Vol. 108, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1982 Pages 454-460

CYTOCHROME b_5 STIMULATES PURIFIED TESTICULAR MICROSOMAL CYTOCHROME P-450 (C_{21} SIDE-CHAIN CLEAVAGE)

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Received July 20, 1982

Cytochrome b_5 purified from neonatal pig testis and that from pig liver stimulated C_{21} steroid side-chain cleavage (progesterone → androstenedione) catalyzed *in vitro* by purified cytochrome P-450 from neonatal pig testicular microsomes. Km of testicular cytochrome b_5 for the P-450 is 6.3-9.1 × 10^{-8}M and the ability of b_5 to stimulate C_{21} side-chain cleavage is different for cytochromes b_5 prepared from different sources.

INTRODUCTION

When reactions catalyzed by cytochrome P-450 are studied in microsomal preparations, NADH may stimulate enzyme activity possibly by providing the second electron of the P-450 cycle via cytochrome b_5 and cytochrome b_5 reductase (1,2). On the other hand, when cytochrome b_5 is added to purified P-450 and P-450 reductase with NADPH but without NADH and without b_5 reductase, a variety of responses have been observed. For example, with the cytochrome P-450 from rabbit liver called LM_2, prostaglandin metabolism is extremely small unless b_5 is added (3). On the other hand, benzphetamine hydroxylation, catalyzed for the same enzyme, is inhibited by addition of b_5 (4). Such observations suggest that cytochrome b_5 may interact with P-450 apart from any influence exerted in microsomes on the process of electron transport from NADH. However, interpretation of these and other related observations is complicated by the fact that a given cytochrome P-450 catalyzes a variety of reactions

This work was supported by a grant from the National Institutes of Health, Bethesda, MD 20205 (Grant 2 R01 AM 28113-03)

0006-291X/82/180454-07$01.00/0

with a number of substrates and may act at several sites on one substrate. By contrast, cytochrome P-450 from testicular microsomes is specific for steroid C_{21} side-chain cleavage and catalyzes two sequential reactions necessary for such cleavage (17α-hydroxylation and $C_{17,20}$ lyase activity) (5,6). This enzyme has been purified to homogeneity (7). It was considered important to determine the possible influence of cytochrome b_5 on these two specific reactions both in intact microsomes and with the reconstituted enzyme system.

EXPERIMENTAL PROCEDURE

Preparation of Microsomes and Microsomal Enzymes. Neonatal pig testes were obtained at castration and microsomes were prepared as described by Imai and Sato (8). Cytochrome P-450 was prepared from the microsomes by a method published from this laboratory (7). P-450 reductase was purified from pig liver and pig testes according to the method of Yasukochi (9); this method, devised for the liver, proved directly applicable to the testis. Cytochrome b_5 was prepared from rabbit liver and pig liver by a published method (10). Cytochrome b_5 was prepared from neonatal pig testicular microsomes as a by-product of the preparation of P-450 as follows. Cytochrome b_5 was eluted from DEAE cellulose after P-450 (7) and the fractions containing cytochrome b_5 were pooled and dialyzed against Buffer A: potassium phosphate buffer (40mM; pH7.25) containing glycerol (20% v/v), EDTA (0.1mM) and dithiothreitol (0.1mM) (final concentrations). The sample was applied to hydroxylapatite in the same buffer. Cytochrome b_5 appeared in the void volume while other proteins remained bound to the column. The fraction containing b_5 was applied to DEAE cellulose in Buffer A and cytochrome b_5 was eluted by 0.3MKCl and sodium cholate 0.6% (w/v) in Buffer A. The fractions containing cytochrome b_5 were pooled and dialyzed against potassium phosphate (100 mM; pH 7.25) with 40% glycerol, EDTA (0.1 mM) and dithiothreitol (0.1 mM) and stored at -20° C. The cytochrome b_5 prepared by this procedure appeared as a single band on SDS acrylamide gels and showed a specific activity of 34.1 n moles heme/mg protein.

