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# Adrenocortical Pregnenolone-Binding Protein: Identification and Antibody Development

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Pregnenolone-binding activity isolated from the cytosol of the guinea pig adrenal cortex appears to correspond to a $M_r$ 34,000 protein when examined by SDS-polyacrylamide gel electrophoresis during different stages of purification. To verify this finding the $M_r$ 34,000 protein band was eluted from the SDS gel and used to generate a polyclonal antibody. Immobilized anti 34,000 IgG on protein A-Sepharose was found to extract pregnenolone-binding activity from solution in contrast to pre-immune IgG and an antibody raised against a $M_r$ 30,000 protein isolated simultaneously. In addition, protein eluted from the protein A-anti 34,000 IgG complex exhibited the expected molecular weight of 34,000 when examined on an SDS gel. These results, thus, confirm that the pregnenolone-binding protein is indeed a protein of $M_r$ 34,000. 

Pregnenolone is a common intermediate in the biosynthesis of all steroid hormones. The synthesis of pregnenolone from cholesterol is the rate-limiting step in steroidogenesis (1-4), and takes place at the inner mitochondrial membrane (5-7). Cholesterol which is poorly soluble in an aqueous medium requires carrier proteins for extra and intracellular transport (8-10). Pregnenolone is also poorly soluble in an aqueous medium and its intracellular transport is poorly understood. An intracellular cholesterol-binding protein that appears to play an important role in adrenocortical cholesterol transport, including the transfer of cholesterol to the inner mitochondrial membrane site of cholesterol side-chain cleavage, has been reported (11-14). It should be noted that the guinea pig adrenocortical cytosol contains cholesterol-binding proteins that have been partially characterized (15). A pregnenolone-binding protein has also been detected in the guinea pig adrenal cortex and partially purified (16,17). Because the pregnenolone-binding activity is not stable, however, a biologically active protein has been difficult to isolate, and the specific protein that binds pregnenolone has not been conclusively identified. This report describes the strategy that has led to the confirmation of a $M_r$ 34,000 protein as the pregnenolone-binding protein.

## MATERIALS AND METHODS

Male guinea pigs (NIH inbred strain 2) weighing 600-800 g were anesthetized with a brief exposure to $CO_2$ and decapitated. The adrenal glands were placed in an iced 20 mM

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Tris-HCl (pH 7.5), 1.0 mM EDTA solution (buffer A). The glands were cleaned of fat and fibrous material, minced and homogenized in buffer A (1 g/5 ml) using a glass-teflon homogenizer. The homogenate was centrifuged at 200,000 x g for 1.5 h. The lipid layer was carefully removed and the supernatant decanted.

Pregnenolone-binding activity was partially purified by ion-exchange and gel permeation chromatography. Cytosol was applied to a column of DEAE-cellulose (15 x 1.6 cm) equilibrated with buffer A. The column was washed with 600 ml of buffer A and the adsorbed proteins desorbed with 500 ml of 0-0.25 M NaCl in buffer A. Fractions containing pregnenolone-binding activity were combined and concentrated. The concentrated protein was incubated with [$^3$H]pregnenolone and applied to an Ultrogel ACA 44 column (100 x 2.5 cm) equilibrated with 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA. The peak radioactive fractions eluted were combined and concentrated.

The proteins contained in the partially purified pregnenolone-binding protein preparation were further resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis using 12% acrylamide (18). After electrophoresis the gels were washed with $H_2O$, stained with Coomassie brilliant blue G (1% in $H_2O$) for 15 min, and destained in $H_2O$ for 20 min. Selected protein bands were excised, electroeluted (19) and concentrated by use of an ISCO apparatus at 6 mA/cup: the buffer system contained 0.1% SDS, 1.0 mM EDTA and sodium borate, pH 8.5 (0.1 M outer chamber, 0.02 M inner chamber and sample cups). Purity of the electroeluted proteins was evaluated by repeat SDS-gel electrophoresis. Antibodies to the purified proteins were developed in rabbits.

