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EFFECT OF HYDROGEN PEROXIDE ON INACTIVATION OF CYTOCHROME P-450

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In rabbit liver microsomes cytochrome P-450 is inactivated in reactions of hydroxylation of substrates I (dimethylalanine, amidopyrine, p-nitroanisole) and II (aniline) types. Inactivation of the enzyme is potentiated in the presence of the catalase inhibitor NaN_3 [1, 3]. This indicates the possibility of damage to the hemoprotein by hydrogen peroxide (H_2O_2), formed in the NADPH-dependent hydroxylase cycle. Inactivation of cytochrome P-450 is accompanied by its decolorization. Inactivation of other electron-carrier enzymes under these same conditions has not been found [4].

The aim of this investigation was to study inactivation of purified and isolated cytochrome P-450 LM_2 under the influence of H_2O_2.

EXPERIMENTAL METHOD

Reagents. The sodium dihydrogen phosphate, phenobarbital sodium, and NaN_3 were obtained from Merck (West Germany), catalase, glucose, β-mercaptoethanol, and sodium dodecylsulfate were from Serva (West Germany), the 5,5-dithio-bis-(2-nitrobenzoic) acid was from Boehringer (West Germany), and emalgen 13 was from Kao-Atlas (Japan). The remaining reagents were of highly pure grade.

Cytochrome P-450 LM_2 was isolated from liver microsomes of rabbits treated with phenobarbital [2]. To remove the dithiothreitol from the preparation of cytochrome P-450, dialysis was carried out overnight at 4°C against 100 mM sodium-phosphate buffer (pH 7.4).

The concentration of cytochrome P-450 was measured on Hitachi-557 and HP-85 spectrophotometers using a two-wave system, by the method in [7].

The glucose-oxidase system (GOS) generating H_2O_2 contained 100 mM sodium-phosphate buffer (pH 7.4), 110 mM glucose, and 1 μM cytochrome P-450. The reaction was started by addition of glucose oxidase (0.18 U/ml) with activity of 93,200 U/g at 37°C. The concentration of glucose and glucose oxidase were chosen so that the rate of H_2O_2 generation corresponded to the rate of its generation in the NADPH-dependent hydroxylase cycle. The H_2O_2 concentration was determined by the thiocyanate method [10]. The content of SH-groups in the protein molecule was determined with the aid of 5,5-dithio-bis-(2-nitrobenzoic) acid [5].

The aggregate state of the cytochrome P-450 was studied by gel-penetrating high efficiency liquid chromatography on TSK-G 3000 SW columns (KLB, Sweden) and also by gel-filtration on TSK-CELTOYOPEARL HW-60 (Toyo Soda, Japan).

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TABLE 1. Content of SH-Groups in Protein Molecule on Inactivation of Oligometric Cytochrome P-450 by GOS (M ± m)

Duration of inactivation, h	Without substrate			0.3 mM benzphetamine		
	A	per cent inactivation	B	A	per cent inactivation	B
0	6,2±0,4	0	6,2	6,2±0,4	0	6,2
3	2,8±0,2	54	6,1	3,6±0,3	40	6,0
5	1,7±0,1	72	6,1	2,9±0,2	53	6,1

TABLE 2. Content of SH-Groups in Protein Molecule on Inactivation of Monomerized Cytochrome P-450 under the Influence of 400 μM H_2O_2 (M ± m)

Duration of inactivation, h	Without substrate			0.3 mM benzphetamine		
	A	per cent inactivation	B	A	per cent inactivation	B
0	6,2±0,4	0	6,2	6,2±0,4	0	6,2
3	1,5±0,15	49	4,0	2,6±0,2	31	4,3
5	0	68	0	1,5±0,15	47	3,8

Legend. Here and in Table 2: A) number of SH-groups per nmole P-450 initially; B) after inactivation.

Monomerized cytochrome P-450 was obtained by incubation of the hemoprotein with 0.025% emalgen 913 overnight at 4°C [4].

