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EFFECTS OF ADDED ALBUMIN ON THE KINETIC ISOTOPE EFFECT OBSERVED IN THE MICROSOMAL OXIDATIVE DEMETHYLATION OF THE METHOXYL GROUP

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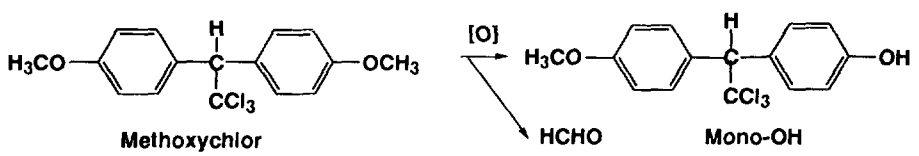
The kinetic deuterium isotope effect on the microsomal oxidative demethylation of methoxyl groups was measured using methoxychlor, an insecticide, as a model compound. When the metabolic reactions were conducted in the presence of bovine serum albumin, the observed isotope effect was smaller than that in its absence. The values were unaltered when the albumin concentration was changed from 1% to 2%. The results were interpreted by the alteration of the ES-complex formation/dissociation rate with the interaction between substrate and albumin.

Isotope effect studies have provided a variety of information on the reaction mechanism of enzyme-catalyzed reactions (1). The values of the observed isotope effect may often be heavily suppressed compared with the intrinsic value, due to various non-catalytic slow steps involved in a reaction sequence. Thus, to investigate the alteration of the observed isotope effect under altered reaction conditions may provide an indispensable type of information on an enzyme-catalyzed reaction.

Methoxychlor, 1,1-bis-(4-methoxyphenyl)-2,2,2-trichloroethane, a biodegradable DDT analog insecticide, is detoxified in mammals mainly through the oxidative demethylation of one of the methoxyl groups at the first step (see scheme 1). The first step is mostly catalyzed by liver microsomal cytochromes P450.

We found (2,3) using rat liver microsomes, that the value of the observed deuterium isotope effect was much smaller than the intrinsic value in this reaction. The magnitude of the intrinsic isotope effect obtained through measuring both the deuterium and tritium effects (2) was about 15, supporting the notion of a radical-scission and



Scheme 1

Abbreviations used:
BSA : bovine serum albumin; GC-MS : gas chromatography-mass spectrometer.

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-recombination mechanism(4), and the observed suppressed isotope effect (2.5 to 3.0) suggests that the reaction should have one or more rate-limiting non-catalytic steps such as ES-complex formation and dissociation (k_1 and k_2 in the simplified scheme 2 in the Discussion section).

In the other stage in the study of methoxychlor metabolism(5), we found that increasing amounts of bovine serum albumin (BSA) in the reaction medium increased the apparent Km values of the same reaction using microsomes from both the untreated and the phenobarbital-treated rat liver, although the V_{max} values were unchanged with various BSA concentrations.

Since V_{max} is expressed as $k_3k_4E_{total} / (k_3 + k_4)$, Km is expressed as $[(k_2 + k_3) / k_1] \times [k_4 / (k_3 + k_4)]$ in the simplified reaction scheme 2, and k_3 remains unchanged with the added BSA, k_4 should also remain constant because of the unchanged V_{max}. Thus the alteration of the apparent Km should depend on the alteration of k_1 and k_2 or either of them, and the effect of added albumin on the value of the observed isotope effect may provide some clues about the kinetic mechanism of the reaction.

Here we describe the effect of added BSA on the values of the observed isotope effect upon the oxidative demethylation of methoxychlor in rat liver microsomes, and discuss the kinetic constants of this reaction.

