Vol. 97, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1980 Pages 582-589

CYTOCHROME b_5 AS ELECTRON DONOR TO RABBIT LIVER CYTOCHROME P-450$_{LM_2}$ IN RECONSTITUTED PHOSPHOLIPID VESICLES

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Received October 14,1980

SUMMARY

When incorporated into phospholipid vesicles containing NADPH-cytochrome P-450 reductase and P-450$_{LM2}$, cytochrome b_5 enhanced the rate of NADPH-supported hydroxylation of 7-ethoxycoumarin or p-nitroanisole about 5-fold. Cytochrome b_5 did not affect the rate of NADPH-oxidation, nor the rate of NADPH-supported formation of the ferrous CO-complex of cytochrome P-450. However, the cytochrome b_5-mediated increase in product formation was found to be correlated with concomitant decreases in the production of H_2O_2 or O_2^- in the system, thus strongly indicating cytochrome b_5 being a more efficient donor of the second electron to cytochrome P-450 than is NADPH-cytochrome P-450 reductase.

INTRODUCTION

The role of cytochrome b_5 in the lower microsomal hydroxylase system has been under much debate. Based on the observation that a reoxidation of reduced cytochrome b_5 in microsomes occurred in the presence of excess NADH when hydroxylation reactions were initiated by the addition of NADPH, Hildebrandt and Estabrook (1) initially suggested that cytochrome b_5 supplies the second electron to cytochrome P-450. Subsequently, completely divergent explanations of the NADH synergism have been raised by others (2,3). Attempts to establish the role of cytochrome b_5 in the cytochrome P-450 system have been carried out by kinetic studies in intact microsomes (1,2,4), by immunochemical studies (5-7) and by experiments in reconstituted systems (8-11), but not resulted in a clear picture about by which mechanism cytochrome b_5 participates in the system.

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[1] Abbreviations used: Cytochrome P-450$_{LM}$, liver microsomal cytochrome P-450; P-450$_{LM2}$, P-450$_{LM3}$, P-450$_{LM4}$, forms of P-450$_{LM}$ designated according to their electrophoretic properties.

0006-291X/80/220582-08$01.00/0

In the present paper we report that, when introduced into the vesicles, cytochrome b_5 efficiently enhances the rate of donation of the second electron to cytochrome P-450, thereby enhancing the catalytic rate of NADPH-supported reactions by a factor of about 5.

MATERIALS AND METHODS

Egg yolk phosphatidylcholine and egg yolk phosphatidylethanolamine, Type III E, were obtained from Sigma and stored in sealed tubes under nitrogen at -20°C. Microsomal phospholipids were prepared according to Bligh and Dyer (12) and were stored as described above. Electrophoretically homogeneous preparations of cytochromes P-450$_{LM2}$, P-450$_{LM3}$, P-450$_{LM4}$ and NADPH-cytochrome P-450 reductase were prepared from rabbit liver microsomes as previously described (13,14). The specific contents were: P-450$_{LM2}$, 16.5 nmol/mg; P-450$_{LM3}$, 12.4 nmol/mg; P-450$_{LM4}$, 11.4 nmol/mg; NADPH-cytochrome P-450 reductase, 12.5 nmol flavin/mg as measured using the absorption coefficient 10.7 $mM^{-1}cm^{-1}$ at 456 nm (15). Electrophoretically homogeneous preparations of cytochrome b_5 were obtained by collecting the narrow red band obtained during DEAE-Sepharose chromatography of the NADPH-cytochrome P-450 reductase fraction (13,14). The preparations were depleted from non-ionic detergent according to Strittmatter (16) and subsequently chromatographed on a Sephadex G-50 column in 50 mM phosphate buffer, pH 7.4. The specific contents were 28.2-34.5 nmol/mg. Succinylated cytochrome c was prepared essentially according to Takemori et al. (17). Unilamellar phospholipid vesicles containing various amounts of cytochrome P-450, NADPH-cytochrome P-450 reductase and cytochrome b_5 were prepared by the cholate gel filtration technique previously described (14,18,19). In experiments using microsomal phospholipids, vesicles were usually prepared devoid of cytochrome b_5. The subsequent addition of cytochrome b_5 to the vesicles at 37°C resulted in a rapid and complete incorporation of the protein into the vesicles as was evident from results obtained by Sepharose 4 B chromatography of the preparations. Cytochrome P-450 was measured according to Omura and Sato (20) using 91 $mM^{-1}cm^{-1}$ as absorption coefficient. Protein was determined according to Lowry (21) using bovine serum albumin as standard. Cytochrome b_5 was quantitated at 424 nm as the absorbance difference between the reduced minus the oxidized form of the protein using 100 $mM^{-1}cm^{-1}$ as absorption coefficient (22). NADPH-oxidation was measured by the decrease of absorption at 340 nm using an absorption coefficient of 6.2 $mM^{-1}cm^{-1}$ and corrected for contribution to the absorption at this wavelength by product or substrate. O-Dealkylation of 7-ethoxycoumarin and p-nitroanisole was quantitated as described elsewhere (23,24).

