ON THE ROLE OF CYTOCHROME *P*-450 IN RABBIT HEPATIC MICROSOMAL 12α-HYDROXYLATION

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1. Introduction

Interest in the hepatic microsomal 12α-steroid hydroxylase stems from the concept that it may regulate the physiological ratio [1] of the two primary bile acids, cholic and chenodeoxycholic acids. The mechanism and regulation of 12α-hydroxylation can best be investigated with a purified preparation of the enzyme. Efforts towards this end may be simplified if the enzyme can be shown to be identical to one of the cytochrome *P*-450 species which has been purified to near-homogeneity [2,3], for both are active in hydroxylating steroidal structures in the presence of oxygen and NADPH [3–6]. The hepatic microsomal 12α-hydroxylase system from the rat appears different from the predominant cytochrome *P*-450 species of the same subcellular origin in its responses to carbon monoxide [7–9], inorganic salts [10], phenobarbital [9], cholesterol [4], cholestyramine [11] and starvation [11]. However, microsomal 12α-hydroxylase from rabbit liver is preferable for study over that from rat liver because of its higher specific activity [12,13]. The following report demonstrates that, unlike most cytochrome *P*-450-dependent reactions, the preparations of microsomal 12α-hydroxylase are barely sensitive to carbon monoxide and metyrapone, and that a solubilized cytochrome *P*-450 fraction which contains 12α-hydroxylase loses the latter activity upon storage under conditions stable to the major species of cytochrome *P*-450.

2. Materials and methods

2.1. *Assay for 12α-hydroxylase*

Substrate ([5α,6α-$^3H_2$]cholestane-3α,7α-diol) and product (5α-cholestane-3α,7α,12α-triol) of the enzymatic reaction were prepared as in [1,14]. Enzymic assay was determined by radioassay of product formed after incubation of 35 nmol substrate for 6 min with appropriate amounts of cofactors and 1.5 mg microsomal protein as in [1]. Identification of the product was confirmed by analysis of the mass spectral pattern of the trimethylsilyl ether obtained by gas chromatography–mass spectrometry (GC–MS) [14].

2.2. *Cytochrome* P-*450 and cytochrome* P-*450 reductase*

Rabbit liver microsomes were harvested as described [1]. Procedures from [5] were employed to prepare cytochrome *P*-450 from rabbits starved for 48 h and cytochrome *P*-450 reductase from male rabbits pretreated with intraperitoneal injection of phenobarbital at 50 mg/kg twice daily for 3 days. Solubilized samples were stored under N_2 at −70°C. Results of assay for solubilized cytochrome *P*-450 [5] for three separate preparations were: 0.55 nmol *P*-450/mg protein associated with ~30% cytochrome *P*-420 (from a female rabbit starved for 24 h), 2.73 nmol/mg with no measurable *P*-420 (from a male rabbit starved for 48 h); and 2.1 nmol/mg with ~3% cytochrome *P*-420

(from a male rabbit not starved). Specific activities [5] of cytochrome *P*-450 reductase from three separations were 385, 184 and 124 units .mg protein^{-1} .min^{-1}. Each contained small amounts of 12α-hydroxylase. ω-Aminooctyl–Sepharose was prepared and used in affinity chromatographic purification of cytochrome *P*-450 as in [15]. Protein was determined by the Lowry method [16].

2.3. *Co studies*

Incubations with microsomal suspension [1] were initiated by the addition of substrate in Tween-80 with phosphate buffer (pH 7.4) containing nicotinamide, to the mixture pre-incubated at 37°C for 3 min. The CO studies were performed in two ways:

(i) CO was bubbled slowly through the mixture for 1 min at 4°C, followed by the addition of substrate. Incubations were then carried out as stated above. Controls contained no added CO.

(ii) The incubation mixture at 4°C was flushed 3 times through a septum with a mixture of 56% N_2, 4% O_2 and 40% CO, with alternate evacuation under vacuum. Under the designated gaseous mixture at atmospheric pressure, a solution of substrate was added via a syringe. Controls utilized either a mixture of 96% N_2 and 4% O_2 or air.

