Comparative studies of four monoclonal antibodies to phenylalanine hydroxylase exhibiting different properties with respect to substrate-dependence, species-specificity and a range of effects on enzyme activity

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Four monoclonal antibodies to phenylalanine hydroxylase are described. Two are inhibitory (PH α 1-1 and PH α 2-1-1 antibodies), one is stimulatory (B5-1 antibody) and one has no effect on enzyme activity (PH α 3-0 antibody). Their properties are compared. Two antibodies (PH α 1-1 and B5-1 antibodies) bind primate and rodent phenylalanine hydroxylase, whereas the other two (PH α 2-1-1 and PH α 3-0 antibodies) bind only the primate enzyme. The binding of PH α 1-1 antibody to phenylalanine hydroxylase is dependent on substrate phenylalanine, whereas the binding of the others is not influenced by phenylalanine. Affinity adsorbents prepared from the four antibodies purified phenylalanine hydroxylase substantially (>80% purity) in one step, except for a PH α 3-0 antibody–Sepharose column, which behaved anomalously. Two previous publications described the isolation and preliminary characterization of B5 and PH α 1-1 antibodies. PH α 2-1-1 and PH α 3-0 antibodies are reported for the first time.

The technique of myeloma–spleen-cell hybridization (Kohler & Milstein, 1975) allows the isolation of monoclonal antibodies each directed against a discrete immunogenic determinant. We have applied the technique to the study of phenylalanine hydroxylase. We have previously reported the isolation of two monoclonal antibodies against mammalian phenylalanine hydroxylase. The first antibody (designated PH α 1-1 antibody) showed an activity-inhibiting property (Cotton *et al.*, 1980). The second antibody (designated B5 antibody) showed an activity-stimulating property (Choo *et al.*, 1980).

In the present study, we report other properties of these and two additional monoclonal antibodies, together with a formal characterization of all four antibodies. The effects these antibodies have on phenylalanine hydroxylase, their species-specificity and the role of phenylalanine in the antibody–antigen interaction are investigated. The properties of all four antibodies are compared.

Materials and methods

Cell fusion and hybrid isolation

The isolation of hybrid cell lines secreting PH α 1-1 antibody (by using a mouse–myeloma–mouse–spleen-cell-fusion system) and B5 antibody (by using a rat–myeloma–rat–spleen-cell-fusion system) has been described previously by Cotton *et al.* (1980)

and Choo *et al.* (1980) respectively. The two additional monoclonal antibody-secreting cell lines characterized in the present study were obtained in the same fusion experiment from which the PH α 1-1 antibody-producing cell line was derived. These two antibodies were designated as PH α 2-0 and PH α 3-0 antibodies. All four antibodies were raised against pure monkey liver phenylalanine hydroxylase.

The following approach was used to test for the presence of phenylalanine hydroxylase-specific antibody (in hybrid culture medium) that did not drastically alter enzyme activity. The culture medium (50 μ l) was incubated with crude monkey liver extract (300 μ g) for 3 h at 4°C, followed by the addition of Ig G Sorb (50 μ l of a 10% cell suspension; Enzyme Center, Boston, MA, U.S.A.) for 15 min at 4°C. After centrifugation at 2000g to remove the Ig G Sorb, the supernatant was assayed for phenylalanine hydroxylase activity. This activity was compared with that of a control supernatant containing no antibody (where the hybrid culture medium was replaced by a freshly prepared culture medium during the initial incubation with crude extract). A significant decrease in phenylalanine hydroxylase activity in the supernatant over that in the control indicated the presence of specific binding antibody in the hybrid culture medium.

Unless otherwise specified, methods for the characterization of the antibodies produced by

PH α 2-0 (or its subclones; see below) and PH α 3-0 cells were as previously described (Cotton *et al.*, 1980; Choo *et al.*, 1980).

In some experiments (where indicated), a control monoclonal antibody (designated RV4-3 antibody) was used. This antibody was produced by a mouse-myeloma-spleen-cell hybrid by using the method as described above but against an antigen that is completely unrelated to phenylalanine hydroxylase. The hybrid cell line producing this antibody was kindly provided by Mr. J. White (Royal Children's Hospital, Melbourne, Vic., Australia).

Chain analysis of secreted immunoglobulins from hybridoma cultures

Immunoprecipitation and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of radiolabelled culture supernatants was performed as described in Choo *et al.* (1980).

