Functional analysis of the effect of monoclonal antibodies on monkey liver phenylalanine hydroxylase

Ian G. JENNINGS,* Richard G. McR. RUSSELL,* Wilfred L. F. ARMAREGO† and Richard G. H. COTTON*

*Birth Defects Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia, and †John Curtin School of Medical Research, Canberra, A.C.T. 2601, Australia

An analysis of the effect of eleven monoclonal antibodies on the functional characteristics of monkey liver phenylalanine hydroxylase is presented. These eleven antibodies have been found to react with eight distinct regions on the phenylalanine hydroxylase protein. PH1 antibody inhibits enzyme activity, is dependent on phenylalanine for its binding, and appears to be related to structural changes occurring during phenylalanine activation of the enzyme activity. PH2 and PH3 antibodies stimulate enzyme activity, their binding is inhibited by lysolecithin and this group apparently is recognizing structures involved in lysolecithin activation of the enzyme activity. PH5, PH10, PH12 and PH6 recognise sites on phenylalanine hydroxylase affected by lysolecithin activation.

INTRODUCTION

The enzyme activity of mammalian liver phenylalanine hydroxylase (EC 1.14.16.1) has been shown to be affected by several mechanisms both reversible and irreversible. Limited proteolysis of the enzyme by chymotrypsin (Fisher & Kaufman, 1973) thiol modification by *N*-ethylmaleimide (Parniak & Kaufman, 1981) and phosphorylation (Abita *et al.*, 1976) all lead to activation of the enzyme activity. Reversible activation occurs in the presence of phospholipids, in particular, lysolecithin (Fisher & Kaufman, 1973) and the enzyme's substrate phenylalanine has also been found to activate the enzyme (Shiman & Gray, 1980). The kinetic characteristics of these activations have been well documented in the above papers along with more recent observations on the catalytic mechanisms within the active site (Wallick *et al.*, 1984). However, information regarding the structural characteristics of both catalytic and activating sites is minimal (Wretborn *et al.*, 1980). Monoclonal antibodies, because of their unique and constant specificity, have been used to determine structure–function relationships both in enzyme active sites (Clark *et al.*, 1981; Thomas *et al.*, 1984) and in receptor-binding sites (Lamers *et al.*, 1982; Moyle *et al.*, 1982; Boulain *et al.*, 1982).

In the present paper we report the production of seven new monoclonal antibodies to phenylalanine hydroxylase in addition to four monoclonal antibodies described previously (Cotton *et al.*, 1980; Choo *et al.*, 1980). These eleven antibodies have been found to react with a minimum of five antigenic regions on the phenylalanine hydroxylase protein. The effect of the antibodies on phenylalanine hydroxylase activity and their binding to phenylalanine hydroxylase in the presence of various ligands are presented.

MATERIALS AND METHODS

Hybridoma production

Production of hybridomas PH6–12 was as published previously by Cotton *et al.* (1980) except that the

X63-Ag-653 mouse myeloma cell line was used (Kearney *et al.*, 1979).

Hybridoma screening

An e.i.a. was used to detect specific anti-(phenylalanine hydroxylase) antibodies in hybridoma supernatants. The protocol was as follows (all additions were of 50 μ l volume). Purified monkey liver phenylalanine hydroxylase (Cotton & Grattan, 1975) at a concentration of 20 μ g/ml in 0.05 M- Na_2CO_3 buffer, pH 9.6, containing 10 mM-phenylalanine was used to coat wells of a poly (vinyl chloride) microtitre tray. The tray was incubated at 37 °C for 1 h followed by 16 h at 4 °C. Non-specific binding was blocked by addition of 1% BSA in PBS containing 10 mM-phenylalanine, followed by 1 h incubation. Hybridoma culture supernatants were then incubated in the wells for 1 h at 45 °C. Following this, the wells were washed three times with PBS containing 0.02% Tween-20. Goat anti-(mouse IgG) conjugated to peroxidase was then added to each well and allowed to incubate for 2 h at room temperature. Antibody-bound peroxidase activity was measured by using a reaction mixture containing 3.7 mM-*o*-phenylenediamine and 0.88 mM- H_2O_2 in 0.1 M-sodium citrate/ K_2HPO_4 buffer, pH 5.4. Wells were incubated, in the dark, for 15 min at room temperature, when the reaction was stopped with 4 M- H_2SO_4 . A_{492} was used to quantify enzyme reaction. Positive hybridomas were subcloned and grown in mice as tumours to obtain ascites fluid (Cotton *et al.*, 1980).

