SUBSTRATE INTERACTION WITH CYTOCHROME P450 OF ADRENAL CORTICAL SUBMITOCHONDRIAL PARTICLES+

John A. Whysner* and Boyd W. Harding**

Departments of Biochemistry and Medicine
University of Southern California, School of Medicine
Los Angeles, California 90033

Received July 23, 1968

Substrate induced difference spectra for cytochrome P450 were first reported in adrenal cortical microsomes (Narasimhulu, et al., 1965). Androstenedione and 17OH-progesterone produced difference spectra with a 420mμ minimum and a 385mμ maximum. A similar difference spectrum has been reported for the substrates deoxycorticosterone (DOC) and 11-deoxycortisol in adrenal mitochondria by Cooper, et al. (1965) and by Oldham, et al. (1968). The results of this investigation show that the oxidized species of P450 gives the substrate induced spectrum in adrenal cortical submitochondrial particles. These particles give the characteristic difference spectrum for 11β-hydroxylation substrates and also give a different type of spectrum with 20αOH-cholesterol having 420, 535 and 570mμ peaks and a 385mμ trough. The substrate dissociation constants for DOC, 11-deoxycortisol and 20αOH-cholesterol are 7, 4-5 and 2μM, respectively. An inhibition of these difference spectra occurs at high substrate concentrations. The present data show that the inhibition of the DOC spectrum is due to DOC binding at a site which is relatively more specific for

---

+ A preliminary report of these studies has been presented, Fed. Proc. 27, Abstract 757 (1968).

* Recipient of American Cancer Society Predoctoral Scholarship PRE-14.
** Recipient of United States Public Health Research Development Award 1-K3-Gm-5532 from the National Institute of General Medical Sciences.

20αOH - cholesterol.

MATERIALS AND METHODS

Bovine adrenal cortex was homogenized with 0.25 M sucrose and centrifuged at 900xg for 10 min. The resulting supernate was centrifuged at 10,000xg for 10 min and the mitochondrial pellet was washed and resedimented. The washed pellet was resuspended, sonified at 3 amp. in a Branson Sonifier for 10 min and centrifuged for 30 min at 105,000xg. The pellet of submitochondrial particles was then resuspended in 0.16 M sodium phosphate buffer at pH 7.4.

Difference spectra were obtained with a Cary Model 14 recording spectrophotometer using the scattered transmission accessory. DOC and 20αOH-cholesterol were obtained from Sigma Chemical Company and Ikapharm, respectively.

RESULTS AND DISCUSSION

The results presented in Figure 1 show that submitochondrial particles gassed with CO for 20 min do not show any P450·CO formation. The small peak at 420mμ is due to hemoglobin contamination. If NADPH is added to the sample and reference cuvettes, P450·CO is formed. These data indicate that there is no endogenous electron donor available to the P450 in these submitochondrial particles and that the P450 is not in a CO binding state, but presumably in an Fe^{3+} state. Figure 2 shows that these submitochondrial particles give the characteristic substrate induced difference spectrum with deoxycorticosterone. Therefore, substrate binding to the oxidized P450 appears to be responsible for the substrate induced difference spectrum.

These findings are in agreement with studies showing that aniline affects the binding of ethylisocyanide to oxidized P450 in liver microsomes

