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CYTOCHROME P-450-DEPENDENT OXIDASE ACTIVITY AND HYDROXYL RADICAL PRODUCTION IN MICELLAR AND MEMBRANOUS TYPES OF RECONSTITUTED SYSTEMS*

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Abstract—In view of conflicting results in the literature regarding the contribution of cytochrome P-450 to hydrogen peroxide production and formation of hydroxyl radicals in the microsomal electron transport chain, experiments were undertaken to evaluate this problem using reconstituted micellar and membranous systems containing NADPH–cytochrome P-450 reductase and cytochrome P-450 LM_2 purified from rabbit liver. It was found that P-450 LM_2 increased the rate of NADPH consumption in the vesicular system, reconstituted with microsomal phospholipids, much more than in the micellar system, based on dilauroylphosphatidylcholine (DLPC) under otherwise similar conditions. At small amounts of Fe(III)–EDTA (1–5 μM), the enhanced oxidase activity was manifested in a much higher dependency on P-450 LM_2 for the production of hydroxyl radicals, as determined by the oxidation of dimethylsulphoxide (Me_2SO) or 2-keto-4-thiomethylbutyric acid (KMBA), in the vesicular than in the micellar system. In the presence of high amounts of Fe(III)–EDTA (10–50 μM), the relative increase due to P-450 LM_2 was less pronounced in both types of reconstituted systems, although the increase in absolute terms was about the same as at small Fe(III)–EDTA concentrations. The data indicate that in the presence of no or small amounts of chelated iron in negatively-charged membranous systems, most of the hydrogen peroxide and superoxide anions necessary for generation of hydroxyl radicals, are produced by cytochrome P-450 LM_2. This appears to be due to a higher affinity between the reductase and P-450 LM_2 in this system. In reconstituted micellar systems or in the presence of high amounts of chelated iron, "uncoupling" at the level of the reductase appears to take place, with a resulting production of hydroxyl radicals and other forms of reactive oxygen species.

In addition to serving as a monooxygenase, the cytochrome P-450 system also exerts an oxidase activity, first observed by Gillette *et al.* [1]:

$$NADPH + H^+ + O_2 \rightarrow NADP^+ + H_2O_2 \quad (I)$$

In the presence of chelated iron the hydrogen peroxide produced might be cleaved in a Fenton reaction yielding hydroxyl radicals:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad (II)$$

Thus, under certain conditions, the oxidase activity of the system might be responsible for generation of reactive oxygen species such as superoxide anions and hydroxyl radicals.

The hydrogen peroxide formation by the microsomal system could in principle occur by a two-electron reduction of dioxygen, either at the level of NADPH–cytochrome P-450 reductase or by the cytochrome P-450 component. In favour of the latter possibility are the findings that compounds that induce the level of cytochrome P-450 in the microsomal membranes like pregnenolone 16α-carbonitrile [2], and phenobarbital [3] also cause an enhanced production of hydrogen peroxide, and that inhibitors of cytochrome P-450 like carbon monoxide [4], H_2-receptor blockers [3], metyrapone [5] and tofenacine [6] also inhibit the microsomal hydrogen peroxide production. Furthermore, treatment of rats with ethanol increases microsomal NADPH consumption, indicative of the oxidase activity of the system, to a similar extent as the amount of P-450, without significantly affecting the level of NADPH–cytochrome P-450 reductase activity [7].

It appears that the microsomal hydrogen peroxide is mainly generated through autooxidation of the oxycytochrome P-450 complex yielding superoxide anions, followed by the dismutation of O_2^- to H_2O_2 [8, 9]. The capability of type II ligands interacting with the heme moiety of cytochrome P-450, like cimetidine and metyrapone, to inhibit microsomal hydrogen peroxide production is probably connected with their ability to prevent oxygen binding and reduction. In fact, a correlation between the ligand-induced low spin formation of cytochrome P-450 and inhibition of hydrogen peroxide production has been described [3]. By the use of the P-450 inhibitor allylisopropylacetamide, evidence has also been given for the dependence on P-450 for production of hydrogen peroxide *in vivo* [10].

In purified systems, the mechanisms for hydrogen peroxide production by the components of the electron transfer system might be different from those occurring in the native microsomal membrane. Thus, with an excess of NADPH–cytochrome P-450 reductase over P-450 in a micellar reconstituted

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system, it appears that the reductase is mainly responsible for the superoxide anions and hydrogen peroxide produced [11]. However, in a membranous reconstituted system evidence was given of cytochrome P-450 as the major component responsible for the superoxide anion and hydrogen peroxide produced [12]. This fact might be of some importance, e.g. in the microsomal oxidation of ethanol, due to the capability of cytochrome P-450 to bind hydrophobic substrates, in combination with the potential generation of reactive hydroxyl radicals from O_2^- and H_2O_2 in a Haber–Weiss reaction, possibly near the active site of P-450 (cf. 12, 13).

