Vol. 80, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

January 13, 1978 Pages 112-118

# INACTIVATION OF AVIAN PROGESTERONE RECEPTOR BINDING TO ATP-SEPHAROSE BY PYRIDOXAL 5'-PHOSPHATE

Hideo Nishigori[1], V.K. Moudgil[2] and David Toft

Department of Molecular Medicine, Mayo Clinic
Rochester, Minnesota 55901

Received September 19,1977

Summary: The affinity of progesterone receptor from hen oviduct for ATP-Sepharose was diminished by preincubation with pyridoxal 5'-phosphate. This effect was specific for pyridoxal 5'-phosphate since the related compounds, pyridoxal, pyridoxine, pyridoxamine and pyridoxamine 5'-phosphate, were not effectors. The inactivation was easily reversed by the addition of the primary amine, Tris. However, in the presence of the reducing agent $NaBH_4$, the inhibitory effect of pyridoxal 5'-phosphate was irreversible. The results suggest that pyridoxal 5'-phosphate forms a Schiff base with a critical amino group, presumably at the nucleotide binding site of the progesterone receptor.

Previous studies from this laboratory have demonstrated an interaction between the avian progesterone receptor and ATP (1-3). This interaction was observed using ATP-Sepharose chromatography and was shown to be reversible and dependent upon ionic strength. While the functional significance of this ATP binding remains obscure, it may represent an important step in the mechanism of steroid hormone action. In an effort to provide additional definition of the receptor-ATP interaction, inhibitors of this process have been sought. Among the compounds which were tested, one of these, pyridoxal 5'-phosphate, indicated promise as an agent to block the nucleotide binding site (4). This observation is documented in the present communication which shows pyridoxal 5'-phosphate to be a potent inhibitor of ATP binding by the progesterone receptor.

## MATERIALS AND METHODS

All reagents were of analytical grade and were made up in glass-distilled water. All procedures were carried out at 4°. Pyridoxal 5'-phosphate, pyridoxal, pyridoxine, pyridoxamine 5'-phosphate and pyridoxamine were purchased from Sigma, and progesterone [1,2-$^3$H], (50 Ci/mmol) from New England Nuclear. ATP-Sepharose containing 4-5 μmoles ATP/ml packed Sepharose was prepared as described previously (1,5).

1. Please direct correspondence to Dr. Nishigori.
2. Present address: Department of Biological Sciences, Oakland University, Rochester, Michigan 48063.

0006-291X/78/0801-0112$01.00/0

Treatment of progesterone receptor. Progesterone receptor was obtained from hen oviduct cytosol and was precipitated with ammonium sulphate (45% of saturation) as described previously (2). The precipitate was stored at -70° until use (no longer than 3 weeks). For the experiments described here, the receptor precipitates were dissolved in 1/10 the original cytosol volume of Barbital buffer (20 mM Barbital-HCl, 10% glycerol, v/v, 5 mM dithiothreitol and 10 mM KCl, pH 8), dialyzed against the same buffer for 2 h and then labeled with $10^{-8}$ M [$^3$H]progesterone for 3 h. Aliquots (0.6 ml) of the labeled receptor were incubated at 4° for 18 h with 0.6 ml of pyridoxal 5'-phosphate or the related compounds dissolved in Barbital-buffer (adjusted to pH 8 with 0.1 M NaOH).

Binding to ATP-Sepharose. Treated samples of progesterone receptor were fractionated on columns of ATP Sepharose as described previously (1-3) except that Barbital buffer was used throughout the procedure. This buffer had no effect on the receptor preparations when compared to Tris-HCl which had been used in our previous studies (1-3). However, since Tris interfers with the action of pyridoxal 5'-phosphate, it could not be used here. Briefly, the receptor sample (0.6 ml) was applied to a 1 ml column of ATP-Sepharose equilibrated with Barbital buffer. After the column was washed with 15 ml of Barbital buffer, the adsorbed receptor was eluted with buffer plus 1 M KCl and ten 1 ml fractions were collected. Aliquots (0.2 ml) from each fraction were transferred to scintillation vials. Five-tenth ml of water and 5 ml of scintillation fluid consisting of toluene (Fisher) Triton X-100 (RPI), and Scintiprep I (Fisher) 190: 99:8 (v/v/v), were added to determine radioactivity with a Beckman LS-250 Liquid Scintillation counter (33% efficiency).

