AFFINITY MODIFICATION OF CYTOCHROME P450 BY IODINE-CONTAINING STABLE IMINOXYL RADICAL

V. V. LYAKHOVICH+, N. E. POLYAKOVA+, V. I. POPOVA, S. I. EREMENKO, S. E. OLKIN and L. M. WEINER

+*Institute of Experimental and Clinical Medicine, USSR Academy of Sciences, Novosibirsk 630091* and *Institute of Chemical Kinetics and Combustion, USSR Academy of Sciences, Novosibirsk 630090, USSR*

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1. Introduction

Stable iminoxyl radicals bound to cytochrome P450 are discussed in [1–4]. Radicals used hitherto have electron-donor groups and are analogous to either substrates or inhibitors of cytochrome P450. These compounds coordinate directly with Fe^{3+} located in the active center, as suggested by the visible spectra; i.e., they behave as type II substrates. The distance between the spin label nitroxide and the Fe^{3+} in such complexes is 8–11 Å [2,3].

It is known from [5] that some stable iminoxyl radicals can be used as analogs of cytochrome P450 substrates to examine electron transfer processes in microsomal systems. In particular, radical 4(3-iodo-2-oxopropylidene)-2,2,3,5,5-pentamethylimidosolydene-1-oxyl (RJ) binds to cytochrome P450 yielding a type I spectral complex [5]. On the other hand, numerous data suggest that the SH-group of cysteine is located in the active center of cytochrome P450 [6–8]. SH-groups are well known to be readily alkylated by halogen-containing compounds [9]. Taking into account this fact, as well as substrate properties of RJ, we anticipated that RJ would interact with the SH-group in the active center of the enzyme. Here the interaction of this radical with microsomal cytochrome P450 by EPR spectroscopy is studied. The distance between the Fe^{3+} and the iminoxyl moiety was estimated at 77 K.

2. Materials and methods

Liver microsomes were prepared as in [10] from Wistar rats (120–140 g). The contents of microsomal protein and cytochrome P450 were determined by conventional methods [11,12]. For EPR experiments the microsomes were filtered through a Chelex 100 column (Bio-Rad Labs).

Aniline (chemically pure) was purified by distillation and naphthalene (Koch-Light Labs) was sublimated. Radicals RJ, 4(2-oxopropylidene)-2,2,3,5,5-pentaimidozolydene-1-oxyl(RH), which is structurally similar to RJ but with H substituted for J, as well as the non-radical analog of RJ with $N–OCH_3$ residue instead of N–O· fragment (NR) were kindly given by Dr L. B. Volodarsky (Institute of Organic Chemistry, Novosibirsk).

The extent of aniline and naphthalene metabolism by microsomal fractions was determined by estimation of the amount of *p*-aminophenol and α-naphthanol as in [13,14]. The content of lipid was determined as in [15].

The difference spectra of cytochrome P450 in the presence of the radicals were measured by a Hitachi-556 scanning spectrometer. The binding of radical RJ was examined with a tandem cuvette device to compensate both the turbidity changes and the absorbance of the RJ radical itself. The spectral constants of the radicals bound to cytochrome P450 (K_s) were determined according to [16].

Stopped flow experiments were performed using a Hitachi-556 spectrometer with a rapid mixing accessory ('dead' time = 1 ms). The cytochrome P450 reduction rate was determined by watching the formation of the CO–ferrous cytochrome P450 complex.

The EPR spectra were taken by a Varian E-109 spectrometer in a capillary tube at room temperature and in a quartz dewar vessel at 77 K.

Address correspondence to V. V. L. and L. M. W.

3. Results and discussion

RJ and RH radicals bind to cytochrome P450 as type I substrates (λ_{max} = 390 nm, λ_{min} = 420 nm) with K_s(RJ) = 66 μM and K_s(RH) = 240 μM. Fig.1 shows the oxidation product yields of type I (naphthalene) and type II (aniline) substrates vs RJ and RH concentrations. As seen from fig.1, RJ, unlike RH, is a highly effective inhibitor of the oxidation reactions catalyzed by cytochrome P450. Note that the degree of inhibition for both substrates becomes constant as soon as [RJ]/[cytochrome P450] ratio reaches unity.

The diminution in the substrate metabolism caused by RJ is not due to the inhibition of the NADPH-cytochrome *c* reductase or NADPH-cytochrome P450 reductase. At $\leqslant$10 μM, RJ does not affect the reactions mentioned. Evidently, the inhibition effect does not result from the conversion of cytochrome P450 into the inactive form (cytochrome P420) since this conversion occurs only if RJ is $\geqslant 10^{-4}$ M.