In a recent paper in this journal, Winston and Cederbaum [14] failed to demonstrate any role for phenobarbital-inducible cytochrome P-450 in hydroxyl radical generation from hydrogen peroxide, determined as ethylene formation from 2-keto-4-thiomethylbutyric acid (KMBA)*, in a micellar reconstituted system containing NADPH–cytochrome P-450 reductase. Since this result was in contrast to previously published data from our laboratory, indicating a pronounced dependency upon cytochrome P-450 on hydrogen peroxide and hydroxyl radical production in a membranous reconstituted system [12], we undertook a study to evaluate the origin of this discrepancy. From the results presented it appears that the coupling efficiency of electrons between NADPH–cytochrome P-450 reductase and cytochrome P-450 is higher when the proteins are reconstituted together in a bilayer membrane, than when present in a phospholipid micelle. As a consequence, cytochrome P-450 contributes much more to hydroxyl radical generation, as measured by the hydroxyl radical scavengers dimethylsulphoxide (Me_2SO) and KMBA, in the membranous rather than in the micellar reconstituted system.

EXPERIMENTAL PROCEDURES

Materials

Ethylene gas was purchased from AGA Specialgas AB (Lidingö, Sweden). Dimethylsulphoxide (Me_2SO)[1], sodium cholate and $FeCl_3$ were obtained from Merck (Darmstadt, Germany). Formaldehyde (37%) was from Mallinckrodt (St. Louis, MO), and EDTA from Fluka AG. (Buchs, Switzerland). NADPH, 2-keto-4-thiomethylbutyric acid, sodium salt (KMBA) and synthetic L-α-dilauroylphosphatidylcholine, (DLPC, 98%) were purchased from Sigma Chemical Co. (St. Louis, MO). Venoject® (Terumo) rubber stoppers were generously provided by Mediplast AB, (Stockholm, Sweden).

Methods

Male rabbits (2.5 kg) were obtained from a local farm and treated with phenobarbital (80 mg/kg) intraperitoneally for two days. Liver microsomes were prepared as previously described [15]. Cytochrome P-450 LM_2 and NADPH–cytochrome P-450 reductase were purified from liver microsomes of phenobarbital-treated rabbits as described elsewhere [15]. Rat liver NADPH–cytochrome P-450 reductase was prepared from microsomes of acetone-treated rats [16] in an identical manner. The specific contents were for P-450 LM_2 10–12 nmol/mg protein and for the reductase 24–30 nmol of flavin per mg of protein as determined by Iyanagi and Mason [17]. It was found that similar results were obtained using either rat or rabbit reductase.

Microsomal phospholipids were extracted from liver microsomes of phenobarbital-treated rabbits according to Bligh and Dyer [18] and stored under nitrogen in sealed tubes at −20°.

Reconstitution of enzymic activities. Cytochrome P-450 LM_2-dependent oxidations were reconstituted in micelles of DLPC and in artificial membranes. For preparation of the DLPC-system, the phospholipid was sonicated at a concentration of 1 mg/ml in potassium phosphate buffer, pH 7.4. The phospholipid suspension was mixed with NADPH–cytochrome P-450 reductase and P-450 LM_2 where indicated, to yield a ratio of 0.25 mg of phospholipid (about 400 nmol) per nmol of total protein. The mixture was incubated for 30 min at room temperature. The content of P-450 and reductase was thereafter determined, before further incubations.

For preparation of the membranous reconstituted system, unilamellar vesicles containing NADPH–cytochrome P-450 reductase, P-450 LM_2 and microsomal phospholipids were formed according to the cholate gel filtration technique [15]. The microsomal phospholipids at a concentration of 10 mg/ml in 15 mM potassium phosphate buffer, pH 7.4, containing 50 mM KCl were sonicated together with sodium cholate (2.4 mg of cholate/mg of lipid) on a water bath, until clarified. Subsequently, the proteins were added at a ratio of 1 nmol total protein per mg of phospholipid (about 1200 nmol). This mixture was incubated at 4° for 30 min prior to gel filtration, performed in 15 mM potassium phosphate buffer, pH 7.4, containing 50 mM KCl.

Assay methods. Protein was quantified according to Lowry, using bovine serum albumin as standard [19]. Cytochrome P-450 was quantified spectrophotometrically [20] and the activity of NADPH–cytochrome P-450 reductase was determined as the rate of reduction of cytochrome *c* at 550 nm and 30° in 0.33 M potassium phosphate buffer, pH 7.6.

