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THE INTERACTION BETWEEN TWO FORMS OF CYTOCHROME P-450 DURING DRUG OXIDATION IN THE RECONSTITUTED SYSTEM CONTAINING LIMITED AMOUNT OF NADPH-CYTOCHROME P-450 REDUCTASE

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Abstract—The activities of drug oxidation in a reconstituted system which contains two forms of cytochrome P-450 and a limiting amount of NADPH-cytochrome P-450 reductase were determined. Cytochrome P-450 (termed MC P-448$_1$ and MC P-448$_2$) purified from liver microsomes of 3-methylcholanthrene-treated rats was active in both 2- and 4-hydroxylation of biphenyl but cytochrome P-450 (termed PB P-450) purified from liver microsomes of phenobarbital-treated rats was active in 4-hydroxylation of biphenyl only. PB P-450, MC P-448$_1$ and MC P-448$_2$ were most active toward benzphetamine *N*-demethylation, aniline hydroxylation and 7-ethoxycoumarin *O*-deethylation, respectively. PB P-450 inhibited the activity of biphenyl 2-hydroxylation supported by MC P-448$_1$ or MC P-448$_2$. On the contrary, no inhibition of PB P-450 supported benzphetamine *N*-demethylation was observed when MC P-448$_1$ or MC P-448$_2$ was added to the system containing PB P-450 and limited amount of the reductase. The apparent K_m of PB P-450 for the reductase obtained from double reciprocal plot of the reductase concentration and the activity of biphenyl hydroxylase or benzphetamine *N*-demethylation was lower than that of MC P-448$_1$ or MC P-448$_2$. These and other results suggest that there is a certain hierarchy among the cytochrome P-450 species for receiving electrons from reductase.

The hepatic microsomal electron transfer system consisting of NADPH-cytochrome P-450 reductase and cytochrome P-450 is responsible for NADPH-dependent monooxygenations of a variety of drugs, toxic compounds including carcinogens and endogenous substrates. Recent studies have shown that there are multiple forms of cytochrome P-450 in rat liver microsomes [1–5]. NADPH-Cytochrome P-450 reductase functions in the transfer of reducing equivalents from NADPH to cytochrome P-450 during catalysis. Furthermore, it has been shown that the activities of drug oxidation are increased upon incorporation of NADPH-cytochrome P-450 reductase into microsomes and that the reductase rather than cytochrome P-450 is the rate limiting component in many drug oxidations catalysed by the microsomal system [6, 7]. In addition, we have also proposed the possibility that multiple forms of cytochrome P-450 compete with each other in functional coupling to the limited amount of the reductase in microsomes [8, 9]. The present study is designed to determine whether or not there is a hierarchy among the cytochrome P-450 species for receiving electrons from reductase. Also the interaction between two forms of cytochrome P-450 during drug oxidation in the reconstituted system in which the reductase is the rate limiting component was investigated.

MATERIALS AND METHODS

Preparation of microsomal enzymes. Liver microsomes from phenobarbital treated (0.1% sodium phenobarbital added to drinking water for 7 days) and 3-methylcholanthrene treated (25 mg/kg), intraperitoneally on the 2nd, 4th and 8th day before sacrifice) male Sprague–Dawley rats weighing 120–160 g were prepared by our standard procedures [10]. Cytochromes P-450 were purified from phenobarbital treated rats, which will be called PB P-450, and from 3-methylcholanthrene treated rats, which will be called MC P-448$_1$ and MC P-448$_2$, by a minor modification of the methods previously described [11–14]. After solubilized supernatant fractions were applied to aminooctyl–Sepharose 4B columns, the columns were washed successively with 10 mM and 100 mM potassium phosphate (pH 7.25) each containing 0.1 mM dithiothreitol, 20% glycerol, 0.4% sodium cholate and 0.2% Emulgen 913, in the case of the purification of PB P-450, or washed successively with 10 mM potassium phosphate (pH 7.25) containing 0.1 mM dithiothreitol, 20% glycerol, 0.4% sodium cholate and 0.08% Emulgen 913, and with 100 mM potassium phosphate (pH 7.25) containing 0.1 mM dithiothreitol, 20% glycerol, 0.4% sodium cholate and 0.2% Emulgen 913, in the case of the purification of MC P-448$_1$ amd MC P-448$_2$. The peak fractions collected from 100 mM washes were pooled in each case. The pooled fractions were diluted four-fold with 20% glycerol containing 0.2% Emulgen 913, and applied to hydroxylapatite columns individually.

