Relationship between the Reduction of Oxygen, Artificial Acceptors and Cytochrome *P*-450 by NADPH–Cytochrome *c* Reductase

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The interaction of NADPH–cytochrome *c* reductase with oxygen, artificial acceptors and cytochrome *P*-450 was studied. The generation of superoxide anion radicals ($O_2^{\cdot -}$) from the oxidation of adrenaline to adrenochrome catalysed by NADPH–cytochrome *c* reductase proceeds independently of the interaction of the enzyme with the artificial anaerobic acceptors cytochrome *c* or 2,6-dichlorophenol-indophenol. Propyl 3,4,5-trihydroxybenzoate inhibited competitively the adrenaline oxidation by isolated NADPH–cytochrome *c* reductase (K_i 3.2–4.7 μM) and inhibited non-competitively the cytochrome *c* reduction (K_i 92–109 μM). In contrast with the process of electron transfer to cytochrome *c*, the rate of reduction of cytochrome *P*-450 and the rate of oxidation of adrenaline in liver microsomal fraction are correlated. Hexobarbital increases the V_{max} of adrenaline oxidation without affecting the K_m value, whereas metyrapone, a metabolic inhibitor decreases V_{max} without affecting the K_m . From the results obtained, some conclusions about NADPH–cytochrome *c* reductase function were made.

Most of the currently proposed schemes for the metabolism of xenobiotics in microsomal preparations from mammalian livers include, as the initial step in the electron-transport chain, the NADPH-specific flavoprotein, NADPH–cytochrome *c* reductase (EC 1.6.2.4). This flavoprotein can transfer electrons to at least three types of acceptors, namely cytochrome *P*-450, anaerobic acceptors (cytochrome *c*, 2,6-dichlorophenol-indophenol and menadione) and molecular oxygen (Mishin *et al.*, 1976; Gnossopelius *et al.*, 1969/1970). The capacity of flavoprotein for one-electron reduction of oxygen has been demonstrated by the inhibitory effect of the enzyme superoxide dismutase (EC 1.15.1.1), which catalyses the dismutation of $O_2^{\cdot -}$.

It has been suggested that the reduction of Fe^{2+} cations during enzymic lipid peroxidation in microsomal fractions also involves $O_2^{\cdot -}$, generated by NADPH–cytochrome *c* reductase (Pederson & Aust, 1972). Cytochrome *c* and 2,6-dichlorophenol-indophenol are reduced under anaerobic conditions without the intermediate formation of radical anions of oxygen; this reduction is not sensitive to added superoxide dismutase (Mishin *et al.*, 1976).

It remains unclear, however, how the electrons of NADPH-specific flavoprotein are redistributed during the interaction with several types of acceptors. We here present an attempt to answer this question.

Experimental

Wistar male rats (150–200 g body wt.) were used. A microsomal fraction from rat livers was separated by

differential centrifugation (Mishin *et al.*, 1976). Spectrophotometric measurements were made at 22°C in 0.15 M-potassium phosphate buffer, pH 8.0, containing 0.1 mM-EDTA. The assay was performed in a 3 ml cuvette. Reduction of 2,6-dichlorophenol-indophenol, cytochrome *c* and Nitro Blue Tetrazolium was measured as described previously (Mishin *et al.*, 1976). Adrenaline oxidation was determined by the rate of formation of adrenochrome at 480–575 nm, by using $\epsilon = 2860$ litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ (Sorgato *et al.*, 1974). Polarographic experiments were performed in 0.15 M-potassium phosphate buffer, pH 8.0, containing 0.1 mM-EDTA. The volume of the polarographic cuvette was 1.4 ml. The concentration of the acceptors of NADPH–cytochrome *c* reductase was the same as in the spectrophotometric experiments.

