Interaction with a monoclonal antibody alters the expression of co-operativity by phenylalanine hydroxylase from rat liver

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Phenylalanine hydroxylase purified from rat liver shows positive co-operativity in response to variations in phenylalanine concentration when assayed with the naturally occurring cofactor tetrahydrobiopterin. In addition, preincubation of phenylalanine hydroxylase with phenylalanine results in a substantial activation of the tetrahydrobiopterin-dependent activity of the enzyme. The monoclonal antibody PH-1 binds to phenylalanine hydroxylase only after the enzyme has been preincubated with phenylalanine and is therefore assumed to recognize a conformational epitope associated with substrate-level activation of the hydroxylase. Under these conditions, PH-1 inhibits the activity of phenylalanine hydroxylase; however, at maximal binding of PH-1 the enzyme is still 2–3-fold activated relative to the native enzyme. The inhibition by PH-1 is non-competitive with respect to tetrahydropterin cofactor. This suggests that PH-1 does not bind to an epitope at the active site of the hydroxylase. Upon maximal binding of PH-1, the positive co-operativity normally expressed by phenylalanine hydroxylase with respect to variations in phenylalanine concentration is abolished. The monoclonal antibody may therefore interact with phenylalanine hydroxylase at or near the regulatory or activator-binding site for phenylalanine on the enzyme molecule.

INTRODUCTION

Phenylalanine hydroxylase (PAH, phenylalanine 4-mono-oxygenase; EC 1.14.16.1) catalyses the initial and presumably rate-limiting step in the hepatic catabolism of phenylalanine, namely the tetrahydropterin-dependent oxygenation of phenylalanine to form tyrosine. PAH purified from rat liver possesses low catalytic activity when assayed with the naturally occurring cofactor (6*R*)-*L*-erythro-tetrahydrobiopterin (BH₄) and shows positive co-operativity in BH₄-dependent activity in response to variations in the concentration of phenylalanine [1]. By contrast, with synthetic cofactors such as 6-methyltetrahydropterin (6MPH₄), the enzyme has high catalytic activity and shows no co-operativity with respect to variations in phenylalanine concentration [1]. However, positive co-operativity appears to be an innate characteristic of PAH, since this co-operativity is observed for the binding of phenylalanine in the absence of any tetrahydropterin cofactor [2].

The low BH₄-dependent activity of native rat liver PAH can be stimulated by a variety of conditions. Incubation with phospholipids [3], limited proteolysis [3,4] and thiol modification [2] result in 20–30-fold activations at low phenylalanine concentrations as well as the abolition of the expression of co-operativity by the enzyme. In contrast, other treatments such as phosphorylation of the enzyme [5] and preincubation of PAH with the substrate phenylalanine [6–9] result in activation with the retention of co-operativity. These activation phenomena are fully observed only when BH₄ is used as cofactor; little effect is noted on the 6MPH₄-dependent activity of the hydroxylase [2–5,9].

Aspects of protein structure involved in the expression of co-operativity and activation of PAH are not well characterized. Gross alterations in secondary structure have been noted upon activation of PAH by limited proteolysis or exposure to phospholipids [4]. Such changes are not observed upon activation of the enzyme by preincubation with phenylalanine, although fluorescence and u.v. difference spectroscopic measurements indicate that some change in conformation of PAH accompanies this activation [10]. Preincubation with phenylalanine has also been observed to alter the oligomeric state of PAH [11,12]. Immunochemical studies with monoclonal antibodies have recently emerged as a useful method for the study of protein structure–function relationships [13]. In the present paper we describe certain functional consequences of the interaction of a monoclonal antibody against hepatic PAH with the enzyme purified from rat liver.