In the case of PB P-450, the hydroxylapatite column was washed with 60 mM potassium phosphate (pH 7.25) containing 0.1 mM dithiothreitol, 20% glycerol and 0.2% Emulgen 913, and then PB P-450 was eluted with 80 mM potassium phosphate

(pH 7.25) containing 0.1 mM dithiothreitol, 20% glycerol, 0.2% Emulgen 913 and 0.1% sodium cholate. The peak fractions collected from the 80 mM wash were pooled and dialysed against 5 mM potassium phosphate (pH 7.4) containing 0.1 mM dithiothreitol, 20% glycerol and 1 mM EDTA. The dialysed solution was applied to a Whatman DE-52 column which had been equilibrated with 5 mM potassium phosphate (pH 7.4) containing 0.1 mM dithiothreitol, 20% glycerol, 0.2% Emulgen 913 and 0.2% sodium cholate, which will be referred to simply as 5 mM Buffer A. After the column was washed successively with 5 mM and 10 mM Buffer A, PB P-450 was eluted with 10 mM Buffer A containing 30 mM potassium chloride. The peak fractions eluted with 10 mM Buffer A containing 30 mM potassium chloride were pooled and applied to second hydroxylapatite column to remove detergent.

In the case of the purification of MC P-448$_1$ and MC P-448$_2$, the hydroxylapatite column was washed with 80 mM potassium phosphate (pH 7.25) containing 0.1 mM dithiothreitol, 20% glycerol, 0.2% sodium cholate and 0.2% Emulgen 913, and then MC P-448$_1$ and MC P-448$_2$ were eluted with 180 mM potassium phosphate (pH 7.25) containing 0.1 mM dithiothreitol, 20% glycerol, 0.1% sodium cholate and 0.2% Emulgen 913. The peak fractions collected from the 180 mM wash were pooled and dialysed against 5 mM Buffer A. The dialysed solution was applied to a Whatman DE-52 column which had been equilibrated with 5 mM Buffer A. After MC P-448$_1$ was eluted as the second peak by washing of the column with 5 mM Buffer A, the column was washed successfully with 10 mM Buffer A and 10 mM Buffer A containing 30 mM potassium chloride, and then MC P-448$_2$ was eluted with 10 mM Buffer A containing 70 mM potassium chloride. The peak fractions eluted were pooled separately and applied to a second hydroxylapatite column to remove the detergent. The second hydroxylapatite column in each case was washed with three to four column volumes of 50 mM potassium phosphate (pH 7.25) and then cytochrome P-450 was eluted with 500 mM containing 20% glycerol. The final preparations used in these experiments had specific contents of 12.0 nmole PB P-450 per mg of protein, 13.9 nmole MC P-448$_1$ per mg of protein and 15.5 nmole P-448$_2$ per mg of protein, respectively. Polyacrylamide gel electrophoresis by the method of Laemmli [15] indicated a single major band in each.

NADPH-Cytochrome P-450 reductase was solubilized with Emulgen 913 from liver microsomes of phenobarbital treated rats and purified by the method of Yasukochi and Masters [16] with minor modifications. The final preparation had a specific activity of 47.1 units/mg of protein. One unit of the reductase activity was defined as the amount of enzyme catalysing the reduction of cytochrome *c* at an initial rate of 1 μmole/min.

Incubation condition. The incubation mixture was composed of the following; 100 mM potassium phosphate (pH 7.25), 25 μg of dilauroyl-3-L-phosphatidylcholine, an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 0.1 unit of glucose 6-phosphate dehydrogenase and 6 mM $MgCl_2$), substrate and variable quantities of purified cytochrome P-450 and the reductase (refer to figures and tables for quantities). The reaction volume and the concentrations of benzphetamine 7-ethoxycoumarin, aniline and biphenyl used were 0.5 ml, 1 mM, 2 mM, 2 mM and 2.5 mM, respectively. The reaction was started by the addition of NADPH-generating system.