MATERIALS AND METHODS

Compounds Methoxychlor, and its mono- and di-demethylated products (mono-OH and di-OH), [1-(4-hydroxyphenyl)-1-(4-methoxyphenyl)-2,2,2-trichloroethane and 1,1-bis-(4-hydroxyphenyl)-2,2,2-trichloroethane] were prepared and purified by the previously reported method (2, 3, 5). [dimethyl-d_6]Methoxychlor (1,1-bis-(4-[d_3]methoxyphenyl)-2,2,2-trichloroethane) was synthesized from di-OH by methylation with [d_3]iodomethane. The compound [monomethyl-d_3]mono-OH was similarly prepared. The degree of deuterium incorporation into each methyl group should be 99% or more, because we used [d_3]iodomethane with a 99% D-atom percent or more, and our check of the degree of incorporation using mass spectrometry showed that the total $d_{5\text{-}0}$ contamination was less than 0.5%. All other compounds were purchased from the Waken-yaku Co. Ltd. (Kyoto) and were of the highest purity commercially available.

Metabolic conditions and assays The reaction mixture (2 ml) consisted of liver microsomes (prepared from untreated male Wistar rats, weighing about 200 g) containing 291 pmol of cytochrome P-450 (323 mg protein), 1 μmol NADPH, and the substrate (in 10 μl ethanol; See Table 1 for the amount and d_0/d_6 ratio.) in 0.2M-potassium phosphate, pH 7.4. The buffer contained 0, 1, and 2% BSA. The cytochrome P450 content was determined by the method of Omura and Sato (6), and protein was determined by the method of Lowry et al.(7).

The reaction was started in a glass-stoppered tube by adding the substrate after the mixture was preincubated for 1 minute at 37°C. After a further incubation for 2 minutes at 37°C, the mixture was rapidly cooled to 0°C, then shaken with 4 ml of hexane/ethyl acetate (4:1 v/v). Triplicate runs were made for each reaction condition.

A 2-ml sample of the organic layer was dried in a V-vial under a stream of nitrogen, to separately determine levels of the [d_0]- and [d_3]-metabolites with a gas chromatography-mass spectrometer (GC-MS). Acetic anhydride and pyridine (each 20 μl) were added to the dried residues.

The vials were tightly capped and warmed in a heating block for 20 minutes at 60°C, then the solvents were dried under a stream of nitrogen. Ethyl acetate (ca. 10 μl)

was added to dissolve the residue, then 0.5- to 1-μl samples were injected into the GC-MS for selected ion monitoring. The ions at m/z 213 and 216 that appeared as base peaks $(M - CH_2CO - CCl_3)^+$ of the acetyl derivatives of the [d_0]- and [d_3]-metabolite were monitored.

A linear standard curve was obtained by plotting the peak area ratio at m/z 213 and 216 against the ratio of the amount of a standard mixture of the acetyl derivative of the [d_0]- and [d_3]-metabolites. The range of the ratio of [d_0]:[d_3] was 1:1 to 4:1. From the curve and the measured peak area ratio of an unknown sample, the ratio of the [d_0]- to [d_3]-metabolites was calculated. This (d_0/d_3) is the rate ratio (v_H/v_D) of metabolite formation, and the value $[(v_H / S_H) / (v_D / S_D)]$ (in which S_H and S_D are the substrate concentrations of unlabeled and deuterium-labeled, respectively) corresponds to the isotope effect on (V_{max} / Km), that is $^D(V / K)$ (For tnotation, see ref.1).

The GC-MS was a Hewlett Packard MSD (model 5970). The gas chromatographic portion was equipped with HP-1, a 12-m capillary column. The oven temperature was programmed from 100° to 210°C.

RESULTS

The observed isotope effects on (V_{max} / Km) for the formation of d_0- and d_3-metabolite from [d_0]- and [d_6]methoxychlor, respectively, are listed as $^D(V / K)$ in Table 1. Under our conditions, the formation of the di-OH metabolite was negligible. The approximate reaction rates of the d_0-substrate are also listed. We previously