EXPERIMENTAL

Materials

Phenylalanine hydroxylase was purified with the use of phenyl-Sepharose CL-4B (Pharmacia) as described [14] from Sprague–Dawley rat livers obtained frozen from Pel-Freez Biologicals (Rogers, AR, U.S.A.). The 6MPH₄-dependent specific activities of the hydroxylase preparations used in these studies ranged from 1.5 to 7 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. The variation in the specific activities of different preparations of purified PAH may be related to the variability of the iron content of the enzyme [15]. However, the interaction of the hydroxylase

Abbreviations used: BH₄, (6*R*)-*L*-erythro-tetrahydrobiopterin; 6MPH₄, 6-methyltetrahydropterin; PAH, phenylalanine hydroxylase; Ab/Ag ratio, antibody/antigen ratio.

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with the monoclonal antibody was essentially identical with all preparations of phenylalanine hydroxylase studied. The methods for the production and cloning of the monoclonal antibody (designated 'PH-1') have previously been described [16,17]. (6*R*)-*L*-erythro-5,6,7,8-Tetrahydrobiopterin and (6*R,S*)-methyl-5,6,7,8-tetrahydropterin were obtained from Dr. B. Shirck's Laboratories (Jona, Switzerland). Catalase and dithiothreitol were purchased from Boehringer-Mannheim. Sheep liver dihydropteridine reductase, *L*-phenylalanine and NADH were from Sigma. Protein A-Sepharose was from Pharmacia. All other reagents were from local suppliers and were ACS reagent grade or better.

Methods

Measurement of PAH activity. The activity of PAH was determined from the decrease in absorbance of NADH at 340 nm in a spectrophotometric assay in which quinonoid dihydropterin, formed during the hydroxylation of phenylalanine, is reduced to tetrahydropterin by dihydropteridine reductase and NADH [18]. Assays were conducted at 25 °C and the assay mixture contained, in a total volume of 0.5 or 1 ml, 0.1 M-potassium phosphate, pH 6.8, 0.1–0.15 mM-NADH, catalase and dihydropteridine reductase in excess, *L*-phenylalanine and BH_4 or 6MPH₄ in the concentrations as indicated in the legends to the Tables and Figures. Reactions were always initiated by the addition of PAH to an otherwise complete assay mixture equilibrated at 25 °C. Initial velocities were determined from the first 15–30 s of the progress curve and were corrected for the rate of tetrahydropterin autooxidation determined from assays in the absence of PAH.

PAH was also measured by a fixed-time assay [19]. The enzyme was added to assay mixtures that contained, in a total volume of 0.5 ml, 0.1 M-potassium phosphate, pH 6.8, 10 mM-dithiothreitol, catalase in excess, *L*-phenylalanine and BH_4 or 6MPH₄ in the concentrations as indicated in the legends to the Tables and Figures. Samples were incubated at 25 °C with shaking for 15–30 min, and the reactions were then quenched by the addition of 1 ml of 10% (w/v) trichloroacetic acid. The amount of tyrosine formed was determined by the colorimetric/fluorimetric nitrosonaphthol procedure [20].

Incubation of phenylalanine hydroxylase with monoclonal antibody PH-1. The concentrations of stock solutions of the purified proteins were determined spectrophotometrically at 280 nm using $A_{\text{cm}}^{1\%}$ of 10 for rat liver phenylalanine hydroxylase [14] and $A_{\text{cm}}^{1\%}$ of 14 for the monoclonal antibody PH-1 [21]. Purified PAH was preincubated for 10 min at 25 °C in 50 mM-potassium phosphate, pH 6.8, in the absence or presence of phenylalanine (0.5–2 mM). Portions of this preincubated enzyme were then added to tubes containing the antibody PH-1 in 50 mM-potassium phosphate, pH 6.8, and an amount of phenylalanine such that the final concentration in the mixture was identical with that in which PAH was preincubated. The mixtures were incubated for 15 min at 25 °C, then for an additional 30 min at 4 °C. Portions of the mixture were then assayed for PAH activity as described above. Trial experiments confirmed that the antibody-antigen interaction was essentially complete under these incubation conditions.

Data analysis. Kinetic parameters were determined with the use of a computer program based on the direct linear plot described by Eisenthal & Cornish-Bowden [22]. Curve-fitting analyses were performed with ASYSTANT Scientific Software (MacMillan Software, New York, NY, U.S.A.).