RESULTS

Effect of cytochrome b_5 on NADPH-supported, cytochrome P-450$_{LM_2}$-catalyzed O-dealkylation of 7-ethoxycoumarin and p-nitroanisole.

When a to P-450 equimolar amount of cytochrome b_5 was incorporated into vesicles prepared from microsomal phospholipids containing phospholipid, NADPH-cytochrome P-450 reductase and cytochrome P-450$_{LM_2}$ in a molar ratio of 800:1:1 the rate of O-dealkylation of 7-ethoxycoumarin (50 mM) and p-nitroanisole (1 mM) increased 5-fold to 3.5 and 5.1 nmol of product formed per nmol P-450 and min, respectively, whereas the rate of NADPH-oxidation remained constant in both cases

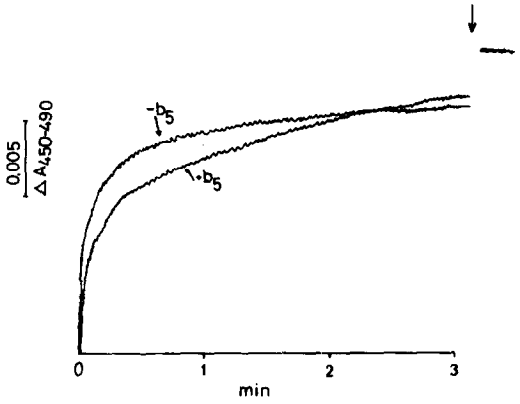

Fig. 1. Time courses of NADPH-dependent reduction of cytochrome P-450$_{LM2}$ at 5°c in reconstituted vesicles prepared from microsomal phospholipids. A solution of vesicles in 2 ml potassium phosphate buffer, pH 7.4, corresponding to 0.2 nmol each of cytochrome P-450, NADPH-cytochrome P-450 reductase and if indicated also of cytochrome b_5 in a 3 ml cuvette was flushed with first nitrogen and then carbon monoxide cleaned by passage through a BASF-catalyst at 100°C. One ml of an NADPH-solution (0.2 mM in phosphate buffer, pH 7.4), treated in the same way, was injected into the cuvette and the absorbance at 450 nm registrated. Arrow indicates addition of a few grains of sodium dithionite.

(7.8 and 20.3 nmol of NADPH oxidized per nmol, min, respectively), indicating no enhanced rate of electron transfer from NADPH to cytochrome P-450 in vesicles containing cytochrome b_5. Similar results have also been obtained when using benzo(a)pyrene as substrate (25) and also by Imai and Sato (8)

Reduction of ferric cytochrome P-450$_{LM_2}$ in the vesicles

In order to evaluate whether the higher catalytic rates in cytochrome b_5-containing vesicles were consequences of a facilitated electron transport from NADPH to cytochrome P-450 in these vesicles, the influence of cytochrome b_5 on the formation of ferrous carbonyl cytochrome P-450$_{LM_2}$ in vesicles prepared from microsomal phospholipids was evaluated. As is evident from Fig. 1, the presence of cytochrome b_5 in the vesicles did not influence the reduction rate, when measured in the presence of CO at 5°C. This indicates NADPH-cytochrome P-450 reductase being the most efficient donor of the first electron to cytochrome P-450$_{LM_2}$ under these conditions.