Table 1.
Alteration of the Deuterium Isotope Effect on V/K by BSA

Substrate conc.		BSA	Observed Isotope Effect	Rate of d_0 (mol/min/ mol P450)
d_0 (μM)	d_6 (μM)	%	$^D(V / K)$ a)	
		0	---	0.522
5.0	0	1	---	2.11
		2	---	2.18
		0	2.96±0.06	0.45
5.0	5.0	1	1.92±0.03	1.29
		2	1.96±0.07	1.24
		0	3.17±0.11	0.65
5.0	2.5	1	2.03±0.07	1.41
		2	1.92±0.03	1.15
		0	3.22±0.10	0.369
2.5	2.5	1	1.75±0.01	1.010
		2	1.76±0.01	0.885
		0	2.90±0.35	0.284
2.5	5.0	1	1.71±0.05	0.507
		2	1.55±0.08	0.378
		0	2.74±0.09	0.133
1.25	5.0	1	1.86±0.02	0.323
		2	1.86±0.04	0.254
	(n=5)	**0**	**3.00±0.20**	
Average	(n=5)	**1**	**1.85±0.13**	**1.83±0.14**
	(n=5)	**2**	**1.81±0.16**	(n=10)

a) Average values of triplicate runs are listed with their standard deviations.

reported (5) that the reactions of methoxychlor in microsomes in the absence of BSA were often heavily suppressed, making the reaction rates smaller than those in the presence of BSA. Here, the same trends were also evident.

When we increased the BSA concentration from 1 to 2%, the rates (except for the runs with d_0-substrate only) decreased, indicating that the proportion of methoxychlor hydrophobically bound to the BSA increased, thus decreasing the substrate proportions on the surface of microsomal enzymes. This is also the same trend as that previously reported (5).

The values of observed isotope effect $^{D}(V / K)$ of the metabolite formation in the presence of BSA were constant throughout the examined range of substrate concentrations and compositions, irrespective of the BSA concentration, 1% (1.85 ± 0.13) (n=5) or 2% (1.81 ± 0.16) (n=5): 1% and 2% 1.83 ± 0.14 (n = 10). The values in the absence of BSA were larger than the above, but were also constant 3.00 ± 0.20 (n = 5) through various substrate concentrations and compositions.

DISCUSSION

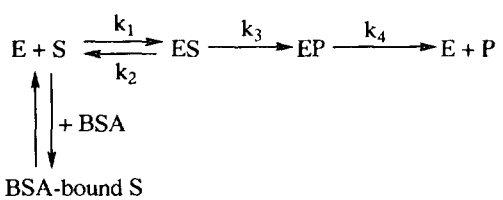
When we increased the BSA concentration in the reaction medium from 1 to 2%, the V_{max} values were not altered but the apparent Km values approximately doubled as previously reported. Thus, we initially thought that, under conditions of a higher Km, the substrate would become "less sticky" to enzyme leading to a lower commitment to catalysis. Therefore, the reaction would give a higher observed isotope effect (1, 8). However, when the BSA concentration was doubled, the observed values on V_{max}/Km were not altered. As described below, it is a different situation from the use of an alternate substrate with a higher Km value that enhances $^{D}(V/K)$ value (1, 8).

As described in the Introduction, the rate constant k_4 should remain unchanged, and k_1 and k_2 or one of them should change depending on the BSA concentration (see scheme 2).

Since $^{D}(V/K) = [^{D}k + (k_3/k_2)] / [1 + (k_3/k_2)]$ (ref. 1), and the value did not change with the increased BSA concentration, k_2 must also be unchanged.

Based upon the expression of Km of the reaction with the respective BSA concentrations, the following relationship is introduced.

$$\{[(k_2 + k_3) / k_1] \times [k_4 / (k_3 + k_4)]\}_{(2\%)} / \{[(k_2 + k_3) / k_1] \times [k_4 / (k_3 + k_4)]\}_{(1\%)} = 1.03 / 0.53.$$



Scheme 2

Therefore, k_1 at BSA 1% = (1.03 / 0.53) x (k_1 at BSA 2%), that is, the (apparent) rate constant with 2% of BSA for the ES-complex formation should become 51.5% of the constant with 1% of BSA(0.53 / 1.03 = 0.515). This simply reflects that the real unbound substrate concentration was reduced to about half when the BSA concentration was doubled (see the scheme 2).