Hormone binding. Bound progesterone was quantitated in samples after inhibitor treatment using the charcoal adsorption method (2). The assay tubes contained 0.1 ml receptor sample (plus labeled progesterone), 0.1 ml ovalbumin (1 mg) and 0.1 ml Barbital buffer. Parallel tubes containing 0.1 µg unlabeled progesterone were included for background determinations. The background tubes were incubated for 1 h at 35° to allow exchange of bound [$^3$H]progesterone for unlabeled hormone. Five-tenths ml charcoal suspension (0.25% charcoal, 0.025% dextran in Barbital buffer) was added to each tube for 5 min at 4°. After centrifugation, the radioactivity of bound progesterone was measured in the supernatants.

## RESULTS

The inhibitory effect of 5 mM pyridoxal 5'-phosphate on binding of the progesterone receptor to ATP-Sepharose is illustrated in Fig. 1. The elution profile of the control sample is as reported previously (1-3). The first peak represents unbound [$^3$H]progesterone, whereas over 80% of the receptor complex is bound to the affinity column and then eluted with 1 M KCl. Pyridoxal 5'-phosphate almost completely abolished receptor binding to the column which could be accounted for by an increase of [$^3$H]progesterone in the first peak (flow-through). Since the progesterone receptor is unstable at elevated temperature, all treatments were performed at 4°. Under these conditions, the effects of pyridoxal 5'-phospate occur gradually and, for convenience, the receptor preparations were incubated overnight (18 h). During this time period, the receptor activity was

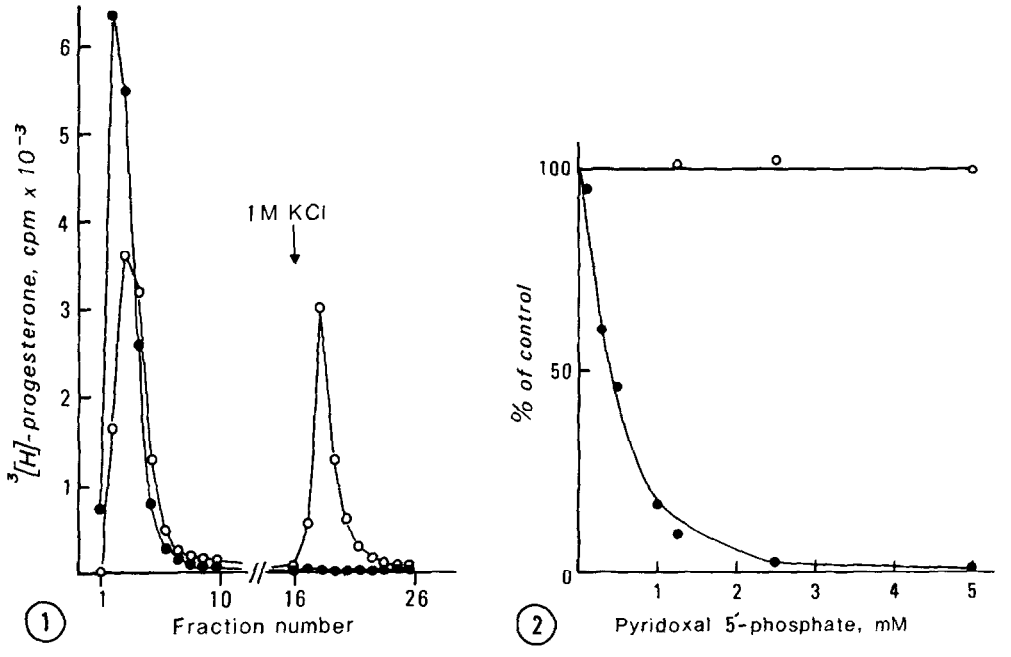


Fig. 1. The effect of 5 mM pyridoxal 5'-phosphate onthe binding of progesterone receptor to ATP-Sepharose. The receptor sample was prepared and labeled with [$^3$H]progesterone as described in Materials and Methods. A portion of this was treated with 5 mM pyridoxal 5'-phosphate for 18 h at 4° and the treated (●——●) and untreated (O——O) samples were fractionated on ATP-Sepharose. In the untreated sample, the first column peak represents primarily unbound hormone, whereas the bound receptor complex is eluted with 1 M KCl (1,2). The amount of receptor complex was equal in both groups as determined by charcoal adsorption assay.