The NADPH–oxidase activity of membranous and vesicular systems was determined spectrophotometrically at 37° by following the decrease of absorption of NADPH at 340 nm using the absorption coefficient 6.2 $mM^{-1}\,cm^{-1}$. The incubation mixtures contained micelles or vesicles corresponding to 0.02–0.1 nmol of reductase in 1 ml of 50 mM potassium phosphate buffer, pH 7.4, and 0.10 mM NADPH.

The oxidation of Me_2SO was determined as the formation of formaldehyde [21] essentially as previously described [7]. Vesicles or micelles corresponding to 0.09 nmol of reductase in 50 mM potassium phosphate buffer, pH 7.4, containing 30 mM Me_2SO and various amounts of Fe(III)–EDTA, added from a fresh stock solution of 0.5 mM $FeCl_3$ containing 1 mM EDTA, were incubated in

* Abbreviations used: KMBA, 2-keto-4-thiomethylbutyric acid; DLPC, L-α-dilauroylphosphatidylcholine; Me_2SO, dimethylsulphoxide; P-450 LM_2, phenobarbital-inducible cytochrome P-450 LM_2 from rabbit liver microsomes.

the presence of 0.25 mM NADPH for 12 min. The reactions were stopped by the addition of 143 μl 60% (w/v) $ZnSO_4$. Control incubations were performed in the absence of NADPH or Me_2SO.

The oxidation of KMBA was detected as the formation of ethylene (cf. 22) using head space gas chromatography. Incubations were carried out in 5 ml glass tubes equipped with gas-tight rubber stoppers. The incubation mixtures contained 5 mM KMBA, various amounts of Fe(III)–EDTA (prepared as described above) and vesicles or micelles corresponding to 0.09 nmol NADPH–cytochrome P-450 reductase. The volume was adjusted to 1 ml with 50 mM potassium phosphate buffer, pH 7.4, and the incubations were started after a preincubation period of 3 min (37°), by the injection of 25 μl 10 mM NADPH through the rubber stopper. The reactions were stopped after 20 min by the injection of 0.1 ml 3 M HCl. The tubes were further incubated at 37° overnight and 1 ml samples of the head space were subsequently analyzed by gas chromatography, utilizing a Shimadzu GC-8A chromatograph equipped with a flame ionization detector and a 1.5 m glass-column filled with Porapak N 80/100. The column temperature was 50° and the carrier gas flow 45 ml/min, yielding a retention time for ethylene of about 1 min. Standard curves were constructed from head space samples of ethylene in water. Control incubations were performed in the absence of substrate or NADPH.

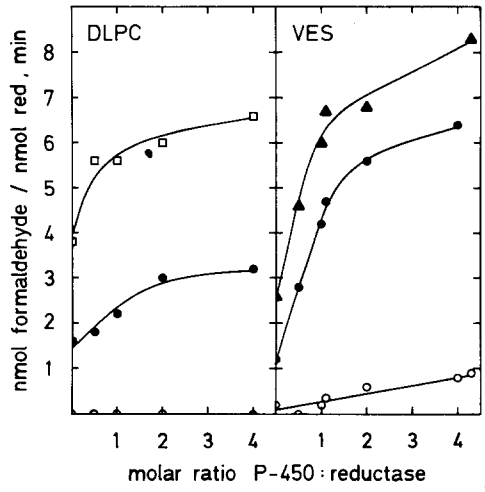


Fig. 1. Effect of the amount of P-450 LM_2 in the presence of various amounts of Fe(III)–EDTA on the rate of oxidation of Me_2SO in DLPC micelles (left) or reconstituted membranes (right). The incubation mixtures contained vesicles or micelles corresponding to 0.09 nmol of NADPH–cytochrome P-450 reductase, indicated amounts of P-450 LM_2, 30 mM Me_2SO and 0.25 mM NADPH in 1 ml pf 50 mM potassium phosphate buffer, pH 7.4. The figure shows the mean values from three different experiments. The standard deviations were generally less than 10%. The results are given in nmol of substrate oxidized per mol of NADPH–cytochrome P-450 reductase, min. The concentrations of Fe(III)–EDTA (1:2) used were, based on the amount of Fe^{3+}: (○---○), 0 μM; (●---●), 2.5 μM; (▲---▲), 5 μM; (□---□), 10 μM.

RESULTS

For the detection of hydroxyl radical production in reconstituted systems containing NADPH–cytochrome P-450 reductase and P-450 LM_2 we used Me_2SO, which is known to scavenge these radicals under the formation of formaldehyde [23] and KMBA which is known to generate ethylene gas a result of interactions with ·OH [22].

