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KINETIC STUDY ON ENZYMATIC S-OXYGENATION PROMOTED BY A RECONSTITUTED SYSTEM WITH PURIFIED CYTOCHROME P-450

Yoshihito Watanabe*, Takashi Iyanagi** and Shigeru Oae*

*Department of Chemistry, University of Tsukuba, Sakuramura, Niiharigun, Ibaraki 305, Japan

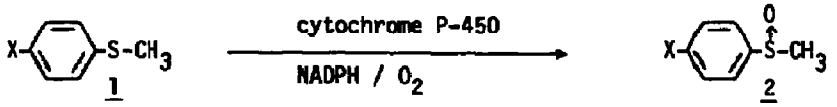
**Division of Biochemistry, School of Medicine, University of Tsukuba, Ibaraki 305, Japan

Abstract; Thioanisole derivatives (1a-e) were found to be oxygenated by a reconstituted system of purified cytochrome P-450 to give sulfoxides (2a-e). Logarithms of Vmax were found to be correlated linearly with one electron oxidation potentials of the sulfides (Ep) suggesting the oxygenation to proceed via one electron transfer from sulfides to the active species of the enzyme.

Enzymatic activities of cytochrome P-450 have drawn considerable attentions in recent years because of its important roles in living bodies.[1] Earlier Hamilton and others postulated as a key species of oxidase[2] an iron bound "oxenoid" which is analogous to Compound I of peroxidase.[3,4] Since peroxidase function of cytochrome P-450 was found,[5] a few more observations have appeared to suport this "oxenoid" intermediate by extensive investigations with enzyme-model system.[6,7] In the enzymatic oxidation of hydrocarbons large primary kinetic isotope effects were observed recently in the hepatic cytochrome P-450 dependent hydroxylations of benzylic position of 1,3-diphenylpropane (k_H/k_D = 11)[8] and norbornane (k_H/k_D = 11.5),[9] which suggest that the rate-determining step of the oxygenation is the hydrogen abstraction from the hydrocarbons with the "oxenoid" intermediate.

Recently we revealed one clear-cut example of one step enzymatic oxygenation of divalent sulfur compounds to the corresponding monooxides by a reconstituted system with purified cytochrome P-450.[10] We also reported that sulfides are good substrates for the oxygenation with cytochrome P-450 enzyme system and oxidized to the corresponding sulfoxides.[11] The same enzymatic system was also found to oxidize acyclic sulfides to optically active sulfoxides by asymmetric oxidation.[11] Since the "oxenoid" intermediate is considered to be highly electron deficient, the S-oxygenation

would be initiated by an electron transfer from a divalent sulfide to the "oxenoid", generating the corresponding sulfide cation radical, as in the oxidative demethylation of aminopyrine with horseradish peroxidase.[13] This paper deals with kinetic evidence to support this electron transfer mechanism for the S-oxygenation of thioanisole derivatives (1a-e) by purified rabbit liver microsomal cytochrome P-450.



(1)

X = a, CH_3O; b, CH_3; c, H; d, Cl; e, NO_2

MATERIALS: Cytochrome P-450 was obtained in purified form from hepatic microsomes of phenobarbital treated rabbit by Imai's method.[14] NADPH-cytochrome P-450 reductase was purified by the method of Iyanagi with some modifications.[15]

KINETIC CONDITIONS: The reconstituted system (1.1 nmole of cytochrome P-450, 1.7 nmole of reductase) was allowed to stand at room temperature for 10 minutes in UV micro-cell, and then was diluted by 0.1 M phosphate buffer (pH 7.7) containing 1 μmole of NADPH and 6.6×10^{-3}% of detergent (Emalgen 913; KAO-ATRAS Chemicals) to 1 ml. After measuring the natural consumption of NADPH by following absorbance at 340 nm due to NADPH, a methanolic solution of substrate (2 - 30 μl, 20 mM) was introduced into the UV micro-cell to initiate the reaction. The oxidation of sulfide was monitored at several time intervals by following the consumption of NADPH.