Monoclonal antibody isotyping

This was performed using the e.i.a. system already outlined, with the following exceptions. All incubations were done at 4 °C. Following the incubation of hybridoma culture supernatant on the phenylalanine hydroxylase-coated wells, rabbit antiserum specific for mouse heavy and light chain classes and subclasses was incubated for 1 h. Peroxidase conjugated to goat anti-(rabbit IgG) was added for 1 h. After washing with

distilled water the peroxidase reaction was allowed to proceed as above.

Purification of monoclonal antibodies from mouse ascites fluid

Mouse monoclonal IgG was purified through a column of protein A-Sepharose 4B as detailed previously (Choo *et al.*, 1981). Purity of the IgG preparations was monitored on 12% polyacrylamide gel electrophoresis containing 0.1% SDS (Laemmli, 1970) and were found to be greater than 90% pure. Protein concentration of purified IgG was calculated from the A_{280} , using $A_{1\%}^{1\text{cm}} = 14.0$.

Purification of monoclonal antibody B5-4 from rat ascites fluid

B5-4 ascites fluid was precipitated with 50% saturated $(\text{NH}_4)_2\text{SO}_4$ (Choo *et al.*, 1979). After dialysis against 10 mM-potassium phosphate buffer, pH 7.2, containing 0.15 M-NaCl, the dialysate was passed through columns of DEAE-cellulose and CM-cellulose equilibrated in the same buffer. In both cases the immunoglobulin was in the void volume and this fraction was 49% pure as found by SDS/polyacrylamide-gel electrophoresis.

Purification of monkey liver phenylalanine hydroxylase

Monkey liver phenylalanine hydroxylase was purified using a PH1-1 monoclonal antibody affinity resin as detailed by Choo *et al.* (1981). Removal of phenylalanine from the column buffer was the basis for the elution of purified monkey liver phenylalanine hydroxylase. Phenylalanine hydroxylase purified by this method was used in all subsequent experiments.

Assay for competition between monoclonal antibodies for antigenic sites

This assay was based on the ability of an unlabelled monoclonal antibody to prevent the binding of a labelled monoclonal antibody to the antigen coated on to plastic wells. All operations were carried out at 4 °C unless otherwise stated. Purified monoclonal antibody was labelled by reductive methylation with KB^3H_4 according to Finger & Choo (1981), except the labelling reaction was allowed to proceed for 16 h instead of 30 min. Termination of the labelling reaction was achieved by dialysing against PBS (2×2 -litres, for 4 h each). Plastic wells were then coated with 1 μg of purified monkey liver phenylalanine hydroxylase for 16 h. Non-specific well binding sites were blocked by a 1 h incubation with PBS containing 10 mM-phenylalanine and 1% BSA. Various amounts (39–2500 ng) of unlabelled purified monoclonal antibody in PBS containing 10 mM-phenylalanine and 1% BSA were then added to the wells for 1 h. Following this, labelled monoclonal antibody was added to the unlabelled monoclonal antibody in the well. After washing with PBS containing 10 mM-phenylalanine, bound labelled monoclonal antibody was eluted from wells with 0.05 M-diethylamine/HCl buffer, pH 11.5, for 1 h at 25 °C. Competition (%) was calculated as:

$100 \times$

$$\frac{\text{Radioactivity in control well} - \text{radioactivity in test well}}{\text{radioactivity in control well}}$$

where the control well is that with no unlabelled antibody present.

Effect of monoclonal antibody on phenylalanine hydroxylase activity

Purified monoclonal antibody was mixed with purified monkey liver phenylalanine hydroxylase in a ratio such that one antibody molecule (M_r 150 000) could react with one phenylalanine hydroxylase subunit (M_r 50 000). This reaction was left at 4 °C for 1 h. The mixture was then assayed for enzyme activity in a reaction mixture containing 0.04 M-potassium phosphate, pH 6.8, 2 mM-dithiothreitol, 0.135 M-phenylalanine, 0.125 μCi of [^{14}C]phenylalanine and 50 μM -tetrahydrobiopterin as detailed by Choo & Cotton (1979). Lysolecithin when included in the reaction mixture was added to a final concentration of 1 mM.