EXPERIMENTAL RESULTS

To study inactivation of cytochrome P-450 changes in the content of the hemoprotein added to the H_2O_2-generating GOS were measured depending on time and the H_2O_2 concentration. H_2O_2 accumulation was found to be accompanied by a marked inactivating effect on cytochrome P-450. Inactivation of the enzyme was not accompanied by an increase in absorption of its complex with CO at 420 nm, evidence of the absence of cytochrome P-420 formation during inactivation of cytochrome P-450, and it agrees with results obtained previously [3]. Table 1 gives data on the content of SH-groups in the protein molecule on inactivation of the hemoprotein in GOS. As Table 1 shows, inactivation of cytochrome P-450 was accompanied by a decrease in the content of SH-groups. Benzphetamine — a specific substrate for this isozyme, used in a concentration of 0.3 mM, gave only a very weak effect, reducing inactivation of cytochrome P-450 and oxidation of SH-groups. In the presence of the substrate, one fewer SH-group in cytochrome P-450 was oxidized.

The study of the aggregate state of cytochrome P-450 showed that inactivation of the enzyme is accompanied by a change in its oligomeric state. Toward 7 h of inactivation, when the cytochrome P-450 concentration is 15-20% of the initial concentration, much of it is in the form of aggregates, with a molecular weight of 1000 kilodaltons or more. Cytochrome P-450 not subjected to the action of H_2O_2 was not inactivated and did not change its oligomeric state.

The results raised two problems: first, to explain whether aggregate formation in the presence of H_2O_2 is the result of formation of covalent molecular cross-linkages, and second, to select conditions of inactivation of cytochrome P-450 without any change in its aggregate state.

The first problem was tackled in experiments in which aggregated cytochrome P-450 was treated with 1% sodium dodecylsulfate (SDS) and 5% β-mercaptoethanol at 100°C for 3 min, and then chromatographed in 0.1% SDS [6]. It was found that the quantity of aggregates was reduced but they did not disappear completely. This is evidence that in the process of inactivation of cytochrome P-450 by the action of H_2O_2, covalent intermolecular cross-linkages are formed.

To study the possibility of inactivation of cytochrome P-450 without any change in its aggregate state, the monomerized hemoprotein was used. The view that aggregate formation is delayed during inactivation of monomerized cytochrome P-450 in a GOS was confirmed. However, it was also found that the control sample of monomerized cytochrome P-450 at 37°C is partially converted into the inactive form, namely cytochrome P-420. Thus in a monomerized system, by contrast with the oligomeric state of the enzyme, thermal inactivation of the hemoprotein takes place. To distinguish between the process of thermal inactivation and inactivation by H_2O_2, experiments were carried out to study the effect of temperature on monomerized cytochrome P-450 in the absence of H_2O_2. It was found that at temperatures of 20°C and below the content of the enzyme was unchanged and it was not converted into the P-420 form. All subsequent investigations of inactivation of the monomerized hemoprotein were therefore carried out at 20°C.

Investigation of inactivation of monomerized cytochrome P-450 in increasing concentrations of H_2O_2 showed that the rate of inactivation of the enzyme was maximal with H_2O_2 in a concentration of 400 μM. Meanwhile, the aggregate state of the enzyme was unchanged by this concentration of H_2O_2. Consequently, in order to obtain inactivation of cytochrome P-450 without any change in its aggregate state, the investigation of inactivation of the monomerized enzyme had to be carried out in the presence of 400 μM H_2O_2 at 20°C. To inhibit the catalase activity of the hemoprotein, 1 mM NaN_3 was added to the incubation mixture consisting of 1 μM cytochrome P-450 in 100 mM sodium phosphate buffer with 0.025% emalgen 913 and 400 μM H_2O_2. Under these conditions the monomerized cytochrome P-450 was inactivated in the course of 5 h. Data on the change in content of SH-groups in the protein molecule during inactivation are given in Table 2. It will be clear from Table 2 that SH-groups in the enzyme molecule could not be detected after 3 h of inactivation. Benzphetamine, as in the case of oligomeric cytochrome P-450, has a protective effect, reducing inactivation of the monomerized hemoprotein and oxidation of SH-groups.

The results are thus evidence that purified, isolated cytochrome P-450 LM_2 is inactivated by H_2O_2. The inactivation is accompanied by a decrease in the content of SH-groups in the protein molecule. Monomerized cytochrome P-450 is more strongly susceptible to the inactivating action of H_2O_2. This can be explained by the greater accessibility of the protein molecules for H_2O_2 in the case of the monomerized enzyme than with its oligomeric form.

It is a noteworthy fact that under the influence of H_2O_2 the aggregate state of cytochrome P-450 can be changed. These changes take place as a result of the formation of covalent intermolecular cross-linkages. This is in agreement with data in the literature on the effect of H_2O_2 on proteins and enzymes [9, 10].

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