GENERATION OF SUPEROXIDE ANION AS A SOURCE OF HYDROGEN PEROXIDE IN A RECONSTITUTED MONOOXYGENASE SYSTEM

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1. Introduction

It was first reported by Gillette et al. [1] and later confirmed by Thurman et al. [2], Hildebrandt et al. [3] and Estabrook et al. [4] that liver microsomes generate hydrogen peroxide during NADPH-oxidation. The stimulating effect of phenobarbital pretreatment as well as a partial inhibition by carbon monoxide suggested the involvement of the cytochrome P450-dependent drug monooxygenase system. Since microsomes in the presence of NADPH have been suggested to form superoxide anions [5–7] the disproportionation of these radicals either nonenzymatically or catalyzed by superoxide dismutase would most likely be the source of hydrogen peroxide. Only indirect evidence exists for the mechanism of O_2^--formation and for its biological significance. Ullrich et al. [8] used high concentrations of azide to dissociate the cytochrome P450-oxycomplex [9] thereby inhibiting the monooxygenase activity and increasing the relative amount of hydrogen peroxide. Sasame et al. [6] first suggested a dissociation of the oxycomplex of cytochrome P450 as a source of O_2^-, but their experiments were based on the adrenochrome test, which generates O_2^- by itself and could be subject to inhibition by NADH. In a recent paper Auclair et al. [7] found an inhibition of superoxide dismutase-sensitive cytochrome *c* reduction by the cytochrome P450 inhibitor metyrapone, but did not succeed in demonstrating an increase in O_2^--formation by substrates as would be expected. It seemed to us that a reconstituted monooxygenase system would be a suitable model to study the source of O_2^- since it lacks superoxide dismutase activity but generates hydrogen peroxide like the microsomal system [10].

Abbreviations: *P*-450, cytochrome *P*-450 from phenobarbital pretreated pigs; F_{pI}, NADPH-cytochrome *P*-450 reductase; SOD, superoxide dismutase; XOD, xanthine oxidase; metyrapone, 2-methyl-1,2-bis-(3-pyridyl)-1-propanone.

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2. Materials and methods

All products were commercially available. Metyrapone was kindly donated by Ciba-Geigy, Basel, Switzerland.

Succinylation of cytochrome *c* was performed by a modified procedure of Takemori et al. [11]. 7-Ethoxycoumarin was synthesized and used as the substrate for O-dealkylation as described earlier [12].

Partially purified NADPH-cytochrome P450 reductase and cytochrome P450 were prepared according to Yasukochi and Masters [13] and Imai and Sato [14], respectively. A typical assay contained 0.9 U reductase (cytochrome *c* reduced) (36 U/mg protein), 0.45 μM cytochrome P450

(6 nmol/mg protein) and 5×10^{-4} M NADPH in 0.65 ml 50 mM phosphate buffer pH 7.6 containing 0.2 mM EDTA. Superoxide anions were determined by two methods: (i) the SOD-sensitive reduction by O_2^- of succinylated cytochrome *c*, similar to the procedure with acetylated cytochrome *c* [15]; (ii) by formation of compound III of lactoperoxidase as indicated by the absorbance increase at 589 nm [16]. Hydrogen peroxide formation was followed by the scopoletin test [17]. Calibration curves for recovery were performed by adding H_2O_2 to the corresponding systems. The H_2O_2 stock solution (1 mM) was standardized at 240 nm using a molar absorption coefficient of 43.6 M^{-1} cm^{-1} [18]. Values are given as means ± SEM from 3–4 experiments.

3. Results and discussion

Succinylated cytochrome *c* was suitable as an electron acceptor for O_2^- in microsomal monooxygenase systems, since it reacted about 200-fold slower with NADPH-cytochrome P450 reductase compared with native cytochrome *c*. In contrast, the efficacy of reduction by O_2^- was 38% of native cytochrome *c*. The rate of inhibition of reduction of succinylated cytochrome *c* by SOD was attributed to superoxide anion. Increasing the amount of SOD did not inhibit further, indicating that all O_2^--ions are disproportionated under these conditions. The trapping of O_2^-, however, by 20 μM succinylated cytochrome *c* was not quantitative as can be seen from the increase of the reduction rate with pH (fig.1). This was not due to an enhanced electron transport since the SOD-insensitive NADPH-succinylated cytochrome *c* reductase activity and the O-dealkylase activity at pH 8.5. In order to obtain a correction factor the reduction of succinylated cytochrome *c* was measured in a xanthine oxidase–xanthine system in which the full rate of O_2^- formation was calculated from the rate of reduction of native cytochrome *c* under saturating conditions.