Kinetic analysis of the effect of PH1 antibody on phenylalanine hydroxylase activity was performed by using the assay detailed by Ayling *et al.* (1974).

Kinetic analysis of the stimulating antibodies effect on phenylalanine hydroxylase activity was performed by using the assay detailed by Shiman & Gray (1980) except that 60 μM -tetrahydrobiopterin replaced 6-methyl-5,6,7,8-tetrahydropteridine.

Monoclonal antibody affinity

The affinity of monoclonal antibody for phenylalanine hydroxylase coated onto wells was determined using varying amounts of reductively methylated antibody (as above). Antibody affinity was calculated by the analysis of Scatchard (1949).

Effect of phenylalanine hydroxylase ligands on monoclonal antibody binding

The aim of this assay was to determine what effect the addition of a ligand to phenylalanine hydroxylase (coated on wells) would have on the binding of the monoclonal antibodies. The assay is an adaption of the e.i.a. system outlined for the detection of hybridomas. All operations, unless stated, were at 4 °C. Coating of antigen onto well and blocking step were carried out as described in the competition assay. Following this, ligand was added at the described concentration and incubated for 1 h.

In reactions where ligand promoted binding i.e. phenylalanine, ligand was included in all following solutions until peroxidase enzyme reaction. For inhibition of binding, ligand was included only in the ligand and antibody binding step. Following incubation of the antibody in the presence of appropriate ligands on the well the procedure was as detailed in hybridoma screening e.i.a. Ligand concentrations required for half-maximum antibody binding were calculated from curves of percentage binding versus ligand concentration; 100% antibody binding was defined as the antibody binding in the absence of any ligand except in the case of phenylalanine, where 100% antibody binding was determined in the presence of 10 mM-phenylalanine.

RESULTS

Production of monoclonal antibodies

The isolation of hybridomas PH1-1, PH2-1 and PH3-0 (Cotton *et al.*, 1980) and B5-1 (Choo *et al.*, 1980) have been described elsewhere. The PH3-0 hybridoma has been successfully subcloned (results not shown) and in all subsequent experiments the hybridoma subclone PH3-1 was used. B5-1, which is a rat hybridoma, was previously

found to secrete the non-specific parental parental myeloma light chain along with the specific anti-(phenylalanine hydroxylase) light chain. Through subcloning of the original isolate, B5-0 hybridoma, we have isolated the hybridoma B5-4 which does not secrete the parental light chain (results not shown). This line will be referred to as PH5 in this paper.

Here we report the production of a further seven hybridomas, PH6–PH12, secreting monoclonal antibodies to monkey liver phenylalanine hydroxylase. The original hybridomas have been successfully subcloned to produce the cell lines PH6-1–PH12-1 which have been used in all subsequent studies.

Monoclonal antibody isotyping

Using rabbit antisera specific to immunoglobulin classes and subclasses the following results were obtained: PH6, 7, 8, 9, 10, 11 and 12 were all IgG₁ with a κ -type light chain.

Monoclonal antibody competition for antigenic sites

Preliminary testing of the labelled antibodies' binding was performed in the absence of unlabelled competing antibody in order to determine the effect of reductive methylation on antibody binding. Using labelled PH6 antibody no radioactivity was found to be bound to the phenylalanine hydroxylase-coated wells despite the fact that its specific radioactivity was in the range observed for the other labelled antibodies. From this we assume that PH6 antibody has been chemically modified during the reductive methylation reaction in such a way that prevents it binding to its corresponding antigenic site. None of the remaining antibodies were affected by the reductive methylation reaction.

Prior to the competition assays, it was established for each individual monoclonal antibody that the amount of labelled antibody bound in the absence of unlabelled competing antibody was below saturation. In addition it was found that > 85% of labelled antibody could be prevented from binding by homologous unlabelled antibody. The antibodies have been divided into groups as shown in Fig. 1 on the basis of their reactions in the competition assay. The groups are based on the ability of an antibody within a group to inhibit by > 50% the binding of another antibody. Two distinct types of competition have been found, and are designated reciprocal and non-reciprocal. Reciprocal competition occurs when two antibodies can compete with each other to the same extent (> 50%). Non-reciprocal competition occurs when one antibody competes with another (> 50%) but the reverse competition does not occur to a similar extent (< 50%). Reciprocal competition is therefore interpreted as antibodies competing for the same antigenic region, whereas non-reciprocal competition could be interpreted as one antibody binding in a region distant from that of the other antibody binding region but affecting the conformation of this region.

