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SHORT COMMUNICATION

THE ROLE OF TYROSINE IN THE BINDING OF STEROID BY PROGESTERONE BINDING GLOBULIN FROM THE GUINEA PIG

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SUMMARY

Treatment of progesterone binding globulin (PBG) with tetranitromethane (TNM) resulted in a loss of steroid binding activity (inactivation) which was dependent on both time and concentration of reagent. Scatchard analysis of binding revealed that inactivation was due to a decrease in binding site number with no effect upon the affinity of PBG for steroid. Incorporation studies demonstrated that the loss of binding activity correlated with the incorporation of 1.3 nitro groups per molecule of PBG. The involvement of the steroid binding site in the reaction was shown by the ability of progesterone, but not cortisol, to protect against inactivation. Treatment with *N*-acetylimidazole did not inactivate PBG nor did the conversion of nitrotyrosyl residues to amino-tyrosines regenerate binding activity, suggesting that the pheolic hydroxyl is not involved in steroid binding. These studies suggest that inactivation was due to the incorporation of a bulky group into the aromatic ring of a tyrosine present at the steroid binding site thus blocking its ability to participate in hydrophobic interactions with the ligand.

INTRODUCTION

Progesterone binding globulin (PBG) is a pregnancy-specific plasma protein of hystricomorph rodents, such as the guinea pig, which binds progestins and androgens with high affinity ($K_D = 10^{-10}$ M). This protein has served as a model system for the study of the nature of the steroid binding site. Extensive studies of binding specificity by Blanford *et al.*[1] have revealed the presence of hydrophobic domains in this site. Modification studies [2] have suggested that tryptophan, lysine, and tyrosine are at or near the steroid binding site. In this communication, we confirm the presence of tyrosine at the steroid binding site and present evidence which more clearly delineates its role in the binding of steroid.

MATERIALS AND METHODS

Progesterone binding globulin (PBG) was purified essentially as described by Cheng *et al.*[3]. The final product was judged to be pure by the presence of a single protein band following SDS gel electrophoresis and by the absence of any free amino terminals as determined by the method of Weiner *et al.* [4]. Tetranitromethane (Sigma Chemical Co.) was dissolved in methanol and was added to PBG (1×10^{-7} M) in 0.05 M Tris to achieve the desired ratio of TNM to protein at a final methanol concentration of 5%. Control incubations received methanol alone. For kinetic studies, aliquots were removed at various times, excess reagent removed by gel filtration, and binding studies performed to determine the number of binding sites and their dissociation constants. All kinetic studies were at 20°C. For incoporation studies, reactions were allowed to proceed to completion by incubation for 18 h at 4°C and reaction products were removed by dialysis prior to assay. Incorporation of nitro groups was determined as the increase in absorbance at the isosbestic point of 381 nm, as described by Riordan *et al.*[5]. Progesterone binding activity was determined by the charcoal adsorption method [6]. Binding data were analyzed according to the method of Scatchard[7] with corrections for nonspecific binding as described by Rosenthal[8].

RESULTS AND DISCUSSION

Reaction of PBG with a 50-fold molar excess of TNM at pH 7.4, and 20°C in the absence of steroid resulted in a pseudofirst order loss of binding sites with a $t_{\frac{1}{2}}$ of 45 min (Fig. 1, dashed line). This confirms the earlier results of Westphal *et al.*[2] who showed that treatment of PBG with TNM resulted in a loss of steroid binding activity. When a 50-fold molar excess of progesterone was included in the reaction mixture, a marked protection against the loss of binding sites was observed (Fig. 1, dotted line). However, a 50-fold molar excess of cortisol, a steroid poorly bound by PBG, afforded essentially no protection (Fig. 1, solid line). It seemed probable, therefore, that the nitration of a tyrosine present at the steroid binding site was responsible for the loss of binding activity. This was further supported by our finding that in order to prepare iodinated PBG which retained steroid binding activity, the iodination reaction had to be performed in the presence of excess steroid (data not shown).

To determine the stoichiometry of inactivation, PBG was reacted for 18 h at 4°C with varying molar ratios of TNM (from 0.5 to 100-fold molar excess). After the removal of excess reagent by dialysis, the degree of modification and its effect upon steroid binding was determined as described in Methods. Figure 2 shows that increasing incorporation resulted in greater loss of binding activity. Extrapolation of the initial linear portion of the curve showed that the incorporation of an average of 1.3 nitro groups per molecule of PBG resulted in the loss of binding activity. Since the the dissociation constant was unchanged at each point, the observed loss was due to a decrease in binding site number and not the production of a molecule with altered affinity. At very high molar ratios of TNM to PBG, all nine tyrosine residues could be iodinated.

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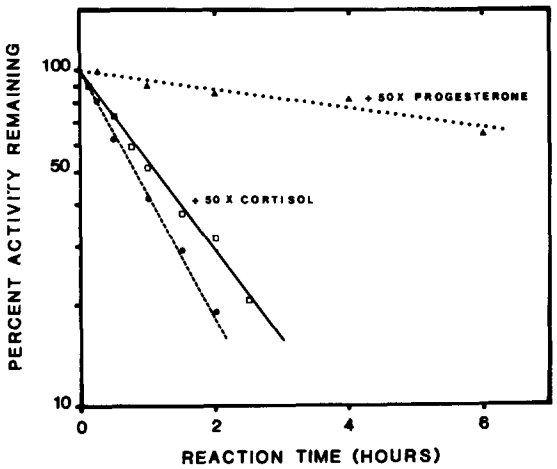

Fig. 1. Effect of steroids upon the reaction of PBG with TNM. PBG in Tris buffer (0.05 M, pH 7.4) was incubated at 20°C with a 50-fold molar excess of TNM in the absence (dashed line) or presence of a 50-fold molar excess of competing steroid (dotted line: progesterone; solid line: cortisol). Aliquots were removed at various times, excess reagent removed by gel filtration, and binding activity determined by Scatchard analysis.