Oxidation of Me_2SO

In the presence of NADPH, NADPH–cytochrome P-450 reductase and P-450 LM_2, but in the absence of Fe(III)–EDTA, Me_2SO was oxidized at a slow rate in reconstituted membrane vesicles, whereas no oxidation occurred in a similar system reconstituted with DLPC (Fig. 1). However, in the presence of very small amounts of Fe(III)–EDTA (2.5 μM) the rate of Me_2SO oxidaztion increased considerable in both types of reconstituted systems. At this concentration, the presence of P-450 LM_2 equimolar to the level of NADPH–cytochrome P-450 reductase, caused almost a 4-fold higher rate of Me_2SO oxidation in the membranous system and a 50% increase of Me_2SO oxidation in the micellar system as compared to data obtained in the absence of P-450 (Figs 1 and 2). The iron-dependent oxidation of Me_2SO, at low concentrations of Fe(III)–EDTA (<10 μM), increases with the amount of P-450 LM_2 up to a molar ratio between reductase and P-450 LM_2 of about 1 in both systems.

The reductase-dependent Me_2SO oxidation is strongly influenced by the amount of chelated iron present and increases in both systems even at higher concentrations of Fe(III)–EDTA (10–50 μM). Especially in the vesicular system, no sign of saturation with regard to the Fe(III)–EDTA concentration, was apparent (Fig. 2). The P-450 LM_2-dependent Me_2SO oxidation, however, regarded as

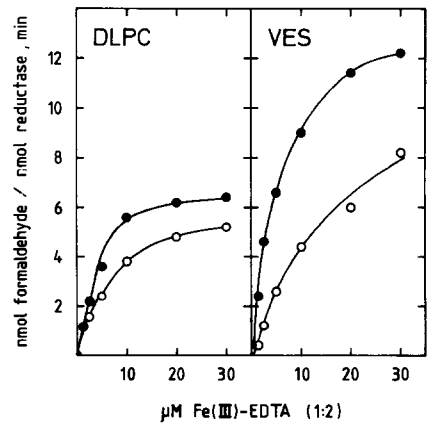


Fig. 2. Effect of the amount of Fe(III)–EDTA on the rate of Me_2SO oxidation in DLPC micelles (left) and reconstituted vesicles (right) in the absence (○---○) or in the presence (●---●) of P-450 LM_2. The incubation mixtures containing micelles or vesicles corresponding to 0.09 nmol of NADPH–cytochrome P-450 reductase and, where indicated, an equimolar amount of P-450 LM_2. Other conditions as described in Methods and in legend to Fig. 1. The results shown are the mean values out of three different experiments. The standard deviations were less than 10%.

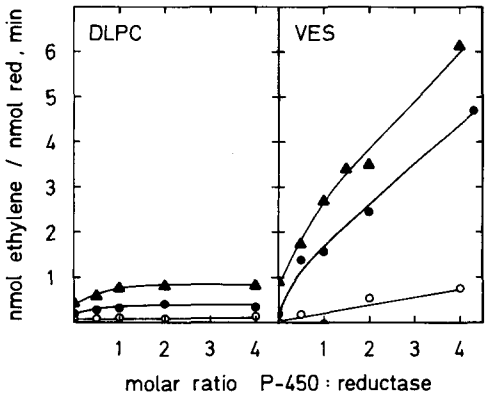


Fig. 3. Effect of the amount of P-450 LM_2 on the rate of NADPH-dependent KMBA oxidation in DLPC micelles (left) or reconstituted membrane vesicles (right). The incubation mixtures contained micelles or vesicles corresponding to 0.09 nmol of NADPH–cytochrome P-450 reductase, 5 mM KMBA, 0.25 mM NADPH and indicated amounts of Fe(III)–EDTA. Other conditions as described in Methods. The figure shows the results from 5 different experiments, given as nmol of substrate oxidized per nmol of NADPH–cytochrome P-450 reductase, min. The standard deviations were less than 10%. The ethylene produced in the absence of NADPH has been subtracted. The concentrations of Fe(III)–EDTA (1:2) used were, based on the amount of Fe^{3+}: (○---○), 0 μM; (●---●), 2.5 μM; (▲---▲), 5 μM.

