EVIDENCE FOR SUPEROXIDE GENERATION BY NADPH-CYTOCHROME C REDUCTASE OF RAT LIVER MICROSOMES[1]

S.D. Aust[2], D.L. Roerig[3] and T.C. Pederson

Department of Biochemistry
Michigan State University
East Lansing, Michigan 48823

Received April 28, 1972

Rat liver microsomes are capable of catalyzing an NADPH-dependent oxidation of epinephrine to adrenochrome that is inhibited by superoxide dismutase. Activity is greater and more sensitive to inhibition by superoxide dismutase at pH 8.5 than pH 7.7. The epinephrine oxidation activity copurifies with NADPH-cytochrome c reductase.

Two analytical tools have made it possible to investigate the generation of the superoxide anion. One is an assay based on the oxidation of epinephrine to adrenochrome by the superoxide anion (1). The second is the discovery of the superoxide dismutase activity of erythrocuprein by McCord and Fridovich (2). Massey et al. (3) have used superoxide dismutase to study the oxygen dependent reduction of cytochrome c by numerous flavoproteins.

Interest in superoxide generation by liver microsomes comes from the fact that microsomes catalyze an NADPH-dependent oxygen uptake in the absence of substrate (4, 5) and Strobel and Coon (6) have demonstrated benzphetamine hydroxylation and ethylmorphine demethylation using a superoxide generator and a solubilized, partially purified preparation of

---

[1] This study was supported in part by EPA Grant No. 8 R01 EP00801 and NIH Training Grant GM 1091 from the National Institute of General Medical Sciences.

[2] To whom inquiries should be addressed.

[3] Present address: Clinical Pharmacology Services, Wood V.A. Hospital, Wood, Wisconsin 53193.

cytochrome P_{450} (7, 8). This communication presents evidence for superoxide generation by rat liver microsomes. The superoxide generation activity copurifies with NADPH-cytochrome c reductase.

METHODS AND MATERIALS: Microsomes were isolated from the livers of control rats and from rats pretreated with phenobarbital as previously described (9) and washed once in 0.1 M pyrophosphate and 0.3 M sucrose (10). NADPH-cytochrome c reductase was solubilized with Bromelain (Dole Company, San Jose, Calif.; 10 μg/mg microsomal protein, 16 hrs at 4° under N_2) and purified and assayed by the methods of Omura and Takesue (11). The freshly purified enzyme would reduce 31 μmoles of cytochrome c/min/mg protein, based on ϵ_{550} (reduced-oxidized) for cytochrome c as $2.10 \cdot 10^4$ cm^2/mmole (12). Epinephrine oxidation was assayed by the method of Mazur et al. (1). Cytochrome c (beef heart, Type IV), NADPH, xanthine oxidase (Grade I from buttermilk), xanthine, nitro blue tetrazolium, and epinephrine were obtained from Sigma Chemical Co.; superoxide dismutase was obtained from Miles Laboratories, Inc.

RESULTS: Neither anaerobiosis nor superoxide dismutase had a significant effect on the NADPH-dependent reduction of cytochrome c catalyzed by rat liver microsomes. Anaerobiosis and superoxide dismutase also had no effect on nitro blue tetrazolium reduction by microsomes. However, microsomes do catalyze an NADPH-dependent oxidation of epinephrine to adrenochrome (Fig. 1). Activity is dependent on the concentration of microsomes and is destroyed by boiling. The assays were characterized by an unexplained lag. The rate of oxidation was higher at pH 8.5 than at pH 7.7. No oxidation could be observed at pH 6.5. Also the sensitivity to superoxide dismutase was highest at the higher pH (Fig. 1). At pH 8.5, 2.5 μg/ml superoxide dismutase inhibited 55% of the oxygen dependent reduction of cytochrome c by xanthine oxidase and xanthine. In the presence of 25 μg/ml superoxide dismutase the oxidation of epinephrine by microsomes (28 μg/ml, pH 8.5) was inhibited 97%. Boiled dismutase had no effect on epinephrine oxidation.

