SUBSTRATE INDUCED SPIN STATE CHANGES IN CYTOCHROME P450

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SUMMARY

Deoxycorticosterone (DOC) produces 385 and 645 nm peaks and 420, 530 and 575 nm troughs, and 20αOH-cholesterol produces 420, 530 and 575 nm peaks and 385 and 645 nm troughs in the difference spectra of adrenal cortical submitochondrial particles. Electron spin resonance studies show that 20αOH-cholesterol produces an increase and 11-deoxycortisol or DOC produce a decrease in the low-spin signal of P450. These results suggest to us that the substrate induced difference spectra may be the result of a spin state shift in cytochrome P450.

The mixed function oxidase, cytochrome P450, is responsible for a number of hydroxylation reactions, the most completely studied being the hydroxylation of steroids in adrenal cortex, drugs in liver and camphor in bacteria. The addition of substrate to this enzyme causes alterations in the absorption spectrum. Cooper, et al. (1965) showed that two substrates for 11β-hydroxylation, deoxycorticosterone (DOC) and 11-deoxycortisol, produced difference spectra with a 420 nm trough in adrenal cortex mitochondria. Oldham, et al. (1968) and Whysner and Harding (1968) demonstrated that

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11-deoxycortisol or DOC produced a peak at 385 nm and additional troughs at 530 and 575 nm, and that the substrate for 22-hydroxylation, 20αOH-cholesterol, produced a different type of spectrum with 420, 530 and 575 nm peaks and a 385 nm trough.

MATERIALS AND METHODS

Submitochondrial particles and mitochondria were prepared as previously described (Whysner and Harding, 1968) except that the final pellet was resuspended in 0.25 M sucrose. Difference spectra were obtained with a Cary Model 14 recording spectrophotometer and the scattered transmission accessory. Low temperature spectra were run using 3 mm path length cuvettes in a liquid nitrogen "cold-finger" arrangement. The contents for the cuvettes were mixed, incubated at room temperature and injected into pre-cooled cuvettes. Electron spin resonance (ESR) studies were done with a Varian E-3 and V-4557 variable temperature accessory. DOC and 11-deoxycortisol were obtained from Sigma Chemical Company and 20αOH-cholesterol from Ikapharm.

RESULTS AND DISCUSSION

The difference spectra presented in Figure 1 show that DOC produces peaks at 385, 500 and 645 nm with a shoulder in the 600-630 nm region and troughs at 420, 535 and 570 nm. Although not shown, 11-deoxycortisol gives an identical difference spectrum. Figure 1 also demonstrates that 20αOH-cholesterol gives a difference spectrum which is a near mirror image of those absorption changes produced by DOC or 11-deoxycortisol.

A previous communication of Whysner and Harding (1968) presented data which indicate that steroid substrates interact with P450 in the Fe^{3+} form and that different P450's act in 11β- and 22-hydroxylation. Omura and Sato (1964) have

