[^{3}H]METYRAPOL AS A TOOL FOR STUDIES OF INTERACTIONS OF DEOXYCORTICOSTERONE WITH ADRENAL CORTEX MITOCHONDRIA

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1. Introduction

Metyrapone (2-methyl-1,2-bis(3-pyridyl)-1-propanone) has been shown to inhibit hydroxylation systems present in adrenal cortex mitochondria [1, 2], in liver microsomes [3–5] or in bacteria [6, 7]. Metyrapone interacts with cytochrome P450, as revealed by typical spectral changes [8, 9]. We have recently tried to study the properties of its binding to adrenal cortex mitochondria by using [^{3}H]metyrapone. [^{3}H]metyrapone was obtained through catalytic exchange in aqueous solution, however it was of limited use because of its low specific activity.

In this paper, it is shown that metyrapol behaves similarly to metyrapone as a competitive inhibitor of 11β-hydroxylation in adrenal cortex mitochondria. Because [^{3}H]metyrapol (but not [^{3}H]metyrapone) can be readily prepared with high specific activity, we are able to report the binding of this inhibitor to adrenal cortex mitochondria.

2. Material and methods

Beef adrenal cortex mitochondria were prepared as previously described [10]. O_2 uptake was measured with a GME oxygraph equipped with a Clark electrode. Mitochondrial protein content was determined by the biuret method.

Unlabelled metyrapol was prepared by reduction of metyrapone with sodium borohydride [11] and purified by chromatography on a silica gel thin-layer plate in chloroform–methanol (100:16, by vol.).

To prepare [^{3}H]metyrapol, 1.2 mg of metyrapone dissolved in 0.6 ml of methanol was added to 5 mCi of solid [^{3}H]sodium borohydride (7.5 Ci/mmole). After 1 hr at room temp. 0.2 ml of acetone was added. [^{3}H]metyrapol was purified by chromatography on a thin layer of silica as mentioned above. Metyrapol was located under UV light, the corresponding zone of silica gel scraped from the plate and extracted with acetone. To complete the purification, another chromatography in the same solvent was carried out. Molarity of metyrapol solution was assayed by absorbancy at 261 nm in ethanol (ϵ 6100). The specific activity of [^{3}H]metyrapol was 2200 dpm/pmole.

Experimental conditions for measuring [^{3}H]metyrapol binding are detailed in the legend of fig. 3.

3. Results

3.1. *Inhibition by metyrapol of the 11β-hydroxylation of the deoxycorticosterone*

The polarographic trace in fig. 1 illustrates the typical stimulating effect of deoxycorticosterone on the cyanide-insensitive respiration of adrenal cortex mitochondria supplemented with malate. This effect is due to the 11β-hydroxylation of deoxycorticosterone into corticosterone [12]. Like metyrapone [12], metyrapol inhibits the deoxycorticosterone-dependent stimulation of O_2 uptake in an apparent competitive manner: the inhibition is released by increasing the concentration of deoxycorticosterone. Titration curves in fig. 2 show that metyrapol and metyrapone are endowed with the same inhibition efficiency to-

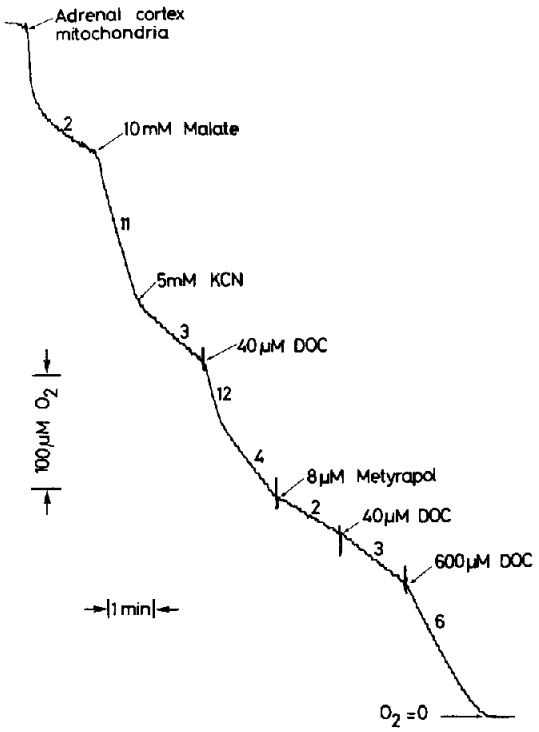

Fig. 1. Effect of metyrapol on the rate of oxygen uptake by adrenal cortex mitochondria. Mitochondria (5.7 mg protein) were added to 1.9 ml of standard saline medium containing 0.12 M KCl, 20 mM HEPES (*N*-2-hydroxyethylpiperazine *N*-2-ethane sulfonic acid) and 10 mM potassium phosphate, pH 7.2 at 25°. L-malate, potassium cyanide, deoxycorticosterone (DOC) were added as indicated. The figures along the trace are respiration rates in nmoles O_2/min/mg protein.