NADPH–cytochrome *c* reductase was separated by using either trypsin or Triton X-100. The microsomal fraction was solubilized with trypsin as described by Ichikawa & Yamano (1970). NADPH–cytochrome *c* reductase was purified by the procedure of Iyanagi & Mason (1973). The purified preparation of this enzyme had an activity of 3180 nmol of cytochrome *c* reduced/min per mg of protein. NADPH–cytochrome *c* reductase was isolated with Triton X-100 by the modified method of Ichihara *et al.* (1972). After the first stage of chromatography on a DEAE-cellulose column, calcium phosphate gel was added to the active fraction (1 g dry wt. of gel to 750 ml); the mixture was stirred for 10 min and then centrifuged at 3000g for 10 min. Reductase was eluted with 25 ml of 0.3 M-potassium phosphate buffer

(pH 7.8) by stirring for 15 min. Subsequently the solution was dialysed for 6 h against 500 ml of 0.05 M-Tris/HCl buffer, pH 7.8, containing 10% (v/v) glycerol and 0.01 mM-dithiothreitol (buffer A). Enzyme solution was diluted with buffer A to a concentration of 1.5 mg of protein/ml and Triton X-100 solution was added to a final concentration of 0.4%. The solution obtained was applied to a DEAE-cellulose column (2.5 cm × 20 cm), previously equilibrated with buffer A containing 0.4% Triton X-100. The column was washed with 300 ml of buffer A containing 0.4% Triton X-100 and 0.1 M-KCl. The active fraction was then eluted from the column with a linear 0.1–0.5 M-KCl gradient (500 ml) in the same buffer. The solution of the enzyme was concentrated by using calcium phosphate gel, as described above, and diluted with buffer A to a protein concentration of 0.3 mg/ml. Then 20% Triton X-100 and KCl were added (final concentrations 0.1% and 0.15 M respectively). The solution was applied to a DEAE-Sephadex A-50 column (2.0 cm × 15 cm) pre-equilibrated with buffer A containing 0.1% Triton X-100 and 0.15 M-KCl. The column was washed with 200 ml of 0.2 M-KCl solution in buffer A containing 0.1% Triton X-100 and then with 500 ml of the same buffer solution, containing a linear 0.2–0.5 M-KCl gradient. The active fraction was collected and concentrated as described above. The activity of this fraction was 1860 nmol of cytochrome *c* reduced/min per mg of protein. Glycerol and dithiothreitol were added to the enzyme solution to a final concentration of 10% and 0.1 mM respectively. Reductase activity was not significantly affected by storage at –20°C for 1 month. Calcium phosphate gel was prepared as described previously (Swingle & Tiselius, 1951).

Superoxide dismutase was isolated from bovine erythrocytes (McCord & Fridovich, 1969). Protein concentration was measured by the method of Lowry *et al.* (1951) or by the biuret method (Gornall *et al.*, 1949), with bovine albumin as standard.

Results

First, an investigation was made of the interaction between the artificial acceptors of NADPH–cytochrome *c* reductase, which accept electrons directly from the flavoprotein (cytochrome *c*, 2,6-dichlorophenol-indophenol), and the acceptors reacting with $O_2^{\cdot-}$ (adrenaline, Nitro Blue Tetrazolium), supplied by NADPH–cytochrome *c* reductase. Cytochrome *c* and adrenaline are more suitable for spectral measurements because they differ considerably in their spectral characteristics. To exclude optical changes at 480 nm (λ_{max} of adrenochrome) during cytochrome *c* reduction, adrenochrome formation was measured at two wavelengths, 480 nm and 562 nm (reference wavelength).

Fig. 1. Effect of cytochrome *c* on the adrenaline oxidation by liver microsomal fraction

Assays were carried out in 0.15 M-potassium phosphate buffer, pH 8.0, containing 0.1 mM-EDTA. Concentrations of added reagents: 0.3 mM-adrenaline, 100 μ M-NADPH, 0.05 mM-cytochrome *c*, superoxide dismutase (15 μ g/ml), 0.5 mg of microsomal protein. A, addition of NADPH; B, addition of cytochrome *c*; C, addition of superoxide dismutase.

As shown in Fig. 1, cytochrome *c* hardly affects the rate of formation of adrenochrome by the liver microsomal fraction, but the reaction is almost completely inhibited by superoxide dismutase irrespective of whether it is added before or after the addition of cytochrome *c*.