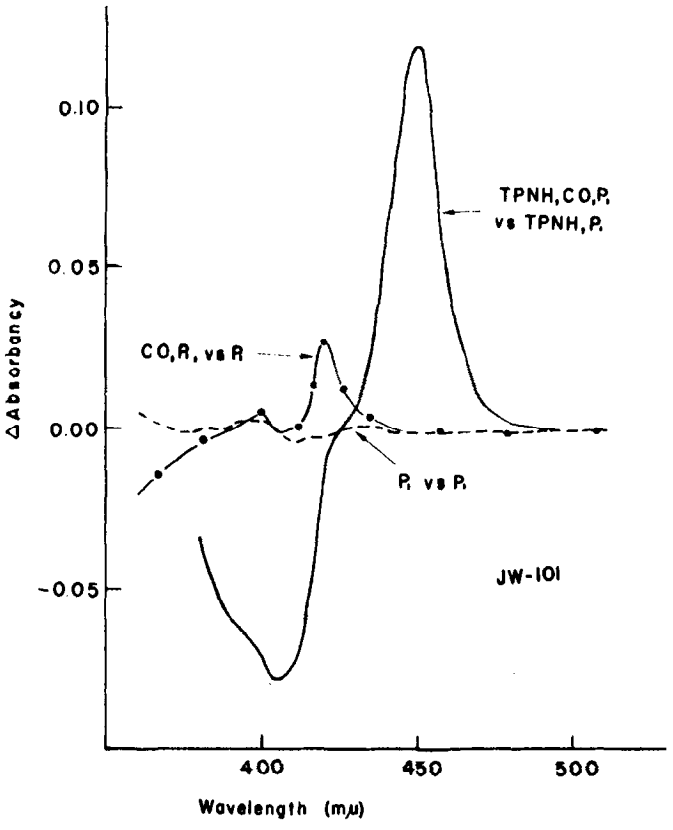

Figure 1. The carbon monoxide difference spectrum of submitochondrial particles. Dashed line: Particles (9.7 mg protein) in 6.0 ml 0.16 M sodium phosphate buffer pH 7.4 were divided equally into sample and reference cuvettes. Broken line with circles: contents of sample cuvette were gassed with humidified CO in a gassing chamber for 20 min. Solid line: 0.02 ml of 20 mM TPNH was added to the sample and reference cuvettes.

(Imai and Sato, 1967). Also, Cammer and Estabrook (1967) found that an anaerobic sample containing adrenal cortical mitochondria and steroid gives the reduced spectrum of P450 instead of the substrate induced difference spectrum. This suggests that the cytochrome is oxidized when it gives the substrate induced difference spectrum.

All reported substrate induced difference spectra for adrenal cortical microsomes or mitochondria have been of the 420, 535 and 570mμ troughs and 385mμ peak type. Various types of spectral changes have been reported

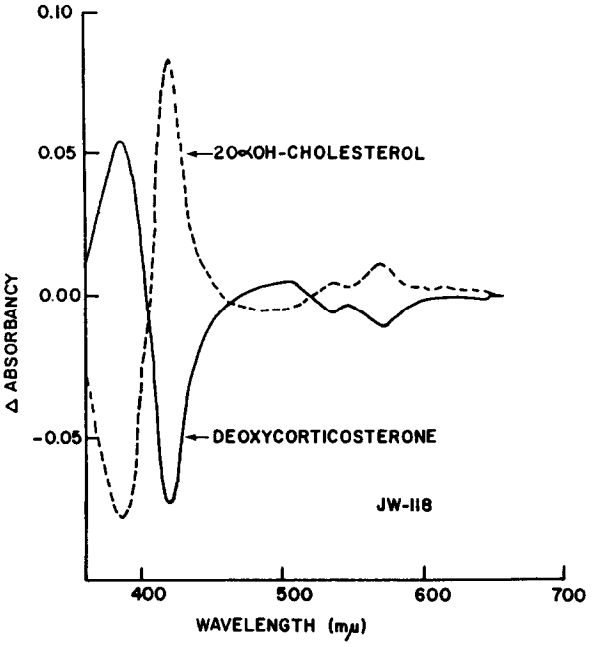

Figure 2. Steroid induced difference spectrum of submitochondrial particles. Deoxycorticosterone spectrum: particles (23.5 mg protein) in 6.0 ml 0.16 M sodium phosphate buffer were divided equally into sample and reference cuvettes and 0.006 ml 10 mM deoxycorticosterone in ethanol was added to the sample cuvette and 0.006 ml ethanol was added to the reference cuvette. 20αOH-cholesterol spectrum: particles (25.6 mg protein) were again diluted and divided and 0.01 ml 10 mM 20αOH-cholesterol was in the sample cuvette and 0.01 ml ethanol was in the reference cuvette.

in liver microsomes (Schenkman, et al., 1967). Figure 2 shows that 20αOH-cholesterol, a substrate for 22-hydroxylation in adrenal mitochondria, gives 420, 535 and 570mμ peaks and a 385mμ trough. This spectrum is a mirror image of all previously reported substrate induced difference spectra in adrenal tissue.