Within group A, which includes PH1, PH5 and PH8 antibodies, no reciprocal competition was observed. That this non-reciprocal competition may be due to different affinities within this group of antibodies is not supported by the relative binding constants of the labelled antibodies measured in the absence of competing antibodies. Binding constants of the labelled antibodies are PH1, 5.52×10^{-10} M; PH5, 8.47×10^{-10} M; PH8, 8.10×10^{-10} M. Group B has only PH7 antibody, with no

		Radiolabelled antibody											
		1	5	8	7	2	3	10	12	9	11	6	
Competing antibody	A	1	■	■		●							*
		5	■	■		●	●	●	●				*
		8	■	■		■	■	■	■	●			*
	B	7	■	●	■	■	■	■	■	●			*
		2	●	●		■	■	■	■	■			*
C	3	3	●			■	■	■	■	■	■		*
		10	●			■	■	■	■	■	■		*
	12	12				■	●	■	■	■	■		*
		9	●			●	●	●		■	■		*
D	11	11				●	●						*
		6											*

Fig. 1. Monoclonal antibody competition for antigenic sites

The data presented represent the maximum competition of radiolabelled monoclonal antibody achieved using 2.5 μ g of unlabelled monoclonal antibody as detailed in the text. The data shown are the average value obtained from four determinations. The four categories of competition of binding are: ■, > 50% competition; ■, 25–50% competition; □, 10–25% competition; □, < 10% competition; * indicates experiment not done.

other antibody able to inhibit > 50% of labelled PH7 binding. Group C consists of two subgroups of antibodies which showed reciprocal competition with each other; the first subgroup comprises PH2 and PH3 and the second comprises PH10 and PH12 (inhibition of antibody binding 68–97%). However, there was non-reciprocal competition between the two subgroups; PH2 and PH3 antibodies competed for PH10 and PH12 antibodies (inhibition 58–69%) but the reverse did not occur (inhibition 0–49%). In group D, PH9 and PH11 antibodies showed reciprocal competition. In group E is PH6, which does not show inhibition of any of the other labelled antibodies' binding. Antibodies which affect PH6 binding could not be determined, as explained above.

Group B antibodies were placed next to group A antibodies because of the similar weak effects of PH7 and PH8 on group C antibodies.

Effect of monoclonal antibodies on phenylalanine hydroxylase activity

The effect of PH1, PH2, PH3 and PH5 monoclonal antibodies on phenylalanine hydroxylase activity have been published previously (Choo *et al.*, 1981). However, these results were obtained using crude preparations of both antibody and phenylalanine hydroxylase mixed in an unknown ratio. Here purified antibody was mixed with purified phenylalanine hydroxylase in a ratio of one antibody molecule (M_r 150000) to one phenylalanine hydroxylase subunit (M_r 50000). In addition the enzyme assay used previously has been altered. The synthetic cofactor 6,7-dimethyl-5,6,7,8-tetrahydropteridine has been replaced by the natural cofactor (6R)-tetrahydrobiopterin. Also lysolecithin, a stimulator of phenylalanine hydroxylase activity (Fisher & Kaufman, 1973), was removed from the enzyme reaction mixture to prevent any competition between it and antibodies stimulating enzyme activity.

Table 1. Effect of monoclonal antibodies on phenylalanine hydroxylase

Purified monoclonal antibody was added to purified phenylalanine hydroxylase in a ratio such that one antibody molecule (M_r 150000) could react with one phenylalanine hydroxylase subunit (M_r 50000). Where indicated the enzyme activity was assayed in the presence of 1 mM-lysolecithin. Enzyme activity is expressed as nmol of tyrosine produced/10 min at 25 °C. The percentage effect of the additions to the enzyme assay were calculated by using the enzyme activity when no additions were made as 0%. + indicates a stimulation of enzyme activity; - indicates an inhibition of enzyme activity. The results shown are the average of four determinations.

