STEREOSPECIFICITY OF CERTAIN REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE-LINKED REACTIONS IN RAT LIVER MICROSOMES *

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1. Introduction

In the presence of NADPH and molecular oxygen rat liver microsomes catalyze the hydroxylation of a number of lipid-soluble compounds including various drugs [1], aliphatic hydrocarbons [2], steroid hormones [3] and fatty acids (ω-oxidation) [2]. They also catalyze the NADPH-linked peroxidation of endogenous lipids [4]. Whereas all these reactions are strongly inhibited by oxidized cytochrome *c*, the hydroxylation processes, but not the lipid peroxidation reaction, are also inhibited by carbon monoxide [5], indicating the involvement of cytochrome P-450 [6-8] in the oxygen-activating step of the hydroxylation reactions. Drugs undergoing hydroxylation have been shown to inhibit the lipid peroxidation activity [9] probably by competition for a common NADPH-oxidizing flavoenzyme involved in both processes. Furthermore, upon treatment of rats with phenobarbital *in vivo* there is a greatly enhanced hydroxylating activity as measured in the isolated liver microsomes with all the above-mentioned hydroxyl acceptors [2,5] which is paralleled by a similar increase [10] in the liver-microsomal content of NADPH-cytochrome *c* reductase [11,12]. Taken together, the available evidence strongly suggests that the microsomal NADPH-cytochrome *c* reductase is involved both in enzymic lipid peroxidation and in the reduction of cytochrome P-450 involved in the various hydroxylation reactions. In the present investigation the stereospecificity with regard to NADPH has been studied in these reactions, i.e. the NADPH-cytochrome *c* reductase, lipid peroxidation, aminopyrine demethylation, testosterone hydroxylation, and the heptane and laurate oxidation reactions. As will be shown the oxidation of NADPH in all these reactions involves the 4*A* hydrogen atom of NADPH.

2. Materials and methods

NADPH labelled with ^3H in the 4*B* position was prepared from D-glucose-1-^3H (New England Nuclear Corp., specific activity 450 millicuries/millimole) by coupling the hexokinase and glucose-6-phosphate dehydrogenase reactions; the latter reaction is known to be 4*B* specific with respect to NADPH [13]. The reaction mixture contained in 1 ml: 100 μmoles tris-HCl buffer, pH 7.5, 10 μmoles $MgCl_2$, 3 μmoles ATP, 0.1 μmole unlabelled $NADP^+$, 0.1 μmole glucose-1-^3H and enough hexokinase and glucose-6-phosphate dehydrogenase to complete the reduction of $NADP^+$ in about 3 minutes at 30°C. The reduction of $NADP^+$ was followed in a Beckman *DK*-2 recording spectrophotometer at 340 mμ. After completion of the reaction, the incubation mixture was heated for one-half minute in boiling water-bath, to destroy enzyme activity, and cooled. Enough solid unlabelled NADPH was then added to the solution to bring the final concentration of NADPH to 0.02 M. The tritiation in the 4*B* position of NADPH resulting from the incubation was controlled by oxidation of the NADPH formed by means of oxidation in the presence of glutathione and glutathione reductase, which involves the 4*B* hydrogen atom of NADPH

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[13]. NADPH-4*A*-^{3}H was prepared from NADPH-4*B*-^{3}H by first oxidizing the latter with cytochrome *c* in the presence of microsomes (thereby removing specifically the 4*A* hydrogen atom; *cf.* table 1), and then reducing the NADP-^{3}H so formed with unlabelled glucose, in the presence of hexokinase, ATP and glucose-6-phosphate dehydrogenase. The detailed procedure was as follows: NADPH-4*B*-^{3}H, prepared as described above, was oxidized to completion by incubation with rat-liver microsomes (ca. 0.1 mg protein) and cytochrome *c* (0.016 mM) at 30°C for 10 minutes in the presence of 10 mM nicotinamide to prevent the degradation of the oxidized labelled $NADP^+$. To keep cytochrome *c* oxidized, cytochrome oxidase was added in the form of submitochondrial particles (0.05 mg protein) [14]. Following dilution of the reaction mixture with an equal volume of distilled H_2O, the suspension was heated for one-half minute in a boiling water bath and centrifuged. The labelled $NADP^+$ of the supernatant was reduced to NADPH-4*A*-^{3}H by the addition of 2 mM unlabelled glucose, 2 mM ATP and enough hexokinase and glucose-6-phosphate dehydrogenase to complete the reaction in about 3 minutes. When the reaction was completed, the incubation mixture was again heated for one-half minute in a boiling water bath to destroy enzyme activity and then cooled. Solid, unlabelled NADPH was added to bring the final concentration of NADPH to 0.01 M.

Rat liver microsomes were prepared as described by Ernster et al. [15]. NADPH-cytochrome *c* reductase activity was measured at 550 mμ in a Beckman *DK*-2 recording spectrophotometer. NADPH oxidase, lipid peroxidation, aminopyrine demethylation, testosterone hydroxylation and heptane and laurate oxidation activities were assayed by measuring the stimulation of NADPH oxidation, caused by the addition of Fe^{+++}-ADP or substrate recorded in an Eppendorf fluorimeter as described by Estabrook and Maitra [16]. At different periods of time after starting the reaction, the samples were frozen in a dry ice-acetone mixture, and water was isolated by sublimation as previously described [17]. A portion of the isolated water was used for counting radioactivity with a Beckman model DPM-100 liquid scintillation counter.

Table 1
Stereospecificity of the NADPH-cytochrome *c* reductase of rat-liver microsomes.