RESULTS

The monoclonal antibody PH-1 was prepared against PAH from monkey liver [16]. The binding of PH-1 to the monkey liver enzyme has been observed only in the presence of phenylalanine [17,23]. The BH_4 -dependent activity of PAH from a variety of sources, such as monkey liver [23] and rat liver [7,8], can be stimulated by preincubation of the enzyme with phenylalanine. In the absence of phenylalanine, PH-1 had no effect on the BH_4 -dependent activity of PAH from rat liver (Fig. 1). However, when the rat liver enzyme was first preincubated with phenylalanine and then incubated with PH-1 (in the continued presence of phenylalanine), a substantial inhibition of the BH_4 -dependent activity of PAH was observed. Titration of PAH with PH-1 under these conditions resulted in a progressive decrease in both BH_4 -dependent activity (Fig. 1) and 6MPH₄-dependent activity (results not shown). However, complete inhibition of PAH activity by PH-1 was not attained. The 6MPH₄-dependent activity of PAH is not generally considered to reflect changes in the state of activation of rat liver PAH [1–5,9]. With 6MPH₄ as cofactor, the

Fig. 1. Titration of BH_4 -dependent activity of PAH with PH-1

Portions (2 μg) of purified rat liver PAH (0.01 nmol with respect to 200 kDa tetramer) were incubated in 50 mM-potassium phosphate, pH 6.8, with various amounts of PH-1 in the absence or the presence of 2 mM-phenylalanine for 15 min at 25 °C and then an additional 30 min at 4 °C. The activity of PAH was then determined in a spectrophotometric assay with 2 mM-phenylalanine and 40 μM -(6*R*)- BH_4 . ■, Activity of PAH incubated with various amounts of PH-1 in the presence of 2 mM-phenylalanine; □, activity of PAH incubated with various amounts of PH-1 in the absence of phenylalanine. Initial activity refers to the activity of PAH preincubated with 2 mM-phenylalanine in the absence of PH-1. In this experiment this activity was 3 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. The broken line describes the activity of PAH incubated as above in either the absence or presence of PH-1, but in the absence of phenylalanine. This activity was 0.25 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$.

Table 1. Kinetic parameters for 6MPH₄-dependent activity of PAH in the absence and the presence of PH-1

Variable substrate*	PH-1	K_m (mM)†	V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)†	Type of inhibition
6MPH ₄	—	0.02	1.8	Non-competitive
	+‡	0.04	0.6	
Phenyl-alanine	—	0.3	1.9	Non-competitive
	+‡	0.8	0.5	

* When 6MPH₄ was the variable substrate, the phenylalanine concentration was maintained at 10 mM. When phenylalanine was the variable substrate, 6MPH₄ concentration was maintained at 0.15 mM.

† Kinetic parameters (K_m and V_{max}) were calculated using a computer program based on the direct linear plot of Eisenthal & Cornish-Bowden [22].

‡ The PH-1/PAH ratio in the samples containing the antibody was 1.6.

residual PAH activity was about 20–25 % of that of the enzyme which had been incubated with PH-1 but in the absence of phenylalanine, or of that of the enzyme which had been incubated with phenylalanine in the absence of PH-1. Alterations in the level of activation of rat liver PAH are most readily observed in the presence of the naturally occurring cofactor, tetrahydrobiopterin [1]. The BH₄-dependent activity of PAH in the presence of both phenylalanine and PH-1 was decreased to about 20 % of that of PAH which had been incubated with phenylalanine only. However, it is noteworthy that the residual BH₄-dependent activity was still 2-fold greater than that of either native PAH (incubated in the absence of both phenylalanine and PH-1) or the enzyme which had been incubated with PH-1 in the absence of phenylalanine (Fig. 1). It is unlikely that the residual 6MPH₄-dependent and BH₄-dependent activities of PAH remaining after complete titration with PH-1 were the result of the presence of PAH molecules which were incapable of interaction with the antibody. The addition of Protein A–Sepharose to mixtures containing PAH and PH-1 [antibody/antigen (Ab/Ag) ratio of 2] in the presence of phenylalanine resulted in the complete removal of PAH activity. By contrast, Protein A–Sepharose was unable to remove PAH activity from mixtures of PAH and PH-1 when phenylalanine was absent (results not shown). The titration of PAH activity by PH-1 in the presence of phenylalanine was complete at an Ab/Ag ratio of 1 (Fig. 1). Ab/Ag ratios were calculated by assuming a molecular mass of 150 kDa for PH-1 and of 200 kDa for PAH, since the enzyme exists entirely as the 200 kDa tetramer after preincubation with phenylalanine [11,12]. The extent of maximum inhibition of PAH activity by PH-1 could not be increased either by increasing the Ab/Ag ratio (ratios as high as 6 were employed) or by increasing the time of incubation of the enzyme with the antibody.

