THE ASSOCIATION BETWEEN CYTOCHROME *P*-450 AND NADPH-CYTOCHROME *P*-450 REDUCTASE IN MICROSOMAL MEMBRANE

Chung S. YANG

Department of Biochemistry, College of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07103, USA

Received 19 March 1975

1. Introduction

The microsomal monoxygenase system catalyzes the biotransformation of fatty acids, steroids, drugs, carcinogens and various other compounds [1]. In this system, the reducing equivalents are transferred from NADPH to cytochrome *P*-450 through a flavoprotein, generally known as NADPH-cytochrome *c* reductase or NADPH cytochrome *P*-450 reductase. In rat liver microsomes, the number of cytochrome *P*-450 molecules is 10–20-fold greater than the reductase [2]. It has been suggested that these enzymes are rigidly organized in individual units of electron transfer complexes with several cytochrome *P*-450 molecules surrounding a reductase molecule [3]. According to current membrane theories [4], it is difficult to envision how the reductase (mol. wt 79 000) [5] can be rigidly associated simultaneously and react efficiently with 10–20 cytochrome *P*-450 molecules (mol. wt 50 000) [6]. Orrenius and Ernster [1] have discussed the possible lateral mobility of these enzymes in the membrane. In the present study, the chemical modification approach of Franklin and Estabrook [3] was used to study the molecular association of these two enzymes in rat liver microsomes. The results do not satisfy the criteria for the rigid organization as proposed previously [3] and indicate that the monoxygenase enzymes may have translational mobility.

2. Materials and methods

Mersalyl (sodium *O*-[(3-hydroxymercuri-2-methoxypropyl)carbamyl] phenoxyacetate) was purchased from Schwarz/Mann Co. NADPH, NADP, isocitric acid, isocitrate dehydrogenase, cytochrome *c*, and bovine serum albumin were purchased from Sigma Chemical Co. Ethylmorphine · HCl and phenobarbital were gifts from Merck and Co. Hepatic microsomes were prepared [7] from male Long-Evans rats (average body weight 75 g) which had been pretreated with three daily intraperitoneal injections of phenobarbital (75 mg/kg). NADPH-cytochrome *c* reductase and ethylmorphine *N*-demethylase activities were assayed as described previously [8]. NADPH-dependent cytochrome *P*-450 reduction was assayed at 30°C in an anaerobic cuvette equipped with side-arms. The cuvette, containing all the reagents was evacuated and refilled with N_2 for eight cycles, and CO was substituted for N_2 finally. Both gases had been deoxygenated with BASF catalyst (Ace Glass, Vineland, N. J.).

3. Results and discussion

Mersalyl is noted for its reactivity with sulfhydryl groups, and NADPH-cytochrome *c* reductase has been suggested as the primary target of its inhibition of the mono-oxygenase system [3]. The effects of mersalyl on the microsomal NADPH-cytochrome *c* reductase and ethylmorphine *N*-demethylase activities are shown in fig.1. A similar pattern of inhibition was observed for both enzymic activities. In agreement with the observation of Franklin and Estabrook [3], the enzyme activities were rather insensitive to lower levels of mersalyl and then responded drastically to further increments of the inhibitor in the concentration range of 25–40 nmol of mersalyl per mg microsomal protein.

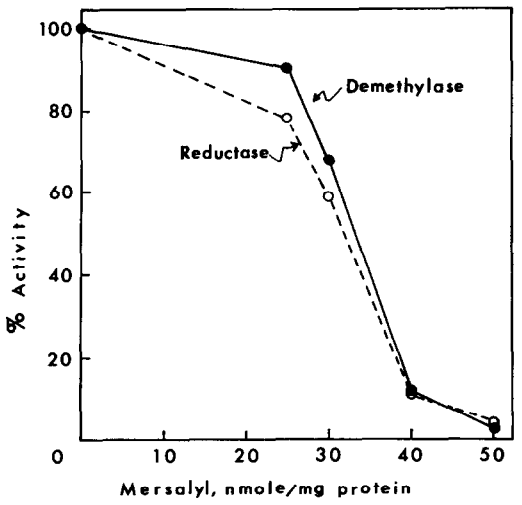

Fig.1. Inhibition of ethylormorphine demethylase and NADPH-cytochrome *c* reductase activities by mersalyl. Microsomes (1 mg protein/ml) were treated with mersalyl at 0°C for 30 min in a medium containing TKM buffer (50 mM Tris · HCl, 150 mM KCl and 10 mM $MgCl_2$, pH 7.4) and 6.5 mM ethylmorphine. The demethylase activity was assayed at 37°C with 0.85 mg microsomal protein per ml and with an incubation period of 15 min [12]. The 100% corresponds to 19 nmol of formaldehyde produced per min per mg of microsomal protein. For the reductase assay, the mersalyl treated microsomes were further incubated at 37°C for 7 min before added to the reductase assay mixture (containing TKM buffer, 1 mM KCN, 0.04 mM cytochrome *c* and 0.2 mM NADPH) to a concentration of 0.05 mg/ml. The initial rate was recorded in the first minute at 37°C. The 100% corresponds to 226 nmol of cytochrome *c* reduced per min per mg microsomal proteins.