Experiments have shown the non-radical analog RJ–NR to be an effective inhibitor of substrate oxidation too, its efficiency being 1.4-times less than RJ. This implies that the RJ inhibition effect is independent of the presence of the unpaired electron.

Thus, the inhibition of aniline and naphthalene oxidation observed (fig.1) can be explained by the covalent binding of the alkylating analog of the substrate to the active center of cytochrome P450. This assumption was verified by the following experiments. Radicals RJ and RH were incubated with microsomes at 4°C, the incubation mixture was then treated with 4% sodium cholate until complete solubilization of the microsomes. The solubilizate obtained was then passed through a column with Sephadex LH-20 to remove the low molecular weight hydrophobic compounds [17]. After centrifugation at 105 000 × *g*, the particles obtained were analyzed by EPR. The EPR signal of immobilized radical arose in particles pre-treated with RJ, while no signal was observed in those treated with RH.

In other experiments the microsomes were treated with RJ then solubilized with detergent. The solubilizate was passed through the column with Biogel. Fig.2 shows the division of the solubilizate into protein and lipid components. Using EPR spectroscopy the radical was found in the protein fraction. The spectrum of the radical was immobilized ($\tau_c \sim 10^{-8}$ s). At the same time, the radical was also observed in the other fractions, its spectrum corresponding to the non-immobilized radical ($\tau_c = 5 \times 10^{-11}$ s). These

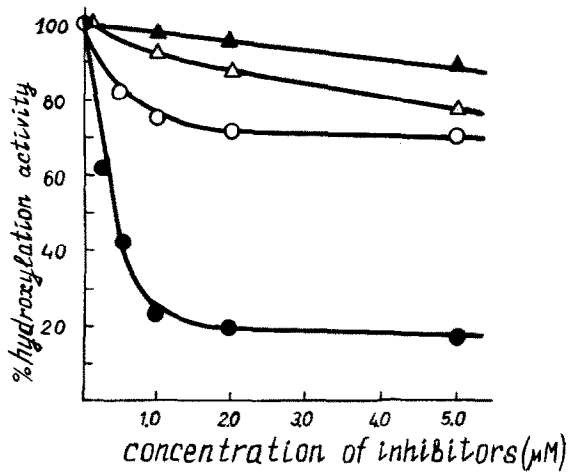

Fig.1. Inhibition of aniline and naphthalene oxidation in microsomes by RJ and RH radicals: protein, 1.5 mg/ml; cytochrome P450, 1.7 × 10^{-6} M; aniline, 10^{-3} M; naphthalene, 5 × 10^{-4} M; HADP.H, 2 × 10^{-3} M. (●) aniline + RJ; (▲) aniline + RH; (○) naphthalene + RJ; (△) naphthalene + RH.

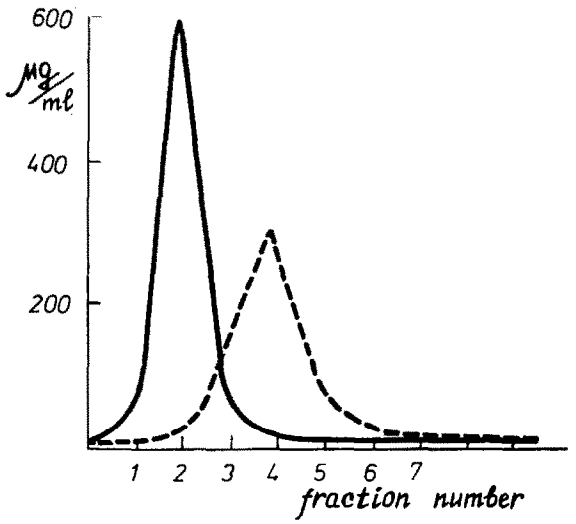

Fig.2. Gel filtration on Bio-gel P-30 of the reaction mixture containing microsomes preincubated with RJ. Reaction mixture: Microsomes, 0.2 ml (38.4 mg protein/ml) were preincubated with RJ at 1.4 × 10^{-4} M, for 30 min, at 310 K, then solubilized with sodium cholate (final conc. 4%) and passed through a column (15 ml). The column was washed with 0.1 M Tris–HCl buffer (pH 7.6) and sodium cholate 4%. The fractions were collected for EPR analysis and protein determination; for lipid determination the fractions were specially concentrated.