The inhibition of PAH by PH-1 was found to be non-competitive with respect to both phenylalanine and tetrahydropterin when 6MPH₄ was used as cofactor (Table 1). The effect of PH-1 on kinetic parameters of PAH with BH₄ as cofactor is somewhat more complex

Table 2. Effect of PH-1 on kinetic parameters of PAH with BH₄ as cofactor

Assays were performed spectrophotometrically with 2 mM-phenylalanine and variable concentrations of (6R)-BH₄.

Enzyme	Ab/Ag ratio	K_m (μM)	V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)
Native	—	2.1	0.24
Activated†	—	14.0	6.5
+ PH-1	0.5	2.2	0.92
	1.5	1.2	0.46

* Kinetic parameters (K_m and V_{max}) were calculated using a computer program based on the direct linear plot of Eisenthal & Cornish-Bowden [22].

† Activated refers to enzyme which had been preincubated with 2 mM-phenylalanine before exposure to antibody.

than with 6MPH₄ as cofactor. The apparent K_m for the natural cofactor (6R)-BH₄ with the native hydroxylase was 2 μM ; activation of the enzyme by preincubation with phenylalanine resulted in a decrease in affinity of PAH for BH₄, with an apparent K_m of 14 μM (Table 2). Addition of PH-1 to this activated enzyme resulted in an apparent increase in affinity for BH₄ concomitant with a decrease in observed velocity. The apparent K_m for BH₄ under conditions of maximal inhibition of the activated PAH by PH-1 was similar to that seen with the native (non-activated) enzyme in either the absence or presence of PH-1. The inhibition of the phenylalanine-activated PAH by PH-1 was non-competitive with respect to BH₄.

The reaction progress curves for BH₄-dependent PAH activity were generally non-linear (Fig. 2). With native PAH, the initial velocity was low and a progressive increase in velocity was observed; this increase was due to phenylalanine-dependent activation of PAH *in situ* in the reaction assay [24]. In contrast, a rapid initial velocity was obtained with PAH which had been activated by preincubation with phenylalanine. This velocity decreased throughout the course of reaction, presumably due to de-activation of PAH *in situ* [7]. The rate of this de-activation was dependent on the concentration of BH₄ employed in the assay, with higher concentrations of this cofactor increasing the rate of de-activation of the enzyme (M. A. Parniak, unpublished work). In the presence of the monoclonal antibody PH-1 (in an amount sufficient to cause maximal inhibition of PAH activity), linear rates of phenylalanine hydroxylation were obtained (Fig. 2). Linearity was noted in assays using either low or high concentrations of BH₄.

Native PAH from rat liver expresses positive co-operativity with respect to phenylalanine in the presence of BH₄ [1]. This positive co-operative relationship was also observed with PAH which had been activated by preincubation with phenylalanine (Fig. 3). Under these conditions, an apparent K_m of 0.03 mM for phenylalanine was calculated. Addition of PH-1 resulted in little effect on the apparent K_m of the enzyme for phenylalanine (0.04 mM), but resulted in a substantial decrease in observed velocity of the PAH reaction. Interaction of the antibody with PAH (at an Ab/Ag ratio sufficient to give maximal inhibition) resulted in the abolition of the