The reaction of mersalyl with the monoxygenase enzymes was not instantaneous. Its inhibitory action was affected by factors such as the type and ionic strength of the buffer, the presence or absence of NADPH [3], and the temperature and duration of the treatment. When the incubation conditions of the cytochrome *c* reductase and ethylmorphine demethylase assays were made as similar as possible, mersalyl inhibited both enzyme activities to about the same extent (fig.1), consistent with earlier observations [3].

The observation that mersalyl inhibited both NADPH-cytochrome *c* reductase and ethylmorphine demethylase to approximately the same extent has been used to support the thesis of a rigid association between cytochrome *P*-450 and the reductase [3]. However, the data in fig.1 are also consistent with the concept that these enzymes are not rigidly organized and have lateral mobility in a fluid mosaic membrane [4]. The mersalyl modification is expected to decrease the amount of functional NADPH-cytochrome *P*-450 reductase and thus inhibits the rate of the reduction of cytochrome *P*-450, which is beleived to be the rate-limiting step in the ethylmorphine demethylation reaction [9].

The effects of mersalyl on the rate and extent of the NADPH-dependent cytochrome *P*-450 reduction are illustrated in fig.2. In the absence of mersalyl, the rate of reduction was fast and most of the reaction was accomplished in the first 30 sec. In the presence of 40 or 45 nmol mersalyl/mg microsomal protein, the rate of the reduction was significantly retarded, but almost all of the cytochrome *P*-450 in the system could still be reducted in a period of 20–25 min. The results of some of this series of experiments are summarized in table 1. The different amounts of cytochrome *P*-450 reduced in the first 30 sec reflect the rate of the reduction which was inhibited by mersalyl;

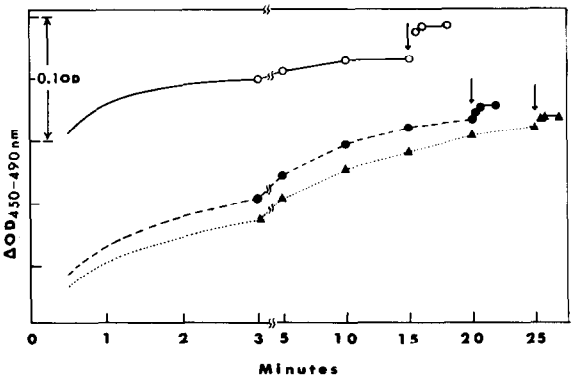

Fig.2. Inhibition of NADPH-cytochrome *P*-450 reductase by mersalyl. Microsomes (1 mg protein/ml) were treated with mersalyl at 0°C for 30 min in a medium containing TKM buffer and 6.5 mM ethylmorphine. After deoxygenation, the reaction was initiated under an atmosphere of CO, by adding NADPH (0.4 mM) to the microsomal suspension in an anaerobic cuvette. The absorbance changes at 450–490 nm was recorded with an Aminco-Chance DW-2 spectrophotometer. The reading was set at the origin at zero time for each experiment. The time between the initial mixing and the first recording was about 30 sec. The arrows indicate the addition of sodium dithionite for the reduction of all the cytochrome *P*-450 in the system. The amounts of mersalyl used in nmol/mg microsomal protein were: 45 (▲ . . . . ▲), 40 (●————●), and 0 (○————○).

Table 1
Effect of mersalyl on the NADPH-dependent reduction of microsomal cytochrome *P*-450 [a]

Mersalyl (nmol/mg)	Reduced in 30 sec (nmol)	% *P*-450 Reducible	*P*-450 content (nmol)
0	4.18	>90[b]	6.59
36	3.43	96	5.14
40	1.04	94	4.81
40[c]	0.69	94	4.45
45	0.82	96	4.56
55	0	0	4.26

a Conditions were similar to those described in fig.2. The *P*-450 contents were determined at the end of the reaction with the method of Omura and Sato [10]. % *P*-450 reducible was calculated from the amount of cytochrome *P*-450 that could be reduced by NADPH and the *P*-450 content.

b Sodium dithionite was added before the NADPH-dependent reduction was complete.

c Microsomes were treated at 0°C for 1.5 hr.

however, mersalyl treatment did not limit the extent of the cytochrome *P*-450 reduction. At 55 nmol/mg, mersalyl inactivated the reductase completely and, hence, no cytochrome *P*-450 could be reduced enzymically. As is shown in the last column of table 1, partial denaturation of cytochrome *P*-450 was also noted under these experimental conditions.

