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17,20-LYASE: ANALYSIS OF THE CYTOCHROME P-450 BINDING SITE USING MULTIPLE INHIBITORS

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SUMMARY

The activity of 17,20-lyase of rat testis microsomes is measured using 17α-hydroxyprogesterone as substrate. Both metyrapone and 20α-hydroxy-4-pregnen-3-one and the 20β-epimer are competitive inhibitors with regard to steroid substrate. Current evidence supports the involvement of cytochrome P-450 in this enzyme activity. The steroid substrate, steroid inhibitors and metyrapone bind to this cytochrome. An analysis of the cytochrome binding of inhibitors was carried out by simultaneous addition of two inhibitors. It was found that when the two steroid inhibitors were added that binding was mutually exclusive and no complex of enzyme–I_1–I_2 was formed. In contrast, a tertiary complex of enzyme–metyrapone–steroid inhibitor was formed although the binding of these structurally dissimilar inhibitors was antagonistic.

INTRODUCTION

One of the steps in the synthesis of androgens and estrogens involves cleavage of the carbon-carbon bond between steroid positions 17 and 20. This microsomal activity is mediated by the enzyme, 17,20-lyase, which employs cytochrome P-450 as the terminal oxygenase. This cytochrome also serves as the steroid substrate binding site [1, 2]. Although there is some controversy regarding the actual substrate of the lyase reaction [3], the initial velocity of cleavage can be accurately measured using 17-hydroxyprogesterone as substrate [4]. Kinetic studies have demonstrated that both the nitrogenous base, metyrapone, and a number of steroids, which are structurally similar to the substrate, compete for the catalytic site of the lyase [5]. Thus, inhibitors with dissimilar structures and functional groups appear to exert an identical effect on the activity of the lyase.

The apparently identical effect of inhibitors with dissimilar structures has previously been studied by Yonetani and Theorell[6]. In this paper they developed the theoretical background for a graphical method to analyze the simultaneous inhibition of an enzyme by two competitive inhibitors. This method of analysis distinguishes whether the inhibitors bind at the same or different sites; whether simultaneous binding of both inhibitors can occur; and whether multiple inhibitor binding is cooperative, antagonistic or no interaction between inhibitors occurs. The degree of interaction may be quantitated by calculation of an interaction constant (α).

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The analysis of multiple inhibition kinetics in the lyase reaction was carried out with a mixture of metyrapone and a steroid inhibitor (20α-hydroxy-4-pregnen-3-one or 20β-hydroxy-4-pregnen-3-one). In other experiments, two steroid inhibitors were added simultaneously. As anticipated, simultaneous binding of both steroid inhibitors to the enzyme did not occur. In contrast, a complex of enzyme, metyrapone and either of the two steroid inhibitors was formed. The interaction of steroid inhibitors and metyrapone was antagonistic, suggesting an overlapping of inhibitor binding sites.

EXPERIMENTAL PROCEDURES

Materials. [21-^{14}C]-progesterone (55.5 mCi/mmol) was obtained from New England Nuclear Corporation.

Methods. Microsomal preparation and assay of enzyme activity has previously been described [4]. The assay for lyase activity is based on partition of the water-soluble, 2-carbon fragment cleaved from the steroid substrate, 17-[21-^{14}C]-hydroxyprogesterone. This steroid substrate was enzymically synthesized as described [4]. The concentration of steroid substrate in the reaction mixture was 2.5×10^{-6} M ($\approx 2.2 \times 10^{-4}$ c.p.m.). The other reactants were: $MgCl_2$, 5×10^{-4} M; glucose:-6-P, 5×10^{-3} M; $NADP^+$, 5×10^{-3} M; glucose 6-P dehydrogenase, 0.6 units; sodium phosphate buffer, 0.05 M; and 1.0 mg of microsomal protein in a final volume of 1.5 ml at 37°C at pH 7.4. The reaction was initiated by addition of $NADP^+$, and at three times (0, 20 and 40 min) an aliquot of the reaction mixture was combined with an equal volume of benzene and shaken. After separation of phases an aliquot of each layer was removed