Measurement of Proteins and Enzymes. Cytochrome P-450 and cytochrome b_5 were measured as described by Omura and Sato (1964) (11). NADPH-cytochrome c reductase, which will be referred to here as P-450 reductase, was measured by the method of Omura and Takesue (1970) (10) and NADH-cytochrome b_5 reductase was measured by the method of Takesue and Omura (1970) (12). The methods for measuring 17α-hydroxylase and $C_{17,20}$-lyase are given in detail elsewhere (7). In the studies to be reported, the two enzyme activities were always measured under conditions in which enzyme activity was proportional to the concentration of cytochrome P-450.

Miscellaneous. Other methods and sources of chemicals have been reported previously (5,6,7).

RESULTS

Electron Carriers and Enzymes in Microsomes from Neonatal Pig Testis. Table 1 shows that microsomes from neonatal pig testis contain cytochrome

TABLE 1

Electron Carriers and Enzymes in Microsomes from Pig Testis

Protein	Content or Activity
Cytochrome P-450	0.32 ± 0.03 n moles/mg Protein
Cytochrome b_5	1.30 ± 0.25 n moles/mg Protein
NADPH-cytochrome c reductase	39.83 ± 7.45 n moles/min/mg Protein
NADH-cytochrome b_5 reductase	6.33 ± 1.32 μ moles/min/mg Protein

The electron carriers and enzymes were measured in microsomes as described under Experimental Procedures. Values represent means and ranges for four separate microsomal preparations.

b_5 and b_5 reductase in keeping with findings from other laboratories (e.g. 13). It should be noticed that there is more b_5 than P-450 and that b_5 reductase is more active than P-450 reductase in these microsomes.

Enzymatic Activity of Microsomes from Neonatal Pig Testis. Figure 1 shows that testicular microsomes catalyze both 17α-hydroxylation and

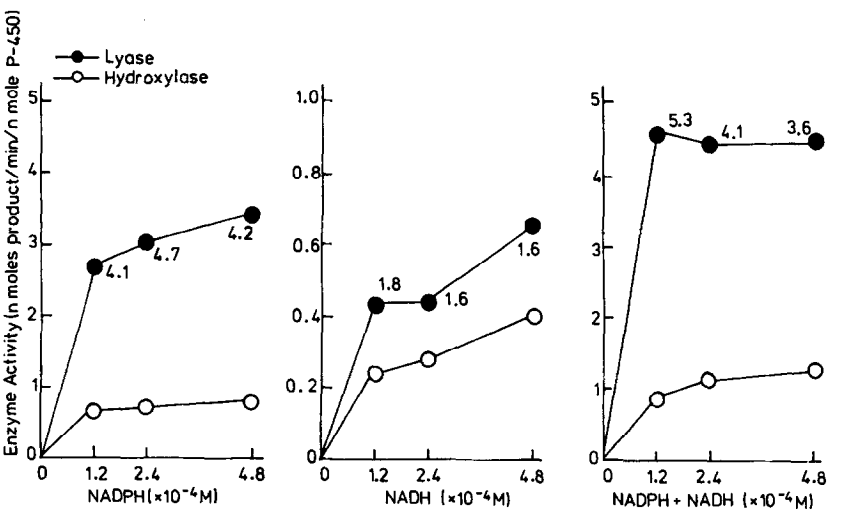

Figure 1. Microsomes from neonatal pig testes were incubated with [4-^{14}C] progesterone (hydroxylase) or 17α-hydroxy [4-^{14}C] progesterone (lyase) with the pyridine nucleotides shown. Values of pyridine nucleotides in the third panel represent total concentrations made up of equal amounts of NADPH and NADH. The microsomes were incubated in potassium phosphate (50mM; pH7.25) with 1mM EDTA for three minutes at 37° C. At this time pyridine nucleotide was added and incubation continued for 20 minutes. Hydroxylase and lyase activities were then measured as described elsewhere (7). The numbers on the upper curves represent the ratio : Lyase/Hydroxylase.

