ELECTRON TRANSFER BETWEEN LIVER MICROSOMAL CYTOCHROME b_5 AND CYTOCHROME P-450 IN THE AZO REDUCTASE REACTION

S. Fujita and J. Peisach

Departments of Molecular Pharmacology and Molecular Biology,
Albert Einstein College of Medicine of Yeshiva University,
Bronx, N. Y. 10461

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SUMMARY: NADPH reduces both liver microsomal cytochrome P-450 and cytochrome b_5. In the presence of CO, ferrous cytochrome P-450 can slowly transfer electrons to amaranth, an azo dye. This reaction is followed by the re-oxidation of cytochrome b_5 which proceeds at essentially the same rate as does cytochrome P-450 oxidation. It is suggested that cytochrome b_5 directly reduces cytochrome P-450 in rat liver microsomes.

INTRODUCTION

There has been considerable speculation regarding the involvement of cytochrome b_5 in the reduction of cytochrome P-450 (1-3). Estabrook and associates (4) showed that the rate of NADH oxidation by liver microsomes is enhanced by the presence of NADPH and a drug substrate, and that the rate of oxidation of NADH is related to the rate of oxidation of the substrate. They also demonstrated spectrophotometrically that the steady state level of reduced cytochrome b_5 in the presence of NADH and NADPH is decreased by the addition of the drug substrate, implicating cytochrome b_5 in the monoxygenase reactions.

A recent study by Sasame *et al.* (5) showed that an antibody against cytochrome b_5 inhibits the monoxygenase reactions when both NADPH and NADH are present, or when NADPH is present alone. This too suggests the involvement of cytochrome b_5 in the reduction of cytochrome P-450.

Other lines of evidence (6) suggest that uncoupled, active oxygen not used for product formation is reduced to water by the NADH-cytochrome b_5 system rather than by the NADPH-cytochrome c reductase system. This electron sparing effect, then, accounts for the synergistic action of NADH on the monoxygenase reactions.

Our previous reports on azo reductase (7,8) indicate that cytochrome P-450

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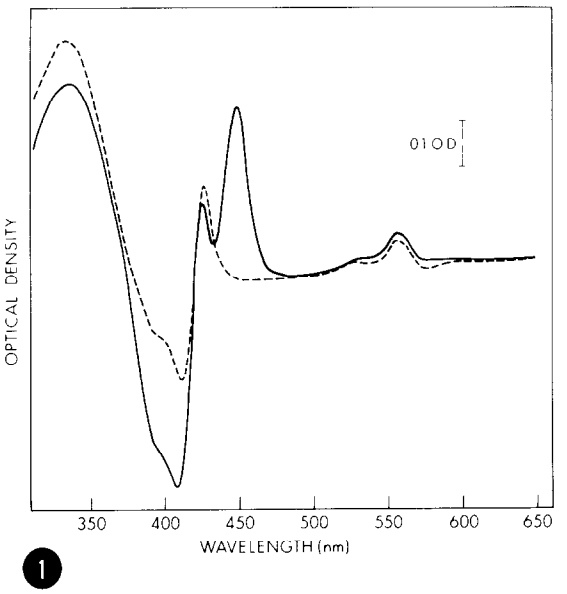
is the sole microsomal reductase engaged in the reduction of both amaranth and neoprontosil. Since these azo compounds receive electrons from cytochrome P-450 and are reduced under anaerobic conditions, the study of these reductase reactions is a powerful tool for the study of microsomal electron transport pathways. We have been able to demonstrate spectrophotometrically, that the gradual oxidation of cytochrome P-450 by an azo dye in the presence of CO is followed by the gradual oxidation of reduced cytochrome b_5 at essentially the same rate.

MATERIALS AND METHODS

Chemicals - Amaranth was purchased from Fisher Chemical Company. Sodium phenobarbital was purchased from Merck and Company, Inc., and 3-methylcholanthrene from Schwarz-Mann Laboratories, Inc. NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Company. Ultra high purity argon and nitrogen and research grade O_2-free carbon monoxide were purchased from T. W. Smith Company.