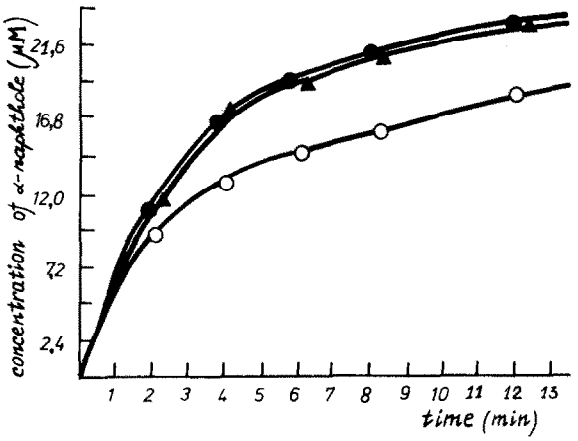

Fig.3. Protection effect of naphthalene in RJ-induced inhibition of microsomal hidroxilation of naphthalene. Microsomes (cytochrome P450, 2.4×10^{-6} M in 0.05 M Tris–HCl buffer (pH 8.1) were preincubated with RJ at 4.2×10^{-7} M for 4 min at 310 K without naphthalene (○) and with naphthalene at 10^{-3} M (▲). The control sample contained the same concentration of reagents without RJ (●). The reactions were started by HADP.H at 3×10^{-3} M.

data show the RJ to covalently bind to the microsomal protein.

To solve the problem of localization of the RJ covalently bound to cytochrome P450, we studied the protection of the active center from substrate inactivation. As seen from fig.3 an amount of naphthalene in the incubation medium prevents inactivation of the enzyme, while aniline has no influence on the interaction of RJ with cytochrome P450.

Fig.4 shows the effect of microsomal fraction on the EPR spectra of RJ and RH radicals. Microsomes induce transformations of both RJ and RH radicals

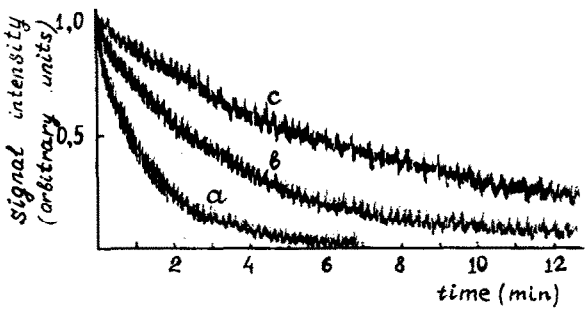

Fig.4. Time evolution of the central component RJ and RH EPR signal in the presence of microsomes. RJ, RH 2×10^{-4} M; cytochrome P450, 5×10^{-5} M; metyrapone, 10^{-3} M; at 303 K. (a) RJ + microsome; (b) RJ + microsome + metyrapone; (c) RH + microsome.

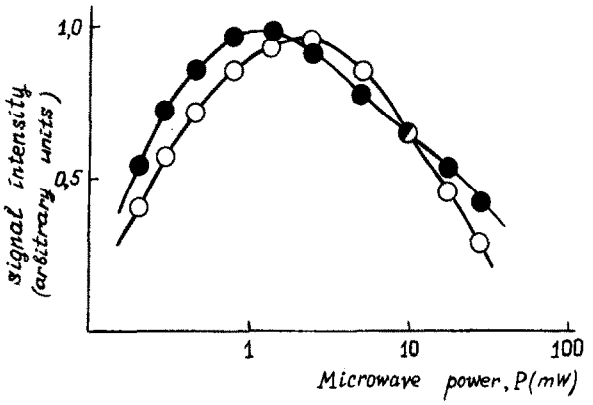

Fig.5. Microwave power saturation curves of RJ at 77 K in microsomes (○) (56 mg protein/ml, 50 μM cytochrome P450, 20 μM spin label conc.), and in liposomes from phosphatidylcholine (●) (40 mg lipid/ml, 70 μM label conc.), EPR modulation amplitude of 4 G, microwave frequency of 8.95 GHz.

leading to the disappearance of EPR spectra. The slow decrease of the peak intensity of the RH EPR spectra may be tentatively explained by reduction of the *N*-oxyl moiety to hydroxylamine by the membrane components [18]. At the same time, the rapid disappearance of the signal in RJ-treated samples is not common. This effect can be explained assuming spin exchange between the radical and the Fe^{3+} localized in the active enzyme center occurring during the covalent binding of RJ to the active center of cytochrome P450 and reducing the RJ EPR signal [19]. Indeed, when the temperature of the sample, with the EPR signal of RJ 'vanished' at 303 K, was decreased to 77 K, the EPR signal appeared again. This signal was the same as the frozen solution of RJ, $g_{zz} = 2.022$, $g_{yy} = 2.008$, $g_{xx} = 1.979$ (frequency $f = 8.93$ GHz). Measurements of the dependence of the peak intensity of the spectra of RJ-treated microsomes at 77 K vs the power of the exciting electromagnetic radiation (microwave power saturation curves, fig.5) made it possible to estimate the distance between the Fe^{3+} and the iminoxyl moiety which appeared to equal 11.5 ± 1 Å as calculated by the method in [20]*.