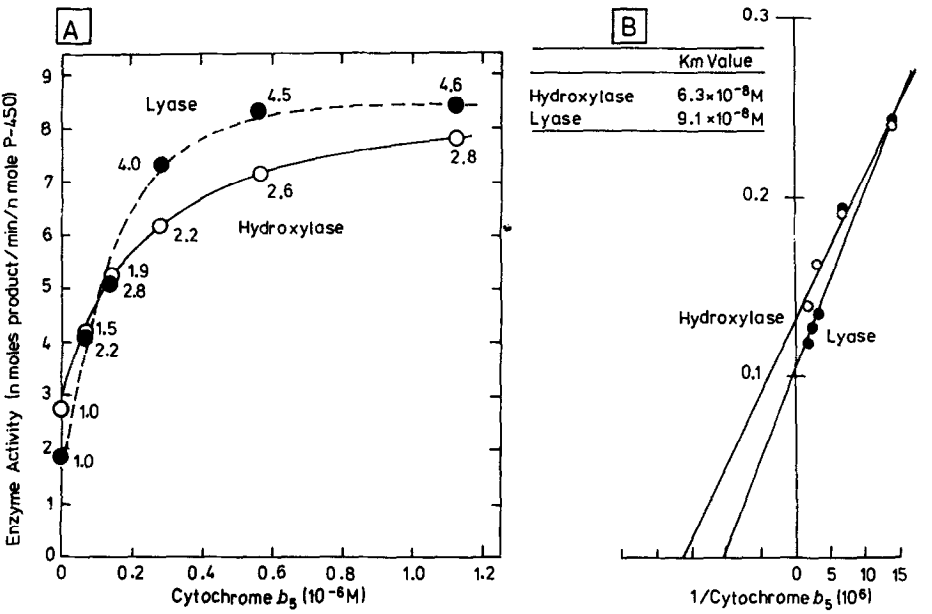

Figure 2. Hydroxylase and lyase activities were measured with purified P-450 from neonatal pig testes (40 p moles/flask) as described elsewhere (7). Cytochrome b_5 was added in the concentrations shown as the last addition prior to preincubation for 3 minutes. Following preincubation, the reaction was started by addition of NADPH (240 n moles) and incubation was continued for 20 minutes.

$C_{17,20}$-lyase activity with considerably greater lyase than hydroxylase activity with exogenous NADPH. Little activity is seen with NADH (scale is expanded) although again lyase exceeds hydroxylase. The two pyridine nucleotides together appear to produce an additive effect; significant synergism was not apparent in four similar studies. Higher concentrations of equal mixtures of the two pyridine nucleotides were without additional effect upon either hydroxylase or lyase activities (not shown).

The Influence of Cytochrome b_5 on Purified P-450. Figure 2 shows that cytochrome b_5 causes considerable stimulation of both hydroxylase and lyase activities of testicular P-450. In the absence of b_5 lyase activity is less than hydroxylase activity. The reverse is true with cytochrome b_5 although the ratio of lyase relative to hydroxylase never reaches levels seen in microsomes (figure 1). Double reciprocal plots yield values of 10^{-8} to 10^{-7} M for the Km of b_5 with cytochrome P-450 (Figure 2B); the two lines shown in Figure 2B are not significantly different ($p > 0.5$).