Preparation of Microsomes - Long Evans male rats weighing 45-50 g were maintained on Purina rat chow for a week. They were then divided into four groups of 5 each. Two groups were used as controls and received i.p. injections of either saline or corn oil for 3 days. The other two groups received i.p. injections of 3-methylcholanthrene, 20 mg/kg/day or phenobarbital 80 mg/kg/day for 3 days. 14-16 hours after the last injections of the drugs, rats were killed by decapitation and the liver was quickly removed. Liver microsomes were prepared by the method of Omura and Sato (9,10). The microsomal fraction was resuspended and centrifuged twice at 100,000 x g for 90 minutes. The microsomal pellet and the fluffy layer were suspended in 0.2 M phosphate buffer and stored in small vials in liquid nitrogen. Microsomal protein concentrations were determined by the method of Lowry (11). Cytochrome P-450 content was determined by the method of Omura and Sato (9,10) using a Cary 14R spectrophotometer.

Spectrophotometric Recording of Simultaneous Decrease of Reduced Cytochrome b_5 and Reduced, CO-Bound Cytochrome P-450 - The mixture in the reference cell (total volume 3 ml) consisted of microsomes prepared from approximately 300 mg liver, containing a final concentration of 5-7 μM cytochrome P-450 in 0.2 M phosphate buffer, pH 7.4. No precaution was taken to remove oxygen from the cell. Amaranth (100 μM final concentration) was added and under these conditions, no reduction of the dye is observed. The sample cell, consisting of a Thunberg cuvette fitted with a rubber septum stopper contained the same concentration of microsomes in 0.2 M phosphate buffer, but under an argon atmosphere. NADPH (final concentration 100 μM) was added anaerobically to the sample and the difference spectrum was then recorded. The Thunberg cell was subsequently gassed with CO (1 atm) and shaken for 5 minutes. It was then placed in a thermoregulatable Cary cell holder adjusted to 37°C for 10 minutes. The spectrum of reduced and CO treated minus oxidized microsomes was recorded. Amaranth, (final concentration 100 μM) was then added to both cuvettes, but anaerobically to the sample cell. The concentration of amaranth added to the reference cell was 10 μM less than that added to the sample cell. Difference spectra were recorded at various time intervals after amaranth addition. The mM extinction coefficients used for the determination of cytochromes P-450 and b_5 concentrations are listed in Table I. The spectral contributions of cytochrome P-450 in the region where cytochrome b_5 absorbs



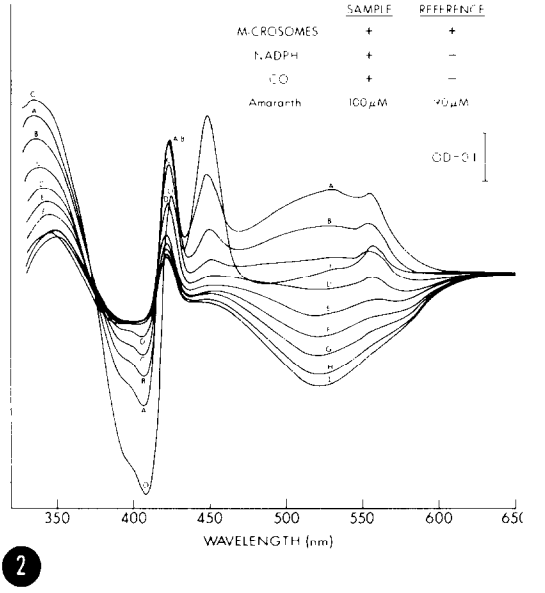


Fig. 1 Optical difference spectrum between NADPH-reduced microsomes versus oxidized microsomes in the presence (_____) and in the absence (--------) of CO. The peak at 340 nm is ascribed to NADPH.

Fig. 2 Optical difference spectrum between CO-treated reduced microsomes versus oxidized microsomes in the presence of amaranth. At zero-time (trace O) 100 μM NADPH and 1 atm CO are added to microsomes in the sample cuvette. Amaranth is added to the sample and reference cuvettes and the optical spectrum is recorded every 7-8 minutes (Spectra A to I).