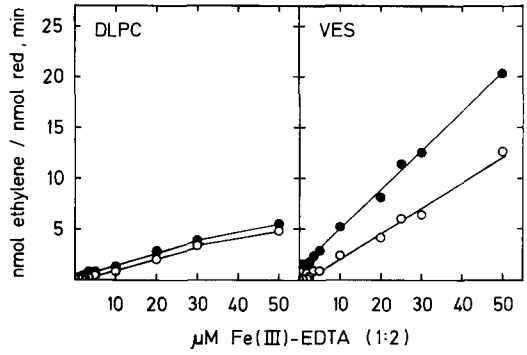


Fig. 4. Effect of the amount of Fe(III)–EDTA on the rate of NADPH-dependent KMBA oxidation in DLPC micelles (left) or reconstituted membrane vesicles (right) in the absence of (○---○) or in the presence (●---●) of P-450 LM_2. Conditions were as described in Methods and in the legend to Fig. 3. The incubation mixtures contained 0.09 nmol NADPH–cytochrome P-450 reductase and, where indicated, an equimolar amount of P-450 LM_2. The ethylene produced in the absence of NADPH has been subtracted. The results shown are the mean values of three different experiments and shown as nmol of substrate oxidized per nmol of NADPH–cytochrome P-450 reductase, min. The standard deviations were less than 10%.

the difference between the two curves in Fig. 2, did not increase at Fe(III)–EDTA concentrations above 10 μM (Fig. 2). Accordingly, in the DLPC-system about one third of the rate of Me_2SO oxidation is accounted for by P-450 LM_2 when the flavoprotein and hemoprotein are present at 1:1 molar ratio and the Fe(III)–EDTA concentration kept below 10 μM. Under similar conditions in the vesicular system, as much as 83% of the total rate of Me_2SO oxidation is dependent on P-450 LM_2 at 1 μM of Fe(III)–EDTA.

Oxidation of KMBA

KMBA was used as a second substrate, in order to quantify the amount of hydroxyl radicals generated in the reconstituted systems containing NADPH–cytochrome P-450 reductase and P-450 LM_2. The effect of P-450 LM_2 on the rate of ethylene gas production at small concentrations of Fe(III)–EDTA was evaluated (Fig. 3). Only a very slight absolute increase of the rate of KMBA oxidation dependent on P-450 LM_2 was seen in the DLPC system at 2.5 and 5 μM Fe(III)–EDTA, respectively. This increase was only evident up to a P-450: reductase ratio of 1:1. By contrast, addition of P-450 LM_2 affected the rate of KMBA oxidation to a great extent in the vesicular system. At equimolar amounts of reductase and P-450, the P-450-dependent oxidation accounted for almost 90% of the total NADPH-dependent KMBA oxidation at small amounts of Fe(III)–EDTA (1–2 μM) and almost 70% of the oxidation at 5 μM Fe–EDTA. At a 4-fold molar excess of P-450 LM_2, the production of ethylene gas in the vesicular system was increased 20-fold at 2.5 μM Fe(III)–EDTA, as compared to vesicles without P-450 (Fig. 3). Higher concentrations of Fe(III)–EDTA (10–50 μM) did not further potentiate the P-450 LM_2-dependent KMBA oxidation in the DLPC system (Fig. 4). The reductase-dependent KMBA oxidation in this system, however, increased with the concentration of chelated iron over the whole concentration range (up to 50 μM). In the vesicular system, the P-450 LM_2-dependent fraction of the total NADPH-dependent KMBA oxidation, decreases at higher Fe(III)–EDTA concentrations, but is still about 50% at 30 μm and 40% at 50 μM.

In the absence of NADPH, there was a considerable formation of ethylene in both systems. This production was not influenced by Fe(III)–EDTA, but increased somewhat with the amount of P-450 LM_2, especially in the membranous systems (data not shown).

Oxidation of NADPH

As previously noted [12, 24], the potential rate of generation of hydrogen peroxide and hydroxyl radicals in a reconstituted cytochrome P-450-containing system is essentially paralleled by the amount of NADPH consumed by the system (cf. eq. I). It was therefore of interest to compare the rate of NADPH consumption under the different conditions employed. In the DLPC system, the rates of NADPH oxidation were generally quite low. At low Fe(III)–EDTA concentrations, a slight increase by the addition of P-450 LM_2 was evident up to a 1:1 molar ratio between the protein components (Fig. 5). In the vesicular system, the rate of NADPH oxidation increased considerably more in response to addition of P-450 LM_2, than in the micellar system (Fig. 5). The reductase-dependent NADPH oxidation was enhanced in both systems by increasing the amount

