Adrenal Mitochondrial Cytochrome P-450 and Cholesterol Side Chain Cleavage Activity

DIFFERENCES IN THE RESPONSE OF THE ZONA GLOMERULOSA AND ZONA FASCICULATA-RETICULARIS TO ADRENOCORTICOTROPIC HORMONE AND ITS WITHDRAWAL*

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SUMMARY

A comparison of the effects of adrenocorticotropic hormone (ACTH) on the cholesterol side chain cleavage system of zona glomerulosa and zona fasciculata-reticularis tissue of the rat adrenal is presented. Following hypophysectomy, the rate of pregnenolone formation from endogenous cholesterol in mitochondria from zona fasciculata-reticularis was lower than in mitochondria from zona glomerulosa. Also, the proportion of high spin, cholesterol-bound side chain cleavage cytochrome P-450 was smaller, as measured by spectrophotometry and electron paramagnetic resonance spectroscopy.

Treatment with ACTH brought about an increase in the rate of cholesterol side chain cleavage and in the proportion of high spin, cholesterol-bound side chain cleavage cytochrome P-450 in mitochondria isolated from both zones. However, the percentage increase found in the zona fasciculata reticularis was much greater than in the zona glomerulosa.

It is concluded that although both zones are affected by ACTH, the cholesterol side chain cleavage system of the zona fasciculata-reticularis is much more sensitive than that of the zona glomerulosa to this hormone.

The effect of adrenocorticotropic hormone in bringing about an increase in corticosteroidogenesis was first observed over 20 years ago (1). It has now been established that this action is mediated through the effect of ACTH on the conversion of cholesterol to pregnenolone (2-4). This cholesterol side chain cleavage reaction is catalyzed by a mixed function oxidase system which involves cytochrome P-450 (5, 6). An increase in the high spin form of this heme protein (7) as well as an increase in cholesterol side chain cleavage activity (8) have been demonstrated following increased ACTH secretion brought about by ether anesthetic stress.

Many studies on the mechanism of action of ACTH and on the adrenocortical cytochrome P-450 system have involved the use of whole adrenal glands, although the functional zonation of the adrenal cortex has been recognized for many years (9, 10). The zona glomerulosa is concerned primarily with the production of aldosterone, whereas the zona fasciculata-reticularis produces mainly corticosterone and 18-hydroxy-11-deoxycorticosterone in the rat. Furthermore, these two functional zones respond to a different extent to ACTH (11) and Tait et al. have shown that this difference in the rate of production for a given steroid is dependent on the zone in which it is synthesized (12).

In the present study, rat capsular tissue, comprised mainly of cells of the zona glomerulosa, has been separated from decapsulate adrenal tissue, containing cells of the zona fasciculata-reticularis. Clear differences in the cholesterol side chain cleavage activity and the high spin forms of cytochrome P-450 were observed between these zones of the adrenal cortex in their response to ACTH and its withdrawal.

EXPERIMENTAL PROCEDURE

Female Sprague-Dawley rats, aged 50 to 60 days, obtained from the Holtzman Co., Madison, Wis., were used in all experiments. They were singly caged in a room at 20 ± 1°C with a 12-hour light-dark cycle. They were maintained on Charles River laboratory chow and tap water ad libitum. Hypophysectomies were performed transaurally, using the Hoffman-Reiter instrument (H. Neuman & Co., Skokie, Ill.).

Twenty-four hours following hypophysectomy, rats were anesthetized with ether and injected intravenously with (a) 0.1 ml of 0.9% NaCl solution (these groups are to be subsequently pregnenolone, 3β-hydroxy-5-pregnen-20-one; deoxycorticosterone, 21-hydroxy-4-pregnen-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnen-3,20-dione.

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The abbreviations and trivial names used are: ACTH, adrenocorticotropic hormone; cyanoketone, 2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one; cholesterol, 5-cholesten-3β-ol;
referred to as untreated rats) or (b) 8 units of ACTH gel (Organon, 80 i.u. per ml). Injections were done 10 min prior to killing by decapitation. Trunk blood was collected for measurement of peripheral corticosterone levels. Adrenal glands were rapidly removed from the rats and trimmed free of adhering fat. An incision was then made across the adrenal and the inner zones were extruded by exerting gentle pressure on the gland with curved forceps (9). Capsular and decapsulate portions of the glands were then placed in ice-cold 0.25 M sucrose. The pooled glands from 20 to 50 rats were used in each group.