for scintillation counting. Steroids were added in ethanol. The concentration of this solvent was the same in all assays. Zero time blanks were measured at less than 10% of the net reaction rates and sequential removal of aliquots for each assay ensured that all rates were initial velocities. None of the inhibitors used had any effect on the NADPH generating system or on cytochrome c reductase activity. Kinetic data was evaluated by the method of Wilkinson[7]. The method of calculation of standard error of α is described by Mellor[8]. The confidence levels for both slopes are used in this calculation.

RESULTS

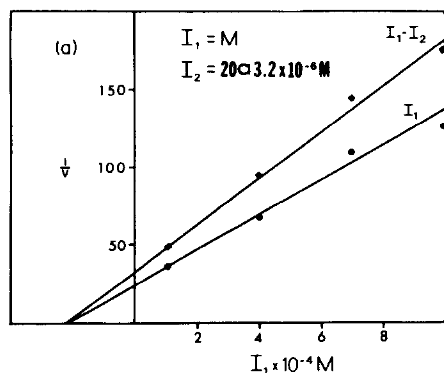
The initial step in these experiments is the measurement of initial velocities with substrate at a single, fixed concentration, but with varying concentrations of an inhibitor (I_1). These assays are then repeated in the presence of a second inhibitor (I_2). The multiple inhibition plots thus generated are used to calculate the interaction constant, α. To enhance confidence in the data, plots are generated with the members of the inhibitor pairs switched. For example, in Fig. 1(a), metyrapone is I_1 and 2C hydroxy-4-pregnen-3-one is I_2. In Fig. 1(b), these assignments are switched. In both cases α values, as noted in Table 1, reveal simultaneous binding of inhibitors to the enzyme, but the interaction of I_1 with the enzyme antagonizes the interaction of I_2. If the 20β-steroid epimer is added with metyrapone (Fig. 1c) simultaneous, antagonistic binding is observed. The disocciation constant reflecting simultaneous binding of inhibitors is an order of magnitude greater than when a single inhibitor binds ($\alpha = K_{I_1}, {}_{I_2}/K_{I_2}$). The interaction constant is calculated from the ratio of slopes (6):

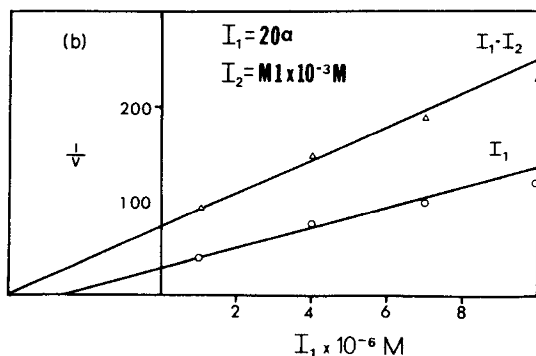
$$\alpha K_{I_2 = I_2} \frac{\text{slope with } I_2}{\text{slope without } I_2} - 1$$

Table 1. Interaction constant (α)

I_1	I_2	α
Metyrapone	20α-	7.9 ± 1.5 (38)
20α-	Metyrapone	15 ± 4 (32)
Metyrapone	20β-	14.4 ± 2.9 (36)
20α-	20β-	95 ± 19 (36)
20β-	20α-	∞ (40)

The numbers in parentheses are the number of assays. 20α- = 20α-hydroxy-4-pregnen-3-one. 20β- = 20β-hydroxy-4-pregnen-3-one.