(between 425 nm and 408 nm) was determined by using mM extinction coefficients obtained for purified rat liver cytochrome P-450 from phenobarbital treated rats (12) generously supplied by Dr. W. Levin, Hoffmann-La Roche. Cytochrome P-420 was prepared from the soluble cytochrome P-450 using deoxycholate. For optical studies of the reduced cytochrome, dithionite was added before CO to simulate experimental conditions.

RESULTS

The addition of NADPH to an aerobic suspension of rat liver microsomes results in a spectral change ascribable to the partial reduction of cytochrome b_5. Under anaerobic conditions, this reaction can be driven to completion as subsequent dithionite addition causes no further change. The optical spectrum shows peaks at 556 nm, 528 nm, and 425 nm and a trough at 408 nm (Fig. 1). When CO is introduced into this system, a new spectral species ascribable to the CO adduct of ferrous cytochrome P-450 appears and overlaps with the spectrum of reduced

TABLE I

Millimolar Extinction Coefficients Used to Determine Cytochromes P-450 and Cytochrome $\underline{b}_5$ Concentrations

Amaranth	$\varepsilon_{520} = 22$ $\varepsilon_{480} = 13.5$ $\varepsilon_{450} = 6.5$
Difference spectrum between CO-bound soluble cytochrome P-450 minus oxidized soluble cytochrome P-450	$\varepsilon_{450-490} = 91$ $\varepsilon_{408-425} = -42.6$
Difference spectrum between CO-bound soluble cytochrome P-420 minus oxidized soluble cytochrome P-420	$\varepsilon_{408-425} = -79$
Difference spectrum between reduced cytochrome $\underline{b}_5$ minus oxidized cytochrome $\underline{b}_5$	$\varepsilon_{408-425} = -185$

cytochrome $\underline{b}_5$. This new species consists of an additional peak at 450 nm and the deepened trough at 408 nm (Fig. 1). Anaerobic addition of amaranth to the microsomal suspension results in a gradual decrease of peaks at 520 nm, 450 nm and 340 nm, as well as a decrease in the difference between 408 nm and 425 nm (Fig. 2). The decrease in the 520 nm peak indicates the gradual reduction of azo dye with the expense of 2.0 ± 0.1 moles of NADPH for each mole of the reduced dye. This stoichiometry is based on the decrease of the 340 nm peak ascribable to NADPH and the extinction coefficient of the dye (Table I). The gradual decrease of the 450 nm peak is due to the oxidation of ferrous cytochrome P-450 caused by transferring of electrons to the dye.

In Fig. 3, we show the gradual decrease of reduced cytochrome P-450 and reduced cytochrome $\underline{b}_5$ plotted against time. The decrease of reduced cytochrome $\underline{b}_5$ is parallel to and is proportional to that of cytochrome P-450. This provides strong evidence for involvement of cytochrome $\underline{b}_5$ in the reduction of cytochrome P-450.

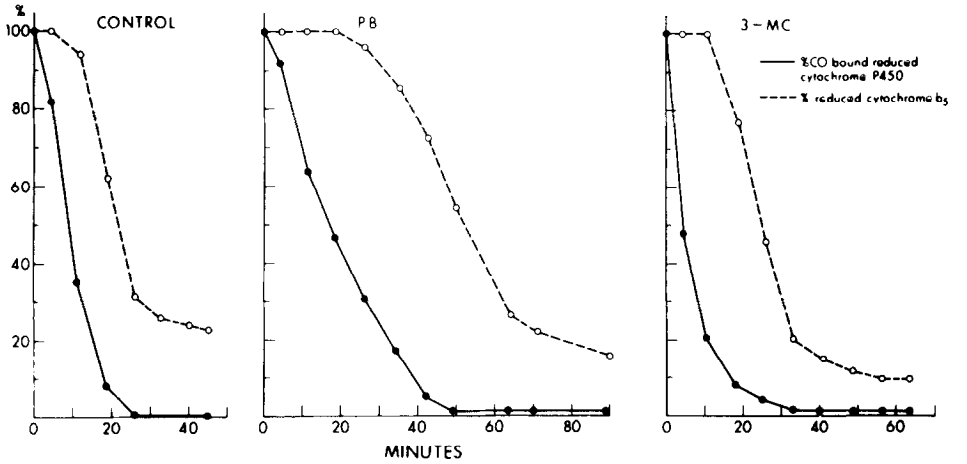


Fig. 3 Relative concentrations of microsomal CO-bound cytochrome P-450 and reduced cytochrome b_5 after the addition of 100 μM amaranth. Spectra were recorded as in Fig. 2. The concentration of CO-cytochrome P-450 is determined from the spectral difference $A_{450-490}$. The concentration of reduced cytochrome b_5 is determined from $A_{408}-A_{425}$, correcting for the CO-cytochrome P-450 contribution at 408 nm.