The adrenal tissue was homogenized in a Potter-Elvehjem type Teflon-glass homogenizer and the homogenate centrifuged at 900 × g for 10 min in a Sorvall RC-2 refrigerated centrifuge to remove unbroken cells, nuclei, and red blood cells. The supernatant was then centrifuged at 10,000 × g for 10 min to give the mitochondrial pellet which was resuspended in 20 ml of 0.25 M sucrose and recentrifuged at 10,000 × g for 10 min. The resulting washed mitochondrial pellet was suspended in a small volume of 0.25 M sucrose to give a concentration of 15 to 30 mg of protein per ml.

For the determination of cholesterol side chain cleavage activity, 0.2 ml of the mitochondrial suspension was added to 3.3 ml of triethanolamine buffer (13), pH 7.3, containing 8 mM 2o-cyanocyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one (cyonoketone), an inhibitor of steroid 3β-ol dehydrogenase (14, 15). After a 5-min preincubation at 28°C, 20 μl of 2 mM isocitrate were added to start the reaction. At 0, 2, 5, and 10 min, 0.2 ml aliquots of incubation medium were removed into tubes containing 10 ml of methanol to stop the reaction. Because of the presence of the cyonoketone, the sole product of cholesterol side chain cleavage activity is 3β-hydroxypreg-5-ene-20-one (pregnenolone) (8, 16). [7-3H]Pregnenolone (0.01 μCi; 25 Ci per mmole; New England Nuclear) was added as internal standard for each sample to be analyzed and steroids were extracted and separated by thin layer chromatography on silica gel (17). Radioactive peaks were located using a Varian Aerograph radio-scanner. The areas corresponding to pregnenolone were scraped off the plates and the steroid extracted from the silica gel with 10 ml of chloroform-methanol (1:1, v/v). The amount of pregnenolone in the extracts was determined by radioimmunoassay using the method described by Veron et al. (18).

Difference spectra were obtained using an Aminco-Chance split beam-dual wave length spectrophotometer. An aliquot of mitochondria was diluted in triethanolamine buffer, pH 6.0, to give approximately 0.6 mg of protein per ml. Cyanoketone (2.5 μM) was added to inhibit steroid 3β-ol dehydrogenase activity, then pregnenolone (20 μM) was added, and the modified type II difference spectrum (A420:A380) was determined. Deoxytocorticosterone (54 μM) was then added to the cuvette and the type I difference spectrum (A450:A420) was determined. Previous studies in our laboratory and elsewhere (19, 20) had shown that pregnenolone does not interfere with the deoxytocorticosterone-induced type I difference spectrum. All steroids were added in small volumes of ethanol.

The concentration of cytochrome P-450 in each sample was determined after reducing the cytochrome with sodium dithionite and gassing with carbon monoxide for 90 s. The levels of cytochrome P-450 were calculated using the millimolar extinction coefficient of 91 determined by Omura and Sato (21).

Aliquots of the concentrated mitochondrial preparations were placed in quartz EPR tubes and frozen in liquid nitrogen. EPR spectroscopy and quantitation of signals were done as previously described (22). Estimates of the amounts of high spin forms of cytochrome P-450 were made by comparison of the product of the heights and the square of the widths of the signals at g = 8, to those obtained from a purified preparation of cytochrome P 450, (22).

Mitochondrial protein was measured by the method of Lowry et al. (23). Blood corticosterone was determined by the fluorescence method of Silber et al. (24).

**RESULTS**

**Serum Corticosterone Levels**—The serum corticosterone levels for the untreated rats was 4.7 ± 0.27 μg/100 ml. The uniformly low serum corticosterone levels in anesthetized untreated rats indicated that the hypophysectomies had been complete. Following treatment with ACTH, the serum corticosterone level rose to 28.3 ± 1.65 μg/100 ml.