Additions	Percent NADPH oxidized	Percent ^{3}H in H_2O
NADPH-4*A*-^{3}H	45	56
NADPH-4*B*-^{3}H	45	1

The incubation mixture contained in a final volume of 3 ml: 150 μmoles Tris-HCl, pH 7.5; 10 μmoles $MgCl_2$; 100 μmoles KCl; 0.2 μmole NADPH (containing ^{3}H in position 4*A* or 4*B*), 5 μmoles KCN; 0.2 μmole cytochrome *c* and 1 mg of microsomal protein. The reduction of cytochrome *c* was followed spectrophotometrically. The temperature was 30°C.

3. Results and comments

Table 1 shows that in the presence of cytochrome *c* and rat-liver microsomes there occurred a substantial detritiation of NADPH-4*A*-^{3}H, and essentially no detritiation of NADPH-4*B*-^{3}H. Although only about one-half of the added NADPH was oxidized during the reaction, the percentage of added NADPH detritiated was roughly equal to that oxidized which suggests that there was no marked isotope effect. However, there was a striking isotope effect in the experiments where the liver-microsomal lipid peroxidation activity was studied. As demonstrated in table 2, during Fe^{+++}-ADP-stimulated lipid peroxidation there was a significant detritiation of NADPH-4*A*-^{3}H although the percentage of detritiation was less than that of NADPH oxidation. This finding indicates that the actual removal of hydrogen from NADPH was rate-limiting. With NADPH-4*B*-^{3}H there was no significant detritiation during the course of the lipid peroxidation reaction.

Upon aerobic incubation of liver microsomes in the presence of NADPH there occurs an oxygen uptake with a concomitant oxidation of NADPH also in the absence of any added hydroxyl acceptor [18]. This reaction has been termed the "NADPH oxidase" of liver microsomes. Its activity is partially inhibited by carbon monoxide, and the CO-insensitive portion of it occurs also under anaerobic conditions [2]. Although the NADPH oxidase reaction has been shown to lead to the formation of hydrogen peroxide [18], the exact nature of the reaction thus

Table 2
Stereospecificity of enzymic lipid peroxidation of rat-liver microsomes.

Additions	Percent NADPH oxidized	Percent ^3H in H_2O
NADPH-4*A*-^3H	25	8
NADPH-4*A*-^3H + Fe^{+++}-ADP	50	21
NADPH-4*B*-^3H	16	5
NADPH-4*B*-^3H + Fe^{+++}-ADP	43	7

The incubation mixture contained in a final volume of 3 ml: 150 μmoles Tris-HCl, pH 7.5; 10 μmoles $MgCl_2$; 100 μmoles KCl; 0.1 μmole NADPH (containing ^3H in position 4*A* or 4*B*); 0.04 μmole Fe^{+++} and 10 μmoles ADP when indicated; and 1 to 2 mg of microsomal protein. NADPH oxidation was followed fluorometrically [16]. The time of incubation was two minutes at 30°C.

remains unclear. When the NADPH oxidase was tested with regard to NADPH stereospecificity there was a marked detritiation upon incubation with the NADPH-4*A*-^3H preparation, whereas there occurred a much less marked detritiation of the 4*B* analogue. Upon addition of various hydroxyl-acceptors, including aminopyrine, testosterone, laurate and heptane, to the microsomes incubated in the presence of NADPH-4*A*-^3H both NADPH oxidation and detritiation were equally stimulated (table 3).

An interesting feature is that like most 4*A*-specific NADH-oxidizing enzymes [13,19] the NADPH-cytochrome *c* reductase does not catalyze an exchange of hydrogen atoms between the reduced pyridine nucleotide and water.

The present results show that the liver-microsomal NADPH-cytochrome *c* reductase, NADPH-linked lipid peroxidation, aminopyrine demethylation, testosterone hydroxylation, heptane oxidation and ω-oxidation of laurate, as well as the major part of the NADPH oxidase, activities are all 4*A*-specific with regard to NADPH oxidation. The findings further support the idea that a common NADPH-oxidizing flavoenzyme – the NADPH-cytochrome *c* reductase – is involved in all these reactions, a hypothesis which is also supported by recent immunological evidence [20]. However, the final proof of this hypothesis must await the isolation and reconstruction of the liver-microsomal hydroxylating enzyme system.

Table 3
Stereospecificity of some NADPH-linked hydroxylation reactions in rat-liver microsomes.

Additions	Percent NADPH oxidized	Percent ^3H in H_2O
NADPH-4*A*-^3H	52	57
NADPH-4*A*-^3H + aminopyrine	77	78
NADPH-4*A*-^3H + testosterone	65	68
NADPH-4*A*-^3H + laurate	65	77
NADPH-4*A*-^3H + heptane	65	77
NADPH-4*B*-^3H	38	10
NADPH-4*B*-^3H + aminopyrine	60	10
NADPH-4*B*-^3H + testosterone	67	14
NADPH-4*B*-^3H + laurate	48	18
NADPH-4*B*-^3H + heptane	76	15

The incubation mixture contained in a final volume of 3 ml: 150 μmoles Tris-HCl, pH 7.5; 10 μmoles $MgCl_2$; 100 μmoles KCl; 0.2 μmole NADPH (containing ^3H in position 4*A* or 4*B*); and 3 mg of microsomal protein. When indicated 2.5 μmoles aminopyrine, 0.06 μmole testosterone, 1.5 μmoles Na-laurate or 1 μmole heptane was added. NADPH oxidation was followed fluorometrically [16]. The time of incubation was 3 minutes and the temperature 30°C.

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