We then studied the effect that the reaction of adrenaline oxidation has on the rate of cytochrome *c* reduction and 2,6-dichlorophenol-indophenol reduction by liver microsomal fraction. As shown in Table 1, adrenaline added just before the beginning of the reaction does not affect the rate of cytochrome *c* reduction by liver microsomal fraction. The addition of adrenaline to microsomal fraction and subsequent incubation with NADPH for 5 min (the time needed to obtain the maximal rate of adrenaline oxidation) also did not affect the reduction of cytochrome *c* or 2,6-dichlorophenol-indophenol.

The results of the polarographic experiments also support the suggestion that the reactions studied are independent of each other (Table 2). The addition of cytochrome *c* does not affect the rate of oxygen consumption during adrenaline oxidation by liver microsomal fraction, although it is inhibited by added Nitro Blue Tetrazolium, which is partially reduced by $O_2^{\cdot-}$ in liver microsomal fraction (Mishin *et al.*, 1976). The duration of Nitro Blue Tetrazolium inhibition depends on its concentration and, judged

Table 1. *Effect of adrenaline on the reduction of cytochrome c and 2,6-dichlorophenol-indophenol in liver microsomal fractions*
For experimental details see the text.

	Enzyme activity (nmol of acceptor/min per mg of protein)	
	Cytochrome <i>c</i> reductase	2,6-Dichlorophenol- indophenol reductase
Control	70	78
+0.3 mM-adrenaline (immediately before the beginning of the reaction)	71	78
+0.3 mM-adrenaline (preincubation for 5 min)	70	79

Table 2. *Effect of various acceptors on oxygen consumption during the adrenaline oxidation*
For experimental details see the text.

	Oxygen consumption during adrenaline oxidation (ng-atoms of oxygen/min per mg of protein)			
	Control	+Cytochrome <i>c</i>	+2,6-Dichlorophenol- indophenol	+Nitro Blue Tetrazolium
Microsomal fraction	19	21	20	2
Flavoprotein isolated with trypsin	840	1920	1800	830
Flavoprotein isolated with Triton X-100	580	1370	1350	590

Table 3. *Activity of NADPH-cytochrome c reductase isolated by different methods*
For experimental details see the text.

	Enzyme activity		
	(nmol of acceptor/min per mg of protein)		(nmol of adrenochrome/ min per mg of protein)
	Cytochrome <i>c</i> reductase	Nitro Blue Tetrazolium reductase	
Microsomal fraction	71	39.5	12.8
Flavoprotein isolated with trypsin	3180	29.6	680
Flavoprotein isolated with Triton X-100	1860	23.6	415

by the result of parallel spectrophotometric measurements, it takes as much time to inhibit with Nitro Blue Tetrazolium as to reduce it completely.

Comparisons of interaction between adrenaline and anaerobic acceptors in isolated flavoproteins and intact microsomal fraction demonstrated some specific features. Thus NADPH-cytochrome *c* reductase obtained by treatment of microsomal fraction with trypsin or Triton X-100 (giving 45- and 27-fold purifications respectively) reduced cytochrome *c* and 2,6-dichlorophenol-indophenol, and oxidized adrenaline to adrenochrome (Table 3). However, the reduction rate of Nitro Blue Tetrazolium by isolated flavoproteins was much lower than that by the intact microsomal fraction. The ratio of reduction rates of cytochrome *c* and Nitro Blue Tetrazolium is 1.8 in the microsomal fraction; the

respective values for assays with trypsin-isolated and Triton X-100-isolated NADPH-cytochrome *c* reductase were 108 and 79 respectively. These results agree with those obtained by Roerig *et al.* (1972) for NADPH-cytochrome *c* reductase isolated with bromelain. Obviously, because of the low interaction rate of Nitro Blue Tetrazolium with isolated flavoproteins, it did not inhibit oxygen consumption during the adrenaline oxidation by isolated NADPH-cytochrome *c* reductase. The addition of lipids or detergents to isolated flavoprotein restores the high activity of the NADPH-Nitro Blue Tetrazolium reductase reaction (Roerig *et al.*, 1972). It is possible that the interaction of Nitro Blue Tetrazolium with the lipids of the microsomal membrane results in changes of redox characteristics of the dye and affinity of Nitro Blue Tetrazolium for reduction by

Table 4. *Effect of cytochrome c on the adrenaline oxidation by isolated NADPH-cytochrome c reductase*
For experimental details see the text.