2.4. *Re-constitution studies*

Determination of 12α-hydroxylase activity in re-constituted solubilized and partially purified preparations [5] was performed as in section 2.1 except that the microsomes were replaced by cytochrome *P*-450 fraction (1 mg protein), cytochrome *P*-450 reductase fraction (75 units), and phospholipid (40 μg dilauroyl GPC, or 10 μg dioleoyl GPC), and that the substrate was solubilized with Tween-80 (0.4 μg) or BSA (1 mg) in the phosphate buffer mixture, or with methanol (10 μl). The order of addition was made according to [17] with NADPH added last.

3. Results

The effect of metyrapone on the activity of rabbit hepatic microsomal 12α-hydroxylase was minimal; at a maximal concentration of 100 μM metyrapone the activity was 93% of the control samples. Bubbling of CO through the incubation mixture caused no loss in enzyme activity (fig.1B). With a gaseous mixture of a fixed composition containing CO a slight decrease in enzyme activity was noted in the dark (fig.1A), but no inhibition was observed in the presence of light

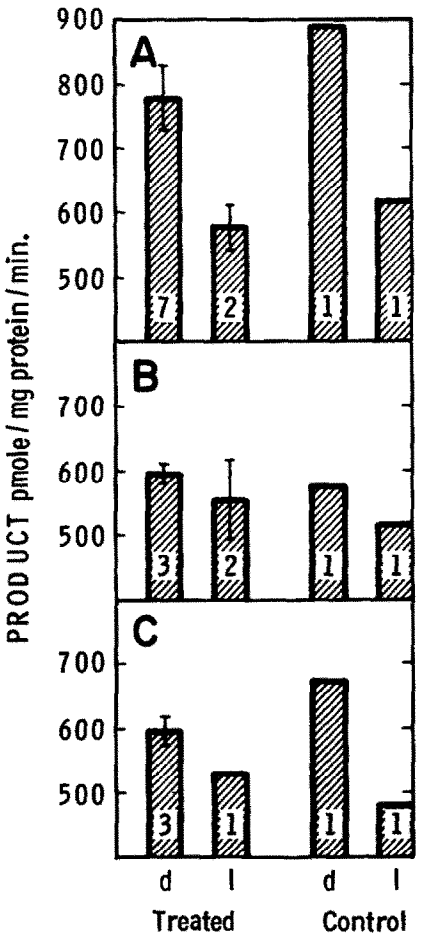

Fig.1. Effect of CO on 12α-hydroxylase activity. 'd' and 'l' stand for dark and light reactions, respectively. 'Control' reactions are those carried out in open air. 'Treated' refers to incubations performed (A) under a gaseous mixture of 56% N_2, 4% O_2 and 40% CO, (B) with CO bubbled through the incubation mixture, and (C) under a gaseous mixture of 96% N_2 and 4% O_2. A single microsomal preparation was used for all experiments which were carried out sequentially over 3–4 h. The height of the bars represents the mean of multiple determinations; the number of measurements is indicated by an Arabic numeral. The SEM values are shown on the top of the bar. Only single measurement of each control was performed because previous experience indicates close agreement between duplicates as in [1].

(fig.1A). Enzyme activities were comparable in the presence of air or a mixture of 96% N_2 and 4% O_2 (fig.1C). Reactions in the light were consistently slower than those in the dark.

Solubilized and partially purified cytochrome *P*-450 contained 12α-hydroxylase activity as demonstrated by the GC–MS identification [14] of the trimethylsilyl derivative of the product (R_T 10.2 min), which was accompanied by two other peaks (R_T 4.44 and 26.4 min) due to non-steroidal substances. Lack of a significant level of radioactivity prevented radioassay of the material present in the GLC peaks. The 12α-hydroxylase activity of the reconstituted solubilized cytochrome *P*-450 system was ~20% that of native microsomes. It was not stimulated by dilauroyl GPC (10–70 μg) or dioleoyl GPC (10–40 μg) when the substrate was solubilized with Tween-80 or BSA (fig.2) but enhancement by dilauroyl GPC was observed when the substrate was added in methanol (fig.2).