Class of immunoglobulin γ heavy-chain and light-chain type were determined by immunodiffusion between concentrated culture supernatants (Choo *et al.*, 1980) and specific antisera.

To determine whether parental non-specific light chains were present or absent, two-dimensional electrophoresis (Choo *et al.*, 1979a) was performed on radiolabelled culture supernatant purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Choo *et al.*, 1979b). The positions of parental non-specific κ light chains of PH α 1-1, PH α 2-1-1 and PH α 3-0 antibodies were identified by using the immunoglobulins produced by mouse myeloma cell line P3-X-63-Ag8 (Kohler & Milstein, 1975), which is the immunoglobulin-secreting parent cell line of the non-secreting (but κ -light-chain-producing) cell line NS1/1Ag4 used in the production of these hybridoma cell lines (Cotton *et al.*, 1980). The position of the parental κ light chains of antibody B5-1 (clone of B5) was identified by using the immunoglobulins produced by the rat myeloma cell line R210.RC.TG1 (Choo *et al.*, 1980).

Affinity chromatography with the use of monoclonal antibody columns

Large quantities of antibodies were obtained from sera or ascites fluid from host animals carrying the hybrid cells (Cotton *et al.*, 1980; Choo *et al.*, 1980). [Although the strain of rat (Wistar) used in our laboratory developed tumours in response to injection with B5 cells, the tumours generally persist for approx. 1 week and then disappear. Over this period an approx. 100-fold increase in the B5 antibody titre in the serum was observed.] The antibodies were partially purified by two successive $(\text{NH}_4)_2\text{SO}_4$ precipitations as previously described (Choo *et al.*, 1979b), except that the final precipitate was redissolved in a minimum volume (2 ml/10 ml of ascites fluid) of coupling buffer (0.1 M-NaHCO₃, pH 8.5,

containing 0.5 M-NaCl). This redissolved material was exhaustively dialysed against the same buffer to remove the $(\text{NH}_4)_2\text{SO}_4$.

The coupling procedure for preparing immuno-adsorbents was as follows. The required amount (1 g yields 3.5 ml of swollen gel) of CNBr-activated Sepharose 4B (Pharmacia) was swollen and washed at the same time on a sintered-glass filter with 1 M-HCl (200 ml/g of gel) for 20 min. The gel was then washed with a small volume of coupling buffer (10 ml/g of gel) and rapidly transferred to the antibody solution (5–10 mg of protein/ml of swollen gel). The antibody and gel were coupled by mixing the suspension end-over-end for 16–18 h at 4°C. After the coupling, excess of protein was washed away and any remaining reactive groups were blocked with 1 M-ethanolamine/HCl, pH 8.0, for 1 h at room temperature. After this, the gel was exhaustively washed with coupling buffer followed by 0.1 M-sodium acetate buffer, pH 4, containing 0.5 M-NaCl, followed by coupling buffer. Finally, the gel was washed with a column buffer (0.1 M-Tris/HCl buffer, pH 7.6, containing 0.5 M-NaCl).

In some experiments (where indicated), the antibody (PH α 1-1) used for coupling was purified through a staphylococcal protein A-Sepharose column (obtained from Pharmacia, Uppsala, Sweden) by using a procedure adapted from that of Ey *et al.* (1978). Protein A-Sepharose was swollen in 10 mM-sodium phosphate buffer, pH 8.0, containing 0.15 M-NaCl, packed into a column with 6 ml bed volume and pre-equilibrated in 0.1 M-sodium phosphate buffer, pH 8.0. Then 5 ml of serum from animals carrying hybrid-cell tumours was applied to the column. The column was washed with 30 ml of 0.1 M-sodium phosphate buffer, pH 8.0, then with 0.1 M-sodium citrate/citric acid buffers of pH 6.0, 4.5 and 3.5. The fractions (approximately in the middle of the pH 4.5 elution volume) giving the highest absorption readings (280 nm) were analysed by immunodiffusion against pure monkey liver phenylalanine hydroxylase to confirm the presence of antibodies. These were then pooled, dialysed against the coupling buffer and used for the synthesis of immuno-adsorbent as described above.

