated at the bottom of each panel. Top, receptor protein A covalently labeled with [3H]R5020 identified by Coomassie blue stain (I) and fluorography (II). Bottom, receptor protein B covalently labeled with [3H]R5020 identified by Coomassie blue stain (III) and fluorography (IV).
Proteolytic Analysis of Progesterone Receptor Proteins

FIG. 5. Peptide maps of 125I-labeled tryptic peptides obtained from pure A and B progesterone receptor proteins. The figure presents photographs of the autoradiographs of the cellulose thin layer sheets after two-dimensional separation (first dimension: electrophoresis in the horizontal direction, left to right; second dimension: chromatography in the vertical dimension, bottom to top) of 125I-labeled tryptic peptides obtained according to the technique of Elder et al. (13). For the rest of conditions see text and "Experimental Procedures." Top, peptide maps of the individual progesterone receptor proteins. A, A protein, 5 μl of the tryptic digest were analyzed; B, B protein, 5 μl of the tryptic digest were analyzed. Bottom, A mix of 5 μl of the tryptic digests of both the A and B proteins was analyzed (left); schematic representation of the spots seen in the map of the mix of A and B (right). Open spots, spots present only in the map of A protein; dotted spots, spots present only in the map of B protein; hatched spots, spots identifiable on the maps of the A and of the B proteins.

doublet of M₀ = 12,000 upon digestion with α-chymotrypsin. Thus, as found with S. aureus V8 protease and trypsin, the patterns of proteolytic degradation of the A and B protein obtained with α-chymotrypsin are indistinguishable by these methods of analysis. Figs. 1-3 thus indicated that these two receptor proteins have a similar, if not identical, structural organization. Both proteins possess protease-sensitive sites exposed at discrete intervals from the labeled hormone site, and their similarities include loci for sensitive glutamic acid residues (S. aureus V8 protease data), lysine or arginine (trypsin data), and aromatic residues (α-chymotrypsin data). It was thus possible that the larger B subunit might be a precursor of the smaller A subunit, thereby differing from A only in the portion of B absent in the smaller protein.

To investigate whether the B protein contains all of the sequence of the A protein, or whether despite the striking similarities in structural organization A and B are distinct and different proteins, we analyzed their tryptic peptide maps. Partially purified A and B proteins were prepared as described previously (10). Starting with 100 g of chick oviducts, approximately 80 μg of A and 56 μg of B were obtained, which were about 8 and 4% pure, respectively. These were photoaffinity-labeled with [3H]R5020 and separated from all contaminants by two-dimensional gel electrophoresis according to the method of O'Farrell (22). The location of A and B proteins on the gels was detected by Coomassie blue staining as shown in Fig. 4 (I and III) and confirmed by fluorography (II and IV). The receptor spots were then cut out of their respective gels. Following the technique described by Elder et al. (13), the receptor proteins in the gel slices were iodinated with 125I as described under "Experimental Procedures" using chloramine-T as the oxidant. Approximately 200,000 dpm of 125I were incorporated into the proteins by this method. The proteins in the gel pieces were then digested with 50 μg/ml of trypsin for 18 h at 37 °C. The tryptic peptides released from the gel slices were concentrated by lyophilization and were subjected to two-dimensional separation as described under "Experimental Procedures." Autoradiographic analysis of the cellulose sheets yielded the tryptic peptide maps of labeled tyrosine-containing peptides of the A and B proteins as shown in Fig. 5: upper left, map of subunit A-derived labeled peptides; upper right, results obtained using subunit B. The two digests were mixed and analyzed as shown in bottom left. A schematic representation, bottom right, highlights the different and common peptides seen in the individual maps. Inspection of the maps shows that there are peptides that appear to be common to the A and B proteins. However, there are also peptides that are unique. Most importantly, there are a number of peptides that are unique to the smaller A protein. This indicates that the A and B proteins do not bear a precursor-product relationship and must be distinct and different from each other.

DISCUSSION

The progesterone receptor system of the chick oviduct contains two proteins, both of which bind progesterone with identical specificity and affinity, and which seem to coexist in a 1:1 relationship in the cytosol. Despite their similarities with respect to progesterone-binding properties, the A and B proteins differ in several respects including not only their chromatographic behavior (11), but also and perhaps most importantly, in regard to their DNA-binding properties. Subunit A binds strongly to DNA-cellulose and to specific DNA fragments flanking the 5′-end of the natural ovalbumin gene (5, 24). The B protein binds very weakly to DNA, i.e. only
when the salt concentration of the medium is below 30 mM (25). Our recent findings that extensive proteolytic fragmentation of the A and B proteins yielded a limit digest of approximate $M_r = 5000$ (H fragments) suggested that the two proteins could share a common progesterone-binding sequence or domain. We extended these investigations to determine whether less extensive proteolytic fragmentation would lead to the formation of larger fragments, of which some were common to the two proteins, with the hope of establishing structural correlates to the known behavioral differences noticed in ion exchange and DNA-binding studies. However, as illustrated in Figs. 1–3 and summarized in Table I, except for the fact that the initial proteolysis of the B protein gave fragments of sizes larger than A, all fragments from A and B below and including $M_r = \sim 60,000$, were indistinguishable.