Antibody group	Additions	Enzyme activity (nmol/10 min)	Effect (%)
-	None	0.91	0
-	Lysolecithin	2.00	+120
A	PH1	0.39	-59
	PH5	1.30	+43
	PH8	0.74	-19
B	PH7	1.17	+29
C	PH2	1.82	+100
	PH3	1.82	+100
	PH10	1.21	+33
	PH12	1.21	+33
D	PH9	1.02	+12
	PH11	1.05	+16
E	PH6	0.86	-6

Table 1 presents the enzyme activities obtained when purified antibody was mixed with purified phenylalanine hydroxylase in the ratio of one antibody molecule to one phenylalanine hydroxylase subunit. Only PH1 antibody substantially inhibited phenylalanine hydroxylase activity, i.e. by 59%. This inhibition was the maximum obtained and was not increased when the antibody:enzyme ratio was increased to 4:1 (results not shown). The inhibition of hydroxylase activity by PH1 antibody was further analysed by the measurement of kinetic parameters in the presence of PH1 antibody. In the absence of PH1 antibody the enzyme showed normal Michaelis-Menten kinetics with respect to the pterin cofactor 6-methyl-5,6,7,8-tetrahydropteridine, producing a K_m value of 0.155 μ M and a V_{max} of 20.5 nmol of pterin oxidized/min at 25 °C. When PH1 antibody was present the V_{max} was decreased by 84% to 3.3 nmol of pterin oxidized/min, but the K_m was unaffected (0.137 μ M). The increased inhibition obtained in this assay as compared with that in Table 1 could be due to the different assay conditions, but this effect has not been investigated further.

From Table 1 it can be seen that the most significant stimulation of hydroxylase activity was observed in the presence of PH2 and PH3 antibodies. These two antibodies, when added to phenylalanine hydroxylase, increased the hydroxylase activity to a similar extent as did lysolecithin, a known stimulator of hydroxylase activity. PH10 and PH12 antibodies, which were found to interact with PH2 and PH3 in the binding site competition assay, stimulated the hydroxylase activity to a lesser extent than PH2, PH3 or lysolecithin. PH5,

Table 2. Effect of stimulating antibodies on phenylalanine hydroxylase initial velocity

Initial velocity was measured at final phenylalanine concentrations of 0.2 mM and 1 mM in the presence of 1 mM-lysolecithin and the stimulating antibodies PH2, PH3 and PH5 individually. Initial velocity results are expressed as μ mol of tyrosine produced/min at 25 °C. The results are the average of three determinations.

Additions	[Phenylalanine] (mM)	Initial velocity (μ mol/min)	
		0.2	1.0
None		1.79	4.64
Lysolecithin		3.42	1.66
PH2		3.28	3.95
PH3		3.68	4.56
PH5		3.41	4.36

previously found to be a potent stimulator when the synthetic cofactor was used (Choo *et al.*, 1980), was a far less efficient stimulator in the presence of the natural cofactor. The stimulation by PH5 at the 1:1 ratio of antibody to antigen in the presence of the natural cofactor was the maximum observed, with no further stimulation observed when the antibody:antigen ratio was increased to 4:1 (results not shown).

The stimulating antibodies PH2, PH3 and PH5 were further analysed. When phenylalanine hydroxylase activity, in the presence of any one of the stimulating antibodies, was measured in an enzyme reaction mixture containing lysolecithin, no further stimulation occurred above the enzyme activity measured when lysolecithin was absent from the reaction mixture (results not shown). This indicated that the antibody-mediated and lysolecithin-mediated stimulations were not additive.

The effect of the stimulating antibodies PH2, PH3, and PH5 and lysolecithin on the initial velocity of the enzyme were measured at 0.2 mM and 1 mM-phenylalanine, as shown in Table 2. Lysolecithin has been shown (when tetrahydrobiopterin is the cofactor) to alter the kinetic characteristics of the enzyme such that the velocity-versus-[phenylalanine] curve changes from sigmoidal to hyperbolic (Fisher & Kaufman, 1973) such that the velocity at low phenylalanine concentration (0.2 mM) is increased, whereas at high phenylalanine concentration (1 mM) the enzyme velocity is inhibited. This has been demonstrated in Table 2 such that lysolecithin has stimulated the initial velocity at 0.2 mM-phenylalanine but has inhibited the initial velocity at 1 mM-phenylalanine. In the case of the stimulating antibodies, they have stimulated the initial velocity at 0.2 mM-phenylalanine but produced only a small inhibition at 1 mM-phenylalanine.