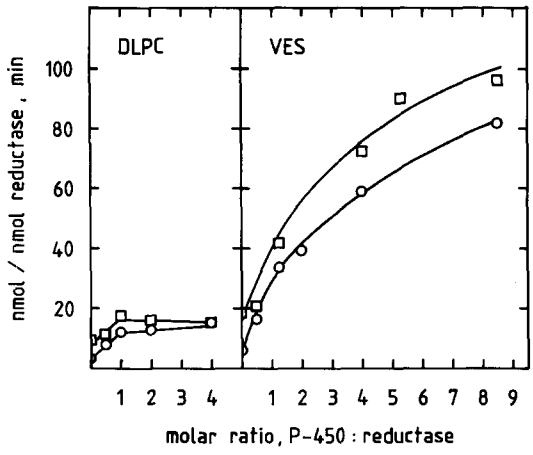


Fig. 5. Effect of the amount of P-450 LM_2 on the rate of NADPH consumption in DLPC micelles (left) or reconstituted membrane vesicles (right). Incubations were carried out as described in Methods with micelles or vesicles corresponding to 0.20–0.1 nmol of NADPH–cytochrome P-450 reductase at 37° in 1 ml of 50 mM potassium phosphate buffer, pH 7.4 in the absence (○---○) or in the presence of 10 μM Fe(III)–EDTA (□---□). The results shown are the mean values of three different experiments. The standard deviations were less than 20%.

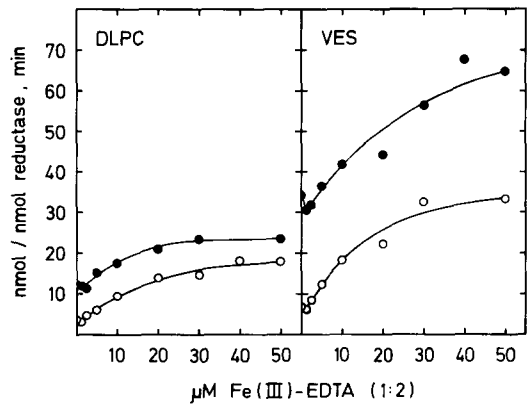


Fig. 6. Effect of the amount of Fe(III)–EDTA on the rate of NADPH consumption in DLPC micelles (left) or reconstituted membrane vesicles (right) in the absence (○---○) or in the presence (●---●) of P-450 LM_2. The reaction mixtures contained 0.02–0.1 nmol of NADPH–cytochrome P-450 reductase and, where indicated, an equimolar amount of p-450 LM_2. The results shown are the mean of three different experiments. The standard deviations were less than 20%.

of Fe(III)–EDTA present (Fig. 6). In the membranous system, this increase was higher than in the micellar one and did not seem to be saturated at higher concentrations of the chelated iron. The P-450 LM_2-dependent NADPH oxidation was apparently not influenced at all by the amount of Fe(III)–EDTA present in either system. Thus, the relative role of P-450 LM_2 for the overall rate of NADPH oxidation decreased with increasing amounts of Fe(III)–EDTA in both systems, i.e. from 70 to 37% in the DLPC system and from 80 to 50% in the vesicular systems with increasing Fe(III)–EDTA from 0 to 30 μM.

Control experiments

Vesicles were prepared with only P-450 LM_2 incorporated at a molar ratio of phospholipids: P-450 of 1200. This preparation was subsequently divided into two parts, one of which was thereafter boiled for 30 min. NADPH–cytochrome P-450 reductase was subsequently added to both preparations and the effect of P-450 LM_2 (3-fold molar excess to reductase) on the rate of oxidation of Me_2SO, KMBA or NADPH was registered. It was found that the increase in oxidation rates caused by P-450 LM_2, i.e. 26-fold, 2–3-fold and 3–18-fold on oxidation of Me_2SO, KMBA and NADPH under these conditions, respectively, was almost totally abolished in membranes containing boiled P-450 LM_2 (Table 1). The absolute turnover values in these series of experiments are lower than in the other experiments presented, since spontaneous incorporation of reductase into preformed vesicles does not give a similar efficiency of incorporation as the cholate gel filtration technique (cf. 15).

Table 1. Effect of boiled P-450 LM_2 on the rate of oxidation of NADPH, KMBA and Me_2SO in reconstituted membranes

Substrate	Rate of oxidation (nmol/nmol reductase, min) Reductase only	Reductase + native P-450	Reductase + boiled P-450
KMBA, 5 μM Fe–EDTA	1.2	3.3	0.8
30 μM Fe–EDTA	3.0	6.9	3.5
Me_2SO, 5 μM Fe–EDTA	0.22	5.8	0.78
NADPH, 0 μM Fe–EDTA	1.3	24	2.2
5 μM Fe–EDTA	4.2	28	6.6
10 μM Fe–EDTA	7.7	28	8.3

Vesicles were prepared containing phospholipids and P-450 LM_2 as described in the text. Half of the preparation was boiled for 30 min, and NADPH–cytochrome P-450 reductase was subsequently introduced into both types of preparations yielding a molar ratio of P-450 LM_2:reductase of 3.2:1. Incubations were also simultaneously performed with vesicles only containing reductase, where the flavoprotein had been incorporated in the same manner, i.e. by incubation with preformed vesicles.