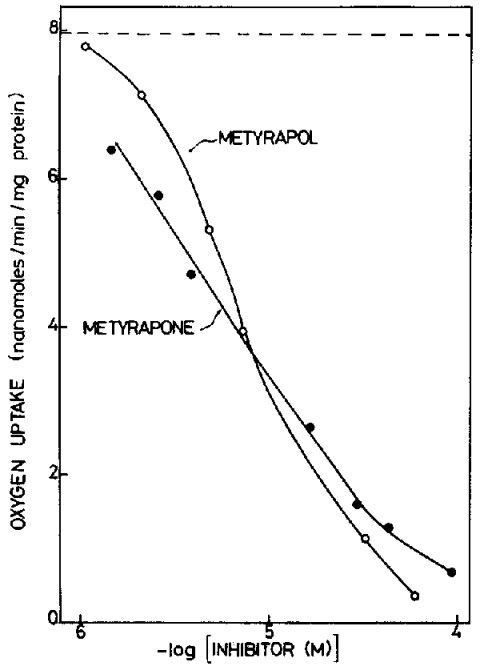

Fig. 2. Inhibition of deoxycorticosterone stimulated oxygen uptake by metyrapone and metyrapol. Adrenal cortex mitochondria (6.2 mg protein) were added to 2 ml of saline medium at 25° containing 10 mM L-malate, 25 μg antimycin A, 600 μM deoxycorticosterone and increasing concentration of metyrapone (●—●—●) or metyrapol (○—○—○). The broken line refers to the rate of oxygen uptake in the absence of inhibitors.

wards the 11β-hydroxylation of deoxycorticosterone.

3.2. *Kinetics of [^{3}H]metyrapol binding to adrenal cortex mitochondria*

The binding of [^{3}H]metyrapol to adrenal cortex mitochondria is a rapid process. At 0°, the saturation of binding sites is completed in less than 1 min. Equilibrium studies allow the determination of a k_d value of about 10 nM.

Bound [^{3}H]metyrapol can be released upon addition of a large excess of unlabelled metyrapol (40 μM in fig. 3); in this condition, it is expected that each molecule of [^{3}H]metyrapol which leaves its binding site is immediately replaced by one unlabelled molecule. The release of bound metyrapol is much slower than its binding and the effect of temperature on the release process is illustrated in fig. 3; the half maximum release occurs in about 5 min at 0° and in less than 30 sec at 30°. Incidentally, it must be noted that the binding capacity of adrenal cortex mitochondria is completely destroyed by heating at 100° for 30 sec.

3.3. *Specificity of metyrapol binding sites*

The specificity of metyrapol binding sites was assayed by the release of bound [^{3}H]metyrapol upon addition of possible competitive ligands: metyrapone, deoxycorticosterone and corticosterone, androstenedione, progesterone and 17α-OH progesterone. Deoxycorticosterone and androstenedione were chosen because they are substrates for the mitochondrial 11β-hydroxylation and 17α-OH progesterone, because it is hydroxylated exclusively by the microsomal system. Metyrapol itself and dimethyl formamide, the solvent used for steroid additions, were included as controls.

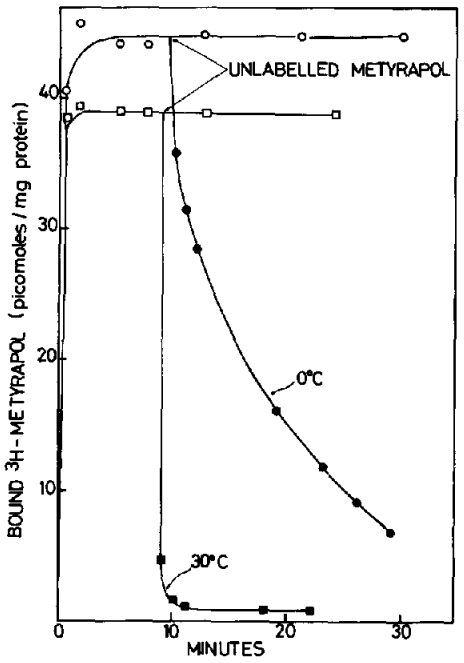

Fig. 3. Kinetics of [^{3}H]metyrapol binding to adrenal cortex mitochondria. Effect of temperature. Mitochondria (12 mg protein) were added at zero time to 15 ml saline medium at 0° containing 4.1×10^{-8} M [^{3}H]metyrapol. Non-radioactive metyrapol was added to a 8 ml portion of the incubation medium to yield a final concentration of 4×10^{-5} M. 1 ml aliquots were sampled at the indicated times and immediately filtered under vaccum through a Millipore filter (pore size: 0.45 μm, diameter: 25 mm). The mitochondria were then quickly washed with 5 ml of saline medium at 0°. Identical experiment was performed at 30°.