According to our previous report (5), the ratio of the substrate distribution in the medium was composed of three components (BSA, buffer solution, and microsomes) can be calculated with the following equation.

$$\frac{\{[S]_f + [SB]\} \times [M]}{[SM]} = 11.3 + 2.80 \times 10^6[B].$$

In this equation, $[S]_f$, [SB], and [SM] correspond to the free substrate (unbound to BSA neither to microsomes), that bound to BSA, and that bound to microsomes, respectively. [B] is the BSA concentration in mol/L, and [M] is the concentration of microsomes in mg/mL. The concentrations of 1 and 2% of BSA correspond to 1.5×10^{-4} and 3.0×10^{-4} mol/L, respectively, and [M] in the present study is 161.5 mg/mL. Therefore, the following equations can be introduced.

[BSA 1%]: $\{[S]_f + [SB]\} / [SM] = (11.3 + 2.8 \times 10^6 \times 1.5 \times 10^{-4}) / 161.5 = 2.67$

and

[BSA 2%]: $\{[S]_f + [SB]\} / [SM] = (11.3 + 2.8 \times 10^6 \times 3.0 \times 10^{-4}) / 161.5 = 5.27.$

Thus, the ratio of the substrate in the solution (free and bound to BSA) versus the substrate bound to microsomes is 2.67 (with 1% BSA) and 5.27 (with 2% BSA). That is, the substrate bound to microsomes is 27.2% [1/(2.67+1)] of the total added substrate in the presence of 1% BSA, and 15.9% [1/(5.27+1)] in the presence of 2% BSA. As previously reported (5), the reaction rate in our system correlated well with the substrate concentration bound to the microsomes; the reduction of [SM] from 27.2% of the total added substrate to 15.9% (reduction of 41.5%) seems to be the most important reason for the reduction of k_1 (apparent rate constant for the ES-formation) in the present study.

In the absence of BSA, the reaction rates were slower than those with the added BSA. On the other hand, the observed isotope effects $^D(V/K)$ in the absence of BSA were larger than those in the presence of 1 and 2% of BSA. Thus, based on eq. [1], the rate constant k_2 of ES-dissociation should be enhanced in the absence of BSA.

$$^D(V/K) - 1 = [^Dk + (k_3/k_2)] / [1 + (k_3/k_2)] - 1 = [^Dk - 1] / [1 + (k_3/k_2)] \qquad [1]$$

Under our reaction conditions, ES-dissociation does not necessarily imply the formation of unbound substrate, but the "dissociated" ES produces the substate bound to non-enzymatic sites of microsomes. In fact, the simple distribution ratio of the substrate between microsomes and the bulk solution is large (5): 21 [= $1/(4.74 \times 10^{-2})$] to 10.2 [= $1/(9.79 \times 10^{-2})$] compared with the ratio in the presence of BSA. For 2% BSA, the ratio

was 0.20 (= 1/5.02) to 0.18 (= 1/5.51). Thus, in the absence of BSA, a large portion of the substrate bound to microsomes must bind to non-enzymatic (non-P-450 system) sites.

In conclusion, the adding BSA to the oxidative demethylation reaction of methoxychlor catalyzed by microsomes caused a reduction in the deuterium isotope effect $^{D}(V/K)$, whereas it enhanced the reaction rates compared with those in the absence of BSA with the same substrate concentrations. These results suggested a reduction of ES-dissociation rate in the presence of BSA. Doubling the BSA concentration generally reduced the reaction rate, but the isotope effect values did not change. This apparently corresponded to the reduction of k_1, but was simply explained by the decrease in the amount of the substrate bound to P-450 in the presence of the increased BSA concentration.

Isotope effect studies with and without added BSA for metabolic reactions of various hydrophobic substrates would give further information upon their kinetic mechanism.

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