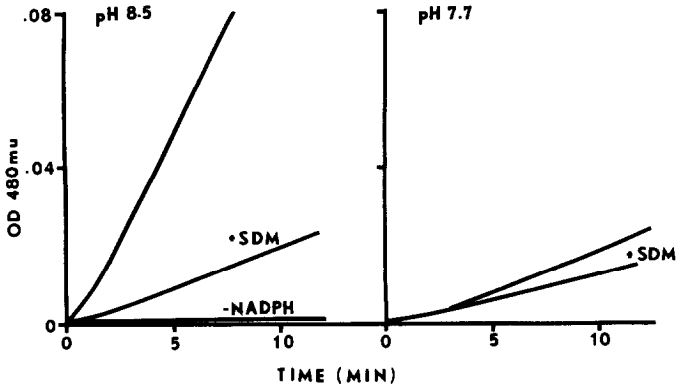

Fig. 1. Oxidation of epinephrine to adrenochrome by rat liver microsomes. Reaction mixtures contained 28 μg/ml microsomal protein, 5 x 10^{-4} M epinephrine, 1 x 10^{-4} M EDTA and 1 x 10^{-4} M NADPH buffered at pH 7.7 or 8.5 in 0.15 M potassium phosphate. Microsomes were isolated from the livers of phenobarbital treated male rats. Optical density was recorded in a Coleman model 124 spectrophotometer at 480 mμ. Superoxide dismutase (SDM) was added at 5 μg/ml.

NADPH-cytochrome c reductase was solubilized with Bromelain and purified by the method of Omura and Takesue (11) and assayed for its ability to generate superoxide and reduce cytochrome c. When all assays were conducted at the same pH and ionic strength, the ratio of cytochrome c reductase to superoxide generation (as judged by ability to oxidize epinephrine) remained constant throughout purification (Table 1). In

Table 1. NADPH-dependent reduction of cytochrome c and oxidation of epinephrine by microsomes and NADPH-cytochrome c reductase isolated from the livers of phenobarbital treated rats. The NADPH-dependent reduction of cytochrome c (11) and the oxidation of epinephrine (1) were assayed in a Coleman Model 124 spectrophotometer at the indicated wavelength in 0.15 M phosphate buffer, pH 8.5, 22°C.

Enzyme	A. Cytochrome c reduction μmoles/min/mg	B. Epinephrine oxidation μmoles/min/mg	A/B
Microsomes	0.2	0.07	2.85
Enzyme from first DEAE column	7.1	2.5	2.84
Enzyme from second DEAE column	16.0	4.75	3.16

addition the ratio of NADPH-cytochrome c reduction to epinephrine oxidation in microsomes isolated from the livers of control rats was identical to that obtained with microsomes from the liver of rats that had been pretreated with phenobarbital (Table 2).

Table 2. NADPH-dependent reduction of cytochrome c and oxidation of epinephrine by microsomes isolated from the livers of control and phenobarbital treated rats. The NADPH-dependent reduction of cytochrome c (11) and the oxidation of epinephrine (1) were assayed in a Coleman Model 124 spectrophotometer at the wavelengths indicated in 0.15 M phosphate buffer, pH 8.5, 22°C.

Microsomes	A. Cytochrome c reduction μmoles/min/mg	B. Epinephrine oxidation μmoles/min/mg	A/B
Control	0.11	0.04	2.75
Induced	0.21	0.075	2.80

DISCUSSION: Evidence has been presented for an NADPH-dependent generation of superoxide by rat liver microsomes. In the presence of NADPH microsomes are capable of oxidizing epinephrine to adrenochrome. Oxidation activity is extremely sensitive to pH, being more active at high pH, as expected since the non-enzymatic dismutation of the superoxide anion occurs more rapidly as the pH is lowered (13). Also the sensitivity to inhibition by superoxide dismutase is higher at high pH.

