The Effect of Metyrapone on the 17,20-Lyase from Rat Testis Microsomes

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Summary

The effect of metyrapone on the activity of the steroid 17,20-lyase from rat testis microsomes was evaluated. A competitive pattern of inhibition was observed with a K_I of $1.3\pm0.12\times10^{-4}$M determined for metyrapone using a least mean squares computer analysis. The substrate for the lyase induced a Type I difference spectrum in an active suspension of Triton treated microsomes. The magnitude of this spectral change was dependent on steroid concentration and was diminished by metyrapone. The effect of metyrapone was abolished at infinite steroid concentration. Aminoglutethimide had no effect on activity or the spectral changes. These results implicate cytochrome P450 as a reactant in the 17,20-lyase reaction.

A number of soluble and particulate enzymes are involved in the conversion of cholesterol to estrogenic steroids. Among these enzymes, only minimal information is available concerning the microsomal enzyme which cleaves the carbon-carbon bond between the 17 and 20-positions of 17-hydroxyprogesterone(17HP). This enzyme is variously known as a desmolase, cleavage enzyme, or as the 17,20-lyase. Although the enzymic mechanism remains to be established, the name 17, 20-lyase will be employed in this communication. A number of studies with the 17,20-lyase from rat testis microsomes and the development of an assay for this enzyme have previously been reported from this laboratory(1).

Because the 17,20- lyase requires molecular oxygen and NADPH(2), it is thought to be a mixed function oxygenase and a report of enzymic inhibition by CO has recently been published(3). Although this finding strongly suggests participation of P450 in the reaction, an earlier report had reported stimulation of the activity by CO(2).

Metyrapone, a nitrogenous base, is an inhibitor of 11β-hydroxylation in the adrenal(4). This inhibition has been reported to be competitive with respect to substrate in mitochondria(5) but a question has been raised regarding the type of inhibition by metyrapone in microsomes(6). The substrate-induced spectral change produced by interaction of deoxycorticosterone with P450 is also inhibited by metyrapone at concentrations which inhibit enzymic activity(5).

This communication reports the effects of metyrapone on 17,20-lyase activity and on the substrate-induced difference spectrum in rat testis microsomes. The

effect of aminoglutethimide, a nitrogenous base which probably inhibits the steroid 20,22-lyase(7), is also reported.

METHODS

Microsomes were prepared and enzymic activity assayed as previously described (1). The assay is based on partition of the water soluble 2-carbon fragment cleaved from the steroid molecule using the radioactive substrate,17-hydroxyprogesterone[21-^{14}C](1). $NADP^+$ (4.2mg) was added with an NADPH generating system utilizing glucose 6-P dehydrogenase. 120μmoles of Na PO_4 buffer were added in a final volume of 1.5 ml (pH7.4). A control experiment revealed no inhibition of the generating system by concentrations of metyrapone used in these studies. Results of rate measurements were analyzed by a computer program obtained from Cleland(8). Spectral studies were performed at 25° using a Cary 14 spectrophotometer. Microsomal subparticles, generated by treatment with Triton N-101(1), were employed for spectroscopy. Metyrapone and aminoglutethimide were gifts of the Ciba company.

RESULTS

Initial velocity inhibition studies using two concentrations of metyrapone resulted in the double-reciprocal plot shown in Fig. 1. The lines were drawn

