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SOME CHARACTERISTICS OF PARTIALLY PURIFIED HUMAN LIVER PHENYLALANINE HYDROXYLASE

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SUMMARY

Phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine: O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) has been partially purified from human autopsy liver. Most of the properties reported for the rat liver phenylalanine hydroxylase are shared by the human enzyme: its activity can be stimulated by endogenous phospholipids as well as by the phenylalanine hydroxylase stimulating protein from rat liver; inhibition of the enzyme is observed with excess phenylalanine in the presence of tetrahydrobiopterin; K_m values vary with the pterin cofactor employed; uncoupling of tetrahydropterin oxidation from phenylalanine hydroxylation is observed with the cofactor, 7-methyltetrahydropterin.

INTRODUCTION

Detailed studies of purified mammalian phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine: O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) have been performed almost exclusively with rat liver phenylalanine hydroxylase[1]. The few properties of the normal human liver enzyme that have been described[2] were determined on crude extracts of liver obtained from small biopsy samples. Since phenylketonuria is associated with a deficiency of this enzyme[3–7], it would be of interest to characterize in detail highly purified human liver phenylalanine hydroxylase. The unavailability of large quantities of fresh human liver, however, has precluded this kind of investigation. Recently we received a portion of necropsy liver from a patient with no evidence of liver disease which was removed and quick-frozen within 30 min of death*. An extract of this liver was found to have 35% as much activity as an extract of normal biopsied liver. With a modification of the purification procedure described for the rat phenylalanine hydroxylase[8], the human enzyme has been purified approximately 100-fold over the extract with a yield of about 10% of the activity. The following properties of this partially purified enzyme are reported here: apparent K_m values for phenylalanine, O_2, and the pterin cofactors; reaction stoichio-

* We are grateful to Dr E. LaBrosse for obtaining the liver sample.

metry with different pterin cofactors; stimulation by phospholipids; and stimulation by a phenylalanine hydroxylase-stimulating protein from rat liver[9]. Whereas the human liver and the rat liver enzymes do differ in some of their characteristics, most of the properties reported for the rat enzyme are shared, at least qualitatively, by the human hydroxylase.

MATERIALS AND METHODS

All of the assay procedures used in the present study have already been described[8]. Materials were obtained from sources that were previously listed[8].

Purification of human liver phenylalanine hydroxylase

The procedure used was essentially the one used for the purification of the enzyme from rat liver[8], with the following modifications: (a) homogenization was with 0.15 M KCl and not with 0.01 M acetic acid; (b) the active ethanol fraction was 8–18% and not 10–21%; (c) a 25–40% first $(NH_4)_2SO_4$ fraction (instead of 29–37%) was taken; (d) the enzyme activity was eluted from calcium phosphate gel with two washes of 0.12 M potassium phosphate buffer at pH 6.8. The DEAE-cellulose and Sephadex G-200 column steps were unchanged. After these steps, the enzyme was completely dependent on phenylalanine, tetrahydropterin, and molecular oxygen for activity. That the reaction product was tyrosine, was checked by including L-[^{14}C]-phenylalanine in the reaction and identifying [^{14}C]tyrosine chromatographically.

RESULTS AND DISCUSSION

Stimulation by phospholipids and K_m values

One of the characteristic properties of rat liver phenylalanine hydroxylase is that its activity is markedly stimulated by certain phospholipids such as lysolecithin[10]. This dramatic stimulation is observed only in the presence of the naturally-occurring cofactor, tetrahydrobiopterin; with the cofactor analogue, 6,7-dimethyltetrahydropterin, a slight stimulation is observed[10]. The major effect of lysolecithin is a 50-fold increase in V; a minor effect is on the K_m for phenylalanine[10]. As can be seen in Fig. 1, lysolecithin also stimulates the human liver hydroxylase, although the effect is not nearly as marked as that seen with the rat liver enzyme. In addition to stimulating the velocity of the hydroxylation reaction, lysolecithin also appears to sensitize the human liver hydroxylase to inhibition by excess phenylalanine. This enhanced sensitivity to inhibition by phenylalanine in the presence of lysolecithin was also observed with the rat liver hydroxylase[10]; with that enzyme, however, substrate inhibition was apparent only at phenylalanine concentrations greater than 0.5 mM[10]. If a similar phenylalanine concentration study is performed on cruder fractions of the human enzyme (*i.e.* the extract or the first $(NH_4)_2SO_4$ fraction), stimulation by lysolecithin is also found*, but the substrate inhibition is less marked; *e.g.* an $(NH_4)_2SO_4$ fraction prepared from the liver extract, which shows maximum

* Approximately the same degree of stimulation is observed at phenylalanine concentrations less than 0.1 mM when the response of a crude extract is compared with that of the purified enzyme.