	Adrenaline oxidase activity (nmol of adrenochrome/min per mg of protein)		
	Control	+Cytochrome c	+Cytochrome c and superoxide dismutase (15 µg/ml)
Flavoprotein isolated with trypsin	680	1430	750
Flavoprotein isolated with Triton X-100	415	950	540

NADPH-cytochrome *c* reductase, leading to more varied reaction rates than in the absence of lipids as found in isolated flavoprotein. 2,6-Dichlorophenol-indophenol and cytochrome *c* produced some increase in oxygen consumption during adrenaline oxidation (Table 2).

Spectrophotometric measurements indicated that adrenaline does not influence the reduction rate of cytochrome *c* and 2,6-dichlorophenol-indophenol by NADPH-cytochrome *c* reductase. Analyses of the effect of cytochrome *c* on adrenochrome formation showed an accelerating effect by cytochrome *c* on the reaction in the presence of purified flavoproteins (Table 4). Misra & Fridovich (1971) have demonstrated that adrenaline autooxidation does not occur at pH 8.0 in the presence of 0.1 mM-EDTA. Under these conditions, adrenochrome is formed at the expense of the $O_2^{\cdot-}$ generated by NADPH-cytochrome *c* reductase, and adrenochrome accumulation is totally inhibited by superoxide dismutase (Aust *et al.*, 1972; Mishin *et al.*, 1976). The presence of superoxide dismutase in the experiments (Table 4) showed that cytochrome *c* added during the reaction of adrenaline oxidation by NADPH-cytochrome *c* reductase does not alter the $O_2^{\cdot-}$ -dependent rate of adrenochrome formation; instead, an $O_2^{\cdot-}$ -independent (i.e. insensitive to the effect of superoxide dismutase) process of adrenochrome formation was initiated. Furthermore, cytochrome *c* added after the complete inhibition of the NADPH-adrenaline oxidase-catalysed reaction by superoxide dismutase also induced adrenochrome formation (Fig. 2). It may be suggested that the acceleration of adrenaline oxidation to adrenochrome is related to the interaction of cytochrome *c* with one of the products of adrenaline oxidation without the participation of NADPH-cytochrome *c* reductase.

The results of the experiments with propyl 3,4,5-trihydroxybenzoate, an inhibitor of NADPH-cytochrome *c* reductase (Torielli & Slater, 1971), are presented in Figs. 3 and 4, which reveal a clear distinction of the differences in the interactions between isolated NADPH-cytochrome *c* reductase and adrenaline and between this enzyme and cytochrome *c*. Propyl 3,4,5-trihydroxybenzoate competitively inhibits the oxidation of adrenaline. From these ex-

Fig. 2. *Effect of cytochrome c on the adrenaline oxidation by isolated NADPH-cytochrome c reductase*

Experimental conditions were as described in Fig. 1. The amount of NADPH-cytochrome *c* reductase present was 0.01 mg of protein. A, Addition of NADPH; B, addition of superoxide dismutase; C, addition of cytochrome *c*.

periments, the values for the K_i range from 3.2 to 4.7 µM. The inhibitory effect of propyl 3,4,5-trihydroxybenzoate on cytochrome *c* reduction is shown in an altered maximal rate of cytochrome *c* reduction; it should be noted that here the value of the K_m remains unchanged. The observed non-competitive inhibition of the cytochrome *c* reduction complies with the experimental results of Torielli & Slater (1971), obtained with the microsomal fraction. The K_i value for the cytochrome *c* reductase reaction is 92–109 µM.