* The contribution of Fe^{3+} to the value of T_1^{-1} of RJ was calculated using the saturation curves by the technique in [20]. $\Delta T_1^{-1} = 2.9 \times 10^4$ s^{-1}. The distance between the Fe^{3+} and the iminoxyl moiety was calculated using the above ΔT_1^{-1}, Fe^{3+} parameter (low spin), and the correlation time $\tau = 3 \times 10^{-9}$ s. The value of τ equal to T_2 of cytochrome P450 Fe^{3+} at 77 K [3] was much shorter than that of T_1 at 77 K [21]

Experiments with metyrapone, a classical competitive inhibitor of reactions catalyzed by cytochrome P450, afford further evidence for the binding of RJ at the active center. Fig.3b shows this compound to slow down the microsome-induced decrease in the peak intensity of the RJ EPR spectrum.

The above experimental data and the comparison of the RH and RJ effects on microsomes prove the covalent binding of RJ to the active center of cytochrome P450. The SH group of the enzyme active site is probably affected by RJ since SH groups are known to be better alkylated by iodine-containing compounds [9]. It should be noted that the oxidation of substrates I and II is unequally inhibited (fig.1). Unfortunately, standard methods of analysis of inhibition curves for covalent binding inhibitors [22] cannot be used in our studies because an appreciable inhibition of substrate oxidation takes place at equal concentrations of the enzyme and the inhibitor. The method to analyze inhibition curves using the exact solution of the equation in [22] is being developed.

Thus, the affinity modification of cytochrome P450 by substrate analogs containing an active group suggests a new approach to the investigation of the geometry of the monooxygenase active center and allows for effective inhibition of reactions catalyzed by this enzyme in vivo.

References

[1] Reichman, L. M., Annaev, B. and Rozantsev, E. G. (1972) Biochim. Biophys. Acta 263, 41–51.
[2] Griffin, B. W., Peterson, I. A., Werringloer, J. and Estabrook, R. W. (1975) Ann. NY Acad. Sci. 244, 107–131.
[3] Ruf, H. H. and Nastainczyk, W. (1976) Eur. J. Biochem. 66, 139–146.
[4] Pirwitz, J., Lassman, G., Rein, H., Ristan, O., Janig, G. R. and Ruckpaul, K. (1977) FEBS Lett. 83, 15–18.
[5] Weiner, L., Eremenko, S., Popova, V., Sagdeev, R., Tsyrlov, I. and Lyakhovich, V. (1979) Proc. XX Congr. AMPERE, Tallin, 520.
[6] Cramer, S. P., Dawson, J. H., Hodgson, K. O. and Hager, L. P. (1978) J. Am. Chem. Soc. 100, 7282–7290.
[7] Lipscomb, J. D., Harrison, J. E., Dus, K. M. and Gunsalus, I. C. (1978) Biochem. Biophys. Res. Commun. 83, 771–778.
[8] Swanson, R. A., Dus, K. M. (1979) J. Biol. Chem. 254, 7238–7246.
[9] Torchinski, J. M. (1971) Sulfhydryl and disulfidic groups of proteins, Nauka, Moscow.
[10] Tsyrlov, I. B., Zakharova, N. E., Gromova, O. A. and Lyakhovich, V. V. (1976) Biochim. Biophys. Acta 421, 44–56.
[11] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[12] Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370–2378.
[13] Imai, Y., Ito, A. and Sato, R. (1966) J. Biochem. 60, 417–425.
[14] Achrem, A. A., Usanov, S. A., Dvornicov, S. S. and Metelitsa, D. I. (1975) Dokl. AN SSSR, 223, 1014–1017.
[15] Batlett, J. R. (1959) J. Biol. Chem. 234, 466–471.
[16] Schenkman, J. B., Remmer, H. and Estabrook, R. W. (1967) Mol. Pharmacol. 3, 113–123.
[17] Mishin, V. M., Grishanova, A. I. and Lyakhovich, V. V. (1979) FEBS Lett. 104, 300–302.
[18] Schreier, S., Polnaszek, C. F. and Smith, I. (1978) Biochim. Biophys. Acta 515, 395–436.
[19] Zamaraev, R. I., Molin, Yu. N. and Salikhov, K. M. (1977) Spin exchange, Nauka, Novosibirsk.
[20] Kulikov, A. B. (1976) Molekul. Biol. 10, 132–141.
[21] Herriek, R. C. and Stapleton, H. J. (1976) J. Chem. Phys. 65, 4778–4785.
[22] Kitz, R. and Wilson, I. B. (1962) J. Biol. Chem. 237, 3245–3249.