Cytosol preparations obtained from the outer and inner zones of the guinea pig adrenal cortex (20) were subjected to SDS-gel electrophoresis (12%) and the proteins transblotted onto nitrocellulose paper overnight (30 V) at 2°C in 25 mM Tris (pH 8.3), 192 mM glycine and 20% methanol. The nitrocellulose paper was blocked with 3% gelatin dissolved in 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl (buffer B) for 1 h and then incubated with antiserum diluted 1:2000 with buffer B containing 1% gelatin for 1 h at room temperature. The antibody-treated nitrocellulose paper was washed 5 times with 0.05% Tween-20 in buffer B and incubated with peroxidase-conjugated goat antirabbit IgG (0.5 μg/ml 1% gelatin in buffer B). Immunoreactive bands were visualized by the addition of $H_2O_2$/4-chloro-1-naphthol reagent.

Protein A-IgG complex was made by applying 0.7-1.0 ml of serum to an Affi-protein A column (0.5 ml packed) equilibrated with 0.1 M potassium phosphate (pH 7.0). The flow through solution was collected and reapplied 5 times. The column was extensively washed with the potassium phosphate buffer followed by buffer A. The resulting Affi-protein A-IgG was transferred to a 1.5 ml conical tube containing 0.5 ml buffer A. A partially purified pregnenolone-binding protein preparation (75 μl, 500 μg) was then added and the mixture incubated overnight at 4°C. The immunoreactive support was repacked into a small column. The supernatant and 0.8 ml wash (buffer A) were collected and analyzed for pregnenolone binding. The column was extensively washed with 0.1 M potassium phosphate buffer (pH 7.0) and the protein bound to the Affi-protein A column eluted with 1.5 ml of 0.1 M glycine (pH 3.5). The eluted proteins were analyzed by SDS-gel electrophoresis.

## RESULTS

### Antibody production

Fractionation of adrenal cytosol for pregnenolone-binding activity by DEAE-cellulose chromatography is shown in Fig. 1, top panel. The SDS-gel pattern of the eluted proteins is depicted in Fig. 1, bottom panel. The intensity of the binding activity appears to correlate with a $M_r$ 34,000 protein. The active fractions from the ion-exchange column were further purified by gel permeation chromatography and the results are shown in Figs. 2 and 3. The binding activity again correlates with a $M_r$ 34,000 protein. The most active Ultrogel fractions were combined and the proteins resolved by SDS-gel electrophoresis. Two proteins corresponding to $M_r$ 30,000 (30 K) and 34,000 (34 K) were eluted and used for antibody production; the purity of the preparations is depicted in Fig. 4.

To monitor antibody production to the 30K and 34K proteins, cytosol was prepared from the inner and outer adrenocortical zones and subjected to SDS-gel electrophoresis. The