Assay method. Cytochrome P-450 and NADPH-cytochrome *c* (P-450) reductase activity were determined according to the methods of Omura and Sato [17], and Phillips and Langdon [18], respectively. The concentration of the reductase was determined from the absorbance at 456 nm in the absolute spectrum using an extinction coefficient of 21.4 mM/cm [19]. Protein was assayed by the method of Lowry *et al.* [20] using bovine serum albumin as a standard. Activities of benzphetamine *N*-demethylation, 7-ethoxycoumarin *O*-deethylation and aniline hydroxylation were measured by the methods of Nash [21], Aitio [22] and Imai *et al.* [23] respectively. Biphenyl hydroxylation was estimated according to a minor modification of the method of Yamazoe *et al.* [24].

Reagents and biochemicals. NADP, NADPH, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Osaka, Japan and cytochrome c (horse heart) was from Boehringer Mannheim. Benzphetamine and Emulgen 913 were kindly provided by the Upjohn Co. and Kao Atlas Co., Japan respectively. All other chemicals were of the highest purity commercially available. Sepharose 4B, DEAE-Sephadex (A-50) and 2′,5′-ADP Sepharose were purchased from Pharmacia Fine Chemicals Co., and hydroxylapatite (Bio-Gel HT) from Bio-Rad.

RESULTS

Figure 1 shows the effect of PB P-450 on MC P-448$_2$ supported biphenyl hydroxylation in the presence of a fixed amount of NADPH-cytochrome P-450 reductase. Although MC P-448$_2$ was active not only in 4-hydroxylation but also in 2-hydroxylation, PB P-450 was active in 4-hydroxylation only. In addition, MC P-448$_2$ was more active in 4-hydroxylation than PB P-450. Furthermore, both 2- and 4-hydroxylation of biphenyl were decreased by increasing the concentration of PB P-450 added to the system containing MC P-448$_2$. The inhibition of MC P-448$_2$ supported biphenyl hydroxylation by PB P-450 could be due to competition between the two cytochrome species for either biphenyl or reductase. However, the inhibition was virtually independent of the concentration of biphenyl used between 1.55 mM and 2.5 mM, suggesting that the inhibitory effect of PB P-450 on biphenyl hydroxylation catalysed by MC P-448$_2$ may not be due to the competition of substrate binding between these two isozymes (data not shown). Therefore, the effect of reductase concentration on the inhibition of MC P-448$_2$ supported biphenyl 2-hydroxylation by PB P-450 was studied.

As can be seen in Fig. 2A, the inhibitory effect of PB P-450 on MC P-448$_2$ supported biphenyl 2-hydroxylation was gradually decreased as the amounts of reductase were increased. On the other hand, PB P-450 supported biphenyl 2-hydroxylation

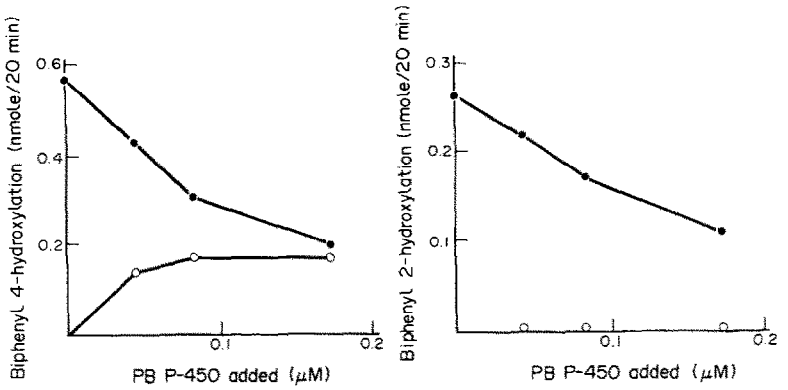

Fig. 1. Effect of PB P-450 on MC P-448$_2$ supported biphenyl 2- and 4-hydroxylation in a reconstituted system. The concentrations of MC P-448$_2$ and the reductase used were 0.05 nmole and 0.1 nmole respectively. Other experimental details are described in Materials and Methods. The rate of biphenyl hydroxylation was determined in the presence (●) and absence (○) of MC P-448$_2$.

was not detected at any concentration of reductase used. As shown in Fig. 2B, the double reciprocal plots of the rate of MC P-448$_2$ dependent biphenyl 2-hydroxylation in the presence or absence of PB P-450 vs the concentration of reductase added indicated that PB P-450 affected the K_m for the reductase but not the V_{max} for MC P-448$_2$ supported biphenyl 2-hydroxylation.