The nitration of tyrosyl residues could produce inactivation by one of two mechanisms. First, the incorporation of a bulky nitro group could result in steric inhibition of interactions between the phenolic ring structure and the steroid. Secondly, nitration of tyrosine results in a lowering of the pK of the phenolic hydroxyl from 10.1 to 7.1 [9]. To test which of these possibilities was responsible for the observed loss of binding activity, two experiments were performed to examine the potential role of the phenolic hydroxyl group. First, PBG was reacted with a 100-fold excess of *N*-acetylimidazole [10]. Despite the modification

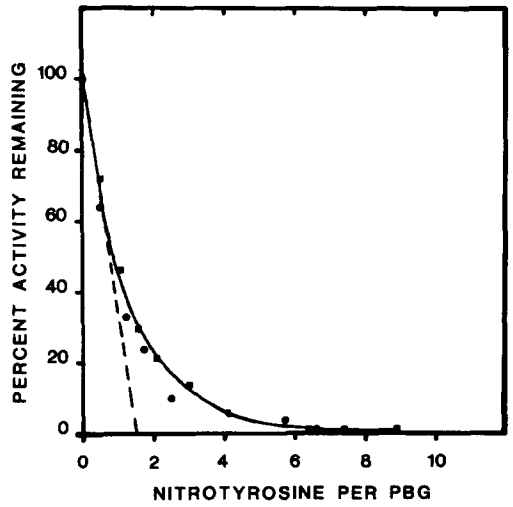

Fig. 2. Loss of steroid binding ability upon the incorporation of nitro groups into PBG. PBG in 0.05 M Tris (pH 7.4) was reacted for 18 h at 4°C with varying molar ratios of TNM to PBG. Following reaction, excess reagent was removed by dialysis against the same buffer. Incorporation in nitro groups was determined by the increase in absorbance at 381 nm and the ability to bind progesterone was determined by Scatchard analysis as described in Methods. The dashed line represents an extrapolation of the initially linear inactivation profile.

of up to 5 tyrosyl residues, no change in either binding site number or affinity could be detected. Thus, acetylation of the phenolic hydroxyl did not produce inactivation. Second, treatment of nitrotyrosine with sodium dithionite converts nitrotyrosines to amino tyrosines [9] and raises the pK of the phenolic hydroxyl from 7.2 to 10.0. When inactive nitrotyrosyl PBG was treated with a 10-fold molar excess of sodium dithionite, no binding activity was regenerated despite the complete conversion of nitrotyrosines to aminotyrosine. Treatment of unmodified PBG with dithionite had no effect on binding activity. The results of these two experiments clearly imply that it is the phenolic ring structure and not the hydroxyl which is involved in steroid binding.

Studies of the relationship of steroid structure and binding affinity have revealed the importance of hydrophobic interactions between the steroid and PBG in the binding reaction [1]. The studies of Westphal and coworkers [2] suggested that a tyrosine is involved in steroid binding. Our work confirms this observation and shows that the modification of a single tyrosine at the steroid binding site is responsible for the observed loss of activity. Furthermore, our studies show that it is the phenolic ring and not the hydroxyl which is involved in the binding reaction. This suggests that one of the important hydrophobic interactions described by Blanford *et al.*[1] is between the steroid and the phenolic ring of a tyrosine present at the steroid binding site of progesterone binding globulin.

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REFERENCES

1. Blanford A. T., Wittman W., Stroupe S. D. and Westphal U.: Steroid–protein interactions—XXXVIII. Influence of steroid structure on affinity to the progesterone-binding globulin. *J. steroid Biochem.* **9** (1978) 187–201.
2. Westphal U., Stroupe S. D., Kute T. and Cheng S.: Steroid interactions with progesterone-binding globulin. *J. steroid Biochem.* **8** (1977) 367–374.
3. Cheng S. L., Stroupe S. D. and Westphal U.: Steroid–protein interactions. Purification of progesterone-binding globulin by affinity chromatography. *FEBS Lett* **64** (1976) 380–384.
4. Weiner A. M., Platt T. and Weber K.: Amino-terminal sequence analysis of proteins purified on a nonomole scale by gel electrophoresis. *J. biol. Chem.* **247** (1972) 3242–3251.
5. Riordan J. F., Sokolovsky M. and Vallee B. L.: The functional tyrosyl residues of carboxypeptidase A. Nitration with tetranitromethane. *Biochemistry* **6** (1967) 3609–3617.
6. Korenman S. G.: Radio-ligand binding assay of specific estrogens using a soluble uterine macromolecule. *J. clin. Endocr. Metab.* **28** (1968) 127–130.
7. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51** (1949) 660–672.
8. Rosenthal H. E.: A graphic method for the determination and presentation of binding parameters in a complex system. *Analyt. Biochem.* **20** (1967) 525–532.
9. Sokolovsky M., Riordan J. F. and Vallee B. L. Conversion of 3-nitrotyrosine to 3-aminotyrosine in peptides and proteins. *Biochem. biophys. Res. Commun.* **27** (1967) 20–25.
10. Simpson R. T., Riordan J. F. and Vallee B. L.: Functional tyrosyl residues in the active center of bovine pancreatic carboxypeptidase A. *Biochemistry* **2** (1963) 616–622.