Temperature Dependence of Cholesterol Binding to Cytochrome P-450_{sec} of the Rat Adrenal

EFFECT OF ADRENOCORTICOTROPIC HORMONE AND CYCLOHEXIMIDE

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A type I absorbance change is observed in suspensions of adrenal cortical mitochondria as the temperature is increased from 0-22°C. This “heat-generated” type I absorbance change is similar in magnitude to the pregnenolone-induced type II absorbance change of these mitochondria. Studies with inhibitors of cholesterol side chain cleavage indicate that the heat-generated type I absorbance change represents the specific interaction of cytochrome P-450_{sec} with endogenous cholesterol in the mitochondria. This finding is confirmed by low temperature EPR spectroscopy on temperature-equilibrated, quick frozen adrenal mitochondrial samples. The EPR resonance at \( g = 8.2 \), which is that of the high spin cholesterol-bound cytochrome P-450_{sec}, is absent in the samples incubated at 0°C and increases in magnitude with increasing temperature of incubation. Studies of the pH dependence of the heat-generated type I and pregnenolone-induced type II absorbance changes reveal that both are diminished by increasing pH over the range 6 to 8. Adrenocorticotropic hormone (ACTH) treatment of rats results in adrenal mitochondria which show a greatly increased heat-generated type I absorbance change. The latter correlates with an increased pregnenolone-induced type II absorbance change and increased EPR \( g = 8.2 \) signal. Prior treatment of animals with cycloheximide eliminated the ACTH-induced increase in the heat-generated type I absorbance change, the pregnenolone-induced type II absorbance change and the EPR \( g = 8.2 \) signal. We estimate that the hydrophobic bonding of cholesterol to cytochrome P-450_{sec} occurs with a \( \\Delta H^o \) of approximately +15 kcal/mol and a \( \Delta S^o \) of approximately +55 cal/mol deg. Our data support the concept of a labile protein which participates directly in this process.

Adrenal mitochondria and smooth endoplasmic reticulum are known to contain a number of P-450 cytochromes which function in oxygen activation for the hydroxylation of steroids (1-5). Mitochondrial cytochrome P-450_{sec} is of particular interest because it catalyzes the rate-limiting step in corticosteroidogenesis, which is the major point at which ACTH exerts control of flux through the steroidalogenic pathways (6-8). Features in the EPR and visible absorption spectra which are characteristic of P-450 cytochromes have been utilized to demonstrate the high specificity of the interactions of these cytochromes with steroids (5, 9-13). In general when a steroid is added to adrenal mitochondria and a type I absorbance change is observed, there is also an increased signal magnitude in the region of the EPR spectrum around \( g = 8 \), where resonances of high spin heme occur. These two spectral changes reflect the specific binding of the steroid to a cytochrome P-450 in the mitochondria (10, 13-15). Other compounds, which when added to adrenal mitochondria displace bound substrates, bring about a type II absorbance change. A similar type II absorbance change is found when Krebs cycle intermediates are added leading to metabolism of bound substrates. This may also occur when lipophilic amines are added which interact directly with the heme center of the cytochrome. The type II absorbance changes are associated with decreased signal in the high spin (\( g = 8 \)) region of the EPR spectrum (14).

Spin state alterations in cytochrome P-450_{sec} have been extensively investigated by EPR and optical difference spectrosopies (14, 16, 17). In rat adrenal mitochondria, the EPR signal of the high spin cytochrome P-450_{sec} is located at \( g = 8.2 \) and is...
with an increase in the pregnenolone-induced absorbance change and in the activity of cholesterol side chain cleavage in these mitochondria (14–17, 18). Bell et al. (19) have also reported an increase in the cholesterol-bound form of cytochrome P-450 \( \text{P}_{450,\text{sec}} \) in adrenal mitochondria following ACTH treatment of rats. Prior treatment with cycloheximide prevented the changes in cytochrome P-450 \( \text{P}_{450,\text{sec}} \) brought about by ACTH (17). Previous studies by Ferguson (20) and Garren et al. (21) have shown that inhibitors of protein synthesis inhibit ACTH action of corticosteroidogenesis; thus a labile protein has been postulated to be required for stimulation of steroid production (21). The possible role of this labile protein in the regulation of intramitochondrial cholesterol pools has been discussed (14–16).

