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Reduction of Cytochrome P-450$_{scc}$ - Substrate Complexes

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Reduction of the mitochondrial cytochrome P-450$_{scc}$ (scc for side chain cleavage) has been investigated. The binding of cholesterol and some steroid substrates perturbs the spin state, midpoint oxidation-reduction potential, and reduction rate of the cytochrome. No correlation was observed among these parameters except between the substrate-induced redox potential and the reduction rate. Results indicate that substrate binding to cytochrome P-450$_{scc}$ regulates the reduction rate through its effect on the redox potential.

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ในการศึกษาปฏิกริยารีดักชันของ cytochrome P-450$_{scc}$ ซึ่งเป็นเอ็นซัยม์ในไมโตคอนเดรียของ adrenal cortex โดยใช้ enzyme-substrate complex ต่าง ๆ ชี้บ่งว่า substrate-induced midpoint oxidation reduction potential ของเอ็นซัยม์ควบคุมอัตราเร็วของปฏิกริยารีดักชันนี้โดยตรง

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Cytochrome P-450$_{scc}$ is a heme containing enzyme which resides in the inner membrane of adrenal cortex mitochondria and catalyzes three sequential mixed function oxidation reactions in the side chain cleavage of cholesterol.[1,4] The first and the second hydroxylation give 22 R-hydroxycholesterol and 20 α , 22R-dihydroxycholesterol intermediates while the third oxidation results in cleavage of cholesterol at the 20-22 carbon-carbon bond to yield pregnenolone.[1,5] Each step requires two electrons which are furnished by NADPH via the flavoprotein adrenodoxin reductase and the iron-sulfur protein adrenodoxin.[6] Adrenodoxin acts as a mobile electron carrier during electron shuttle,[6,7] forming a one-to-one complex first with NADPH reduced adrenodoxin reductase from which it accepts an electron, then dissociating and finally reassociating with and donating an electron to the membrane-bound cytochrome P-450$_{scc}$. Physiological and some non-physiological steroid substrates bind to the cytochrome with different affinities and perturb the spin state and oxidation-reduction potential of the heme iron thus influencing the reduction by adrenodoxin.[8,9] The relationship of substrate binding, spin state, oxidation-reduction potential, and reduction rate was tested herein.

Procedures

Cytochrome P-450$_{scc}$ and adrenodoxin were purified from bovine adrenal cortex mitochondria as described previously.[10,11] The purification of cytochrome P-450$_{scc}$ was carried out by cholate solubilization from the mitochondrial pellet, ammonium sulfate fractionation, and hexylagarose chromatography. Concentration of the cytochrome was determined from the diferences in absorbancies of reduced complex with carbon monoxide minus reduced cytochrome alone using an extinction coefficient of 91 mM^{-1} cm^{-1} for 450 minus 490 nm.[10] Concentration of adrenodoxin was determined using an extinction coeffient of 11 mM^{-1} cm^{-1} at 414 nm.[11]

Reduction of cytochrome P-450$_{scc}$ - substrate complex by adrenodoxin was monitored by stopped-flow spectrophotometry. Stopped-flow experiment utilized a modified Update Instrument interfaced to a Northstar Horizon computer (model 3820 On Line Instrument System). Spectra were stored on magnetic diskettes for later analysis. Complexes of cytochrome P-450$_{scc}$ and series of hydroxysteroid were prepared by including saturated concentration of each steroid under conditions as described in Figure Legend. Complexes were fully saturated with substrates, as judged by complete conversion to the expected spin state previously determined by spectrophotometric titration experiment.[8] Cytochrome P-450$_{scc}$ - substrate complex was reduced anaerobically at 20 °C by dithionite reduced adrenodoxin. Spectral changes were recorded at 450 nm (for the reduced carbon-monoxide cytochrome complex) using an extinction coefficient of 91 mM^{-1} cm^{-1}.

The first-order rate constant for reduction of the cytochrome was calculated manually using a semilogarithmic fit of absorbance change versus time in which the rate constant was determined from half time of the reduction process. A computerized exponential fit and linear fit to a semilogarithmic plot of the data were also used to calculate the rate constant.