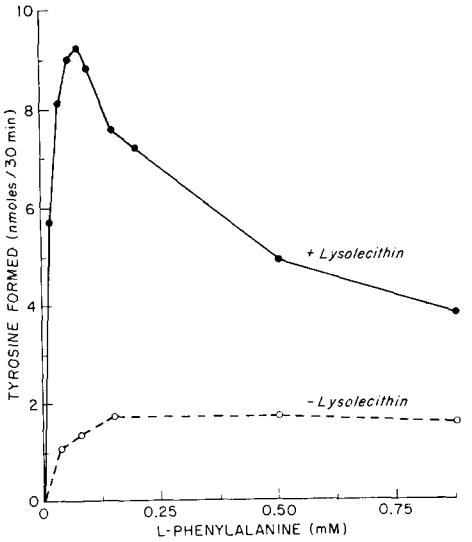

Fig. 1. Effect of phenylalanine concentration on hydroxylase activity with tetrahydrobiopterin as cofactor. Each assay tube (total volume 0.5 ml) contained the following components (in μmoles unless otherwise stated): potassium phosphate buffer, pH 6.8, 50; NADPH, 0.05; glucose 6-phosphate, 0.5; glucose-6-phosphate dehydrogenase (EC 1.1.1.49), catalase (EC 1.11.1.6), and dihydropteridine reductase in excess. Incubation was for 30 min at 25 °C. Tetrahydrobiopterin concentration was 24 μM. The enzyme used (280 μg) was from a DEAE-cellulose peak fraction. Lysolecithin concentration was 1.2 mM when present. Tyrosine formation was determined by the nitrosonaphthol method[11,12].

activity with lysolecithin at a phenylalanine concentration between 0.1 and 0.2 mM, is inhibited only 20–25% at 0.8 mM phenylalanine.

A K_m value for L-phenylalanine of 0.04 mM was obtained from the data in Fig. 1. This value is significantly lower than the value of 0.2 mM obtained with the rat liver enzyme in the presence of lysolecithin[10]. It is of interest that this K_m value for L-phenylalanine (0.04 mM), obtained with the human enzyme in the presence of lysolecithin, is quite close to normal human plasma phenylalanine levels (0.047–0.058 mM)[13,14]. An approximate K_m value for phenylalanine in the absence of lysolecithin, calculated from the data in Fig. 1, indicates that this value (0.05 mM) is close to that obtained in the presence of lysolecithin. It should also be noted that the V for the human enzyme with tetrahydrobiopterin in the presence of lysolecithin is about one-half the V with 6,7-dimethyltetrahydropterin in the absence of lysolecithin (data not shown).

K_m values for tetrahydrobiopterin and O_2 were also determined in the presence of lysolecithin. These values, together with the corresponding values for 6,7-dimethyltetrahydropterin and 6-methyltetrahydropterin, are shown in Table I. The low activity of the hydroxylase in the presence of tetrahydrobiopterin but in the absence of lysolecithin, and the limited amounts of the purified human enzyme available, precluded an accurate determination of K_m values of tetrahydrobiopterin, and of phenylalanine and oxygen in the absence of lysolecithin. Since the effect of lysolecithin on the rat liver hydroxylase in the presence of 6,7-dimethyltetrahydropterin and 6-methyltetrahydropterin is minimal[10], the differences in K_m values for the sub-

TABLE I

SUMMARY OF APPARENT K_m VALUES

Assay conditions are as described in Fig. 1. The hydroxylase source was either DEAE-cellulose or Sephadex G-200 peak fractions. The ranges over which the substrates studied were varied are indicated in the second column for each cofactor. When tetrahydrobiopterin was the cofactor, assays were run with 1.2 mM lysolecithin.

Substrate	*Cofactor:*					
	Tetrahydrobiopterin		*6,7-Dimethyltetrahydropterin*		*6-Methyltetrahydropterin*	
	K_m	*Substrate range*	K_m	*Substrate range*	K_m	*Substrate range*
L-Phenylalanine	0.04 mM	0.02–0.875 mM	1.6 mM	0.4–4 mM	0.9 mM	0.2–2 mM
Pterin	0.003 mM	3.2–40 μM	0.05 mM	0.032–0.256 mM	0.04 mM	0.025–0.200 mM
O_2	0.95%	0 to 100%	3.40%	0 to 100%	—	—

strates determined in the presence of the different pterins is probably not due to the presence or absence of lysolecithin.

Just as with the rat liver enzyme[1,15], the apparent K_m values of the human enzyme for its substrates, phenylalanine and oxygen, vary with the pterin cofactor used. Thus, the K_m for phenylalanine in the presence of tetrahydrobiopterin is only one-fortieth as great as it is in the presence of 6,7-dimethyltetrahydropterin (Table I). Similarly, the K_m for oxygen in the presence of tetrahydrobiopterin is only one-third as great as it is in the presence of 6,7-dimethyltetrahydropterin. It should be noted, however, that the K_m of the human enzyme for oxygen in the presence of tetrahydrobiopterin (0.95%) is larger than it is for the rat liver enzyme (0.35%)[15]. In addition, whereas inhibition of the human enzyme's activity by high O_2 levels is evident (in the presence of tetrahydrobiopterin), it is much less marked than that seen with the rat enzyme[15]; at 40% and 100% O_2, inhibition of the human enzyme (compared with peak activity) is only 5 and 25%, respectively (data not shown). Inhibition of the rat enzyme at 40% O_2 is about 35–40%[15].