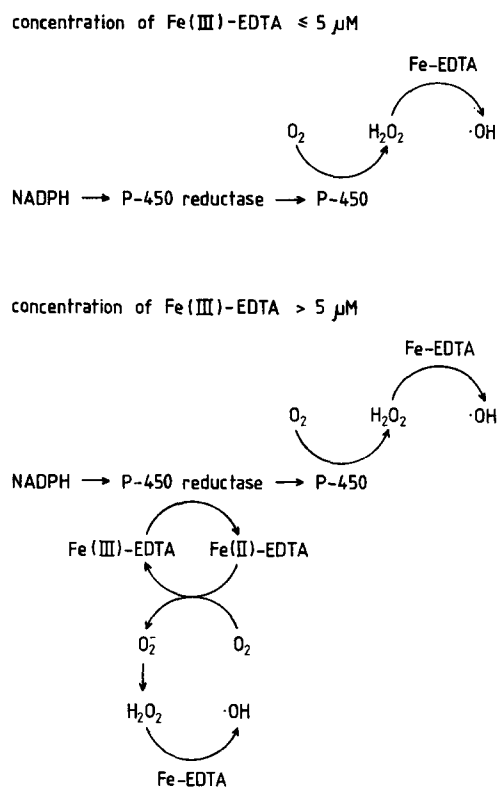
DISCUSSION

The results presented indicate that, under otherwise similar conditions, the rate of electron transport in the cytochrome P-450 system is higher when the components are embedded in a bilayer membrane, than when reconstituted in DLPC micelles. This is manifested in a 4-fold higher rate of NADPH-consumption in the membranous system containing a 4-fold molar excess of P-450 LM_2 relative to reductase than in the micellar system (Fig. 5). This fact appears to contribute to a higher rate of Me_2SO and KMBA oxidation in P-450-containing membranous systems than in the corresponding micelles (Figs 1 and 3). We have previously found that the rate of reduction of P-450 LM_2 to a great extent is dependent on a negative surface charge of the membrane [25, 26]. The reason has been shown to be a 10-fold lower association constant between NADPH–cytochrome P-450 reductase and P-450 LM_2 in negative, than in neutral membranes [27]. This appears to be of importance for the higher rate of NADPH consumption and rate of electron transport in the present case, using the membranous system based on negatively-charged phospholipids.

A better coupling between the reductase and P-450 LM_2 in the membranous system would be expected to cause a higher rate of oxidase activity dependent on P-450 LM_2. In the presence of Fe(III)–EDTA this would result in the generation of hydroxyl radicals. Accordingly, a much more pronounced dependency on P-450 LM_2 for oxidation of Me_2SO (Fig. 1) and KMBA (Fig. 3) was observed in the membranous system. The effect was most prominent in membranes at small amounts (2.5–5 μM) of Fe(III)–EDTA, where at a 4-fold molar excess of P-450 relative to reductase, the rate of KMBA and Me_2SO oxidation was 3.5–22 times that of membranes containing only P-450 reductase. In micelles, under otherwise similar conditions, a 4-fold molar excess of P-450 only increased the oxidation of the hydroxyl radical scavengers by 60–100% (Figs 1 and 3). Higher amounts of Fe(III)–EDTA (10–50 μM) did not significantly alter the P-450 LM_2-dependent oxidation rate, but since the reductase-dependent oxidation was greatly enhanced by these amounts of chelated iron, the P-450-dependent oxidation accounts for a much smaller portion of the total NADPH-dependent oxidation in this concentration range. This is especially true in the micellar system with KMBA as substrate. In fact, no effect of P-450 LM_2 was observed on KMBA oxidation in the DLPC micellar system at these Fe(III)–EDTA concentrations (Fig. 4), a finding in agreement with that of Winston and Cederbaum [14].

Fe(III)–EDTA (5–50 μM) causes an enhanced rate of NADPH oxidation in reconstituted systems containing NADPH–cytochrome P-450 reductase as the only protein component (Fig. 6). The increase in NADPH oxidation is especially pronounced in the membranous system and at an Fe(III)–EDTA concentration of 50 μM, the reductase-dependent rate approaches that of the vesicular system containing P-450 LM_2 without added Fe(III)–EDTA (Fig. 6).