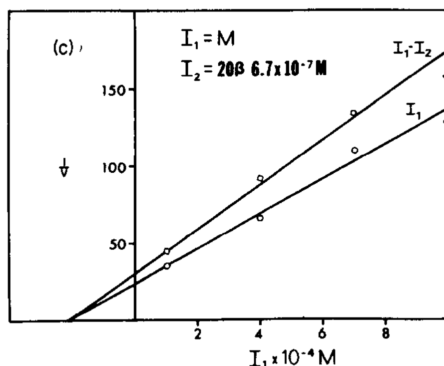
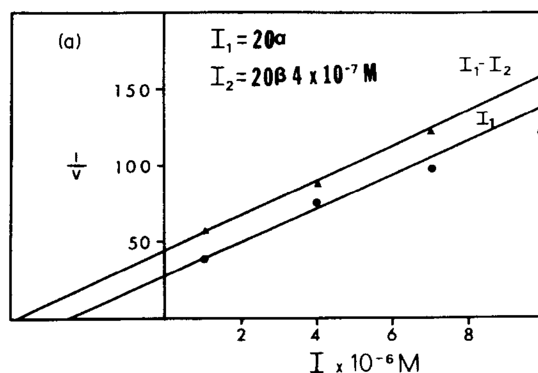


Fig. 1. (a) Metyrapone (M) as varying inhibitor I_1, 20α-hydroxy-4-pregnen-3-one (20α-) as I_1, M as I_2. (c) M as I_1, 20β-hydroxy-4-pregnen-3-one (20β-) as I_2.



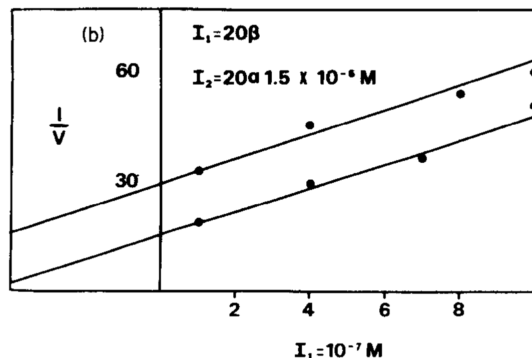


Fig. 2. (a) 20α- as I_1, 20β- as I_2. (b) 20β- as I_1, 20α- as I_2.

where K_{I_2} is the disocciation constant of I_2 [published elsewhere, ref. 5]. I_2 is the concentration of the second inhibitor. Thus, when the slopes are parallel (Figs 2a and 2b), α approaches infinity, and simultaneous binding of both inhibitors does not occur.

DISCUSSION

The mutual exclusivity of the two steroid inhibitors could be expected from their structural similarity with each other and with the steroid substrate. Analogous findings were reported by Yonetani and Theorell[6] for lactate dehydrogenase. In this case, ADP and AMP were mutually exclusive competitive inhibitors with regard to NAD^+. It is of interest that the absorption site for steroids will accomodate only one of these molecules at a time. The fact that a number of steroids [9] will bind to testis cytochrome P-450 as evidenced by spectral perturbation suggests that the interaction is relatively non-specific or that multiple sites are involved. The data presented here are concomitant with the interpretation that steroid absorption occurs at a single, limited site on the cytochrome.

The relationship between the binding sites of steroids and metyrapone is speculative. Several possibilities exist. Steroid and metyrapone could bind on opposite sides of the heme ring. Current evidence suggests that this binding would result in displacement of a thiolate anion on one side of the pyrrole ring and an imidazole nitrogen from the other ring surface [10]. An alternative consideration entails the coordination of the nitrogen of metyrapone to the iron of the cytochrome with the steroid inhibitor binding at a site distant from the iron. The steroid interaction must, however, be close enough to the iron for efficient catalysis and must also be in a position to effect perturbation of the heme chromophore. In contrast to the placental cytochrome P-450 (11), steroids do not displace CO from the testis cytochrome [9]. Since the ability to displace CO has been suggested as a criterion for direct interaction of a ligand with iron [12], the binding of steroid at a site distant from iron would seem to be a likely possibility in the testis microsomal system. Metyrapone, which yields a type II spectrum with the testis cytochrome [9], probably binds to the iron by one of its pyridine nitrogens [13]. The overlapping binding sites of both metyrapone and steroids are probably in a hydrophobic pocket [13].

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