Formation of superoxide anions and hydrogen peroxide in the vesicles

In the presence of molecular oxygen, cytochrome P-450, upon reduction, is readily oxidized, thereby producing superoxide anions which in the subsequent

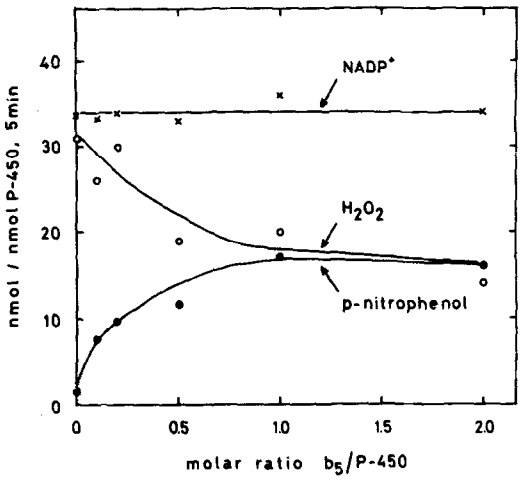

Fig. 2. Cytochrome P-450_{LM2}-dependent formation of p-nitrophenol, hydrogen peroxide and $NADP^+$ by vesicles prepared from microsomal phospholipids. Vesicles containing phospholipid, cytochrome P-450 and NADPH-cytochrome P-450 reductase in a molar ratio 800:1:1 were titrated with cytochrome b_5. NADPH-oxidation and p-nitrophenol formation were measured simultaneously at 360 nm and 417 nm, respectively. The absorbance difference at 360 nm due to NADPH-oxidation was calculated after compensation for the contribution by decreasing amounts of substrate and increasing amounts of product, respectively. After 5 min the contents of the sample and reference cuvettes (the latter not containing NADPH) were distributed into two tubes, diluted with 10% (w/v) trichloroacetic acid and the H_2O_2 formed was subsequently quantitated using thiocyanate and ferrous ammonium sulfate (29).

dismutase reaction give hydrogen peroxide (26-28). Since the presence of cytochrome b_5 in the vesicles did affect the catalytic rate of the cytochrome P-450_{LM_2}-catalyzed reactions, but not the rate of NADPH consumption, a more efficient utilization of the electrons in the cytochrome b_5-containing vesicles seemed plausible and it was therefore considered of interest to investigate whether this was reflected in a lower production of the autooxidation products by these vesicles. Liposomes containing cytochrome P-450_{LM_2} and NADPH-cytochrome P-450 reductase in a molar ratio of 1:1 were prepared and titrated with cytochrome b_5. The production of superoxide anions and hydrogen peroxide was measured and compared to the number of substrate molecules oxidized. As is evident from Figs. 2 and 3, increasing amounts of cytochrome b_5 in the vesicles resulted in an impaired autooxidation of cytochrome P-450 and a concomitant enhancement in product formation when the cytochrome P-450_{LM_2}-catalyzed O-demethylation of para-nitroanisole was assayed. During titration the rate of NADPH-

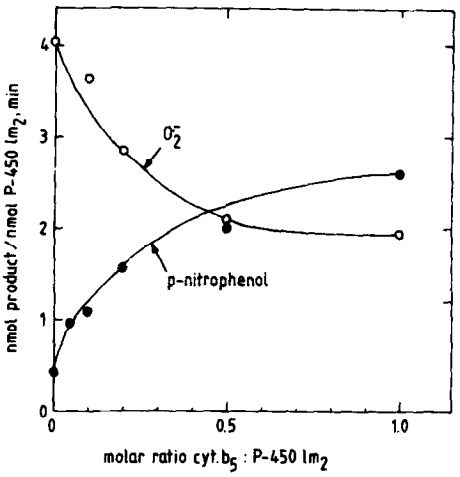

Fig. 3. Cytochrome P-450$_{LM2}$-dependent formation of p-nitrophenol and O_2^- by vesicles prepared from microsomal phospholipids. Vesicles, containing phospholipid, NADPH-cytochorme P-450 reductase and cytochrome P-450 in a molar ratio of 800:1:1 were titrated with cytochrome b_5 and O-demethylation of p-nitrosanisole was measured as described in legend to Fig. 1. In parallel experiments the superoxide anions produced during the incubations were quantitated using succinylated cytochrome c (17). The amount of O_2^- indicated in the figure was obtained by subtracting the O_2^- produced in similar vesicles devoid of cytochrome P-450, i.e. in vesicles containing NADPH-cytochrome P-450 reductase and different amounts of cytochrome b_5, from the amount of superoxide anions formed in the complete system.

oxidation remained constant, whereas H_2O_2-production decreased from 30 to 18 nmol per nmol P-450 and 5 min (Fig. 2). The rate of O-demethylation was enhanced concomitantly in a stoichiometric manner.