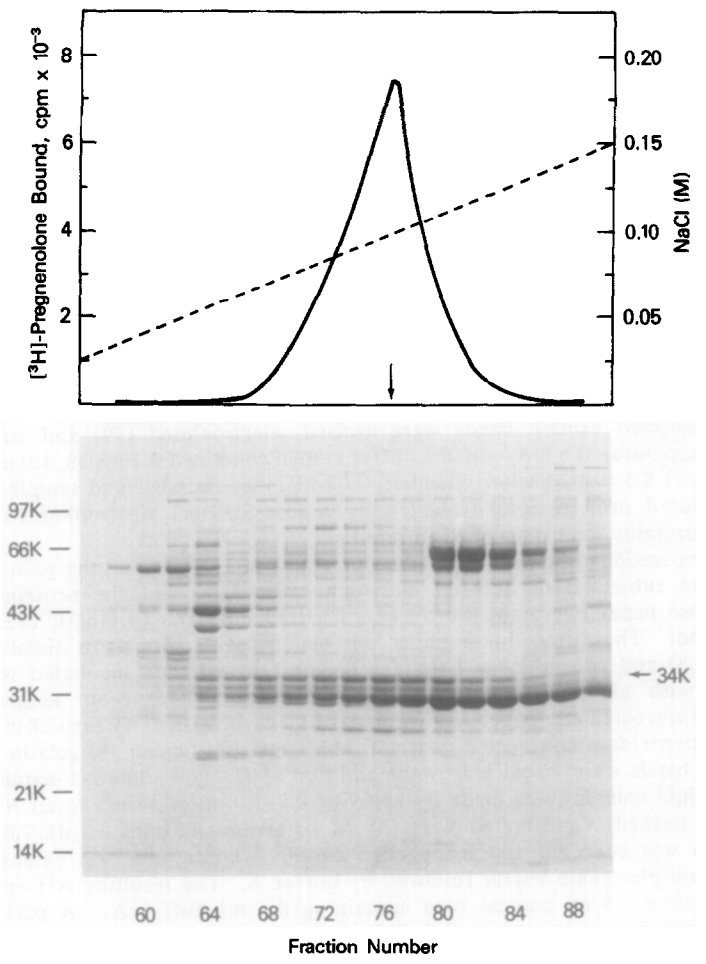


**Fig. 1.** DEAE-cellulose column chromatography. Adrenal cytosol was applied, the column washed and 5 ml eluted fractions collected. Aliquots (200 μl) were assayed for pregnenolone binding using small columns of Sephadex G-25 (upper panel); additional aliquots (30 μl) were subjected to SDS-polyacrylamide gel electrophoresis (lower panel). The arrow indicates the SDS gel fraction corresponding to the most active pregnenolone-binding fraction off the DEAE column.

resolved proteins in the gel were transblotted to nitrocellulose paper and subjected to immunoblotting. The immunoblot with anti-34K serum showed a single protein band of 34K (Fig. 5, lanes 3 and 4). The antiserum produced against the 30K protein recognized the corresponding antigen and several other minor proteins (Fig. 5, lanes 1 and 2).

Identification of the pregnenolone-binding protein

In order to determine if the 34K protein was indeed the pregnenolone-binding protein, the 30K and 34K antisera were tested for their ability to remove pregnenolone-binding activity from a partially purified preparation. The Affi-protein A-preimmune IgG complex failed to adsorb pregnenolone-binding activity (Fig. 6, 1) as was essentially the case for the Affi-protein A-anti 30K IgG complex (Fig. 6, 2). In contrast, the Affi-protein A-anti 34K IgG complex adsorbed more than 80% of the pregnenolone-binding activity (Fig. 6, 3). Proteins

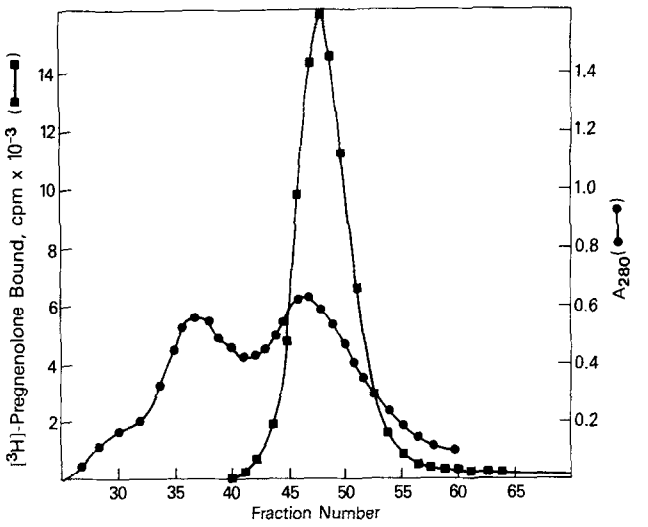


**Fig. 2.** Ultrogel ACA 44 column chromatography. The most active pregnenolone-binding fractions off the DEAE column were pooled, concentrated, incubated with [$^3$H]pregnenolone and applied to the Ultrogel column. The radioactivity in a 200 µl aliquot of each 5 ml fraction was determined.

adsorbed by the Affi-protein A- IgG complexes were eluted and analyzed by SDS-gel electrophoresis. No major band was eluted from the preimmune complex (Fig. 7, lane 3). Elution of the Affi-protein A-anti 34K IgG complex revealed a protein of $M_r$ 34,000 (Fig. 7, lane 2), while a major protein of $M_r$ 30,000 was eluted from the Affi-protein A-Anti 30K IgG complex (Fig. 7, lane 1).