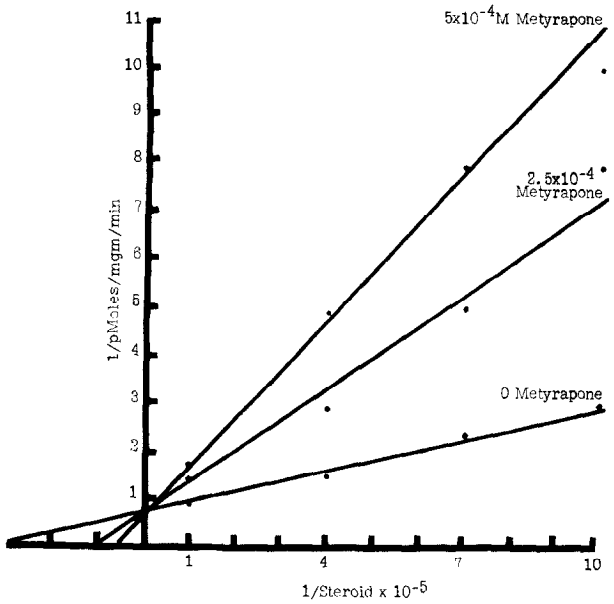

Fig. I. Double reciprocal plot of 17,20-lyase velocities at various steroid substrate concentrations. The levels of metyrapone were 0; 2.5x10^{-4}M; and 5x10^{-4}M. Temperature was 37°.

from the computer calculated kinetic constants. K_I for metyrapone was 1.3±0.12 x10^{-4}M. Values of kinetic constants and standard errors were calculated from 66 data points. These measurements were repeated twice with competitive inhibition being observed each time although the precision of the reported data was somewhat higher than that for the other experiments.

In contrast to metyrapone, aminoglutethimide demonstrated no inhibition of the 17,20-lyase reaction at concentrations of 10^{-6}M to 10^{-3}M.

Spectral studies - The 17,20-lyase substrate, 17HP, induced a difference spectrum in Triton treated microsomes with a maximum at 388 nm and a minimum at 422 nm (Fig.2.). This corresponds to a Type I spectrum(9) which has been inter-

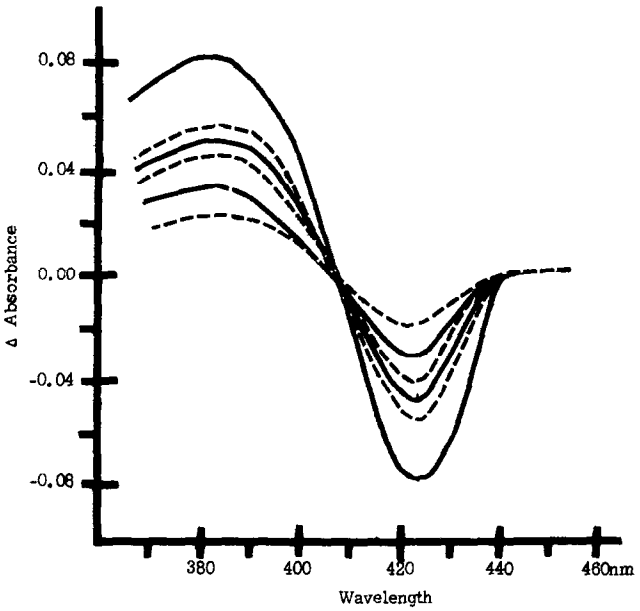

Fig. II. Difference spectra of Triton treated microsomes. The sample cuvette contained 17α-hydroxyprogesterone in concentrations varying from 2x10^{-6}M to 1x10^{-5}M added in 100µl of ethanol. The reference cuvette contained 100µl of ethanol. Protein concentration was 5 mg/ml.

preted as the interaction of the substrate with P450(10). The magnitude of the spectral change was dependent on the concentration of steroid substrate. A double reciprocal plot of ΔOD 407-422 nm vs substrate concentration is shown in Fig. 3. The substrate concentration at which ΔOD was one-half maximal was 2.0x10^{-5}M, which corresponds well with a Km of 1.5x10^{-5}M for Triton treated