**Cholesterol Side Chain Cleavage**—The cholesterol side chain cleavage activity of capsular adrenal mitochondria from untreated rats was considerably greater than that of decapsulate mitochondria. Both capsular and decapsulate adrenal mitochondria from ACTH-treated rats showed enhanced activity of side chain cleavage when compared with corresponding mitochondria from untreated animals (Fig. 1). The side chain cleavage activity of the decapsulate adrenal mitochondria from ACTH-treated rats was 11-fold greater than that from the untreated animals during the initial 2-min rapid phase of pregneno-

![Fig. 1. Pregnenolone formation from endogenous precursors in mitochondria isolated from capsular and decapsulate portions of adrenals from untreated and ACTH-treated hypophysectomized rats.](http://www.jbc.org/)

**Fig. 1.** Pregnenolone formation from endogenous precursors in mitochondria isolated from capsular and decapsulate portions of adrenals from untreated and ACTH-treated hypophysectomized rats. Mitochondria were incubated at 28°C in triethanolamine buffer, pH 7.3, in the presence of cyonoketone and isocitrate as described under "Experimental Procedure." Protein concentrations were: untreated capsular (□), 1.38 mg per ml; untreated decapsulate (○), 1.85 mg per ml; ACTH-treated capsular (■), 1.40 mg per ml; ACTH-treated decapsulate (●), 1.75 mg per ml.
alone formation. In contrast, capsular adrenal mitochondria only showed a 5-fold increase at this time period.

**Absorption Spectroscopy**—Data from the difference spectra of adrenal mitochondria from capsular and decapsulate glands are shown in Table I. The pregnenolone-induced modified type II difference spectrum is characterized by an increase in absorbance at 420 nm and a decrease in absorbance at 390 nm. This type of absorbance change is indicative of a transition of the ferrous heme protein from a high spin to a low spin form (20). The magnitude of the change upon addition of pregnenolone is an indirect measure of the cholesterol side chain cleavage cytochrome P-450 which is present in the mitochondrial preparation in the high spin form (7, 8).

A larger type II spectral change was observed in the capsular mitochondria from untreated rats than in the decapsulate mitochondria from these animals. Following ACTH treatment, there was a much greater increase in the type II spectral change in the decapsulate mitochondria than in the capsular mitochondria. As can be seen in Table I, the type II spectral change for the decapsulate mitochondria from ACTH-treated rats was 6 times larger than that for the decapsulate mitochondria from untreated rats. On the other hand, there was only a 3-fold increase in the type II spectral change of the capsular mitochondria from ACTH-treated as compared to untreated animals.

The type I spectral changes observed upon the addition of deoxycorticosterone to the mitochondrial preparations is also shown in Table I. This spectral change is characterized by a decrease in absorbance at 420 nm and an increase in absorbance at 390 nm, and is indicative of a low to high spin transition of the heme (26). The type I spectral change is a measure of the 11β-hydroxylase cytochrome P-450 which is present in the low spin form. There was usually a small decrease in this spectral change in the decapsulate and capsular mitochondria following ACTH treatment. The type I spectral change was consistently smaller for the capsular mitochondria than for the decapsulate mitochondria.

Based on adrenal mitochondrial protein concentration, the cytochrome P-450 levels were always lower in the capsular than in the decapsulate preparations. However, there was no change in the levels of cytochrome P-450 following treatment of the animals with ACTH for the short period of time used in these experiments. The amount of capsular tissue is much smaller than that of the decapsulate tissue such that only about 15% of the total cytochrome P-450 of adrenal mitochondria was present in the capsular portion of the gland. This percentage is undoubtedly an overestimate due to contamination of capsular tissue with decapsulate tissue.

**EPR Spectroscopy**—Fig. 2 depicts the low field portion of the EPR spectra of the mitochondria from capsular and decapsulate adrenal tissue from untreated and ACTH-treated animals. Present evidence supports the concept that the g = 8.2 signal corresponds to the high spin cholesterol side chain cleavage cytochrome P-450 and the g = 7.9 signal represents the high spin 11β-hydroxylase cytochrome P-450 (27).

The g = 8.2 signal was very small in spectra obtained with the decapsulate mitochondria from untreated rats and was only slightly larger in the mitochondria from the capsular tissue of these animals. There was a large increase in this signal in the decapsulate and capsular glands following treatment with ACTH. As can be seen in Table II, the increase in g = 8.2 signal was about 8-fold following ACTH treatment in the decapsulate mitochondria and about 2-fold in the capsular mitochondria. Even after ACTH treatment, it is noticeable that the percentage of mitochondrial cytochrome P-450 which is the high spin cytochrome P-450 cholesterol complex is not high.