Fig. 2. The effect of different concentrations of pyridoxal 5'-phosphate on the progesterone-receptor complex and its binding to ATP-Sepharose. Samples of [$^3$H]progesterone-receptor complex were treated with pyridoxal 5'-phosphate and the extent of ATP-Sepharose binding (●——●) was measured as illustrated in Fig. 1. The total progesterone binding activity (O——O) was also measured in a portion of each sample by the charcoal adsorption method. A sample without pyridoxal 5'-phosphate treatment served as the control.

stable and the effects of the inhibitor were complete. Fig. 2 illustrates the concentration dependency of pyridoxal 5'-phosphate inhibition. The inhibition is half-maximal at 0.5 mM pyridoxal 5'-phosphate and is complete at 2.5 mM. At these concentrations of inhibitor, the progesterone-receptor complex remains intact (Fig. 2). However, higher concentrations have been found to reduce

hormone binding and this effect is more obvious when pyridoxal 5'-phosphate is included prior to hormone addition (results not shown).

The inhibition of ATP binding by progesterone receptor was specific for pyridoxal 5'-phosphate (Table 1). Four other related compounds, pyridoxal, pyridoxine, pyridoxamine 5'-phosphate and pyridoxamine, did not have a significant inhibitory effect on ATP binding even at a high concentration of 5 mM.

In other systems, it has been demonstrated that pyridoxal 5'-phosphate reacts with amino groups of proteins through Schiff base formation which is easily reversed by the addition of excess primary amines (6-9). This is also true of the present system where the addition of 100 mM Tris-HCl (pH 8) abolished the inhibition caused by pyridoxal 5'-phosphate (Fig. 3). However, when the reducing agent, $NaBH_4$ was added prior to the Tris, the Tris could not reverse the inhibition of ATP binding. In separate experiments, $NaBH_4$ alone was found to have no effect on the steroid-receptor complex or on ATP binding by receptor. These observations indicate the existence of a Schiff base between the receptor and pyridoxal 5'-phosphate which is converted to a more stable covalent bond through the action of $NaBH_4$.

## DISCUSSION

The results presented above demonstrate that the binding ability of the avian progesterone receptor to ATP-Sepharose can be inactivated by pyridoxal 5'-phosphate, presumably through the reversible formation of a Schiff base. Since the related compounds, pyridoxal, pyridoxine, pyridoxamine and pyridoxamine 5'-phosphate, are not inhibitory, both the aldehyde and the phosphate groups seem to be required for the inactivation. Although the mechanism of inhibition is not clearly defined in the present report, it is likely that pyridoxal 5'-phosphate is bound to a critical lysine or arginine amino acid at the nucleotide binding site of the receptor. Pyridoxal 5'-phosphate has recently been used as a specific chemical modifer of ε-aminolysyl residues of several proteins. These include a variety of nucleotide-binding proteins such as fructose 1,6-diphosphatase (10), pyruvate kinase (9), RNA polymerases (6-8), and

TABLE 1.

The effect of pyridoxal 5'-phosphate and related compounds on the binding of progesterone receptor to ATP-Sepharose

| Additive (5mM) | ATP-Sepharose bound % of control |
|---|---|
| Control | 100 |
| Pyridoxal 5'-phosphate | 1 |
| Pyridoxal | 82 |
| Pyridoxine | 93 |
| Pyridoxamine | 94 |
| Pyridoxamine 5'-phosphate | 80 |

some DNA polymerases (11).

It is likely that pyridoxal 5'-phosphate would be an effective inhibitor in other steroid-receptor systems. This is indicated by a recent preliminary report by Litwack and Cake (12). These investigators have observed an inhibitory effect of pyridoxal 5'-phosphate on the binding of glucocorticoid receptor to DNA-cellulose.