OTHER MEASUREMENT: Cyclic voltammograms of substrates were obtained as 0.1 mM solution of acetonitrile containing 0.1 M of nBu_4NClO_4 (scane rate; 300, 150 and 50 m volt/sec). Maesurement of the difference spectra caused by the addition of sulfides to the purified cytochrome P-450 was taken place as described in recent papers.[10,11]

When 40 - 600 μM of sulfides (1a-e) were incubated in the reconstituted system with cytochrome P-450, characteristic NADPH consumption was observed. In the oxidation of sulfides by liver microsomal cytochrome P-450 containing NADPH generating system,[10] corresponding sulfoxides were found to be produced as the sole products (analyzed by GLC and TLC), and the co-production of hydroxylated derivatives of aromatic ring was not observed. And this result is in agreement with the recent work.[16] Since a small amount of NADPH was found to be devoured by substrates even with a complete system minus cytochrome P-450, rates of oxygenation were calculated by subtracting the rate of this undesired side reaction from the overall rate of the NADPH consumption. Vmax and Km were calculated from the Lineweaver-Burk plots of the rate

constants of the S-oxygenation, obtained in a linear line, and are listed in the Table. All the sulfides were found to be compounds of typical type I.[17a,b]

Table Comparison of kinetics of oxidation of NADPH in the reconstituted system of purified cytochrome P-450 with one electron oxidation potential (Ep)

substrate	Km (μM)	Vmax (μM/min)	Vmax (nmole/min/nmle P-450)	Ep[a] (volt vs SCE)	σ^+	binding type
1a	63	20	18	1.26	-0.78	I
b	77	19	17	1.41	-0.31	I
c	110	15	14	1.53	0	I
d	135	23	25	1.55	0.11	I
e	31	12	11	1.85	0.79	-[b]

a) Oxidation potentials were measured in 0.1 M of nBu_4ClO_4 / CH_3CN.
b) It is not measurable, see ref. 17b.

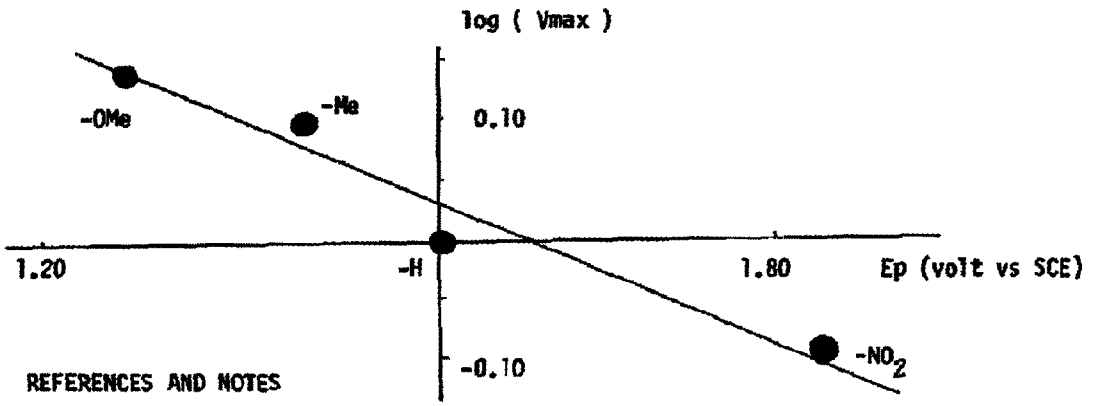
Inspection of the data in Table reveals obviously that Vmax of the oxygenation increases with the increase of electron donating ability of the substituent in accordance with the expected electrophilic nature of the "oxenoid" intermediate.[18] While the rates of the oxidations of these sulfides (1a-e) with hydrogen peroxide are already known to be correlated nicely with Hammett σ-values (ρ= -1.17),[19] Vmax in Table were found to be correlated better with σ^+-values rather than σ-values (ρ^+ = -0.16). These results cannot be explained by simple nucleophilic attack of divalent sulfur atom on the electrophilic centre of the active oxygen species. In order to shed light on the electron transfer mechanism, one electron oxidation potentials of the sulfides were measured by cyclic voltammetry and found to be correlated with σ^+-values. Thus log(Vmax)'s of enzymatic oxidations of the sulfides are correlated linearly with one electron oxidation potentials as shown in Fig. except 1d. These results suggested clearly that the S-oxygenation is initiated by one electron transfer from the divalent sulfide to the "oxenoid" as illustrated below (eq. 2). The rather small substituent effect on the Vmax can be rationalized in term of the extremely facile nature of the one electron transfer process