We next studied the interaction between adrenaline oxidation and cytochrome *P*-450 reduction. In the following experimental series, we used hexobarbital as hydroxylation substrate and metyrapone [2-methyl-1,2-di-(3-pyridyl)propan-1-one] as inhibitor of xenobiotic metabolism with the intention of affecting the reduction rate of cytochrome *P*-450 in

Fig. 3. *Propyl 3,4,5-trihydroxybenzoate inhibition of the adrenaline oxidation by isolated NADPH-cytochrome *c* reductase*

Experiments were carried out with an assay medium containing 0.15M-potassium phosphate buffer, pH 8.0, 0.1mM-EDTA, 100 μ M-NADPH. $\circ$, Without propyl 3,4,5-trihydroxybenzoate; $\bullet$, with 2.5 μ M-propyl 3,4,5-trihydroxybenzoate; $\blacksquare$, with 5 μ M-propyl 3,4,5-trihydroxybenzoate. The amount of enzyme present was 0.01 mg of protein.

Fig. 4. *Inhibitory effect of propyl 3,4,5-trihydroxybenzoate on the reduction of cytochrome *c* by isolated NADPH-cytochrome *c* reductase*

Experimental conditions were as described in Fig. 3. $\circ$, Without propyl 3,4,5-trihydroxybenzoate; $\bullet$, with 100 μ M-propyl 3,4,5-trihydroxybenzoate.

microsomal fractions. We showed that metyrapone inhibits considerably not only cytochrome *P*-450 reduction, but also the oxidation of adrenaline to adrenochrome (Fig. 5). Hexobarbital, an agent capable of accelerating the rate of NADPH-cytochrome *P*-450 reductase, also increases that of adrenaline, whereas metyrapone inhibits this acceleration. Fig. 5 shows how hexobarbital and metyrapone affect the values of the kinetic constants of adrenaline

Fig. 5. *Effects of hexobarbital and metyrapone on the adrenaline oxidation by liver microsomal fraction*

Experimental conditions were as described in Fig. 3. $\circ$, Control; $\bullet$, with 1.2mM-hexobarbital; $\blacksquare$, with 0.1mM-metyrapone.

Table 5. *Effect of cytochrome *c* and hexobarbital on the rate of NADPH consumption during adrenaline oxidation by liver microsomal fraction*

For experimental details see the text.

NADPH consumption (nmol/min per mg of protein)		
Adrenaline oxidase	+Cytochrome <i>c</i> (20 μ M)	+Hexobarbital (3.2mM)
5.4	32	7.2

oxidation by liver microsomal fraction. Because the addition of hexobarbital or metyrapone to purified flavoproteins, used for oxidizing adrenaline, had no effect on the rate of conversion of the latter into adrenochrome, it seemed possible that the substrates were not directly involved in the stimulation of the oxidative conversion of adrenaline.

While measuring NADPH consumption, we found that the addition of cytochrome *c* or hexobarbital during the reaction of adrenaline oxidation by the microsomal fraction increases the rate of NADPH consumption (Table 5). However, it should be noted that NADPH consumption during the adrenaline oxidation by the microsomal fraction may be related to the stimulating effect of one of the so far unknown products of oxidative conversion of adrenaline (Augusto *et al.*, 1973).

Discussion

These results led to several conclusions about the function of NADPH-cytochrome *c* reductase in liver

microsomal fraction. Obviously, flavoprotein is capable of one-electron reduction of oxygen, thereby forming $O_2^{\cdot-}$, as well as such anaerobic artificial acceptors as cytochrome *c* and 2,6-dichlorophenol-indophenol, the reduction of which, under the conditions tested, is insensitive to the inhibitory effect of superoxide dismutase. Support for these conclusions comes from the experimental results presented in Fig. 1 and Tables 1 and 2. Microsomal NADPH-specific flavoprotein reacts with oxygen and with artificial acceptors, concomitantly added to the aerobic incubation medium, without affecting appreciably the rate of each reaction. The conclusion made is in accordance with our previous results concerning the capacity of the enzyme to reduce Nitro Blue Tetrazolium by a mechanism sensitive to the effect of superoxide dismutase as well as by direct electron transfer to the dye (Mishin *et al.*, 1976).