Although the possible role of ATP binding in a function of the progesterone receptor has not been clearly established, its involvement in an enzymatic activity of the receptor has been indicated. A recent report from this laboratory showed that highly purified preparations of progesterone receptor contain an ATP-pyrophosphate exchange activity (13), and the possibility that this relates to a nucleotidyl transferase activity has been discussed. Pyridoxal 5'-phosphate should be a very useful agent in further characterization of the nucleotide binding site. It may be applied both as a reversible inhibitor of the binding process and as an irreversible affinity label of this site on the receptor.

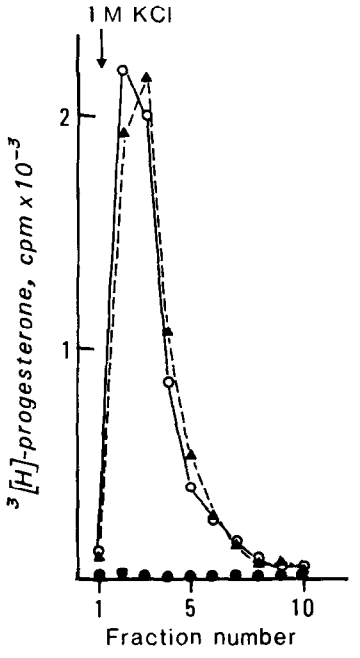


Fig. 3. The effect of Tris and $NaBH_4$ on the inhibition of receptor by pyridoxal 5'-phosphate. A receptor sample (plus labeled progesterone) was incubated with 5 mM pyridoxal 5'-phosphate for 18 h at 4°. $NaBH_4$ ( 5 mM) was added to half of the receptor preparation for 15 min, 4°. Both samples were then dialyzed for 1 h against Barbital buffer and combined with Tris-HCl (pH 8, 100 mM final concentration) for 2 h, 4°. They were then fractionated on ATP-Sepharose. A sample with no additives served as the control. Only the 1 M KCl elution profiles are illustrated: control, O——O; plus pyridoxal 5'-phosphate, $NaBH_4$, then Tris, ●——●; plus pyridoxal 5'-phosphate and then Tris (no $NaBH_4$), (▲---▲).

Acknowledgements: The technical assistance of Vernon Summerlin and Bridget Stensgard is greatly appreciated. This work was supported by NIH Contract HD-3-2769 and by the Mayo Foundation.

REFERENCES

1. Moudgil, V.K. and Toft, D.O. (1975) Proc. Nat. Acad. Sci. USA 72, 901-905.
2. Moudgil, V.K. and Toft, D.O. (1977) Biochem. Biophys. Acta 490, 477-488.
3. Toft, D.O., Moudgil, V.K., Lohmar, P. and Miller, J. (1977) Ann. N.Y. Acad. Sci. 386, 29-42.
4. Moudgil, V.K., Lohmar, P.H., Toft, D.O. (1976) The Endocrine Soc., 58th Annual Meeting, San Francisco, Abstr. 47.
5. Lamed, R., Levin, Y. and Wilcheck, M. (1973) Biochem. Biophys. Acta 304, 231-235.
6. Venegas, A., Martial, J. and Valenzuela, P. (1973) Biochem. Biophys. Res. Commun. 55, 1053-1059.
7. Martial, J., Zaldivar, J., Bull, P., Venegas, A. and Valenzuela, P. (1975) Biochemistry 14, 4907-4911.

8. Bull, P., Zaldivar, J., Venegas, A., Martial, J. and Valenzuela, P. (1975) Biochem. Biophys. Res. Commun. 64, 1152-1159.
9. Johnson, G.S. and Deal, W.C., Jr. (1970) J. Biol. Chem. 245, 238-245.
10. Marcus, F. and Hubert, E. (1968) J. Biol. Chem. 243, 4923-4926.
11. Modak, M.J. (1976) Biochem. Biophys. Res. Commun. 71, 180-187.
12. Litwack, G. and Cake, M.H. (1977) Fed. Proc. 36, 911.
13. Moudgil, V.K. and Toft, D.O. (1976) Proc. Nat. Acad. Sci. USA 73, 3443-3447