Effect of phenylalanine hydroxylase ligands on monoclonal antibody binding

In order to define further the relationship between the antibody site and any functional region on the phenylalanine hydroxylase protein, experiments were carried out to determine the effect on monoclonal antibody binding of preincubation of phenylalanine hydroxylase with various ligands. Quantification of antibody binding was performed by using the e.i.a. detailed earlier. The ligands involved were the hydroxylase

Fig. 2. Phenylalanine-dependent PH1 antibody binding to phenylalanine hydroxylase

Antibody binding was measured by using the e.i.a. detailed in the text. Antibody bound is calculated as the ratio of antibody binding at the various phenylalanine concentrations to the antibody binding in the presence of 10 mM-phenylalanine, and is expressed as a percentage. The values are the average of two assays performed in triplicate.

substrate phenylalanine, hydroxylase cofactor tetrahydrobiopterin and hydroxylase stimulator lysolecithin.

Phenylalanine. Fig. 2 shows the effect of increasing phenylalanine concentrations on PH1 antibody binding. This demonstrates more quantitative evidence of the phenylalanine-dependent binding of PH1 than that found by Choo *et al.* (1981). The concentration of phenylalanine required for half-maximum binding was found to be 0.135 mM. None of the other ten antibodies showed this type of dependency on phenylalanine for binding or showed inhibition of binding by phenylalanine.

Tetrahydrobiopterin. Preincubation of phenylalanine hydroxylase with tetrahydrobiopterin (0–10 mM) inhibited to some extent the binding of antibodies in groups A, C and E. However, in all cases the concentration for half-maximum binding was in the range 1–5 mM. As the K_m of tetrahydrobiopterin for this enzyme is 2 μ M (Parniak & Kaufman, 1981), these effects have not been investigated further.

Lysolecithin. Table 3 details the inhibition of binding by 10 mM-lysolecithin. No effect was observed on the binding of antibodies in group B (PH7) and group D (PH9 and PH11). Varied inhibition (30–70%) was observed within group A antibodies. The decreased concentration required for PH1 inhibition may be due to the presence of phenylalanine in this reaction. Group C antibodies binding (PH2, PH3, PH10 and PH12) and PH6 antibody binding were completely inhibited by lysolecithin. The half-maximum lysolecithin concentrations were similar to the half-maximum concentration required to activate the enzyme (0.4 mM-lysolecithin).

Species reactivity

Antibodies were tested for their ability to bind to purified phenylalanine hydroxylases from four species, monkey, man, rat and mouse, by using the e.i.a. system.

Table 3. Effect of lysolecithin on antibody binding to phenylalanine hydroxylase

Antibody binding was measured by using the e.i.a. described in the text. The maximum inhibition by 10 mM-lysolecithin of antibody binding is the percentage decrease in antibody binding when compared with the antibody binding in the absence of lysolecithin. The lysolecithin concentration, expressed in mM, is for half-maximum antibody binding and was calculated from curves of antibody-binding-versus-[lysolecithin] (0–10 mM). The results are the average of two assays performed in triplicate.

Group	Antibody	Maximum inhibition of antibody binding (%)	Half-maximum [lysolecithin] (mM)
A	PH1*	31*	0.12*
	PH5	69	0.40
	PH8	49	0.54
B	PH7	0	—
C	PH2	95	0.43
	PH3	97	0.45
	PH10	93	1.0
	PH12	92	0.80
D	PH9	0	—
	PH11	0	—
E	PH6	96	0.44

*Experiment performed in the presence of 10 mM-phenylalanine

It was found that antibodies in groups A, B, and E reacted with all species. In group C PH2 did not recognize rat and mouse phenylalanine hydroxylase but PH3, PH10 and PH12 recognized all species tested. In group D PH9 and PH11 showed no reaction with rat and mouse phenylalanine hydroxylase, but recognized the human and monkey enzyme.

DISCUSSION

In an attempt to isolate monoclonal antibodies which will react with binding sites on phenylalanine hydroxylase important in the mechanism and regulation of the enzyme's activity, we have extended our range of antibodies from those published previously. Purification of the monoclonal antibodies from ascites fluid was deemed necessary to obtain quantitative data for both the antibody competition assay and the effect of antibody on enzyme activity.