Preliminary experiments from this laboratory have indicated that there is a type I absorbance change as adrenal mitochondria warm from 0 to 22°C (22). The present studies were undertaken to investigate the nature of the temperature dependence of the cholesterol-cytochrome P-450 \( \text{P}_{450,\text{sec}} \) interaction, especially as this might apply to investigations of the mechanism of action of ACTH on steroidogenesis.

**EXPERIMENTAL PROCEDURES**

Female Sprague-Dawley rats (Holtzman Co. or Charles River Laboratories) weighing approximately 250 g were used throughout. Rats were singly caged and maintained on Purina Lab Chow ad libitum. All rats were hypophysectomized through the ear using the Hoffman-Reiter hypophysectomy instrument. Experiments were carried out 22 to 24 h post-hypophysectomy. Control animals received 0.1 ml of placebo injected into the jugular vein 15 min prior to killing. ACTH-treated groups received 8 units of ACTH (0.1 ml) by the same route, also 15 min prior to killing. Some groups (CYCLO) received 10 mg of cycloheximide by intraperitoneal injection followed 5 min later by intravenous ACTH. These rats were killed 15 min following the ACTH injection. Rats were killed by decapitation and blood collected from the neck for corticosterone assay (23). Adrenal glands were quickly dissected and rapidly enucleated in situ (24). This method of adrenal cortex collection yields zona fasciculata-reticularis tissue essentially free from zona glomerulosa.

Adrenal tissue was pooled by group in cold 0.25 M sucrose and homogenized by three passes of a Potter-Elvehjem type homogenizer. Mitochondria were isolated by differential centrifugation as previously described (17). The washed mitochondrial pellet was resuspended in a small volume of cold, buffered sucrose (pH 6.0). Aliquots of this concentrated preparation were taken for protein assay by the method of Lowry et al. (25). The remaining mitochondria were either placed directly into matched EPR tubes or diluted with triethanolamine buffer, pH 6.0, for optical studies.

The pregnenolone-induced type II absorbance change (PII) and the 11-deoxycorticosterone-induced type I absorbance change (DOCII) were determined at 22°C unless otherwise specified and under aerobic conditions as previously described (17). Cytochrome P-450 concentration was determined by the method of Omura and Sato (26). Absorbance changes were observed in the Amino-Chance Dual Wavelength/ Split Beam Spectrophotometer. The heat-generated type I absorbance change (HGI) was measured by taking the mitochondrial suspensions from ACTH-treated animals. The spectrophotometer was operating in the dual wavelength mode (420 - 390 nm) as the temperature of the

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**RESULTS**

A suspension of adrenal mitochondria from ACTH-stimulated rats was prepared in triethanolamine buffer, pH 6.0, and placed in matched cuvettes. The sample cuvette was equilibrated for 20 min at 22°C while the reference cuvette was maintained at 0°C. The difference spectrum was obtained using the Amino-Chance spectrophotometer operating in the split beam mode. A baseline scan was obtained after both the reference and the sample cuvette had been equilibrated to 22°C. Subtraction of the 22°C baseline from the 22°C - 0°C difference spectrum yielded the type I absorbance spectrum shown in Fig. 1. The spectral maximum was 386 nm and the minimum was at 418 nm.

**Heat-generated and Steroid-induced Absorbance Changes**—Fig. 2a illustrates a typical recording in the dual wavelength mode (420 - 390 nm) as the temperature of the...
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A sample of adrenal cortical mitochondria rises from 0°-22°. The negative tracing represents the HGI. The subsequent positive deflections (type II absorbance changes) were observed upon the addition of CTA and pregnenolone and represent the PI. CTA was added prior to pregnenolone to inhibit mitochondrial steroid Δ3-3β-ol dehydrogenase activity. In six experiments the HGI and PI1 (measured at 22°) per mM cytochrome P-450 were identical in magnitude within experimental error. The DOCI per mM cytochrome P-450 was found to be an independent constant with an approximate value of 50/mM total cytochrome P-450. When steroid induced absorbance changes were obtained in cuvettes maintained at 0°, a tracing like that in Fig. 2b resulted. The DOCI remained constant but the PI1 was absent.