Results

Table I summarized the rate constants for reduction of various cytochrome-substrate complexes and also from substrate free cytochrome. Both the presence or absence and the nature of the bound substrate had significant effects on the rate constant, resulting in a 35-fold range in rate constant. This table also shows the estimated percentage of the cytochrome in high spin form for various substrate-bound and substrate-free enzyme species from a previous experiment.[12] Both cholesterol and 20 α, 22R-dihydroxycholesterol yeild completely high spin cytochrome, where as 22R-hydroxycholesterol and 20 α-hydroxycholesterol provided entirely low spin heme. The 25-hydroxycholesterol resulted in a mixture of low and high spin forms and the substrate-free enzyme are low spin. Though the nature of substrate effected the spin state and the rate of reduction by reduced adrenodoxin, there appeared to be no correlation between these parameters.

Table 1 Effect of Bound Substrates on the Spin State, Midpoint potential, and Reduction Rate of Cytochrome P-450$_{scc}$

Bound Substrate	%High spin	Midpoint Potential (mV)	Reduction rate (s^{-1})
25-Hydroxycholesterol	25	-265	3.17
20α, 22R - Dihydroxy-cholesterol	> 95	-270	2.24
Cholesterol	> 95	-282	2.20
22R-Hydroxycholesterol	< 5	-285	1.19
20α-Hydroxycholesterol	< 5	-350	0.56
Substrate free	< 5	-412	0.09

Midpoint oxidation-reduction potentials for various cytochrome-substrate as determined previously[9,12] were also summarized in table I. Comparison of the midpoint potential for each steroid complex to the predominant spin state again failed to reveal any correlation between them.

In Figure 1, the logarithm of the rate constant for reduction of each cytochrome-substrate complex was plotted as a function of midpoint potential for each complex. Data for substrate-free form of the enzyme were also included. Inspection of Figure 1 reveals an excellent correlation between the rate of reduction of cytochrome P-450$_{scc}$ by adrenodoxin and midpoint potential of the heme iron. Not only the presence or the absence of substrate, but also the structure of the particular bound substrate exert parallel effects on both the midpoint potential and the reduction rate of the cytochrome.

Discussions

The ability of different steroid substrates to modulate the spin state of cytochrome P-450$_{scc}$ has provided a convenient method to test the relationship of spin state and other phenomena in the reduction of the hemoprotein. In different cytochrome P-450 systems, the spin state of heme iron has been proposed to modulate the sub-