In previous studies, where we examined the rate of P-450 LM_2-dependent O_2^- formation and lipid peroxidation [29], we observed that addition of Fe(III)–EDTA caused an NADPH–cytochrome P-450 reductase-dependent "uncoupling" of the electron transport system at a concentration of about 10 μM, thereby generating oxygen species capable of initiating lipid peroxidation. In combination with the present data it appears that the site of formation of hydroxyl radicals, especially in the vesicular system, is dependent on the concentration of chelated iron (Scheme I). With small amounts of Fe(III)–EDTA present, the chelated iron will preferentially cause a cleavage of the hydrogen peroxide formed by P-450 LM_2. At higher amounts of Fe(III)–EDTA, the chelated iron will also "uncouple" the electron transport chain, resulting in the reductase-dependent formation of hydroxyl radicals.



Scheme I.

In conclusion, the data here presented, in combination with results previously published, indicate that in the presence of no or only small amounts of chelated iron, cytochrome P-450 is mainly responsible for production of hydrogen peroxide and superoxide anions necessary for the generation of hydroxyl radicals, both in microsomal systems and in reconstituted membranous systems. In reconstituted systems of micellar nature and/or in the presence of high amounts of chelated iron, "uncoupling" at the level of NADPH–cytochrome P-450 reductase can occur, with the resulting production of hydroxyl radicals and other forms of reactive oxygen. The toxicological importance of these kinds of reactions remains to be established (cf. [13]).

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REFERENCES

1. J. R. Gillette, B. B. Brodie and B. N. LaDu, *J. Pharmac. exp. Ther,* **119**, 532 (1957).
2. A. G. Hildebrandt and I. Roots, *Archs Biochem. Biophys.* **171**, 385 (1975).
3. A. Bast, E. M. Savenije-Chapel and B. H. Kroes, *Xenobiotica* **14**, 399 (1984).
4. H. Kuthan and V. Ullrich, *Eur. J. Biochem.* **126**, 583 (1982).
5. D. Rössing, R. Kahl and A. G. Hildebrandt, *Tox. Lett.* **34**, 67 (1985).
6. A. Bast, J. W. Brenninkmeijer, E. M. Savenije-Chapel and J. Noordhoek, *FEBS Lett.* **151**, 185 (1983).
7. G. Ekström, T. Cronholm and M. Ingelman-Sundberg, *Biochem. J.* **233**, 755 (1986).
8. H. Kuthan, H. Tsuji, H. Graf, V. Ullrich, J. Werringloer and R. W. Estabrook *FEBS Lett.* **91**, 343 (1978).
9. H. Kuthan and V. Ullrich, *Eur. J. Biochem.* **126**, 583 (1982).
10. N. Premereur, C. van der Branden and F. Roels, *FEBS Lett.* **199**, 19 (1986).
11. G. W. Winston and A. I. Cederbaum, *J. biol. Chem.* **258**, 1508 (1983).
12. M. Ingelman-Sundberg and I. Johansson, *J. biol. Chem.* **259**, 6447 (1984).
13. A. Bast, *Trends Pharmac. Sci.* **7**, 266 (1986).
14. G. W. Winston and A. I. Cederbaum, *Biochem. Pharmac.* **35**, 4053 (1986).
15. M. Ingelman-Sundberg and H. Glaumann, *Biochim. biophys. Acta* **599**, 417 (1980).
16. I. Johansson, E. Eliasson, C. Norsten and M. Ingelman-Sundberg. *FEBS Lett.* **196**, 59 (1986).
17. T. Iyanagi and H. S. Mason, *Biochemistry* **12**, 2297 (1973).
18. E. G. Bligh and W. J. Dyer, *Can. J. Biochem. Physiol.* **37**, 911 (1959).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
21. T. Nash, *Biochem. J.* **55**, 416 (1953).
22. R. E. Heikkila, B. Winston and G. Cohen, *Biochem. Pharmac.* **25**, 1085 (1976).
23. S. M. Klein, G. Cohen and A. I. Cederbaum, *Biochemistry* **20**, 6006 (1981).
24. M. Ingelman-Sundberg and I. Johansson, *J. biol. Chem.* **256**, 6321 (1981).
25. M. Ingelman-Sundberg, T. Haaparanta and J. Rydström, *Biochemistry* **20**, 4004 (1981).
26. M. Ingelman-Sundberg, J. Blanck, G. Smettan and K. Ruckpaul, *Eur. J. Biochem.* **134**, 157 (1983).
27. J. Blanck, G. Smettan, O. Ristau, M. Ingelman-Sundberg and K. Ruckpaul, *Eur. J. Biochem.* **144**, 509 (1984).
28. Y. Terelius and M. Ingelman-Sundberg, *Eur. J. Biochem.* **161**, 303 (1986).
29. G. Ekström and M. Ingelman-Sundberg, *Eur. J. Biochem.* **158**, 195 (1986).