These results show that the A and B proteins have very similar conformational organizations where domains sensitive to proteases are interspersed with less sensitive domains. In our digestion experiments, we noted that not all fragments appear simultaneously. Rather, larger size fragments appear to serve as precursors for smaller ones (Figs. 1–3). It would seem, therefore, that not all proteolytically sensitive bonds that are hydrolyzed under mild conditions are exposed in the intact molecules. Although other explanations are possible, the results obtained are consistent with the view that the availability of proteolytically sensitive peptide bonds whose hydrolysis yields the H-type fragments requires partial unfolding of the protein and that such unfolding is favored by previous proteolytic fragmentation. The same reasoning is applicable to the sequential formation of mero- and Form IV-type fragments. As summarized in Table I, the findings reported here using photoaffinity labeling and detailed size analysis (Figs. 1–3) showed that Form IV and mero receptor observed under nondenaturing conditions are each a family of fragments.

At least a partial amino acid sequence identity between the 79,000 A protein and the 108,000 B protein would be expected from the studies thus far discussed. However, a more sensitive analysis revealed that a significant proportion of the iodinated tryptic peptides of the smaller A protein (obtained by iodinating and trypsin-treating after extensive denaturation with NaDodSO$_4$), were found to be absent in the B protein. This result demonstrates that the smaller A protein cannot be a proteolytic product derived from B. This conclusion is further substantiated by earlier studies (26) where we were unable to convert B to A using endogenous protease activity or various incubations of B with oviduct tissue fractions. The differences in the tryptic two-dimensional peptide maps shown in Fig. 5 indicate that A and B are distinct proteins and, most likely, are derived from separate mRNA sequences. If the proteins arise from separate mRNA molecules, the similarities in structure and the indistinguishable hormone-binding sites would lead one to speculate that they were evolved originally by gene duplication.

The results obtained by analyzing the proteolytic fragmentation of the A and B proteins in their native conformation, showing practically identical fragmentation patterns with three different enzymes, coupled to the results obtained by analyzing the proteolytic fragmentation of the denatured A and B proteins, indicate that upon folding the proteins resemble each other in spite of differences in their primary structure and suggest that homologies exist between them. Whether these similarities in the folding patterns between the two proteins are also associated with similarities in function is being investigated.

As reported elsewhere we have localized the DNA-binding activity of the A protein to one 15,000 proteolytic fragment obtained upon treatment with $S.$ aureus V8 protease (27). In that study, the proteolytic fragments were separated by NaDodSO$_4$-PAGE and transferred onto nitrocellulose paper by the blotting technique described by Bowen et al. (28), and the paper was incubated with $[^{3}P] $DNA. We found that while the Form IV-type $M_r = \sim 43,000$ peptide that contains the hormone-binding site was able to bind $[^{3}P] $DNA, $S.$ aureus V8 protease treatment under conditions which produced the $M_r = 29,000-26,000$ mero fragment led to the appearance on the blots of a new $[^{3}P] $DNA A-binding band of an approximate $M_r = 15,000$. Thus, it appears that the conversion of the Form IV-type peptides to mero-type peptides is associated with the splitting off of a discrete DNA-binding peptide (D fragment) from that portion of the molecule which is responsible for the hormone-binding properties.

Although smaller than the $M_r = 90,000$ glucocorticoid receptors of rat liver (29, 30) and lymphoma cell lines (31, 32), the $M_r = 79,000$ A progesterone receptor protein appears to have a proteolytic fragmentation pattern that resembles that described, under nondenaturing conditions, for glucocorticoid receptors. Wrang and Gustafsson (33), working with rat liver and Stevens and Stevens (34), working with a glucocorticoid-sensitive lymphoma cell line, found that controlled proteolytic fragmentation leads to the formation of hormone-binding fragments with masses ranging from 36,000 to 40,000 daltons which were capable of binding DNA, analogous to the Form IV-type fragment of the progesterone A receptor protein. Hormone-binding fragment(s) with a mass of approximately 23,000 daltons were no longer capable of binding DNA and hence are analogous to the mero-type fragments of the A protein. Moreover, it is of interest that a glucocorticoid-resistant variant of this lymphoma cell line has a glucocorticoid receptor of the type which can be classified as Form IV-type, i.e. having $M_r = 40,000-46,000$ and capable of binding DNA (34). Structural similarities must underlie the remarkable similarities in these proteolytic fragmentation patterns of steroid receptors. It is tempting to suggest that steroid receptors may all be similarly organized, not only with respect to their proteolytic fragmentation, but also with respect to their various functional domains.

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**REFERENCES**


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Assessment of structural similarities in chick oviduct progesterone receptor subunits by partial proteolysis of photoaffinity-labeled proteins.
M Birnbaumer, W T Schrader and B W O'Malley


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