Fig. 2. Reaction progress curves for PAH in the absence or presence of PH-1

Portions (5 μ g) of purified rat liver PAH (0.025 nmol with respect to 200 kDa tetramer) were preincubated in 50 mM-Tris (pH 6.8, 25 $^{\circ}$ C)/0.2 M-KCl with various additions for 10 min at 25 $^{\circ}$ C and then 30 min at 4 $^{\circ}$ C. Aliquots of these preincubated samples (corresponding to 4.5 μ g of enzyme) were then added to otherwise complete spectrophotometric assay mixtures containing 2 mM-phenylalanine, 5 μ M-(6R)-BH₄ and other components as described in the Experimental section. A, PAH preincubated in the absence of phenylalanine and in the absence of PH-1; B, PAH preincubated with 2 mM-phenylalanine in the absence of PH-1; C, PAH preincubated with 2 mM-phenylalanine and 5.7 μ g of PH-1 (0.038 nmol, Ab/Ag ratio 1.5). The broken lines describe the initial velocities of the PAH reaction, determined as described under 'Methods'.

expression of positive co-operativity in the variation of the BH₄-dependent activity of the enzyme with respect to phenylalanine concentration (Fig. 3).

DISCUSSION

PAH from rat liver is an oligomeric protein composed of 50 kDa subunits which are identical in primary structure [25]. The native enzyme exists as a mixture of 100 kDa dimers and 200 kDa tetramers, with the latter accounting for about 75% of the native hydroxylase protein at pH 6.8 and 25 $^{\circ}$ C [12]. Preincubation of PAH with the substrate phenylalanine results in a number of changes in the enzyme: (i) a 10–20-fold increase in BH₄-dependent activity, but with little change in 6MPH₄-dependent activity [2,7,9,10]; (ii) alterations in the tertiary conformation of the enzyme [10]; and (iii) complete conversion of the enzyme into a 200 kDa tetramer [11,12]. The monoclonal antibody (PH-1) binds to PAH only when the enzyme has been incubated with phenylalanine.

The concentration of phenylalanine required for the half-maximal binding of PH-1 to monkey liver PAH is about 0.1 mM [23]. This concentration is similar to that required both for the half-maximal activation of the BH₄-dependent velocity and for the phenylalanine-dependent changes in tertiary conformation of the rat liver enzyme [10]. We have recently found that 0.1 mM is also the half-maximal concentration of phenylalanine required for the formation of 200 kDa tetramer from the 100 kDa dimer of rat liver PAH (M. A. Parniak, unpublished work). Although PH-1 must bind to the 200 kDa tetramer, since this is the only oligomeric form of the enzyme in the presence of phenylalanine, it is unlikely that this quaternary structure alone is sufficient to enable interaction with the antibody. PH-1 had no effect on native PAH (i.e. in the absence of phenylalanine), even though the native enzyme consists predominantly of 200 kDa tetramers. It therefore appears that only PAH which has undergone some alteration in tertiary structure in response to preincubation with phenylalanine is capable of interaction with the antibody.

The positive co-operativity with respect to phenylalanine expressed by PAH, as well as the activation of the enzyme upon preincubation with the amino acid, have led to proposals that rat liver PAH possesses both catalytic and regulatory (activator) binding sites for phenylalanine [2,8–10,26,27]. It has been observed that PAH can bind 1.5 mol of phenylalanine/mol of 50 kDa subunit [2]; however, the enzyme binds only 1 mol of BH₄/mol of subunit [10]. Since the tetrahydropterin cofactor is absolutely essential for catalysis, it is assumed that each identical subunit of PAH has one catalytic site, and the equivalent of 0.5 regulatory or activator site for phenylalanine [12]. The inhibition of PAH activity upon maximum binding of PH-1 is not complete and is non-competitive with respect to tetrahydropterin cofactor. Therefore it is unlikely that the antibody binds to the catalytic region of PAH. The inhibition was accompanied by the abolition of the co-operativity of PAH with respect to phenylalanine. We therefore feel that PH-1 may bind to a region at or near the activator- or regulatory binding site for phenylalanine on the PAH molecule. We have proposed that the regulatory binding site for phenylalanine may exist at a subunit/subunit interface of PAH [12]. The titration data presented in Fig. 1 indicate that maximal inhibition of PAH activity was noted at an Ab/Ag ratio of 1. It is possible that the epitope responsible for PH-1 binding may consist of regions from two separate subunits of PAH. Consistent with this possibility is the observation that PAH binds to a non-linear epitope of PAH, since no binding of the antibody to PAH is noted upon Western blot analysis (I. G. Jennings & R. G. H. Cotton, unpublished work). However, it is also possible that PH-1 recognizes linearly separate regions of the same subunit which come into close proximity due to conformational change resulting from preincubation of PAH with phenylalanine. Resolution of the PH-1 binding domain must await more extensive structural analysis of the PAH molecule.