### Table I

Cytochrome P-450 concentrations and steroid-induced spectral changes of capsular and decapsulate adrenal mitochondria

<table>
<thead>
<tr>
<th>Group</th>
<th>Cytochrome P-450 (nmole/mg protein)</th>
<th>Type II spectral change</th>
<th>Type I spectral change</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ΔA_{nmol/nmole} P-450/10^6</td>
<td>ΔA_{nmol/nmole} P-450/10^6</td>
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<tr>
<td>Untreated Capsular</td>
<td>0.97</td>
<td>8.6</td>
<td>45.6</td>
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<tr>
<td></td>
<td>0.90</td>
<td>10.3</td>
<td>44.2</td>
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<tr>
<td></td>
<td>1.20</td>
<td>6.6</td>
<td>49.3</td>
</tr>
<tr>
<td>Decapsulate</td>
<td>1.30</td>
<td>3.7</td>
<td>53.6</td>
</tr>
<tr>
<td></td>
<td>1.30</td>
<td>3.7</td>
<td>48.9</td>
</tr>
<tr>
<td></td>
<td>1.55</td>
<td>3.5</td>
<td>51.7</td>
</tr>
<tr>
<td>ACTH-treated Capsular</td>
<td>1.15</td>
<td>20.6</td>
<td>45.1</td>
</tr>
<tr>
<td></td>
<td>0.89</td>
<td>26.7</td>
<td>44.2</td>
</tr>
<tr>
<td>Decapsulate</td>
<td>1.31</td>
<td>27.9</td>
<td>49.5</td>
</tr>
<tr>
<td></td>
<td>1.18</td>
<td>20.8</td>
<td>49.1</td>
</tr>
<tr>
<td></td>
<td>1.57</td>
<td>19.5</td>
<td>49.4</td>
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Fig. 2. Low field portion of EPR spectra obtained from mitochondria of the capsular and decapsulate portions of the hypophysectomized rats, which had been pretreated with 0.9% NaCl solution or ACTH. Conditions of EPR spectroscopy were as follows: microwave power, 9 milliwatts; frequency, 9.18 GHz; modulation amplitude, 12.5 Gauss; temperature, 8°K; scanning rate, 100 Gauss per min; time constant, 1 s. The final amplifier gain was the same for all samples. The protein concentrations of individual samples in milligrams per ml were: capsular, hypophysectomized (Hypox), 14.5; capsular, hypophysectomized + ACTH, 25.3; decapsulate, hypophysectomized, 22.7; decapsulate, hypophysectomized + ACTH, 30.5. A signal-averaging computer was used to accumulate four spectra from each sample.
The action of ACTH to stimulate corticosteroidogenesis apparently involves activation of adrenal cortical adenyl cyclase (28, 29) and of a protein kinase (30). These events evidently precede any modulation of the steroid biosynthetic pathway. Subsequently it appears that it is the cholesterol side chain cleavage step of corticosteroidogenesis which is then activated (2-4). Recent studies (7, 27) have indicated that adrenal mitochondria isolated from stressed rats show an increased EPR signal around g = 8 as well as an increased rate of pregnenolone formation from endogenous cholesterol. This signal is similar to that of the high spin substrate-bound cytochrome P-450 isolated from Pseudomonas putida (22). Closer examination of this low field signal of rat adrenal mitochondria (27) has shown that it consists of two components, one appearing at g = 7.9 and the other at g = 8.2. The magnitude of the signal at g = 7.9 is greatly enhanced when deoxycorticosterone is added, a finding similar to that of Whysner et al. (26). On the other hand, addition of high concentrations of pregnenolone prior to EPR spectroscopy causes the disappearance of the signal at g = 8.2 in rat adrenal mitochondria. It has been further observed that addition of cholesterol to acetone-extracted adrenal mitochondria from hypophysectomized rats causes an increase in the signal at g = 8.2. These data are consistent with the interpretation that the signal at g = 7.9 is that of 11β-hydroxylase cytochrome P-450 in the high spin form, i.e. substrate-bound, whereas the signal at g = 8.2 is that of cholesterol side chain cleavage cytochrome P-450 in its high spin form. In the light of these findings, the increase in the g = 8.2 signal brought about by an increase in the blood ACTH level can now be interpreted as indicating that ACTH action on the adrenal cortical cell leads to an increase in flux of cholesterol through the side chain cleavage cytochrome P-450 system.