Substrate titrations of adrenal mitochondria are difficult because endogenous electron donors support hydroxylation, thereby, changing the substrate concentration. As shown in Figure 1, these particles contain no available endogenous electron donor, thus permitting satisfactory substrate titrations. Figure 3 presents a double reciprocal plot of a titra-

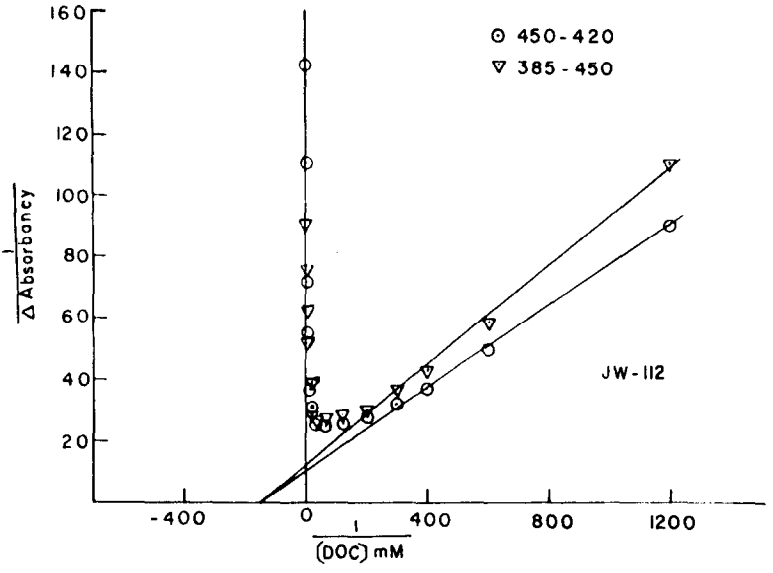

Figure 3. Deoxycorticosterone titration of submitochondrial particles. Particles (11.7 mg protein) in 6.0 ml 0.16 M sodium phosphate buffer were divided equally into sample and reference cuvettes and solid deoxycorticosterone was added to the sample cuvette.

---

tion of DOC giving a substrate dissociation constant, Ks, of 7μM. Similar data give Ks=4-5μM for 11-deoxycortisol and Ks=2μM for 20αOH-cholesterol. These dissociation constants are lower than the Km values determined for DOC and 11-deoxycortisol determined for acetone powders of adrenal mitochondria (Sharma, et al., 1962).

Figure 3 also shows that high concentrations of DOC cause a reversal of the substrate induced spectral change. This reversal is also found for 11-deoxycortisol and to a lesser extent for 20αOH-cholesterol. The possibility that high steroid concentrations act similarly to many organic solvents (Ichikawa and Yamano, 1968) to convert cytochrome P450 to its inactive P420 derivative is excluded by the following experiment. DOC at a concentration high enough to cause a 50% inhibition of the maximum difference spectrum was incubated with submitochondrial particles for one hour.

The ratio of P450 to P420 was measured by the method of Omura, et al. (1967). No significant difference in this ratio occurs between the steroid treated and control particles.

Another explanation of the inhibition is that at high concentrations DOC binds to a site specific for 20αOH-cholesterol binding giving a peak at 420mμ and a trough at 385mμ, as does 20αOH-cholesterol. This binding would have the effect of apparently reversing the spectrum of low DOC concentrations. This explanation is supported by the data presented in Figure 4. The two curves labelled A represent two wavelength pairs which measure the trough and the peak of the difference spectrum produced by DOC titration. The curves labelled B show the titration of DOC measured in the presence of a concentration of 20αOH-cholesterol in the sample cuvette high