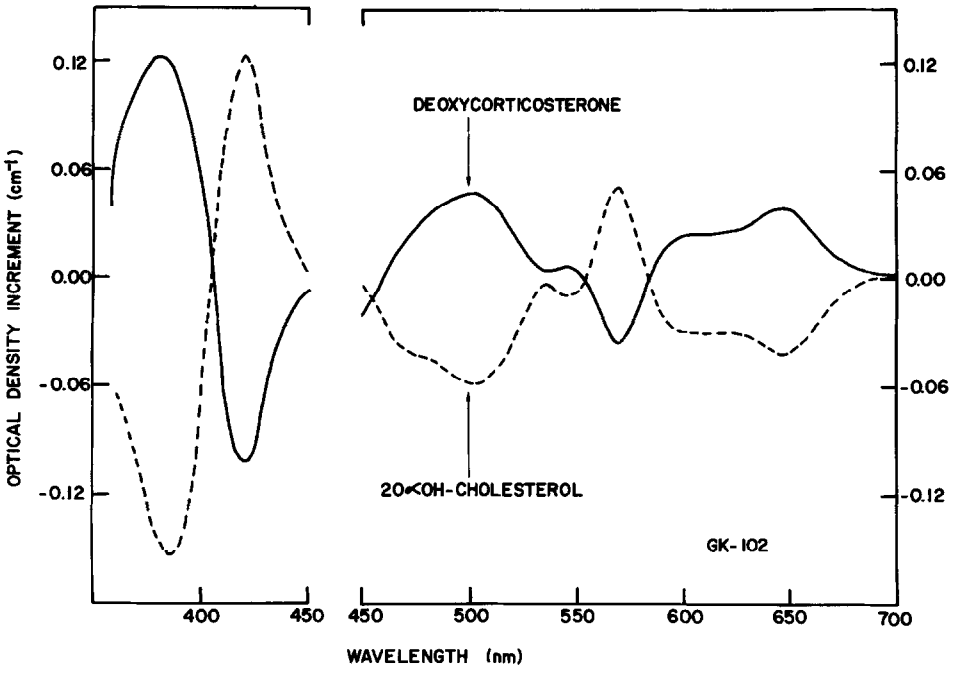

Figure 1. Steroid Induced Difference Spectra of Submitochondrial Particles. Protocol: 6 ml of 0.14 M sodium phosphate buffer, pH 7.4, containing particles were divided equally and placed in 1 cm path length, 3 ml volumn cuvettes. Particle protein was 40.4 mg for 360-450 nm and 121 mg for 450-700 nm. The additions were 160 nmoles of solid DOC, and 80 nmoles of 20∝OH-cholesterol in 4λ of ethanol (4λ of ethanol in reference cuvette).

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shown that in liver microsomes P450 is a hemoprotein with a protoheme prosthetic group. The absorbancy changes produced by DOC in Figure 1 are an increase in the charge transfer absorption regions (460-520 nm and 600-670 nm), a shift of the Soret absorption maximum to a shorter wavelength and a decrease in the α and β (535 and 570 nm) bands. Absorbancy changes of this type in a Fe^{3+} hemoprotein are strongly suggestive of a low spin (S=1/2) to a high spin (S=5/2) change in the ferric iron (Brill and Williams, 1961). In contrast, the decrease in charge transfer absorption, the shift of the Soret absorption to longer wavelengths and the increase in

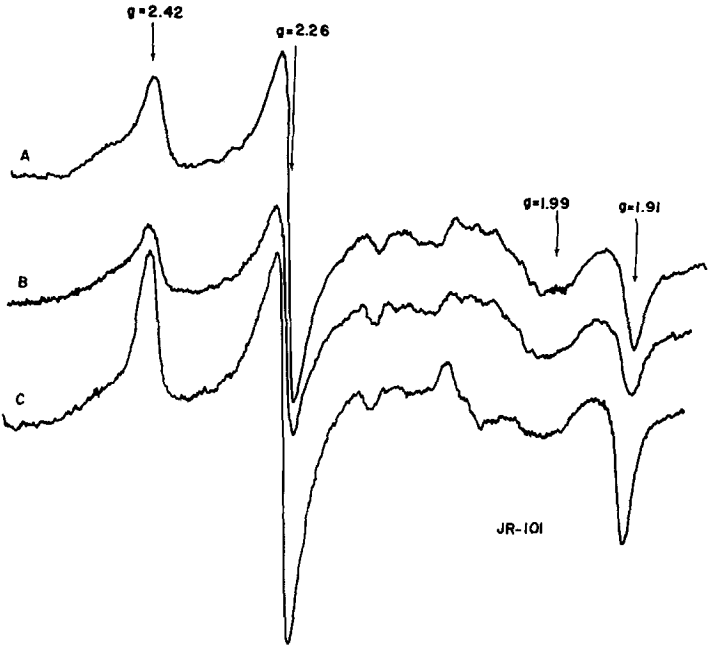

Figure 2. Effects of Substrates on the ESR Spectrum of P450. A, particles; B, particles and 100 uM DOC; C, particles and 100 uM 20αOH-cholesterol. All samples contained 1% ethanol; concentration of submitochondrial particles, 80.7 mg/ml; power, 200 mW; modulation amplitude, 20 gauss; modulation frequency, 100 KH_Z. g values were determined by comparison with a DPPH standard.

α and β bands produced by 20αOH-cholesterol are indicative of a high to low spin transition.

A low spin hemoprotein ESR spectrum of P450 was reported by Mason, et al. (1965). Figure 2 presents the low spin hemoprotein signal of P450 in adrenal cortex submitochondrial particles (A). DOC causes a decreased (B) and 20αOH-cholesterol causes an increased (C) intensity in each of the three anisotropic signals. Table I presents quantitation of the low spin P450 present under control conditions and subsequent to substrate interaction.*

*The previous, variable ESR data from this laboratory (Oldham, et al., 1968) were obtained from studies not controlled well enough for quantitation.

