CYTOCHROME P-450 AND 18-OXYGENASE SYSTEM FROM BEEF ADRENOCORTICAL MITOCHONDRIA. - SPECTRAL AND KINETIC STUDIES

J.-G. Lehoux and J.C. Forest

Département d'Obstétrique et Gynécologie
Université de Sherbrooke
Sherbrooke, Québec, Canada, J1H 5N4

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SUMMARY: The transformation of corticosterone to aldosterone and the binding of corticosterone to cytochrome P-450 were studied in beef adrenal mitochondria. The corticosterone concentration giving a half maximal rate of aldosterone synthesis was found to be 1.5 X 10^{-5}M in beef adrenal mitochondrial preparations. 7.0 X 10^{-5}M was the concentration at which half maximal binding of corticosterone to cytochrome P-450 was obtained. pH 7.0 was found optimal for both the binding of corticosterone to cytochrome P-450 and its transformation to aldosterone. 2M urea diminished the binding of corticosterone to cytochrome P-450 by 78% and the conversion of corticosterone to aldosterone by 72%. It seem that *in vitro* factors favouring the binding of corticosterone to cytochrome P-450 also favour the transformation of corticosterone to aldosterone.

The conversion of corticosterone to aldosterone in the mitochondria of adrenal glomerulosa appears to be an important step in the regulation of mineralocorticosteroid synthesis. Potassium and ACTH enhance the *in vitro* transformation of corticosterone to aldosterone in rat adrenal glomerulosa tissue (1,2) and capsular adrenal glands of potassium deficient rats convert much less corticosterone to aldosterone than controls (3). Sodium depletion on the other hand, increases the rate of this reaction (4) while potassium depletion reduces it (5,6).

The steps in the transformation of corticosterone to aldosterone are not clear. It appears that in mammals, 18-hydroxycorticosterone might be an intermediate step of the conversion of corticosterone into aldosterone (6,7), while in non mammals, conversion of corticosterone into aldosterone could occur without intermediary product (8-10). This important step in the biosynthesis of aldosterone has been investigated under numerous experimental conditions (6) but relatively little attention has been paid, as yet, to the study of the interaction between corticosterone and the 18-oxygenating system responsible for carrying out this reaction.

The present paper deals with the results of our in vitro study on the properties of the 18-oxygenating system of beef adrenal glomerulosa and on the interactions of corticosterone with the mitochondrial cytochrome P-450 of glomerulosa which has already been shown to be a component of the 18-oxygenase system (9,10,11).

Materials and Methods

A glomerulosa rich fraction of beef adrenals was used for these experiments. The tissue was homogenized with twice its weight of ice cold 0.25 M sucrose solution buffered to pH 7.0 with 0.05 M TRIS-HCl or 0.01 M phosphate. Mitochondria were obtained by ultracentrifugation according to the Schneider and Hogeboom method (12); these organelles were suspended in the sucrose solution and used without any further purification.

Incubation medium used contained $MgCl_2$ (8.5 mM), $CaCl_2$ (2.7 mM), KCl (3.13 mM), NaCl (7.59 mM), bovine serum albumin (2%) and was buffered to pH 7.0 with 0.05 M TRIS-HCl or with 0.01 M phosphate. Mitochondria were incubated in the presence of 1 µCi of tritiated corticosterone and of NADPH (0.5 mM) or a NADPH generating system (9) for 20 min at 37°C in a Dubnoff metabolic shaker.

The isolation of aldosterone was performed using the technique outlined by Sandor and Lanthier (13) with slight modifications. Known amounts of ^{14}C-aldosterone were added to incubation media to monitor losses occuring during isolation procedures. Extractions were performed with a chloroform: ethyl acetate (1:1; V:V) solution. The biosynthetized ^{3}H-aldosterone was purified by paper partition chromatography and derivatives formation. Aldosterone was isolated first by three consecutive paper partition chromatographies using the solvent systems E_2B (14), A/B (15) and B_1 (16); the aldosterone fraction was acetylated and chromatographed with the solvent system B_1. Aldosterone diacetate was then transformed to aldosterone monoacetate gamma-lactone by oxidation with chromic acid according to the Kliman and Peterson technique (17) and chromatographed in systems B_1 and cyclohexane: benzene: methanol 80%

(4:3:7, V/V). The identity of aldosterone was accepted according to the identification criteria of Sandor and Idler (18) when three consecutive paper chromatographies yield $^{3}H/^{14}C$ ratios with a coefficient of variation not greater than ± 5%.

Induced spectra due to the binding of substrate to cytochrome P-450 were analyzed as previously described using a Unicam SP-800 recording spectrophotometer equipped with a temperature control cell holder (9,19). Temperature was maintained at 0°C except for the experiments on the effect of temperature on the binding of corticosterone to cytochrome P-450 where temperature was kept at 0°, 25° and 37° C respectively. Mitochondria were suspended in 0.05 M TRIS-HCl 0.01 M or in phosphate buffer pH 7.0 and difference spectra induced by the binding of substrates to cytochrome P-450 were recorded by scanning between 370 and 500 nm.