## DISCUSSION

It was suggested in a previous report that the pregnenolone-binding protein in the soluble fraction of the guinea pig adrenal cortex had a molecular weight of 34,000 (17). This

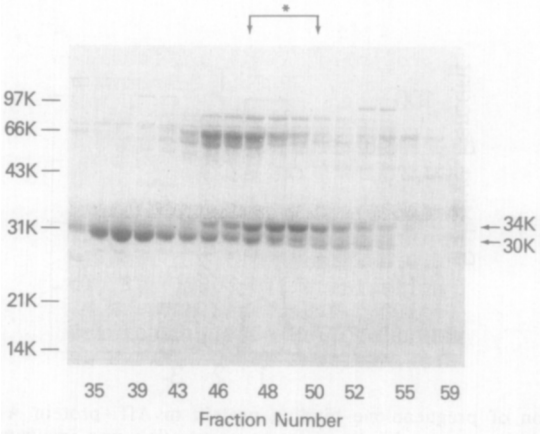


**Fig. 3.** SDS-polyacrylamide gel electrophoresis. Aliguots (30 µl) of Ultrogel ACA 44 column fractions were analyzed between each side of the radioactive peak shown in Fig. 2. *Indicates the most active pregnenolone-binding fractions bracketed by the arrows.

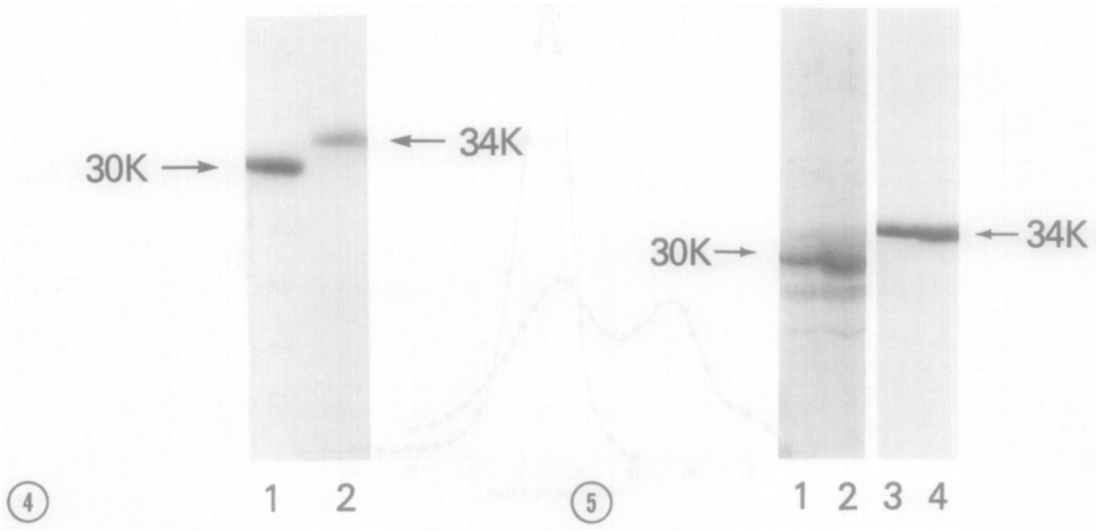


Fig. 4. SDS-polyacrylamide gel electrophoresis of isolated 30K and 34K protein preparations. Refer to the text for details.