The catalytic and the regulatory binding sites for phenylalanine on PAH presumably encompass different regions of the enzyme molecule, as is evident from the non-competitive nature of the inhibition by PH-1. The dynamic relationships between these binding sites which are involved in the co-operativity and the substrate-level activation of PAH are not yet well understood. It appears, however, that the full expression of activation and co-

Fig. 3. Effect of PH-1 on BH_4 -dependent activity of rat liver PAH as a function of phenylalanine concentration

Portions ($2\text{ }\mu\text{g}$) of purified rat liver PAH (0.01 nmol with respect to 200 kDa tetramer) were preincubated with 0.5 mM -phenylalanine in 50 mM -potassium phosphate, $\text{pH } 6.8$, in the absence or the presence of $2.4\text{ }\mu\text{g}$ (0.016 nmol) of PH-1 for 15 min at 25°C , then for an additional 30 min at 4°C . The activity of PAH was then determined spectrophotometrically by assay with $40\text{ }\mu\text{M}$ -($6R$)- BH_4 and the concentrations of phenylalanine as indicated. Initial rates of reaction were determined from the first $15\text{--}30\text{ s}$ of the progress curves. □, PAH preincubated with phenylalanine in the absence of PH-1. The line is calculated for a K_m of 0.03 mM and a Hill coefficient (h) of 2.0 . ■, PAH preincubated with phenylalanine in the presence of PH-1. The line is calculated for a K_m of 0.04 mM and h value of 1.0 . The data are expressed as a percentage of maximum velocity for simplicity in graphic representation. However, V_{max} in absence of PH-1 was $4.5\text{ }\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, and in the presence of PH-1 it was $0.24\text{ }\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. The inset shows Hill-type replots of the data from the main Figure.

operativity by PAH must require some degree of conformational flexibility in the regulatory domain of the protein. The interaction of PH-1 with PAH appears to 'freeze' the enzyme into a conformation such that information concerning the binding of phenylalanine to the regulatory site is unable to be completely transmitted to the catalytic region. The linearity of the reaction progress curve shown by the PH-1-treated enzyme is consistent with this concept. In the absence of the antibody, the PAH reaction progress is non-linear, owing to continual changes in the amounts of at least two different conformational states of the enzyme, the low-activity native conformation, which may be promoted or stabilized by the binding of BH_4 [8,10,28], and a high-activity species, resulting from the initial binding of phenylalanine, presumably to the regulatory site [6–10]. The residual activity of PAH upon complete binding of PH-1 was about 20% of that of the enzyme which had been activated by preincubation with phenylalanine. However, this residual activity was still 2-fold higher than that of native, unactivated, PAH. Thus the interaction of PH-1 with PAH in the presence of phenylalanine appears to 'stabilize' an activity state intermediate between the low-activity native enzyme and the fully activated enzyme. Recent studies have suggested that the binding of phenylalanine to the catalytic sites of PAH may be non-co-operative; co-operativity and the full expression of activation may be due to interactions between the regulatory sites of the enzyme (M. A. Parniak, unpublished work). The monoclonal-antibody

PH-1, by binding at or near the regulatory domain, may prevent this interaction between the regulatory sites and thereby inhibit the expression of co-operativity by PAH.

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