After precipitation with trichloroacetic acid (10), proteins were solubilized in 1 N NaOH and the concentration determined by the Lowry technique (20) using bovine serum albumin as a standard.

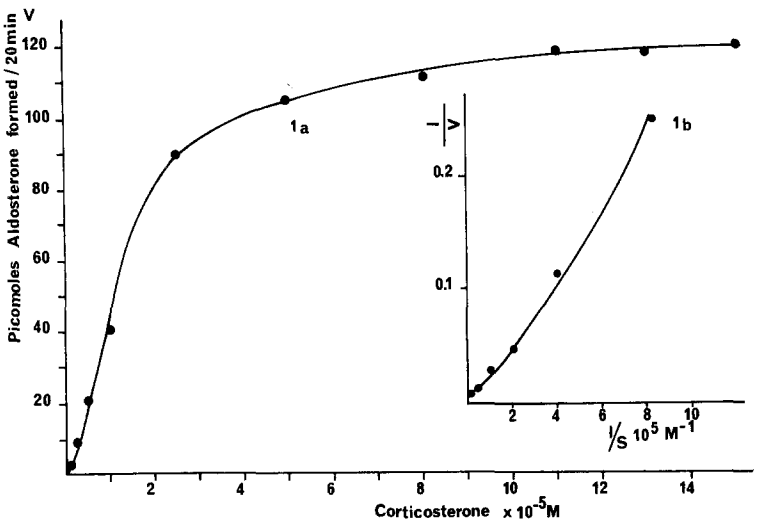
Trivial names used: aldosterone = 11β, 21-dihydroxy-4-pregnene-3, 20, dione-18-al; corticosterone: 11, 21-dihydroxy-4-pregnene-3, 20 dione; 18-hydroxycorticosterone = 11β, 18, 21-trihydroxy-4-4pregnene, 3, 20-dione-20-18-cyclic hemiketal.

Results and Discussion

Initial velocity patterns

Mitochondrial fraction from beef glomerulosa rich adrenals was incubated with ^{3}H-corticosterone in the presence of a NADPH generating system. ^{3}H-aldosterone formed was isolated and well identified as specified in the materials and methods section. The reaction was linear with time for at least

^{3}H-corticosterone (specific activity 50 Ci/mM) and ^{14}C-aldosterone (specific activity 55 mCi/mM/ were purchased from New England Nuclear Co.; non radioactive corticosterone and aldosterone were obtained from Ikapharm Co.

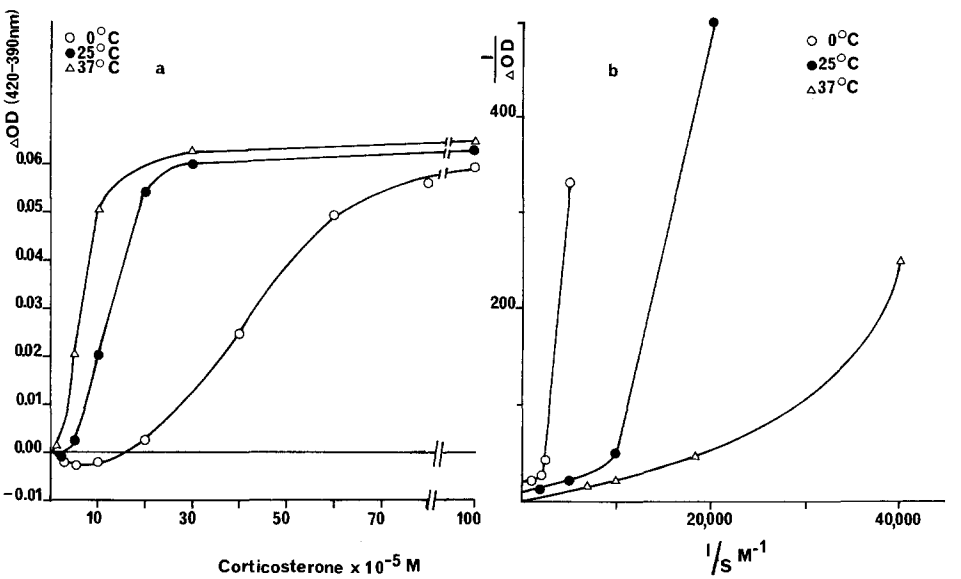


Figures 1 a and b

Effect of substrate (corticosterone) concentration on the formation of aldosterone. Mitochondria equivalent to 2.01 mg protein/ml were incubated with ^{3}H-corticosterone (1 μCi) and various amounts of non radioactive corticosterone for 20 minutes in air and in the presence of a NADPH generating system. PH was buffered with 0.01 M potassium phosphate. 1 a = Michaelis-Menton plot. 1 b = Double reciprocal plot.

60 min. and increasing mitochondria concentration up to 2.75 mg protein per ml of incubation medium.