From the results of the antibody competition assay we have divided the antibodies into five groups according to their reaction in this assay. However an analysis of this data shows that, from the 11 antibodies, we have generated antibodies to eight regions of the protein, with PH2 and PH3, PH10 and PH12, and PH9 and PH11 competing directly. Although this assay has limitations due to its failure to account for differences in antibody affinity and steric hindrance, those antibodies directly competing with each other showed good correlation in their effect on enzyme activity and reaction with phenylalanine hydroxylase in the presence of ligands.

The effect of the monoclonal antibodies on enzyme activity is of great importance when searching for those reacting with the active site or the activation sites, as described in the Introduction. An antibody reacting at the active site would be expected to inhibit enzyme activity completely at equimolar concentrations. PH1 was the only antibody inhibiting phenylalanine hydroxylase to a significant extent but this inhibition of enzyme activity was not complete. Furthermore kinetic analysis showed that this inhibition was non-competitive with respect to the pterin cofactor. Therefore it is concluded that PH1 is not binding at the active site of the enzyme. However the phenylalanine-dependent binding of PH1 to phenylalanine hydroxylase yielded a half-maximum phenylalanine concentration of 0.135 mM for PH1 antibody binding. This compares favourably with the half-maximum concentration found for phenylalanine activation of rat phenylalanine hydroxylase by Shiman & Gray (1980) of 0.06 mM and by Kaufman & Mason (1982) of 0.07 mM. Thus two possible binding sites can be proposed: either PH1 antibody binds directly to the activation site to which phenylalanine is bound or to a unique site generated by phenylalanine activation. The reason for the PH1 antibody inhibition could be its blocking of the change to the active site caused by phenylalanine activation.

Of the antibodies stimulating phenylalanine hydroxylase activity, PH2 and PH3 appear the most interesting. PH2 and PH3 stimulate the enzyme activity to the same extent as did lysolecithin, with this stimulation being observed at a phenylalanine concentration of 0.2 mM, as has been found for lysolecithin (Fisher & Kaufman, 1973). In addition the antibody-mediated and lysolecithin-mediated stimulations of the enzyme are not additive and PH2 and PH3 antibody binding is prevented by preincubation of phenylalanine hydroxylase with lysolecithin. Thus we have demonstrated competition between the two antibodies and lysolecithin in their interaction with phenylalanine hydroxylase. Therefore we propose that PH2 and PH3, which competed with each other for binding to phenylalanine hydroxylase, are binding at or near the lysolecithin binding site on phenylalanine hydroxylase. Two other categories of antibodies which are involved in the lysolecithin activation process have been found. PH5, PH10, and PH12 stimulated enzyme activity to a lesser extent than did lysolecithin and their binding was completely inhibited by lysolecithin. PH6 did not stimulate activity, but its binding was completely inhibited by lysolecithin; PH1 and PH8, although not stimulating activity, had their binding partially blocked by lysolecithin. So lysolecithin when bound to phenylalanine hydroxylase can affect the binding of six out of the eight antigenic regions identified. This finding is in agreement with the large conformation change in phenylalanine hydroxylase caused by lysolecithin binding, as observed by Abita *et al.* (1984) using c.d. measurements. It has been shown from kinetic and spectral data (Abita *et al.*, 1984) that activations produced by phenylalanine and by lysolecithin are functionally different. Here we found structural evidence that these two activators have a different effect. The phenylalanine-dependent PH1 antibody binding site is not dependent on lysolecithin for binding, and PH2 and PH3 antibodies binding is inhibited by lysolecithin but not by phenylalanine. In

addition, PH1 antibody does not directly compete with PH2 and PH3 antibody for a binding site on phenylalanine hydroxylase.

In conclusion, future work will involve the determination of the protein structures involved in antibody binding. As preliminary results have shown that PH1, PH2 and PH3 antibodies all recognise unstable epitopes this will not be straightforward. However, the techniques of X-ray crystallography (Smith-Gill *et al.*, 1984) and protein cross-linking (Emini *et al.*, 1982) have been shown to be valuable. The relationship between the structure of the antibody binding site and the functional effect of the antibody should yield valuable information about the site and mechanism of activation of phenylalanine hydroxylase by phenylalanine and lysolecithin.

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