Epinephrine oxidation activity copurifies with NADPH-cytochrome c reductase. Also, the ratio of NADPH-cytochrome c reduction to epinephrine oxidation in microsomes isolated from the livers of control animals and animals pretreated with phenobarbital remains constant. These results suggest that the superoxide generation can be ascribed to NADPH-cytochrome c reductase.

There has been some question as to the identity of the flavoprotein in the microsomal hydroxylation system. Lu, Junk, and Coon (14) found that their detergent-solubilized NADPH-cytochrome c reductase would support laurate hydroxylation when combined with their cytochrome P-450 fraction

in the presence of lipid, but that the pure, lipase-solubilized reductase of Williams and Kamin would not. However, Ichihara et al. (15) found that a reductase fraction that would support laurate hydroxylation and reduce cytochrome c would only reduce cytochrome c after brief incubation with trypsin. Several investigators have shown that antisera against purified NADPH-cytochrome c reductase will inhibit hydroxylations by intact microsomes (15-17). In collaboration with Dr. M. J. Coon we have found that the enzyme used in this study will anaerobically reduce cytochrome P-450, but will not substitute for the detergent-solubilized reductase in drug hydroxylation. The enzyme will also catalyze the peroxidation of phospholipids (19).

ACKNOWLEDGMENT: We are grateful to Dr. M. J. Coon and H. W. Strobel for collaborative experiments involving cytochrome P_{450} reductase assays.

REFERENCES:

1. Mazur, A., Green, S. and Shorr, E., J. Biol. Chem., 220, 227 (1956).
2. McCord, J.M. and Fridovich, I., J. Biol. Chem., 244, 6049 (1969).
3. Massey, V., Strickland, S., Mayhew, S.G., Howell, L.G., Engel, P.C., Matthews, R.G., Schuman, M., and Sullivan, P.A., Biochem. Biophys. Res. Commun., 36, 891 (1969).
4. Orrenius, S., Ericsson, J.L.E. and Ernster, L., J. Cell Biol., 25, 627 (1965).
5. Orrenius, S., J. Cell Biol., 26, 713 (1965).
6. Strobel, H.W. and Coon, M.J., J. Biol. Chem., 246, 7826 (1971).
7. Lu, A.Y.H., Strobel, H.W. and Coon, M.J., Biochem. Biophys. Res. Commun., 36, 545 (1969).
8. Lu, A.Y.H., Strobel, H.W., and Coon, M.J., Mol. Pharmacol., 6, 213 (1970).
9. Pederson, T.C. and Aust, S.D., Biochem. Pharmacol., 19, 2221 (1970).
10. Sachs, H., Biochim. Biophys. Acta, 21, 188 (1956).
11. Omura, T. and Takesue, S., J. Biochem., 67, 249 (1970).
12. Massey, V., Biochim. Biophys. Acta, 34, 255 (1959).
13. Knowles, P.F., Gibson, J.F., Pick, F.M. and Bray, R.C., Biochem. J., 111, 53 (1969).
14. Lu, A.Y.H., Junk, K.W. and Coon, M.J., J. Biol. Chem., 244, 3714 (1969).
15. Ichihara, K., Kusunose, E. and Kusunose, M., FEBS Letters, 20, 105 (1972).
16. Wada, F., Shibata, H., Goto, M. and Sakamoto, Y., Biochim. Biophys. Acta, 162, 518 (1968).
17. Glazer, R.I., Schenkman, J.B. and Sartorelli, A.C., Mol. Pharmac., 7, 683 (1971).
18. Masters, B.S.S., Baron, J., Taylor, W.E., Isaacson, E.L. and LoSpalluto, J., J. Biol. Chem., 246, 4143 (1971).
19. Pederson, T.C. and Aust, S.D., Fed. Proc., 31, 894 (1972).