TABLE I

TUBE CONTENTS		g=2.25 SIGNAL			
Experiment		Totals			Average
1	Mitochondria (70.0 mg protein/ml)	1.00	1.00	1.09	1.03
	Mitochondria + 100 μM 11-deoxycortisol	0.82	0.70	0.73	0.75
	Mitochondria + 100 μM 20∝OH-cholesterol	1.32	1.37	1.44	1.38
2	Particles (85.0 mg protein/ml)	1.00	1.10	0.98	1.03
	Particles + 100 μM DOC	0.84	0.76	0.98	0.86
	Particles + 100 μM 20∝OH-cholesterol	1.50	1.50	1.42	1.47
3	Particles (80.7 mg protein/ml)	1.00	0.99	1.01	1.00
	Particles + 100 μM DOC	0.83	0.82	0.83	0.83
	Particles + 100 μM 20∝OH-cholesterol	1.32	1.32	1.36	1.33

Table 1. Effects of Substrates on the Amount of Low Spin P450. The amount of low spin P450 was determined by double integration of the g=2.25 signal, and normalized to one of the controls. Each result represents a separate reaction mixture, and all tubes contained 1% ethanol. Tube volumns were corrected by DPPH calibration and instrument parameters are as in Figure 2.

If the hypothesis of substrate induced spin state changes is correct, it should be possible to see a high spin g=6.0 signal for P450 with amplitude changes complementary to the observed changes in the low spin signal. As yet, we have been unable to resolve the g=6.0 signal at liquid nitrogen temperatures. However, the ESR changes observed for low spin P450 do correspond to the spin state changes suggested by visible difference spectroscopy.

Temperature is known to affect the high to low spin equilibrium of hemoproteins. Therefore, the substrate induced difference spectra were observed at liquid nitrogen temperature (Figure 3) and found to be qualitatively similar

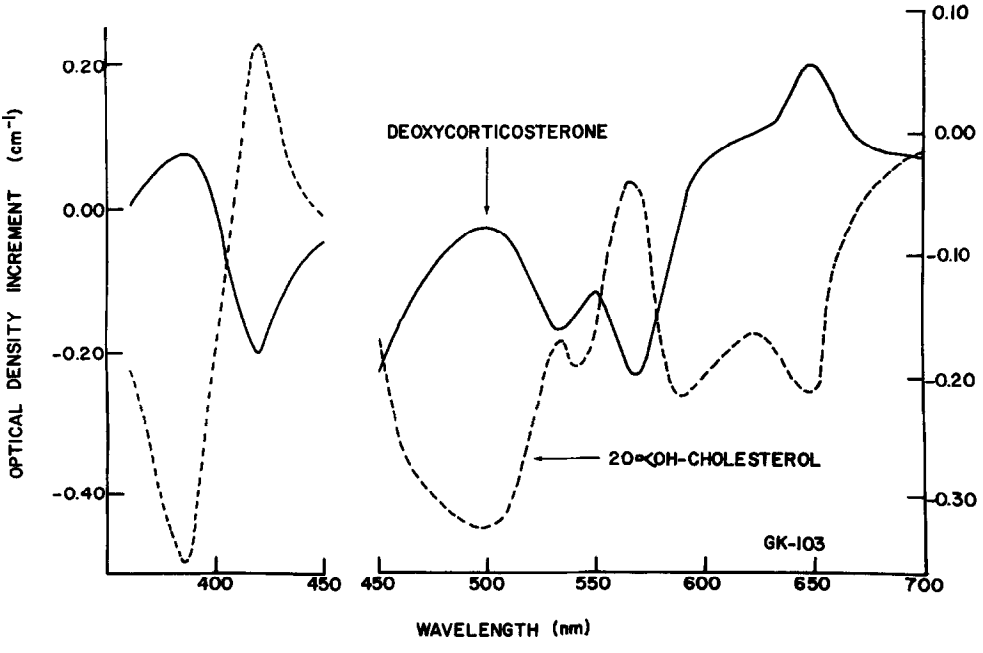

Figure 3. Low Temperature Steroid Induced Difference Spectra of Submitochondrial Particles. Protocol: Same as in Figure 1 except that temperature was near liquid nitrogen, cuvette path length was 3 mm and cuvette volumn 1 ml. Particle protein was 20.2 mg for 450-700 nm and 8.1 mg for 360-450 nm. The additions were 40 nmoles of solid DOC and 40 nmoles of 20∝OH-cholesterol in 2λ of ethanol (2λ of ethanol in reference cuvette).

to the room temperature spectra.

Spin state changes may also be responsible for the substrate induced difference spectra in other tissues. Substrates and various organic solvents induce spectral alterations in liver microsomes similar to the substrate induced difference spectra of adrenal cortex mitochondria (Imai and Sato, 1967). Hildebrandt, et al. (1968) concluded from difference absorption spectroscopy that different spin states of liver microsomal P450 existed which changed spin state upon substrate interaction.

In a purified bacterial camphor hydroxylase system Gunsalus (1969) has found that substrate interaction caused a shift in the absolute P450 spectrum from 418 to 385 nm and a decrease in the ESR low spin signal intensity.* These changes are compatible with a low to high spin shift.

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