In discussing the molecular organization of the microsomal electron transport system, Franklin and Estabrook [3] have advanced the criterion that in a 'rigid' system the structural organization precludes an accessibility for the interaction between electron carriers from one complex to electron acceptors of another complex; whereas in a 'non-rigid' system, inter-complex electron transport is possible. In a mersalyl treated microsomal sample, when the rate of NADPH-dependent cytochrome *P*-450 reduction is inhibited by 50% as a result of the inactivation of the reductase, the 'rigid' model predicts that only 50% of the cytochrome *P*-450 can be reduced by NADPH. On the other hand, the 'non-rigid' model predicts that all of the cytochrome *P*-450 can be reduced enzymically. The data in fig.2 and table 1 definitely favor the 'non-rigid' over the 'rigid' model. Results obtained from similar chemical modifications studies on the cytochrome b_5 system have lent support to the random distribution and rapid lateral mobility of the cytochrome b_5 system [11]. The observed slow rate of cytochrome *P*-450 reduction in the mersalyl modified sample (fig.2) suggests that cytochrome *P*-450 and its reductase do not have translational movements as fast as have been reported for cytochrome b_5 and cytochrome b_5 reductase [11, 12]. It may be argued that inter-complex electron transfer is also possible in a rigid system, if there is electron transfer reaction between cytochrome *P*-450 molecules. Although this type of electron transfer, if it can occur at all, would not be expected to take place to a great extent in a 'rigid' system, it cannot be completely ruled out at the present time.

In the light of the present observations, we have to consider that a non-rigid oranization of the monooxygenase enzymes in the membrane is quite possible. The rate and extent of their mobilities in the membrane remain to be determined. Cytocrhome *P*-450 is known to be present in large molar excess to NADPH-cytochrome *P*-450 reductase. A non-rigid association and the lateral mobility of these enzymes would enable the reductase to react efficiently with all of the cytochrome *P*-450 molecules and would also allow them to interact with the cytochrome b_5 electron transfer system.

Acknowledgements

This work was supported by USPHS Grant CA-16788. I am grateful to Drs F. P. Guengerich, T. A. van der Hoeven, and M. J. Coon of University of Michigan for the use of their Aminco-Chance DW-2 spectrophotometer, to Mr F. S. Strickhart for his capable technical assistance and to Drs A. Y. H. Lu, R. H. Marks and W. R. Frisell for helpful discussions.

References

[1] Orrenius, S. and Ernster, L. (1974) in: Molecular Mechanisms of Oxygen Activation (Hayaishi, O., ed.) pp. 215–244, Academic Press, New York.
[2] Estabrook, R. W., Hildebrandt, A., Remmer, H., Schenkman, J. B., Rosenthal, O., and Cooper, D. Y. (1969) in: 19th Colloq. Ges. Biol. Chem. (Hess, B. and Staudinger, H., eds.) pp. 142–177, Springer-Verlag, Berlin.
[3] Franklin, M. R. and Estabrook, R. W. (1971) Arch. Biochem. Biophys. 143, 318–329.

[4] Singer, S. J. and Nicolson, G. L. (1972) Science 175, 720–731.
[5] Vermilion, J. L. and Coon, M. J. (1974) Biochem. Biophys. Res. Commun. 60, 1315–1322.
[6] Lu, A. Y. H., West, S. B., Ryan, D., and Levin, W. (1973) Drug Metab. Dispos. 1, 29–39.
[7] Yang, C. S. (1974) Arch. Biochem. Biophys. 160, 623–630.
[8] Yang, C. S. and Strickhart, F. S. (1974) Biochem. Pharmacol. 32, 3129–3138.
[9] Gillette, J. R., Davis, D. C. and Sasame, H. A. (1972) Ann. Rev. Pharmacol. 12, 57–84.
[10] Omura, T. and Sato, R. (1967) Methods Enzymol. 10, 556–561.
[11] Rogers, M. J. and Strittmatter, P. (1974) J. Biol. Chem. 249, 895–900.
[12] Rogers, M. J. and Strittmatter, P. (1974) J. Biol. Chem. 249, 5565–5569.