When either dithionite or NADH is used as a reducing agent in the same system, the 450 nm peak decreases but the 425 nm peak and 408 nm trough ascribable to cytochrome b_5 do not. This indicates that both dithionite and NADH reduce cytochrome b_5 reductase which can in turn reduce cytochrome b_5 far more efficiently than does cytochrome c reductase. Therefore, the reduction of cytochrome b_5 can catch up with its oxidation and the steady state level of reduced cytochrome b_5 is maintained.

DISCUSSION

There have been many reports on the so-called "NADH synergism" in cytochrome P-450-mediated monoxygenation reactions (2,13,14). Since there is an electron transport system leading electrons from NADH to cytochrome b_5 in microsomes, the involvement of cytochrome b_5 in the reduction of cytochrome P-450 has been suggested (2,3). The most convincing, though indirect, evidence for such involvement, was obtained by Sasame et al. (5) by use of an antibody to cytochrome b_5. This antibody inhibits a number of monoxygenase reactions catalyzed by cytochrome P-450, thereby implicating cytochrome b_5 in the re-

duction of cytochrome P-450. One interesting observation which escaped these authors' attention is the fact that the faster the rate of oxygenation, the greater the inhibition. If the monoxygenase rate is less than 20 nmoles/mg/min, the inhibition by the antibody becomes very small. This implies that when the reaction rate is so fast that the rate limiting step becomes the reduction of cytochrome P-450, the electron transport from cytochrome $\underline{b}_5$ to cytochrome P-450 becomes more important.

Our observation clearly indicates that cytochrome $\underline{b}_5$ can transfer electrons to ferric cytochrome P-450 in microsomes. In the presence of a reducing agent, CO becomes bound to cytochrome P-450 eliciting the 450 nm characteristic chromophore. In the presence of dye, reduced cytochrome P-450, which is in equilibrium with the CO-bound form, can transfer an electron to the dye and becomes ferric. When excess reducing equivalents are present, the ferric cytochrome P-450 formed by transferring electrons to the dye will be reduced back to the ferrous form and once more bind CO. However, when the reduction is slower than the oxidation, there is an accumulation of ferric cytochrome P-450 which is unable to bind CO. This causes the decrease of the 450 nm peak, and the decrease of the negative contribution of the difference spectrum at 408 nm. However, the decrease in depth of the trough at 408 nm is not only due to the decrease of negative contribution of the cytochrome P-450-CO difference spectrum, but also to oxidation of reduced cytochrome $\underline{b}_5$ (Table I). With time the peaks at 425, 528 and 556 nm ascribable to cytochrome $\underline{b}_5$ decrease as well. The difference between 425 and 408 nm becomes small and the 425 nm peak shifts towards 420 nm, indicating almost complete oxidation of cytochrome $\underline{b}_5$ [1]. With excess dye, the reaction can be driven towards the complete oxidation of both cytochromes P-450 and cytochrome $\underline{b}_5$. The oxidation of cytochrome P-450, however, always precedes that of cytochrome $\underline{b}_5$ (Fig. 3).

Our results do not suggest that cytochrome $\underline{b}_5$ is obligatory in the reduction

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[1] The remaining spectral peak at 420 nm is ascribed to cytochrome P-420.

of cytochrome P-450. Since we can observe a decrease in reduced cytochrome b_5 even when cytochrome P-450 turnover is largely reduced by the presence of CO, the contribution of cytochrome b_5 to the microsomal electron transport chain is a significant one. For maximal enzymatic activity, electron supply from both NADPH cytochrome c reductase and from NADH via cytochrome b_5 may be necessary. This clearly explains the synergistic effect of NADH and NADPH (13,14) in reactions involving cytochrome P-450.

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