Stoichiometry

Reaction stoichiometry, the ratio of pterin cofactor oxidized to tyrosine formed, was also determined (Table II). With the rat liver phenylalanine hydroxylase, this

TABLE II

STOICHIOMETRY WITH VARIOUS COFACTORS

Tetrahydropterin oxidized was measured indirectly from NADPH oxidation using a previously described spectrophotometric assay[16]. Assay was for 45 min at 25 °C in a 1-ml volume. Tyrosine was determined after 45 min by the nitrosonaphthol procedure[11,12]. The enzyme used (1.05 mg) was from a DEAE-cellulose peak fraction. Tetrahydropterin concentrations are indicated in parentheses. When tetrahydrobiopterin was the cofactor, lysolecithin (1.2 mM) was present.

Pterin	*NADPH oxidized (nmoles)*	*Tyrosine formed (nmoles)*	*Ratio NADPH/tyrosine*
Tetrahydrobiopterin (4.8 μM)	17.9	17.7	1.01
6,7-Dimethyltetrahydropterin (80 μM)	42.8	36	1.19
7-Methyltetrahydropterin (80 μM)	33.6	6.6	5.10

ratio with either tetrahydrobiopterin or 6,7-dimethyltetrahydropterin is equal to 1.0[1,16]; with the human enzyme, the ratio with tetrahydrobiopterin is equal to 1.0 and is close to 1.0 with 6,7-dimethyltetrahydropterin. With 7-methyltetrahydropterin, uncoupling (*i.e.* more tetrahydropterin oxidized than tyrosine formed) is observed with the human enzyme as it is with the rat enzyme[17,18].

Effect of phenylalanine hydroxylase stimulating protein

A phenylalanine hydroxylase-stimulating protein has recently been partially purified from rat liver[9]. With tetrahydrobiopterin as cofactor, the protein maximally stimulates the activity of the rat liver phenylalanine hydroxylase at about pH 8 with high concentrations of the enzyme[9]. As shown in Fig. 2, highly purified rat liver phenylalanine hydroxylase-stimulating protein can also stimulate the activity of the human hydroxylase. The stimulation observed is only 2-fold, but it should be noted that rather low concentrations of enzyme have been used here; more pronounced stimulation might be expected at higher enzyme concentrations.

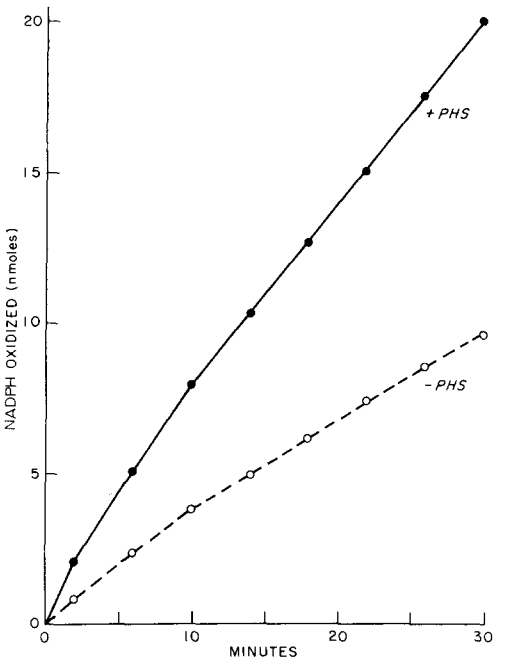

Fig. 2. Effect of phenylalanine hydroxylase-stimulating (PHS) protein on the activity of human phenylalanine hydroxylase. Assay conditions are exactly as has been described[9]; the pH was 8.2. Tetrahydrobiopterin (4.8 μM) was the cofactor; lysolecithin (1.2 mM) was present. The enzyme used (2.1 mg) was from a DEAE-cellulose peak fraction. In the experiment in which it was present, 15 μg of highly purified phenylalanine hydroxylase-stimulating protein from rat liver was used.

Although our observations are limited, they do serve to emphasize the qualitative similarities between the purified human and rat liver phenylalanine hydroxylases with regard to some of their kinetic properties and potential regulatory properties (*i.e.* substrate inhibition by excess phenylalanine, stimulation by endogenously occurring phospholipids, stimulation by phenylalanine hydroxylase-stimulating pro-

tein). As yet unexplained quantitative differences do exist. Conceivably, some of the quantitative differences that have been observed would be minimized if the hydroxylase from fresher human liver samples could be studied.

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