From these results, it is suggested that the competition between PB P-450 and MC P-448$_2$ for reductase may be responsible for the inhibition of biphenyl hydroxylation by PB P-450.

To determine if there is an order in cytochrome P-450 species for receiving electrons from the reductase, the activities of benzphetamine *N*-demethylation and 7-ethoxycoumarin *O*-deethylation in the reconstituted system, which contains limited amounts of the reductase and equimolar amounts of two cytochrome P-450 isozymes, were determined.

Table 1 shows the interaction between PB P-450 and MC P-448$_2$ during drug oxidations in the presence of a limited amount of reductase. MC P-448$_2$ was more active than PB P-450 in 7-ethoxycoumarin *O*-deethylation as in the case of biphenyl hydroxylation. On the contrary, PB P-450 was more active toward benzphetamine *N*-demethylation than MC P-448$_2$. When PB P-450 was added to the reconstituted system which contains MC P-448$_2$ and limited amount of reductase, 7-ethoxycoumarin *O*-deethylation activity was found to be lowered as compared with the activity observed in the system containing MC P-448$_2$ alone. Furthermore, the decrease in the activity due to PB P-450 was diminished with increasing the concentration of the reductase added. On the other hand, no inhibition of PB P-450 supported benzphetamine *N*-demethylation by MC P-448$_2$ was observed even when reductase was the rate limiting component.

As shown in Table 2, MC P-448$_1$ was active toward

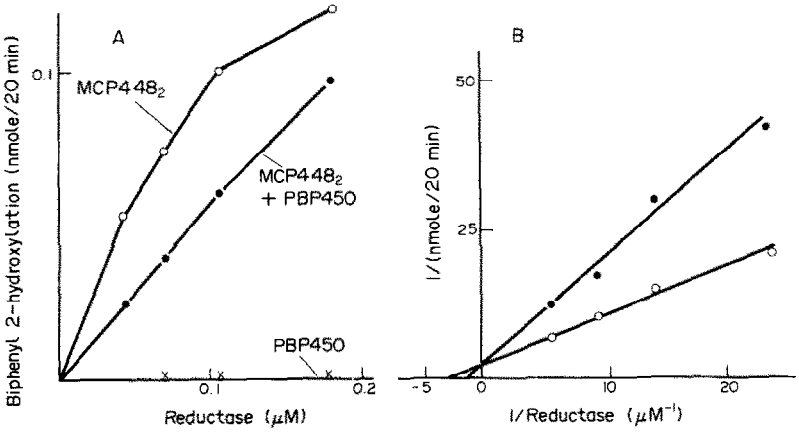

Fig. 2. Effect of PB P-450 on MC P-448$_2$ supported biphenyl 2-hydroxylation in the presence of various amounts of reductase. The concentrations of PB P-450 and MC P-448$_2$ employed were 0.043 and 0.036 nmole respectively. Other experimental details are described in Materials and Methods. The rate of biphenyl 2-hydroxylation was determined in the presence of both cytochromes (●) or either of them (PB P-450; ×, MC P-448$_2$; ○).

Table 1. The interaction between PB P-450 and MC P-448$_2$ during drug oxidations in the presence of limited amounts of reductase

Reactions	Reductase (μM)	Cytochrome P-450		
		PB P-450	MC P-448$_2$ (nmole product/20 min)	PB P-450 + MC P-448$_2$
Benzphetamine	0.03	2.91 ± 0.71	ND	2.75 ± 0.49
N-demethylation	0.20	31.40 ± 4.45	1.92 ± 0.11	34.13 ± 5.35
7-Ethoxycoumarin	0.06	0.75 ± 0.07	12.75 ± 0.21	5.03 ± 0.71
O-deethylation	0.11	1.50 ± 0.14	22.54 ± 0.85	16.80 ± 0.71
	0.23	2.20 ± 0.14	26.55 ± 0.48	26.65 ± 0.07
Biphenyl	0.05	ND	0.166 ± 0.001	0.058 ± 0.011
2-Hydroxylation	0.09	ND	0.175 ± 0.031	0.095 ± 0.011
4-Hydroxylation	0.05	0.146 ± 0.014	0.268 ± 0.007	0.227 ± 0.035
	0.09	0.195 ± 0.013	0.410 ± 0.058	0.285 ± 0.014

The concentration of cytochrome P-450 was 0.1 μM in the case of benzphetamine *N*-demethylation or 7-ethoxycoumarin *O*-deethylation and was 0.04 μM in the case of biphenyl hydroxylation. Each value represents mean ± S.D. of duplicate determinations.