The Δ20-300 absorbance changes resulting from warming (0-22°) and subsequent cooling (22°-0°) is illustrated in Fig. 3. The HGI was demonstrated in this way to be reversible through several warming and cooling cycles. This absorbance change is also partially reversed by the addition of 1.0 mM isocitrate at 22°.

Effect of Various Steroids on the Heat-Generated Absorbance Change Aminoglutethimide, CTA, pregnenolone, and deoxycorticosterone were each added to separate adrenal mitochondrial suspensions and equilibrated at 0° for several minutes. The mitochondrial sample was then transferred to the spectrophotometer and the heat-generated and steroid-induced absorbance changes recorded. The first three compounds were potent inhibitors of the development of the heat-generated absorbance change. Inhibition of the HGI and PI1 absorbance changes was greater than 50% at an aminoglutethimide concentration of 2 μM whereas inhibition of the DOCI was less than 15%. Inhibition with CTA was less than 50% at a final concentration of 8 μM, a concentration sufficient for complete inhibition of steroid Δ3-3β-ol dehydrogenase. Inhibition with pregnenolone was complete at 30°. Concentrations of deoxycorticosterone as high as 60 μM produced only a small inhibition of the HGI. The magnitude of the PI1 paralleled the HGI in all studies.

Effect of ACTH and Cycloheximide – The HGI and PI1 were observed to be 3 to 5 times larger in magnitude in the adrenal mitochondria of ACTH-treated rats when compared to those from CYCLO or placebo groups. Table I summarizes the data obtained in four experiments. The coefficient of correlation between the HGI and PI1 was observed to be greater than 0.95 in ACTH-stimulated groups and approximately 0.7 in placebo and CYCLO groups.

Blood corticosterone levels were 4.3 ± 0.6 μg/100 ml in the placebo groups, 6.4 ± 0.9 μg/100 ml in the CYCLO animals and 73.6 ± 8.2 μg/100 ml in the ACTH-treated groups.

In further experiments optical and EPR spectral changes were compared. In Fig. 4, EPR spectra in the region of g = 8 of mitochondrial samples from placebo or ACTH-treated groups are shown. Spectra were obtained from samples pre-equilibrated at 0° and 22°. In another experiment (Fig. 5), EPR spectra were obtained of adrenal mitochondria from CYCLO and ACTH-treated groups. In both of these experiments separate aliquots of mitochondria were diluted with triethanolamine buffer, pH 6.0, for parallel spectral studies and cytochrome P-450 analysis. Values obtained from EPR spectra appear with values obtained via optical spectroscopy (Table II).

In samples equilibrated at 22° the placebo and CYCLO groups were observed to have little or no g = 8.2 (P-450<sub>ccc</sub>) or g = 7.9 (P-450<sub>1ab</sub>) electron paramagnetic resonances. Samples from ACTH-treated groups, on the other hand, demonstrated large g = 7.9 and g = 8.2 signals. The identical samples from ACTH-treated groups when equilibrated at 0° prior to freezing in EPR tubes, still demonstrated an increased g = 1.9 signal, but the g = 8.2 signal was virtually absent. The g = 8.2 signal was also diminished in mitochondrial samples from placebo and CYCLO groups, when the samples were pre-equilibrated at 0°.

A third EPR study was performed with ether-stressed rats. Adrenal mitochondrial samples for EPR spectroscopy were equilibrated at 0, 7, 14, 20, and 36°. Fig. 6 shows the low field EPR spectra of the samples. At 0° there was no detectable g = 8.2 signal, but as the pre-equilibration temperature was increased, the magnitude of this signal also increased until at 36° it was greater than that of the g = 7.9 signal. The g = 7.9 signal was apparently diminished by 25% as the temperature was raised from 0-36°. Table III presents the numerical values for optical absorbance changes and EPR data.