The initial rate of aldosterone formation was measured at varied concentrations of corticosterone at pH 7.0 (19) and at 37°C. Figures 1 a and 1 b show results obtained. The maximal rate of conversion of corticosterone to aldosterone by the mitochondrial fraction from the glomerulosa of beef adrenals is comparable to the results published by Raman et al. (7). The concentration of corticosterone giving a half maximal rate of synthesis of aldosterone was found to be 1.5×10^{-5} M in this series of experiments as compared to 2.0×10^{-5} M in the case of the 18-oxygenating system from sheep (7), 6.6×10^{-6}M for the duck adrenal 18-oxygenase system (9) and 7.1×10^{-6}M in the case of the same system from bullfrog (11).



Figures 2 a and 2 b

Effect of temperature on corticosterone binding to cytochrome P-450 by mitochondrial preparations from beef adrenal glomerulosa. Mitochondria were suspended to 1.2 mg/ml 0.01 M phosphate, pH 7.0 and divided between two cuvettes. To the sample cuvette were added increasing concentration of corticosterone and the difference in optical density between 420 nm and 390 nm (ΔO.D. 420-390 nm) was recorded.

Spectral changes

Effect of corticosterone concentration. - Since cytochrome P-450 is a component of the 18-hydroxylating system (8,9), properties of the binding of corticosterone, the precursor of aldosterone, to cytochrome P-450 were also investigated. The binding of corticosterone to cytochrome P-450 of a mitochondrial fraction isolated from beef adrenal glomerulosa induced spectral changes characterized by the appearance of a peak at about 420 nm and a trough at 390 nm typical of the inverted type I difference spectrum (21).

Effect of temperature. - Fig. 2 a shows the changes in absorbance between 420 nm and 390 nm in relation to increasing concentrations of corticosterone

in the mitochondrial cytochrome P-450 preparation at 0°, 25° and 37°C. The double reciprocal plot of the data of Fig. 2 b shows concave upward lines. The concentration at which half maximal binding of corticosterone to cytochrome P-450 is obtained was found to be 4.5 x 10^{-4}M at 0°C and 7.0 x 10^{-5}M at 37°C. This latter value is comparable to the apparent Km calculated for the 18-oxygenating system.

Effect of pH. - PH has a marked effect on the binding of corticosterone to cytochrome P-450. Fig. 3 shows the changes of optical density between 420 nm and 390 nm in relation to corticosterone concentration and under various pH's. Optimal pH for the binding of corticosterone was found to be 7.0 under these conditions. The transformation of corticosterone to aldosterone was also found to be optimal at pH 7.0.

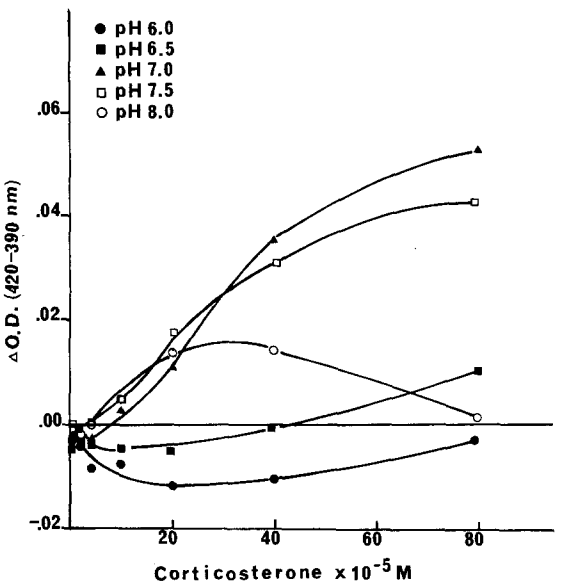


Figure 3

Effect of pH on corticosterone binding to P-450 by mitochondrial preparations from beef adrenal glomerulosa. Mitochondria were suspended to 1.5 mg/ml TRIS-HCl 0.05 M and divided between two cuvettes. To the sample cuvette was added increasing amounts of corticosterone and the difference in optical density between 420 and 390 nm was recorded at 0°C.

Effect of urea. - Urea reduced at about the same extent both the binding of corticosterone to cytochrome P-450 and the conversion of corticosterone to aldosterone by the 18-oxygenase system. At a concentration of 2 M, urea reduced corticosterone-P-450 binding by 78% and the conversion of corticosterone to aldosterone by 72%.

The sigmoidal effect reported here for both aldosterone formation and corticosterone binding to cytochrome P-450 is most interesting. These results may be attributed to anyone or a combination of the following factors: 1) in mammals, the transformation of corticosterone to aldosterone may be a two staged reaction proceeding through 18-OH-corticosterone (6,7). 2) The fact that the work was carried out with relatively intact mitochondria may bring about interactions of the steroid with other components of the mitochondria or limitations on the transport of corticosterone through the membrane. 3) Finally the results are also suggestive of a positive cooperative type of reaction compatible with an enzyme with interacting substrate-binding sites. This possibility is in agreement with the recent demonstration of the polymeric nature of mitochondrial cytochrome P-450 of adrenals. Indeed Shikita and Hall have demonstrated that mitochondrial cytochrome P-450 from bovine adrenal cortex has a molecular weight of 850,000 and dissociates into 16 subunits of a molecular weight of 53,000 daltons each (22). These results are preliminary and work is now being done to purify the 18-oxygenase system from beef adrenal in order to elucidate these findings.

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