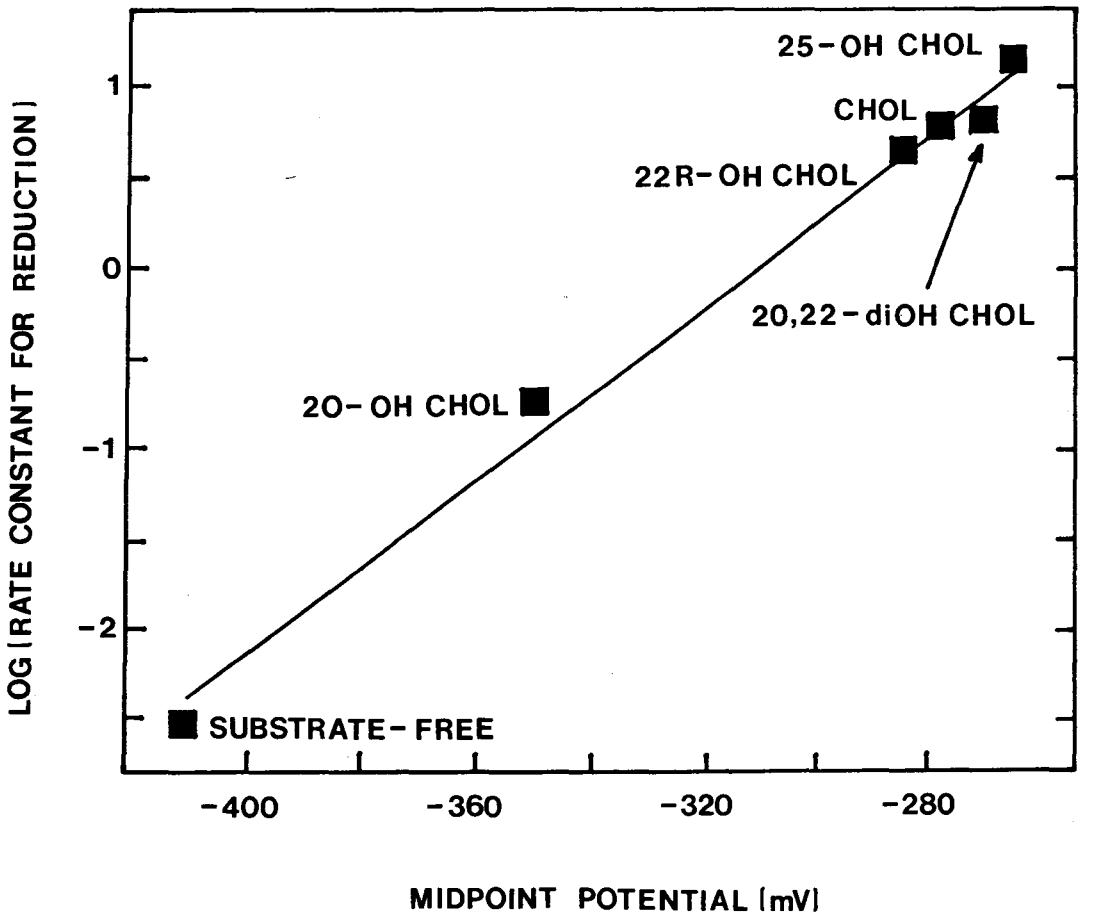

Figure 1 Dependence of reduction rate of cytochrome P-450$_{scc}$ - substrate complex on substrate-induced midpoint oxidation reduction potential. Experiments were carried out with cholesterol and various hydroxycholesterol at substrate concentration high above their K_m for side chain cleavage reaction. Cytochrome P-450$_{scc}$ was incorporated into detergent micelles containing 1000 μM steroid substrate and 40 μM cardiolipin. The final concentration of the cytochrome and adrenodoxin were 1 μM. Reactions were carried out at 20° C, 1.2 mM dithionite with 0.1% Tween 80 (w/v) in 25 mM HEPES, pH 7.0.

strate binding, the heme midpoint potential,[13] and also the reduction rate of the cytochrome.[14] The order of strength of binding of substrates to the cytochrome P-450_{scc} has been shown : 22R-hydroxycholesterol > 20 α, 22R-dihydroxycholesterol >20 α -hydroxycholesterol > 25-hydroxycholesterol ≫ cholesterol. This order failed to correlate with the substrate-induced spin state. Similar lack of correlation between spin state and substrate binding has also been observed with benzphetamine analogues binding to the liver microsomal cytochrome.[14] In the latter study, there is an exelllent correlation between substrate-induced spin state and the rate of reduction of the cytochrome by NADPH. The present studies failed to reveal any correlation between substrate-induced spin state to either the midpoint potential or the reduction rate. However, there existed a correlation of substrate-induced midpoint potential with the rate of reduction as in Figure 1. Correlation between the rate constant of reduction and the redox potential has been noted in nonbiological electron transfers and predicted in various theoretical treatments.[15,16] Recently such a relationship has been observed with reduction of various electron transfer proteins by photoreduced flavins.[16] The present studies provide direct demonstration of redox potential/electron transfer rate correlation in a biological protein-protein electron transfer complex. Results indicate that in this system, the energetic contribution of coupling of the spin state to kinetic (reduction rate) and thermodynomic (midpoint potential, substrate binding) processes must be relatively minor, and other factors (e.g., protein conformation, protonation state of the residue, steric factor, etc.) must dominate these processes.

It is likely that the steric requirement and bonding properties of residues of the substrate in the steroid binding site rather than electronic properties of the heme iron regulate the strength and specificity of steroid binding. The bound steroid then modulates both the midpoint potential/reduction rate and spin state by independent mechanisms.

Since the oxidation of cholesterol to form pregnenolone is the rate limiting step in adrenal steroidogenesis, the present findings reveal a possible area in which to investigate its regulatory mechanisms. Ultimately, certain cholesterol substrate analogs may be used to modulate steroid synthesis both in experimental animals and in clinical research.

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