Effect of pH on Spectral Changes – The HGI and PI1 were found to be pH-dependent. In the range of pH of 6.0 to 8.0, the two absorbance changes decreased in magnitude with rising pH value (Fig. 7). Mean values at pH 8.0 of both absorbance changes were reduced approximately 70% when compared to values obtained at pH 6.0. The progressive reduction in magnitude of both absorbance changes with increase in pH, was observed to occur in parallel. An apparent pK of 6.8 was obtained for both phenomena.

Thermodynamics of Cholesterol Binding to Cytochrome P-450<sub>ccc</sub> – We do not at present have extensive data on the temperature dependence of the occurrence of the high spin

<table>
<thead>
<tr>
<th>Group</th>
<th>Spectral change</th>
<th>Heat generated type I</th>
<th>Pregnenolone-induced type II</th>
<th>Deoxycorticosterone-induced type I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0°</td>
<td>22°</td>
<td>0°</td>
<td>22°</td>
</tr>
<tr>
<td>Placebo</td>
<td>6.6 ± 2.7</td>
<td>6.8 ± 1.6</td>
<td>49.2 ± 2.1</td>
<td>48.6 ± 3.3</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>8.7 ± 2.2</td>
<td>7.9 ± 3.0</td>
<td>47.3 ± 3.6</td>
<td>48.0 ± 2.6</td>
</tr>
<tr>
<td>ACTH</td>
<td>26.0 ± 5.1</td>
<td>25.2 ± 5.7</td>
<td>46.6 ± 3.1</td>
<td>45.7 ± 2.9</td>
</tr>
</tbody>
</table>

* Standard error of the mean.
Temperature Effects on Adrenal Cortical Cytochrome \( P-450_{sec} \)

8.2

7.9

I

IF.

4. EPR spectra between 720 and 920 G at 9.2 GHz (\( g = 9.1 \) to 7.1) of concentrated adrenal mitochondrial samples from ACTH-treated and placebo-treated animals. Samples were: a, ACTH-treated, preincubated at 22\(^\circ\); b, placebo-treated, preincubated at 22\(^\circ\); c, ACTH-treated, preincubated at 0\(^\circ\); and d, placebo-treated, preincubated at 0\(^\circ\). In ACTH-treated groups the instrument gain was 5,000 whereas in placebo-treated groups the gain was 10,000. Total cytochrome \( P-450 \) concentration was adjusted to 54.4 \( \mu \)M in both groups. Samples were prepared in triethanolamine buffer, pH 6.0. The conditions of EPR spectroscopy were, microwave frequency, approximately 9.2 GHz; microwave power, 10 milliwatts; modulation frequency, 100 KHz and amplitude, 10 G; temperature, 13 K; time constant, 1 s; and scanning rate, 50 G/min.

Form of \( P-450_{sec} \), but if certain assumptions are made, the data of Table III can be analyzed to yield estimates of the thermodynamic parameters associated with cholesterol binding. For the reaction

\[
P-450_{sec} + \text{cholesterol} \rightleftharpoons P-450_{sec-\text{cholesterol}}
\]

the equilibrium constant may be calculated on the assumption that the cholesterol and \( P-450 \) are dissolved in a phase composed of the mitochondrial protein plus phospholipid, i.e., in the mitochondrial membrane. If the density of the protein is 1.4, the density of the lipids is 0.8, and the membranes are 80% protein, the calculated membrane density is 1.28. The concentration of protein is then approximately 1 g/ml of membrane, and if the value of 1.5 \( \mu \)mol of cytochrome \( P-450/g \) of protein and 55 \( \mu \)mol of cholesterol/g of protein are used, then the equilibrium constant is

\[
K_{eq} = \frac{f}{[\text{cholesterol}][0.5 - f]},
\]