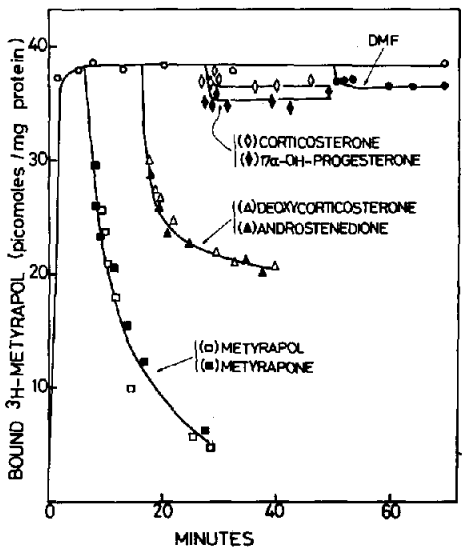

Fig. 4. Specificity of the dissociation of metyrapol. Adrenal cortex mitochondria (110 mg) were added at zero time to 100 ml saline medium at 0° containing 4.1×10^{-8} M [^{3}H]metyrapol. At the indicated times 10 ml portions of the incubation medium were transferred in another vessel and incubated with 3.3×10^{-5} M metyrapol or 5×10^{-5} M metyrapone or 2×10^{-4} M androstenedione, deoxycorticosterone, corticosterone or 17α-OH progesterone or 0.5% dimethyl formamide (DMF) (identical to the amount of DMF introduced with the steroids). At given periods of incubation 1 ml aliquots were sampled and processed as described in fig. 3.

The rates of release of bound [^{3}H]metyrapol upon addition of metyrapone or metyrapol were similar, pointing to the identical behavior of the two ligands (fig. 4). As shown in the same figure, deoxycorticosterone and androstenedione displace bound metyrapol in contrast with corticosterone and 17α-OH progesterone which are virtually ineffective. In other experiments not shown here, cholesterol, 20α-OH cholesterol and progesterone were found inactive.

Displacement of bound metyrapol by deoxycorticosterone (fig. 4) is in agreement with the fact that deoxycorticosterone competes with [^{3}H]metyrapol for binding to adrenal cortex mitochondria. The k_d for deoxycorticosterone binding determined with inner membrane of adrenal cortex mitochondria was found to be 1.3 μM (unpublished experiments).

3.4. *Effect of dithionite and carbon monoxide on [^{3}H]metyrapol binding*

Addition of dithionite and CO to adrenal cortex mitochondria are required for the formation of an inactive cytochrome P450–CO complex. The combined effect of dithionite and CO resulted in a 20% decrease of the capacity for binding [^{3}H]metyrapol and in a 50% decrease of the rate of [^{3}H]metyrapol release upon addition of unlabelled metyrapol (fig. 5).

4. Discussion

Inhibition and binding experiments reported here as well as other experiments bearing on optical and

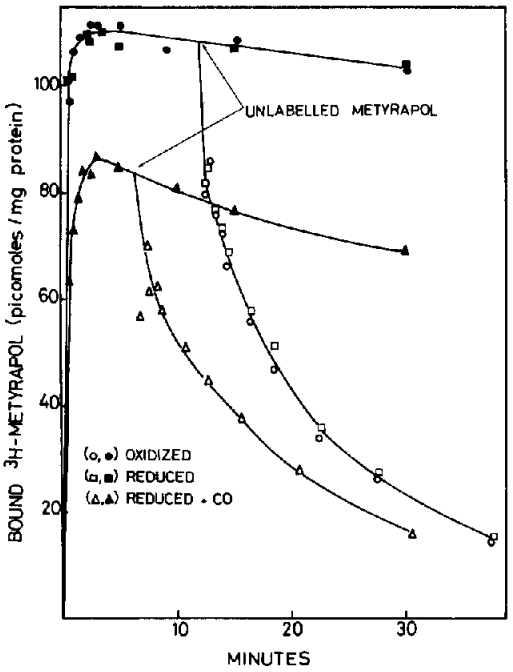

Fig. 5. Kinetics of metyrapol binding to adrenal cortex mitochondria. Effect of sodium dithionite and carbon monoxide. Mitochondria (48 mg protein) were suspended in 100 ml saline medium at 0° and divided in three portions. The first part was kept as an oxidized control; the second was reduced with 10 mM $Na_2S_2O_4$; the third was reduced with 10 mM $Na_2S_2O_4$ and bubbled with CO for 3 min. Incubation was started by addition of 5.5×10^{-8} M [^{3}H]metyrapol. As shown, nonradioactive metyrapol was added to 12 ml portions of incubation medium to yield a final concentration of 2.7×10^{-5} M. At given periods of incubation 1 ml aliquots were processed as described in fig. 3.

EPR spectra (to be reported elsewhere) allow us to conclude that metyrapone and metyrapol interact with the same efficiency at the same binding site on adrenal cortex mitochondria.

Other results reported in this paper indicate that metyrapol (or metyrapone) on one hand and deoxycorticosterone and androstenedione on the other, bind to the same site or to two closely related and interacting sites. The second alternative appears more likely since there is no obvious similarity in structure of metyrapol (metyrapone) and steroids. The effect of CO plus dithionite on the metyrapol binding site confirms that metyrapol binds to cytochrome P450 [2, 5–9] or to a protein in the close neighbourhood. Efforts towards isolation and identification of a mitochondrial membrane protein binding specifically [^{3}H]metyrapol are in progress.

Acknowledgements

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