$$Ar\text{-}\overset{\cdot\cdot}{S}\text{-}CH_3 \xrightarrow[\text{rate determining step}]{\text{cyt. P-450 } (FeO)^{3+},\ e^-} Ar\text{-}\overset{+\cdot}{S}\text{-}CH_3 + (FeO)^{2+} \longrightarrow Ar\text{-}\overset{\overset{O}{\uparrow}}{S}\text{-}CH_3 + Fe^{III} \qquad (2)$$

because of the highly electron-demanding character of the "oxenoid".

An alternative mechanism involving a sulfide dication is unlikely since ^{18}O-labeled oxygen is scarecely incorporated in sulfoxide 2c when 1c was oxygenated by liver microsomes in a phosphate buffer solution using $H_2{}^{18}O$.[20]

Figure Relationship between Ep and log Vmax of p-substituted phenyl methyl sulffdes



REFERENCES AND NOTES

1) R. Sato and T. Omura,"Cytochrome P-450", Kodansha Ltd., Tokyo (1978), 2) G.R. Schonbaum and S. Lo, J. Biol. Chem., 247, 3353 (1972), 3) L.P. Hager, D.L. Doubek, R.M. Silverstein, J.H. Hargis and J.C. Martin, J. Am. Chem. Soc., 94, 4364 (1972), 4) G.A. Hamilton,"Molecular Mechanisms of Oxygen Activation", O. Hayaishi Ed., Academic Press New York, p 405 (1974), 5) G.D. Nordblom, R.E. White and M.J. Coon, Arch. Biochem. Biophys., 175, 524 (1976), 6) C.K. Chan and Ming-Shang Kuo, J. Am. Chem. Soc., 101, 3413 (1979), 7) J.T. Groves, T.E. Nemo and R.S. Myers, ibid., 101, 1032 (1979), 8) L.M. Hjelmeland, L. Aronow and J.R. Trudell, Biochem. Biophys. Res. Commun., 76, 541 (1977), 9) J.T. Groves and G.A. McClusky, ibid., 81, 154 (1978), 10) D. Fukushima, Y.H. Kim, T. Iyanagi and S. Oae, J. Biochem., 83, 1019 (1978), 11) T. Takahashi, D. Fukushima, K. Fujimori, T. Iyanagi and S. Oae, Heterocycles, 10, 229 (1978), 12) T. Takata, Y.H. Kim, M. Yamazaki, K. Fujimori, S. Oae and T. Iyanagi, unpublished results, 13) B.W. Griffin abd P.L. Ting, Biochem., 17 2206 (1978), 14) Y. Imai and R. Sato, Biochem. Biophys. Res. Commun., 60, 8 (1974), 15) H. Taniguc Y. Imai, T. Iyanagi and R. Sato, Biochem. Biophys. Acta, 550, 341 (1979), 16) J.E. Tomaszewski, D.M. Jerina and J.W. Daly, Biochem., 14, 2024 (1975), 17) a; 1e has so large absorbance in the region (350 - 500 nm) that the difference spectrum cannot be observed. b; W. Nastainzcyk, H.H. Ruf and V. Ullrich, Eur. J. Biochem., 60, 615 (1975), 18) V. Ullrich and W. Doppel,"The Enzymes", P.D. Boyer Ed., Vol 12, Academic Press New York, p 284 (1975), 19) G. Modena and L. Mariola, Gazz. Chi. ital., 87, 1306 (1957), 20) When the oxidation of sulfide 1c by rabbit liver microsomes was carried out in ^{18}O-labeled phosphate buffer sola., only 1.3% of oxygen was found to be incorporate into the sulfoxide 2c from heavy water, $H_2{}^{18}O$ used.

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