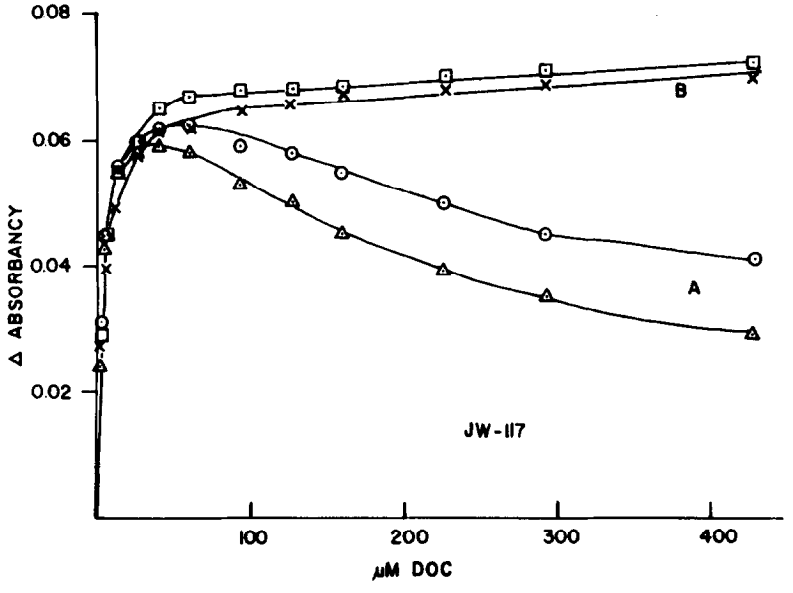

Figure 4. Effect of 20αOH-cholesterol on deoxycorticosterone titration. Conditions and procedure were the same as in Figure 3 except that 24.5 mg of protein was used, and 0.01 ml of ethanol was in the sample cuvette and in the reference cuvette. DOC titration: 450-420mμ, ⊙ ; 385-450mμ, ▲ . DOC titration with 0.01 ml 4 mM 20αOH-cholesterol dissolved in ethanol in the sample cuvette and 0.01 ml ethanol in the reference cuvette: 450-420mμ, x; 385-450mμ ⊡ .

enough to produce a maximal 20αOH-cholesterol difference spectrum as the baseline. Under these conditions no inhibition of the DOC spectrum occurs at high concentrations.

The model of substrate binding to P450 which is most consistent with these data requires that there are at least two P450's in adrenal cortical mitochondria. One P450 is specific for DOC, and another is specific for 20αOH-cholesterol but can bind DOC at high substrate concentrations. In Figure 4B prior addition of 20αOH-cholesterol has saturated its binding site so that DOC cannot bind to this site.

These studies suggest that substrate induced difference spectra of DOC, 11-deoxycortisol and 20αOH-cholesterol are due to binding of steroid substrate to a form of P450 which does not bind CO, presumably an Fe^{3+} state. Also, the data are consistent with the presence of at least two P450's in adrenal cortical mitochondria, one specific for 20αOH-cholesterol and the other specific for DOC.

This work was supported by American Cancer Society grant P-294 and United States Public Health Service grant CA 07057.

REFERENCES

Cammer, W., and Estabrook, R. W., Arch. Biochem. Biophys., 122, 735 (1967).
Cooper, D. Y., Narasimhulu, S., Slade, A., Raich, W., Foroff, O., and Rosenthal, O., Life Sci., 4, 2109 (1965).
Ichikawa, Y., and Yamano, T., Biochim. Biophys. Acta, 147, 518 (1967).
Imai, Y., and Sato, R., J. Biochem., 62, 239 (1967).
Narasimhulu, S., Cooper, D. Y., and Rosenthal, O., Life Sci., 4, 2101 (1965).
Oldham, S. B., Wilson, L. D., Landgraf, W. L., and Harding, B. W., Arch. Biochem. Biophys., 123, 484 (1968).
Omura, T., and Sato, R., in S. P. Colowick and N. O. Kaplan, Methods in Enzymology, Vol. 10 (R. W. Estabrook and M. E. Pullman, Ed.), Academic Press, New York, 1967, p. 556.
Schenkman, J. B., Remmer, H., and Estabrook, R. W., Molec. Pharmacol., 3, 113 (1967).
Sharma, D. C., Porchielli, E., and Dorfman, R. I., J. Biol. Chem., 237, 1495 (1962).