where \( f = [P-450_{sec-\text{cholesterol}}]/[P-450_{total}] \) and \([\text{cholesterol]}_{free} = [\text{cholesterol]}_{total} - [\text{cholesterol]}_{bound} \), assuming that 0.5 of the total \( P-450 \) is \( P-450_{sec} \). Values of these constants are found in Table IV. From \( \Delta G^o = -RT \ln K_{eq} = \Delta H^o - T \Delta S^o \) and a plot of \( \Delta G^o/T vs 1/T \) the values of \( \Delta H^o \) and \( \Delta S^o \) can be extracted (Table IV). There was no evidence for a bend or break in the plot of \( \Delta G^o/T vs 1/T \) over the range 7-36\(^\circ\) unlike the corresponding case for \( P-450_{sec} \) interaction with camphor reported by Griffin and Peterson (29).

DISCUSSION

The striking temperature dependence of cholesterol binding to \( P-450_{sec} \) is probably a reflection of a special case of "hydrophobic character"; both in the cytochrome and the steroid. The rate at which equilibrium is attained is obviously very slow compared to the rate at which we froze the EPR samples, so that these samples represent "trapped" equilibria fairly close to the situation present prior to freezing. The exact thermodynamic parameters obtained depend on the assumptions of unit activity coefficients as well as the assumptions used to derive the volumes accessible to the
Comparison of absorbance and EPR spectral changes in adrenal mitochondria from placebo and ACTH-treated rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Heat-generated type I absorbance change</th>
<th>Pregnenolone-induced type II absorbance change</th>
<th>Deoxycorticosterone-induced type I absorbance change</th>
<th>Deoxycorticosterone-induced type II absorbance change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0°</td>
<td>22°</td>
<td>0°</td>
<td>22°</td>
</tr>
<tr>
<td>ACTH</td>
<td>26.8</td>
<td>31.2</td>
<td>-0</td>
<td>0.076</td>
</tr>
<tr>
<td>Placebo</td>
<td>5.8</td>
<td>4.8</td>
<td>-0</td>
<td>0.015</td>
</tr>
</tbody>
</table>

* Absorbance changes are ΔA_{404-420}/μM P-450.

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**TABLE III**

Dependence of EPR signals arising from high spin hemes on incubation temperature of rat adrenal mitochondria

<table>
<thead>
<tr>
<th>Temperature</th>
<th>μmol spins (EPR)/μmol P-450</th>
<th>g = 8.2</th>
<th>g = 7.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°</td>
<td>0</td>
<td>0.071</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.010</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.034</td>
<td>0.059</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.046</td>
<td>0.054</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>0.116</td>
<td>0.054</td>
<td></td>
</tr>
</tbody>
</table>

* Samples at this indicated temperature were rapidly (<2 s) frozen by immersion in a vigorously stirred bath of isopentane at 130 K.

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**TABLE IV**

Equilibrium constant data for P-450_{res} + cholesterol = P-450_{res}·cholesterol

<table>
<thead>
<tr>
<th>Temperature</th>
<th>K_{eq}</th>
<th>ΔG°</th>
<th>ΔH°</th>
<th>ΔS°</th>
</tr>
</thead>
<tbody>
<tr>
<td>7°</td>
<td>0.38</td>
<td>+0.53</td>
<td>15</td>
<td>52</td>
</tr>
<tr>
<td>14</td>
<td>1.34</td>
<td>-0.16</td>
<td>15</td>
<td>53</td>
</tr>
<tr>
<td>30</td>
<td>1.89</td>
<td>-0.37</td>
<td>15</td>
<td>52</td>
</tr>
<tr>
<td>36</td>
<td>5.68</td>
<td>-1.05</td>
<td>15</td>
<td>52</td>
</tr>
</tbody>
</table>

* Calculated from a straight line fit to the data, on a plot of ΔG°/T versus 1/T.