The visible absorbance spectrum of partially purified cholesterol side chain cleavage cytochrome P-450 isolated from the bovine adrenal cortex (26) is almost identical with that of the high spin cytochrome P-450 sme (31). Titration of this soluble adrenocortical cytochrome P 450 with pregnenolone yields an absolute absorbance spectrum with a maximum shifted from 393 to 416 nm. This latter spectrum very closely resembles that of substrate-free low spin cytochrome P-450 sme. This shift in absorbance maximum results in a modified type II difference spectrum, i.e. one with a peak at 420 nm and a trough at 390 nm. For rat adrenal mitochondria, an increase in the type II spectral change is associated with stress (7, 8) and this is correlated with an increase in the high spin form of cholesterol side chain cleavage cytochrome P-450 as measured by EPR spectroscopy (7). Thus the substrate-bound form of side chain cytochrome P-450 can be measured both by visible absorbance spectral changes and by EPR spectroscopy.

We have studied the effects of ACTH and its withdrawal on the cholesterol side chain cleavage system in the different functional zones of the rat adrenal cortex. The greater sensitivity of the zona fasciculata-reticularis to the withdrawal of ACTH was evident from the visible spectral changes and the EPR measurements of adrenal mitochondria from hypophysectomized rats. The type II spectral change and the g = 8.2 signal were much smaller in the mitochondria from the decapsulate gland when compared to those from the capsular gland. Following ACTH treatment, however, the decapsulate mitochondria showed a greater increase in both type II spectral change and in the g = 8.2 signal than did capsular adrenal mitochondria. This difference was also apparent in the rates of pregnenolone formation by the capsular and decapsulate adrenal mitochondria isolated from the two groups of animals (Fig. 1). These differences were evident despite the known contamination of the capsular adrenal tissue by decapsulate tissue (12) when these zones are separated by the method of Giroud et al. (9).

ACTH treatment of hypophysectomized rats also led to an increase in the magnitude of the g = 7.9 EPR signal (Fig. 2) especially in the adrenal mitochondria isolated from decapsulate tissue. An explanation for this may be that ACTH increases the concentration of endogenous deoxycorticosterone (32) or progesterone in the mitochondria. Both of these steroids bind to the low spin form of 11β-hydroxylase cytochrome P-450 to give an increase in the g = 7.9 signal. However, despite this increase in binding of substrate to 11β-hydroxylase cytochrome P-450, the activity of steroid 11β-hydroxylase is not affected by ACTH (8), in contrast to our finding that the rate of cholesterol side chain cleavage is increased.

The greater effect of ACTH on the decapsulate adrenal mitochondria would be expected since the evidence is that the zona fasciculata-reticularis is wholly under the control of this hormone whereas the zona glomerulosa is affected by a number of other factors (33-38). The increase in the cholesterol side chain cleavage reaction that is observed in the capsular tissue is probably responsible for the increased aldosterone production which has been reported following acute ACTH treatment (39, 40). It seems clear that differences in rates of steroid production by the different zones of the adrenal gland in response to ACTH are a reflection of differences in the response of the cholesterol side chain cleavage reaction to this hormone.

**Acknowledgments**—We wish to acknowledge the assistance of Mr. L. M. Joseph who performed the hypophysectomies, Mr. R. E. Hansen and Mr. W. D. Hamilton who assisted with the electron paramagnetic resonance measurements, and Mr. R. Linsmair for the photography. The help of Dr. Samuel Gallant with the animal experiments was greatly appreciated.

**REFERENCES**


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**Table II**

High spin cytochrome P-450 in capsular and decapsulate adrenal mitochondria as determined by EPR

<table>
<thead>
<tr>
<th>Group</th>
<th>g = 8.2 signal</th>
<th>g = 7.9 signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Capsular</td>
<td>0.031</td>
<td>0.039</td>
</tr>
<tr>
<td>Decapsulate</td>
<td>0.008</td>
<td>0.024</td>
</tr>
<tr>
<td>ACTH-treated Capsular</td>
<td>0.063</td>
<td>0.087</td>
</tr>
<tr>
<td>Decapsulate</td>
<td>0.064</td>
<td>0.191</td>
</tr>
</tbody>
</table>

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