Two possibilities may be envisaged. The existence of two independent functional centres in the flavoprotein molecule is one likely possibility. The other is the presence of two different NADPH-specific enzymes, one activating O_2 with the formation of $O_2^{\cdot-}$, the other directly transferring electrons to cytochrome *c* or 2,6-dichlorophenol-indophenol. The method used for the isolation of NADPH-cytochrome *c* reductase (treatment with trypsin or the detergent Triton X-100) does not interfere with the distribution of electrons between these acceptor groups, although the enzyme solubilized by trypsin or Triton X-100 differs very much in molecular weight, the respective values being 69000 and 400000 (Iyanagi & Mason, 1973; Ichihara *et al.*, 1973). It has also been shown that when isolated with trypsin, NADPH-cytochrome *c* reductase is incapable of functioning in a reconstituted system containing cytochrome *P*-450 (Lu & Levin, 1974). Hence it is quite plausible that the independence of the two reactions, interaction with anaerobic acceptors and with oxygen, is not related to the presence of two different NADPH-specific flavoproteins in the microsomal membrane. It is rather a property of the enzyme itself. This assumption gains support from the results of inhibition experiments with propyl 3,4,5-trihydroxybenzoate, which indicated that the interaction between isolated NADPH-cytochrome *c* reductase and adrenaline or cytochrome *c* differs not only in the K_i values, but also in the type of inhibition (competitive for adrenaline oxidation, and non-competitive for cytochrome *c* reduction). Moreover, both active centres are located in the hydrophilic moiety of the molecule of flavoprotein, whereas trypsin, as Depierre & Dallner (1975) have suggested, removes the hydrophilic moiety, leaving the presumably hydrophobic moiety binding to the microsomal membrane. The interactions with two acceptor types are quite likely to be related to either

different redox states of flavoprotein or to different flavins (FAD or FMN) that are contained in NADPH-cytochrome *c* reductase (Iyanagi & Mason, 1973).

Interaction of NADPH-cytochrome *c* reductase with cytochrome *P*-450, a natural acceptor of electrons appears to be essential for the function of the enzyme. By demonstrating the inhibitory effect of cytochrome *c* on xenobiotic metabolism, Ernster & Orrenius (1965) have proved that cytochrome *P*-450 reduction and reduction of cytochrome *c* are related. Hence there is definite competition of electron flows distributed by NADPH-cytochrome *c* reductase between cytochrome *P*-450 and artificial anaerobic acceptors. The experiments described in the present paper permit us to trace the relation between the reduction rate of cytochrome *P*-450 and oxygen activation on the flavoprotein portion of the microsomal electron-transfer chain (Fig. 5, Table 5). It is noteworthy that unlike the transfer process of electrons to cytochrome *c*, the rates of cytochrome *P*-450 reduction and adrenaline oxidation (a superoxide dismutase-sensitive reaction) are directly related. The accelerating effect of the hexobarbital on the oxidation rate of adrenaline to adrenochrome was observed only in experiments with intact microsomal fraction. With flavoprotein (irrespective of the isolation procedure used), the same concentrations of hexobarbital had no effect on adrenochrome formation.

Fig. 5 shows that hexobarbital increases the V_{max} of the reaction of adrenaline oxidation without affecting the K_m value, whereas metyrapone decreases the V_{max} and does not affect the K_m . It may well be that electron transfer from NADPH-cytochrome *c* reductase to cytochrome *P*-450 and then directly to oxygen involves interrelated redox states of flavoprotein. In this connexion, it should be noted that $O_2^{\cdot-}$, generated by NADPH-cytochrome *c* reductase, may be one of the decisive factors in the induction of the microsomal hydroxylase system, as proposed by Paine & McLean (1974).

In conclusion, it should be emphasized that NADPH-cytochrome *c* reductase is a polyfunctional component of the microsomal electron-transfer chain, reacting with different acceptor types and also competent for complex intramolecular electron redistribution between various functional groups.

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