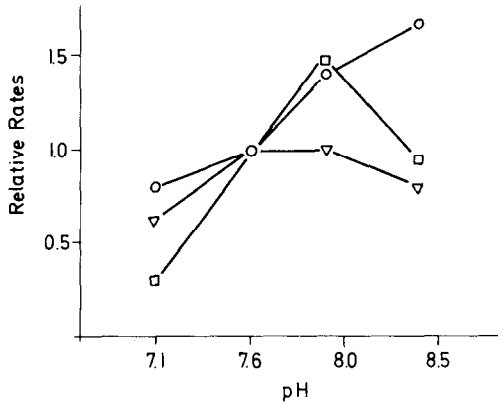

Fig.1. pH-profiles for O_2^--mediated succinylated cytochrome *c* reduction in comparison with NADPH-dependent reductase and O-dealkylation activity. Assay conditions: 0.9 U F_{pI}, 0.45 μM P450, 21 μM succinylated cytochrome *c* in 0.65 ml 50 mM phosphate buffer, pH as indicated. ○—○, O_2^--formation; △—△, O-dealkylation (7-ethoxycoumarin); □—□, cytochrome *c* reduction.

A second test for O_2^--generation based on compound III formation of lactoperoxidase [16] was also employed. In this system the calibration by the XOD-method proved unsuccessful and therefore the numbers only reflect the relative rates of O_2^--formation. Table 1 contains the values obtained for the O-dealkylation of 7-ethoxycoumarin, for the O_2^--formation as determined by the two methods and for hydrogen peroxide formation. The autoxidation of the cytochrome P450 reductase in the presence of NADPH caused a basal value of O_2^- and H_2O_2-production. The presence of cytochrome P450 and substrate greatly increased both rates. Carbon monoxide completely inhibited this increase, which clearly points to the involvement of P450. Metyrapone also caused an inhibition to a similar extent as for the O-dealkylation reaction. If one subtracts the basal rates of O_2^-- and H_2O_2-formation mediated by the reductase from the values in the complete system a ratio of 3.15/1.87 is obtained. Considering the errors of the methods one can conclude that the cytochrome P450-mediated H_2O_2 formation proceeds entirely via O_2^--radicals. These can only originate from a dissociation of the oxycomplex. The second mechanism of O_2^--formation, by autoxidation of the flavoprotein, may depend on the integrity of the isolated flavoprotein and may be of lesser significance under physiological conditions. The dissociation of the oxycomplex seems to represent a major pathway of O_2^-- and H_2O_2-formation in the cell. It should be noted, that this oxidase function of P450 must be differentiated from the 'uncoupling' phenomenon [19], in

Table 1
Comparison of the rates of 7-ethoxycoumarin O-dealkylation, O_2^--formation and hydrogen peroxide formation in a reconstituted monooxygenase system

	7-Ethoxycoumarin-dealkylation	O_2^--formation detected by		H_2O_2-formation
		succinylated cyt. *c*	lactoperoxidase	
F_{pI} + substrate	0	3.91 ± 0.21	1.31 ± 0.20	0.46 ± 0.09
F_{pI} + P450	0	4.79 ± 0.62	1.25 ± 0.15	0.91 ± 0.15
F_{pI} + P450 + substrate	0.88	7.10 ± 0.45	3.24 ± 0.23	2.33 ± 0.19
F_{pI} + P450 + substrate + CO/O_2 (80 : 20, v/v)	0	3.92 ± 0.30	1.43 ± 0.04	0.95 ± 0.08
F_{pI} + P450 + substrate + 10 μM metyrapone	0.26	5.40 ± 0.32	–	–

All assays were performed in 50 mM K-phosphate buffer pH 7.6, containing 0.2 mM EDTA in a total volume of 0.65 ml. T = 294°K. P450, 0.45 μM; F_{pI}, 0.9 U (cytochrome *c* reduced), 0.14 mM NADPH. For O_2^--detection 21 μM succinylated cytochrome *c* and 3.6 μM lactoperoxidase were present, respectively. 2 μM SOD was added after 2 min. Since formation of compound III of lactoperoxidase could be inhibited by SOD to only 92% a corresponding correction factor was introduced for these values. For the CO-inhibition the substrate–buffer solution was pregassed with the gas mixture for 10 min, followed by 4 min gassing of the complete assay before addition of NADPH. All activities are expressed as nmol/min · nmol P450 ± SEM

which the active oxygen complex is reduced to water without hydrogen peroxide formation.

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