N.D., not detectable.

Table 2. The interaction between PB P-450 and MC P-448$_1$ during drug oxidations in the presence of limited amounts of reductase

Reactions	Reductase (μM)	Cytochrome P-450		
		PB P-450	MC P-448$_1$ (nmole product/20 min)	PB P-450 + MC P-448$_1$
Benzphteamine				
N-demethylation	0.03	2.89 ± 0.71	0.55 ± 0.21	3.30 ± 0.71
Biphenyl	0.05	ND	0.136 ± 0.007	0.104 ± 0.006
2-hydroxylation	0.07	ND	0.209 ± 0.011	0.166 ± 0.011
	0.30	ND	0.360 ± 0.020	0.363 ± 0.029
4-hydroxylation	0.05	0.105 ± 0.016	1.244 ± 0.110	1.080 ± 0.040
	0.07	0.128 ± 0.025	1.690 ± 0.156	1.390 ± 0.127
	0.30	0.355 ± 0.021	4.655 ± 0.148	4.700 ± 0.240
Aniline	0.03	0.24 ± 0.05	0.68 ± 0.09	0.58 ± 0.05
hydroxylation	0.40	1.13 ± 0.10	6.62 ± 0.57	7.28 ± 0.49

The concentration of cytochrome P-450 was 0.1 μM in the case of aniline hydroxylation. Other experimental conditions are the same as described in Table 1. Each value represents mean ± S.D. of duplicate determinations.

NO, not detectable.

Table 3. The interaction between MC P-448$_1$ and MC P-448$_2$ during drug oxidations in the presence of limited amounts of reductase

Reactions	Reductase (μM)	Cytochrome P-450		
		MC P-448$_1$	MC P-448$_2$ (nmole product/20 min)	MC P-448$_1$ + MC P-448$_2$
7-Ethoxycoumarin	0.11	0.49 ± 0.06	21.55 ± 4.60	17.35 ± 1.20
O-deethylation	0.32	1.11 ± 0.01	34.60 ± 2.26	41.75 ± 2.19
Aniline	0.02	0.40 ± 0.09	0.09 ± 0.04	0.19 ± 0.03
hydroxylation	0.05	1.12 ± 0.17	0.15 ± 0.03	1.04 ± 0.06
	0.40	6.62 ± 0.57	0.79 ± 0.07	6.70 ± 0.57

Experimental details are the same as described in Table 1 and 2.

Table 4. The apparent K_m of cytochrome P-450 for reductase in the presence of various drug substrates

Reactions	Cytochrome P-450					
	PB P-450		MC P-448$_1$		MC P-448$_2$	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Benzphetamine *N*-demethylation	0.16 ± 0.02	55.8 ± 5.7	0.63 ± 0.14	18.5 ± 4.9	0.54 ± 0.13	6.2 ± 1.6
7-Ethoxycoumarin *O*-deethylation	0.21 ± 0.04	5.4 ± 0.1	0.55 ± 0.09	2.8 ± 0.5	0.41 ± 0.07	87.3 ± 13.9
Biphenyl						
2-hydroxylation	—	—	0.28 ± 0.02	0.8 ± 0.1	0.59 ± 0.14	2.2 ± 1.1
4-hydroxylation	0.18 ± 0.07	0.6 ± 0.2	0.25 ± 0.05	7.6 ± 2.4	0.50 ± 0.15	4.6 ± 1.4
Aniline						
4-hydroxylation	0.85 ± 0.20	4.1 ± 0.8	0.48 ± 0.04	15.2 ± 2.5	0.34 ± 0.02	1.3 ± 0.1