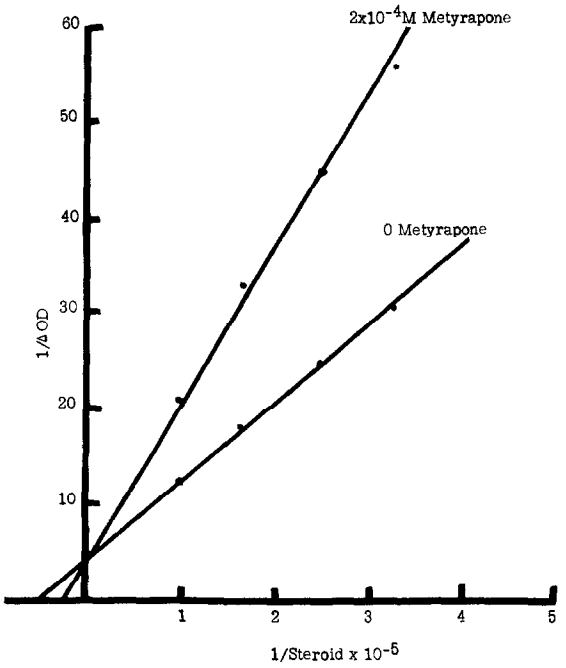

Fig. III. The lower line represents the double reciprocal plot of ΔOD 409-422 from Fig. II. The upper line represents ΔOD 409-422 in the presence of $2x10^{-4}$M metyrapone.

microsomes assayed at 25°. The metyrapone inhibition of the spectral change is no longer evident at infinite substrate concentration, again suggesting competition between substrate and metyrapone for a common P450 binding site. Addition of aminoglutethimide at a concentration of 10^{-3}M had no effect on the difference spectrum induced by 17HP.

DISCUSSION

The kinetic studies reported in this communication indicate that metyrapone and 17HP compete for the same form of the enzyme. Fitting the initial velocity data to Cleland's competitive inhibition program results in a K_I of the slope with a S.E. less than 10%. Standard errors for inhibition constants of up to 20% are adequate to insure precision of measurements(8). The precision for the Km and Vmax of zero and $2.5x10^{-4}$ metyrapone concentrations are excellent with standard errors of 10%, but despite repeated measurements the S.E. of the lower velocity line ($5x10^{-4}$ metyrapone) is greater than desirable. In spite of this difficulty, the lower velocity line confirms that infinite substrate concentration abolishes inhibition. The most plausible interpretation of Fig. 1 is that substrate and metyrapone compete for the same enzymic binding site.

It is believed(11) that substrates are bound to P450 prior to enzymic hydroxylation, and that the difference spectrum induced by adding substrate to a clarified microsomal preparation(9) reflects this interaction. These spectra are designated Type I(12) in contrast to the spectra induced by non-substrates (Type II) such as metyrapone(5). The substrate for the 17,20-lyase induces a Type I difference spectrum, the magnitude of which is proportional to steroid concentration. The binding constant for this interaction, determined by spectral studies is comparable to the Km determined by initial velocity studies under the same conditions. As demonstrated in Fig. 3, metyrapone at concentrations near its K_I, also diminishes the spectral change induced by substrate. Analogous to the kinetic studies, the spectral inhibition by metyrapone is competitive with the steroid. In contrast to metyrapone, aminoglutethimide inhibits neither the enzymic reaction nor the interaction of substrate with P450. These results are interpreted to mean that metyrapone inhibits the 17,20-lyase reaction by competition with 17HP for the P450 binding site. Although these experiments yield insight into the mechanism of metyrapone inhibition, the primary purpose of this investigation was to identify microsomal components involved in the 17,20-lyase reaction. These data provide strong support for the participation of P450 in this reaction.

Hildebrandt(6) has reported that in liver microsomes the inhibition of a number of hydroxylase reactions by metyrapone is not abolished by increased substrate concentration suggesting inhibitor binding at a site not occupied by substrate or binding at two sites. A number of explanations for the differences in kinetic results are possible. There is evidence that metyrapone can bind to both reduced and oxidized P450(13) which would result in non-competitive inhibition. As our rate measurements were initiated by addition of $NADP^+$, it might be anticipated that the oxidized cytochrome is already bound to substrate or metyrapone before the reaction proceeds with no reduced P450 available for binding. An alternative explanation for the difference in inhibition patterns might be the affinity of P450 for metyrapone in the tissue studied. A marked difference (100 fold) in K_I for metyrapone is apparent in comparing measurements with adrenal mitochondria(5) and testis microsomes. It is concievable that a second binding site for metyrapone in testis microsomes has such low affinity that only competitive inhibition is observed with binding of the inhibitor limited to the substrate binding site.

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