Cytochrome *P*-450 fractions derived from 45% and 50% ammonium sulfate precipitations of the same solubilized microsomal preparation contained higher 12α-hydroxylase activity per nanomole of cytochrome *P*-450 in the former fraction (13.9 and 13.3 nmol product/min) than in the latter (1.6 and 2.8 nmol/min, respectively) when assayed in two separate experiments in the presence of BSA. Upon storage at −70°C, the amount of solubilized cytochrome *P*-450 remained at 2.2 nmol/ml after 6.5 months whereas the accompanying 12α-hydroxylase activity, assayed in the presence of Tween-80, decreased from 0.15–0.06 nmol product/mg protein/min over the same period. The hydroxylase was also strongly inhibited by the detergent Emulgen 913 with loss of >80% of the activity in the presence of 0.5% of the detergent.

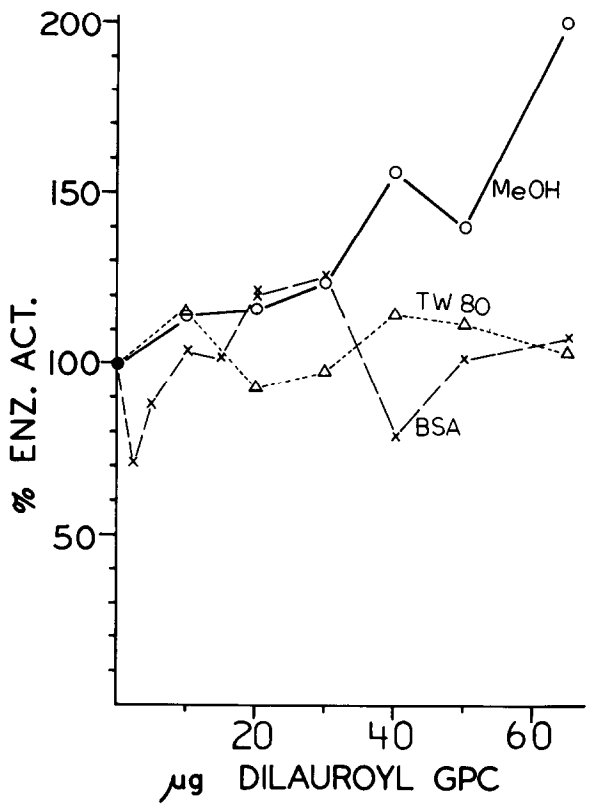

Fig.2. Effects of added dilauroyl GPC on 12α-hydroxylation of [5α,6α-$^3H_2$]cholestane-3α,7α-diol solubilized with methanol (MeOH), BSA or Tween-80 (TW 80); the solubilized enzyme was prepared as in [5].

4. Discussion

Similarities between preparations of microsomal 12α-steroid hydroxylase and cytochrome *P*-450 from rabbit microsomes in their requirement of oxygen and NADPH in hydroxylating steroids [1,3–6,14] would suggest that cytochrome *P*-450 catalyzes the 12α-hydroxylation of 5α-cholestane-3α,7α-diol. However, these preparations of 12α-hydroxylase are only slightly affected by either CO or metyrapone, both potent competitive inhibitors of hepatic microsomal drug hydroxylation mediated by cytochrome *P*-450 [18,19]. The human placental microsomal aromatase [18,19] behaves likewise. Other dischordant behavior of 12α-hydroxylase and cytochrome *P*-450 is found in the solubilized and partially purified preparations [5] in which the former is more active in the 45% than the 50% ammonium sulfate fraction, and it is not as stable to storage under N_2 at −70°C. Finally, the various fractions including cytochrome *P*-450 obtained from chromatography of solubilized microsomes through ω-aminooctyl–Sepharose [2,15] contained no detectable 12α-hydroxylase activity and inhibits microsomal 12α-hydroxylation.

In view of these results, it is reasonable to assume that none of the major species of rabbit hepatic microsomal cytochrome *P*-450 is likely to be the

12α-hydroxylase. This conclusion is in agreement with that drawn from studies made with the rat by more diverse approaches [4,7–11], but it does not exclude the possibility that the major species of cytochrome *P*-450 are indirectly involved, as, for instance, in electron transport, or that one of the minor species of cytochrome *P*-450 which have not yet been subjected to closer investigation, may be identical to the 12α-hydroxylase [20].

This study indicates that the procedures for the isolation of 12α-hydroxylase should differ from those devised for the purification of the major species of cytochrome *P*-450. It has also clarified some of the parameters for the assay of the crude solubilized hydroxylase to cytochrome *P*-450 could be answered unequivocally with a purified enzyme, an objective which is actively pursued in this laboratory.

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