Similar results were obtained when the amount of superoxide anions formed were quantitated using succinylated cytochrome c (Fig. 3). A stoichiometric relationship was found between the number of para-nitrophenol molecules and the number of superoxide anions formed; the sum always being equal to about 4.5 nmol/nmol LM_2,min.

In both cases, titration with cytochrome b_5 was performed in vesicles only containing NADPH-cytochrome P-450 reductase and the amount of superoxide anions or hydrogen peroxide produced was subtracted from the values obtained in the complete system. Without cytochrome b_5 in the vesicles 1.5 nmol of O_2^- was formed per nmol of NADPH-cytochrome P-450 reductase; this value increased up to 2.4 when cytochrome b_5 was present at a two-fold molar excess.

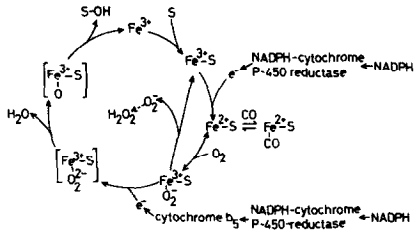

Fig. 4. A schematic representation of cyclic function of cytochrome P-450 illustrating the introduction of the two electrons and the formation of superoxide anions by autooxidation of oxycytochrome P-450.

Effects of cytochrome b_5 in different types of reconstituted systems

When 0.1 nmol of NADPH-cytochrome P-450 reductase and 0.1 nmol of cytochrome P-450_{LM_2} were reconstituted with 50 µg dilauroylphosphatidylcholine, no significant effect was seen after the introduction of cytochrome b_5 into the system when the P-450_{LM_2}-catalyzed O-demethylation of para-nitroanisole or O-deethylation of 7-ethoxycoumarin were examined.

When the proteins were reconstituted into egg yolk phosphatidylcholine vesicles, the presence of cytochrome b_5 resulted in a 4-fold increase in the rate of O-deethylation of 7-ethoxycoumarin; a turnover of 0.42 nmol of product per nmol LM_2, min was obtained in these vesicles. In mixed egg yolk phosphatidylcholine:egg yolk phosphatidylethanolamine (2:1, w/w) vesicles, cytochrome b_5 enhanced the rate of the P-450_{LM_2}-catalyzed O-demethylation of para-nitroanisole 5-fold to 2.7 nmol/nmol, min.

Cytochrome b_5 did not affect the apparent K_m-value of 7-ethoxycoumarin for cytochrome P-450_{LM_2} (80 µM), nor the activation energy of the O-demethylation of para-nitroanisole (78 KJ/mol) in reconstituted vesicles prepared from microsomal phospholipids.

DISCUSSION

During the cyclic function of cytochrome P-450, superoxide anions are produced as a result of autooxidation of oxycytochrome P-450 (Fe^{3+}-O_2^-), i.e. before the introduction of the second electron, and subsequently dismutate to form hydrogen peroxide (27,28, cf. Fig. 4). Incorporation of cytochrome b_5 in-

to phospholipid vesicles containing NADPH-cytochrome P-450 reductase and cytochrome $P\text{-}450_{LM_2}$, resulted in a lower production of superoxide anions and hydrogen peroxide and in a stoichiometrically corresponding increase in product formation. By contrast, the number of NADPH molecules oxidized remained constant. In addition, cytochrome b_5 was shown not to affect the rate of formation of the ferrous carbonyl complex of P-450 upon addition of NADPH to the vesicles. The results thus strongly indicate that cytochrome b_5 enhances the rate of product formation by donating the second electron to cytochrome P-450 more efficiently than NADPH-cytochrome P-450 reductase, thereby preventing the autooxidation of the $Fe^{3+}\text{-}O_2^-$-complex and thus the formation of superoxide anions. The introduction at this stage of one electron, will give one hydroxylated products instead of one superoxide anion (cf. Fig. 4). The molar ratio of cytochrome b_5 to cytochrome P-450 in liver microsomes is 0.55-0.9 (30), depending on the animal species. It can therefore be concluded that cytochrome b_5 probably plays an important role in the liver microsomal hydroxylase system in vivo. The stereospecificities of the hydroxylation reactions will be determined by the type of cytochrome P-450 present in the microsomes (cf. 31,32), whereas the amount of cytochrome b_5, on the other hand, may regulate the rate of the cytochrome P-450-catalyzed reactions.

ACKNOWLEDGEMENTS

We are indebted to Ms. Ann-Louise Edvardsson for purification of the enzymes and other valuable aid. This work was supported by the Swedish Cancer Society.

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