Each value was obtained by means of the double reciprocal plot of the activity of drug oxidation vs the concentration of reductase.

both 2- and 4-hydroxylation of biphenyl and was less active in benzphetamine *N*-demethylation than PB P-450. The addition of PB P-450 to the MC P-448$_1$ system containing 0.05 μM of reductase resulted in a decrease in the activity of biphenyl 2-hydroxylation by about 70% of the activity in the control experiment. On the other hand, the activity of benzphetamine *N*-demethylation in the system containing both cytochromes was comparable to that observed in the system containing PB P-450 alone. In addition, MC P-448$_2$ was more active in 7-ethoxycoumarin *O*-deethylation than MC P-448$_1$, but MC P-448$_2$ was less active in aniline hydroxylation than MC P-448$_1$ (Table 3). When both cytochromes were present in a reconstituted system, MC P-448$_1$ was found to be inhibitory on MC P-448$_2$ supported 7-ethoxycoumarin *O*-deethylation depending on the concentration of the reductase added. On the other hand, MC P-448$_2$ had an inhibitory effect on MC P-448$_1$ supported aniline hydroxylation when both cytochromes were present in the system.

To determine if the affinity of cytochrome P-450 for the reductase depends on the cytochrome species, the affinity was measured using the double reciprocal plots of the activity of drug oxidation vs the concentration of the reductase (Table 4). The apparent K_m of PB P-450 for reductase was lower than those of two other isozymes when benzphetamine and 7-ethoxycoumarin were used as substrates ($P < 0.05$). Furthermore, PB P-450 had a tendency to show lower values than two other isozymes when biphenyl was used as a substrate. On the other hand, MC P-448$_1$ and MC P-448$_2$ exhibited lower values than PB P-450 when aniline was used as substrate ($P < 0.01$). In addition, the apparent K_m of MC P-448$_1$ was similar to that of MC P-448$_2$ when benzphetamine, 7-ethoxycoumarin or aniline was used as substrates but not when biphenyl was used. From these results, it seems possible to assume that the apparent K_m of cytochrome P-450 for the reductase is dependent on the substrate used, and there is no correlation between apparent K_m and V_{max}.

DISCUSSION

The inhibition of MC P-448$_2$ supported biphenyl hydroxylation or 7-ethoxycoumarin *O*-deethylation by PB P-450 could be reversed by the addition of more reductase indicating that PB P-450 and MC P-448$_2$ may be competing for the reductase in the reaction mixture during drug oxidations. Similar results were obtained when MC P-448$_1$ was used in place of MC P-448$_2$. These results were consistent with the studies previously reported by West and Lu [25]. The formation of a binary complex between cytochrome P-450 and reductase has been shown to be essential for catalysis in a reconstituted hydroxylation system [26]. In addition, when reductase is the rate limiting component as is the case in the present study, the concentration of cytochrome P-450 reductase complex which is catalytically active will be dependent on the concentration of the reductase. It seems, therefore, reasonable to assume that the decrease in the rate of reaction catalysed by MC P-448$_1$ or MC P-448$_2$ in the presence of PB P-450 may be due to the decrease in the concentration of the catalytically active complex between MC P-448$_1$ or MC P-448$_2$ and reductase. On the contrary, neither MC P-448$_1$ nor MC P-448$_2$ inhibited PB P-450 supported benzphetamine *N*-demethylation under similar conditions suggesting that the concentration of the binary complex between PB P-450 and reductase may be virtually unchanged with or without MC P-448$_1$ or MC P-448$_2$. At present, the reason why neither MC P-448$_1$ nor MC P-448$_2$ affects PB P-450 dependent reaction when reductase is present in limiting amount can not be explained. However, the apparent K_m of PB P-450 for reductase obtained using biphenyl, 7-ethoxycoumarin or benzphetamine as a substrate was lower than that of MC P-448$_1$ or MC P-448$_2$ suggesting that the inhibition of MC P-448$_1$ or MC P-448$_2$ supported reaction by PB P-450 appears to be at least in part due to the difference in the affinity for reductase. Since it has been demonstrated [19] that in a reconstituted system

cytochrome P-450, reductase, phospholipid and substrate are mutually interacting to form the functional enzyme complex, more precise kinetic measurements are required before this relationship can be firmly established.

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