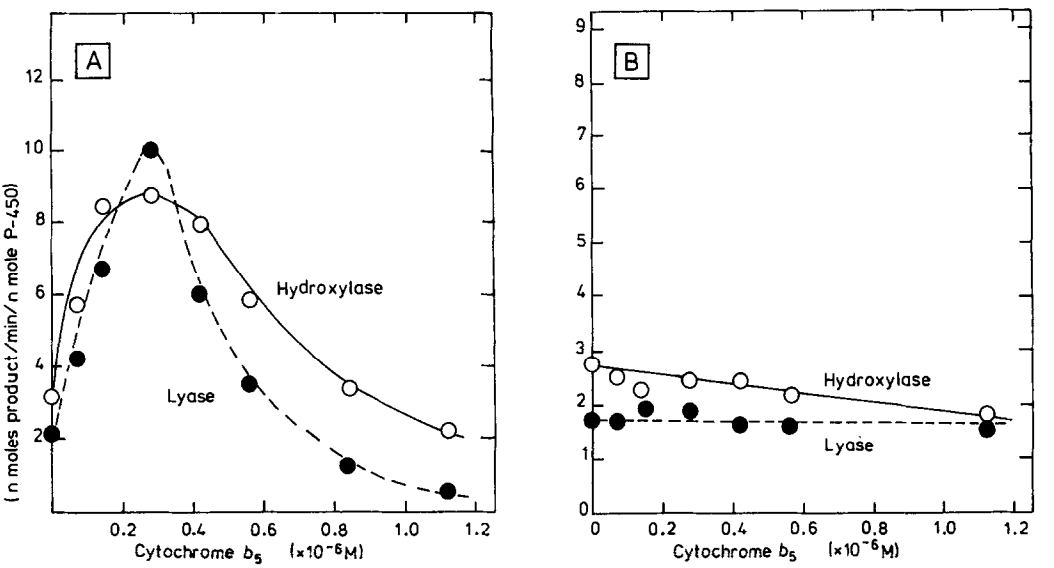

Figure 3. The effects of cytochromes b_5 on the enzymatic activities of testicular microsomal cytochrome P-450. These experiments were performed as described in the legend to Figure 2 except for the source of cytochrome b_5 which was pig liver (A) or rabbit liver (B).

When cytochrome b_5 from pig liver is added to the reconstituted system (Figure 3A) the response is different. Stimulation at low concentrations is followed by inhibition at high concentrations. On the other hand, cytochrome b_5 from rabbit liver is without obvious effect on the activities of the cytochrome P-450 (Figure 3B).

None of the results was altered by addition of phosphatidylcholine (30µg/flask) to the incubation system.

DISCUSSION

The present studies show that microsomes from neonatal pig testes contain cytochrome b_5 in keeping with results from other laboratories with rat Leydig cells (13). Since neonatal pig testis consists largely of Leydig cells and since the studies of Ohba *et al.* (13) were performed on microsomes from rat Leydig cells, it is likely that the cytochrome b_5 measured in our studies with microsomes from neonatal pig testis is localized largely if not entirely in the steroidogenic cells of the pig testis. In addition, these microsomes also contain cytochrome b_5 reductase. Moreover, cytochrome b_5 is present in higher concentration than

P-450 and b_5 reductase is several orders of magnitude more active than NADPH-cytochrome c reductase (P-450 reductase) (Table 1).

In considering the possible relationship of cytochrome b_5 to C_{21} side-chain cleavage, it is important to consider the fact that in microsomes NADH permits very little C_{21} steroid side-chain cleavage activity in contrast to NADPH which produces considerable activity. Again, the two reduced pyridine nucleotides in the highest concentration tested, produce only slightly greater activity than that seen with the same concentration of NADPH alone (Figure 1). It is also clear that NADPH stimulates lyase activity to a greater extent than hydroxylase while NADH stimulates both activities to approximately the same extent. These results do not suggest an important role for NADH as an electron donor for steroidogenesis in testicular microsomes. Any contribution of cytochrome b_5 to C_{21} side-chain cleavage is therefore unlikely to proceed via NADH and b_5 reductase. Since the observations of Ohba *et al.* suggest that cytochrome b_5 may be involved in microsomal steroid production (13), it was important to determine whether or not this cytochrome influences the activity of the purified enzyme system.

It is clear from Figure 2 that cytochrome b_5 produces considerable stimulation of C_{21} side-chain cleavage under conditions in which P-450 activity is limiting. Moreover, the effect of b_5 on lyase activity is greater than that on hydroxylase. This action of cytochrome b_5 shows some specificity in that the cytochrome b_5 from rabbit liver is without effect (Figure 3B) whereas the cytochrome b_5 from pig liver is highly effective (Figure 3A), although qualitatively different in its effect when compared to testicular cytochrome b_5. These observations support the suggestion (13) that cytochrome b_5 may play a physiological role in sertoid C_{21} side-chain cleavage *in vivo*. Presumably b_5 interacts with P-450 itself.

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