Fig. 5. Immunoblots of adrenal cytosol prepared from the outer and inner cortical zones. Outer zone cytosol (90 μg) in lanes 1 and 3 and inner zone cytosol (77 μg) in lanes 2 and 4 were immunoblotted with anti-30K serum (lanes 1 and 2) and with anti-34K serum (lanes 3 and 4).

was based on the analysis of protein bands on SDS gels after partial purification, including gel electrophoresis under non-denaturing conditions and pregnenolone affinity chromatography (17). Since the pregnenolone-binding protein was not purified to complete homogeneity, however, conclusive identification of the active protein was not possible. The experiments reported in this paper confirm that the $M_r$ 34,000 protein is indeed the pregnenolone-binding protein in

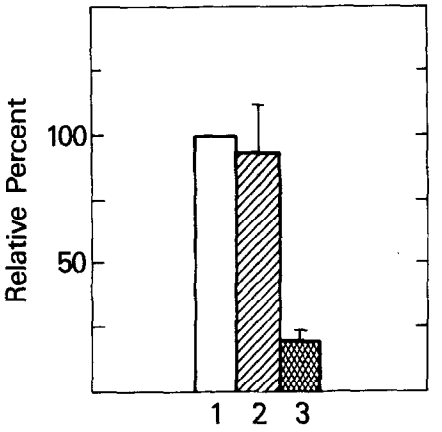


Fig. 6. Adsorption of pregnenolone-binding protein to Affi-protein A-IgG complexes. A partially purified pregnenolone-binding protein preparation was incubated with various IgG complexes and the non-adsorbed material assayed for pregnenolone-binding activity. The pregnenolone-binding activity in the non-adsorbed material when pre-immune-IgG was tested was used to establish the 100% reference value. Pre-immune-IgG complex (1), anti 30K-IgG complex (2), anti 34K-IgG complex (3).

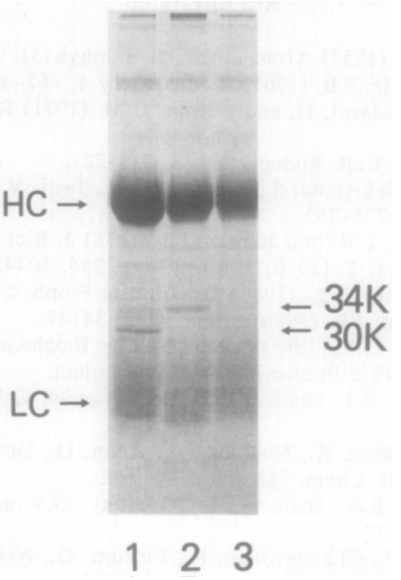


Fig. 7. Proteins desorbed from Affi-protein A-IgG complexes used in the experiment noted in Fig. 6. Proteins adsorbed to the various complexes were eluted at pH 3.5 and analyzed by SDS-polyacrylamide gel electrophoresis. Anti 30K-IgG complex (lane 1), anti-34K-IgG complex (lane 2), pre-immune-IgG complex (lane 3). HC = heavy chain of IgG and LC = light chains of IgG.

that the removal of pregnenolone-binding activity was coincident with the adsorption of a single protein of $M_r$ 34,000 by the Affi-protein A-anti 34 K IgG complex.

It was previously noted that pregnenolone-binding activity was primarily located (80-90%) in the inner zone of the adrenal cortex (20). When cytosol from the outer and inner zones, however, was examined by immunoblotting, the binding protein appeared to be present to about the same extent in the outer as in the inner zone (Fig. 5). The exact explanation for this finding is not understood but there are several possible reasons. It is possible that the binding protein in the outer zone is essentially saturated with endogenous pregnenolone, or that the binding protein is activated and/or inactivated by some modification such as phosphorylation/dephosphorylation. The former seems to be an unlikely explanation since the content of pregnenolone is 10-20 times higher in the inner zone than in the outer zone (21). These possibilities still remain to be examined.

The function of the pregnenolone-binding protein is not presently known; no catalytic activity has been found associated with the protein (17). Since pregnenolone is very insoluble in an aqueous medium, an intriguing possibility is that the binding protein could serve as a transporter. It is also conceivable that the binding protein might play a role in the regulation of steroid synthesis by some direct action on the mitochondrial cholesterol side-chain cleavage enzyme system or more indirectly by some action on the nuclear genome. These considerations will require a careful examination. Now that an antibody to the pregnenolone-binding protein is available several important areas of investigation can be undertaken: these will include development of a radioimmunoassay, isolation of the specific mRNA, etc.

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