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**FIG. 6**

EPR spectra in the g = 8 region of adrenal cortical mitochondria incubated at the indicated temperatures between 0 and 36° in triethanolamine buffer, pH 6.0. The adrenal mitochondria were prepared from ether-stressed animals. The instrument gain was identical in all spectra. The total cytochrome P-450 concentration of all samples was 60.3 μM. The conditions of EPR spectroscopy were as in Fig. 4.

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**FIG. 7**

The pH dependence of the HGI and PII. The data were normalized such that the HGI at pH 6.0 was considered to be 100% of the achievable absorbance change. Squares represent the normalized HGI. Circles represent the normalized PII.

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reactants, but the conclusions, i.e. that the equilibrium constant is near unity and that the binding is accompanied by a large increase in both enthalpy and entropy are relatively insensitive to the assumptions. Griffin and Peterson (29), examining the analogous binding of camphor to P-450_{can}, concluded there was a hydrophobic interaction driving that reaction, as is reasonable on consideration of the character of camphor. However, in our case the vanishingly low solubility of cholesterol in water makes it unlikely that the structural properties of water underlie the entropy-driven nature of cholesterol binding to P-450_{res}. Rather, it is likely that when cholesterol leaves the phospholipid phase to bind to the protein, the restrictions in the motions of the acyl chains of the phospholipids are removed, leading to at least part of the observed large increase in entropy. One may also speculate that the binding of cholesterol to P-450_{res} is accompanied by a net breaking of several hydrogen bonds, leading to the large
positive enthalpy change. It is thought (30) that cholesterol in mixed cholesterol-phospholipid micelles restricts the motions of the phospholipid side chains. In addition to this, there may be contributions to the entropy change from the interaction of the steroid with the protein. The above suggestions as to the origins of the observed entropy and enthalpy changes are thus only qualitative. It is unlikely that the temperature effects reported here are in any way related to the observed influence of temperature of isolation on the response of adrenal cortical tissue to ACTH added in vitro (31).

The pH studies demonstrate a high degree of parallelism between the heat-generated type I and pregnenolone-induced type II absorbance change. The only adrenal cytochrome P-450 reported to have a large pH dependence is the P-450 (14, 32, 33). These studies thus point again to P-450 as the enzyme involved. These experiments further demonstrate that pregnenolone displacement of endogenous cholesterol from the P-450 is not the phenomenon which is pH sensitive, rather it is the ability of the P-450 to form the high spin complex which is altered.

Throughout these studies the PI1 and the HGI were found to be nearly identical in magnitude. This indicates that the displacement of cholesterol by pregnenolone is nearly quantitative and is consistent with the interpretation that these two optical phenomena arise from interactions on the same enzyme. Further verification of this interpretation of the optical data is provided by the EPR data which show that the g = 8.2 resonance, which has been identified as belonging to the P-450, is the one which increases with increasing incubation temperature and is diminished by the addition of pregnenolone at high temperature.

In these studies mitochondria from ACTH-treated groups demonstrated HGI and PI1 which were 4 to 5 times larger than the same changes obtained in either cycloheximide-treated or control groups. This difference between groups is larger than previously reported (15-17). This is probably due to incomplete equilibration of the P-450 with cholesterol in earlier studies.

The present studies confirm the previous findings that ACTH increases the high spin form of P-450 and that this increase is prevented by cycloheximide (15, 17, 34). The finding that the HGI is reversible through several cycles of warming and cooling adds new dimensions to these well established findings. The increased high spin P-450 can no longer be considered to be cytochrome P-450 which is "caught" in the bound form at the time of homogenization. At the temperature of homogenization (0-3°C) little, if any, binding of cholesterol can occur. Thus the binding of cholesterol to the P-450 observed upon warming is determined by some other factor. If the postulated labile protein (21) is in fact the effector of this increased binding, as is supported by evidence (11-13), the effector of this increased binding is the one which increases with increasing incubation temperature and is diminished by the addition of pregnenolone at high temperature.

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Temperature Effects on Adrenal Cortical Cytochrome P-450

Temperature dependence of cholesterol binding to cytochrome P-450scc of the rat adrenal. Effect of adrenocorticotropic hormone and cycloheximide.

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