Crude monkey liver extract was prepared in 0.1 M-Tris/HCl buffer, pH 8.6, containing 0.5 M-NaCl, as described before (Choo & Cotton, 1979). This was applied to each of the four antibody-Sepharose columns. The columns were then washed with column buffer (see above), before an elution buffer [50 mM-diethylamine, pH 11.5 (Sunderland *et al.*, 1979)] was applied. The fractions collected in the latter buffer were immediately neutralized to pH 7 with various volumes of 0.5 M-Tris/HCl buffer, pH 7.6, and the columns were equilibrated with column buffer.

In some experiments (where indicated), crude

extract prepared in 0.1M-Tris/HCl buffer, pH 8.6, containing 10mM-phenylalanine was used. In this case, after extract application, the columns were extensively washed with column buffer containing 10mM-phenylalanine. Elution of the columns used column buffer with no added phenylalanine. The columns were regenerated with 0.1M-sodium acetate buffer, pH 4, containing 0.5M-NaCl, followed by 0.1M-Tris/HCl buffer, pH 9, containing 0.5M-NaCl, and finally the column buffer.

Fractions from the columns were assayed for phenylalanine hydroxylase activity by using the nitrosonaphthol method of tyrosine assay (Cotton & Grattan, 1975).

Control adsorbents were used as described in the text.

Testing for cross-reaction against different mammalian phenylalanine hydroxylase species

The antibody-Sepharose resins prepared above were used as a solid-phase immunoabsorbent to test for the removal of phenylalanine hydroxylase activity from crude extracts of different mammalian livers. A 100 μ l sample of extract (20–30 μ g of protein/ μ l) was added to 50 μ l of packed resin. This was mixed and incubated for 1h at 4°C. The adsorbent was then centrifuged at 500g for 5min, and a sample of the supernatant was assayed for phenylalanine hydroxylase activity. The assay measured the conversion of [14 C]phenylalanine into [14 C]tyrosine (Choo & Cotton, 1979).

Results

Isolation and preliminary characterization of PH α 2-1-1 and PH α 3-0 antibodies

The procedure for the initial screening of cell hybrid clones producing antibodies to phenylalanine hydroxylase involved assaying culture supernatants for the inhibition of enzyme activity in monkey liver extract (Cotton *et al.*, 1980). One of the clones, designated PH α 2-0, was identified as a positive because its supernatant consistently gave 10–15% inhibition of enzyme activity. This hybrid was subcloned twice by limiting dilution. The following studies used either the subclone PH α 2-1, or PH α 2-1-1, which was a subclone of PH α 2-1, which in turn was a subclone of PH α 2-0.

A second clone, PH α 3-0, which gave no detectable inhibiting activity against phenylalanine hydroxylase in crude monkey liver extract, was initially retained as a control (AA8 in Cotton *et al.*, 1980), but this proved to be a hybrid producing an antibody that exhibited a null effect on phenylalanine hydroxylase activity (see below). This hybrid was subcloned, and the subclones were tested for the production of phenylalanine hydroxylase-specific

antibody as described in the Materials and methods section. Testing of seven randomly picked subclones indicated that these were all positive. Further characterization was performed on clone PH α 3-0.

Karyotype analysis of PH α 2-1 and PH α 3-0 cells confirmed that these were chromosomally hybrids. Whereas the myeloma cell line NS1/1Ag4 had a mean chromosome number of 63, PH α 2-1 and PH α 3-0 cells showed mean chromosomal numbers of 99 and 126 respectively.

Characterization of immunoglobulins secreted by the hybrids

When radioactively labelled cell-culture media from PH α 2-1-1 and PH α 3-0 cells were immunoprecipitated by using rabbit anti-(mouse immunoglobulin) antibodies and analysed on a sodium dodecyl sulphate/polyacrylamide gel (Fig. 1), both the heavy and light immunoglobulin chains were demonstrated by determination of molecular weight. The heavy chains from the two sources both belonged to the γ -globulin class (mol.wt. 56000). In the present study, the heavy chains from PH α 1-1 cells were found to be of the γ class; this had not been defined previously (Cotton *et al.*, 1980) (Fig. 1).

The heavy chains of the four monoclonal antibodies were subclassed by immunodiffusion with antisera to the relevant mouse heavy chains. The subclasses of PH α 1-1, PH α 2-1-1, PH α 3-0 and B5-1 were found to be γ 3, γ 2a, γ 2a and γ 1 respectively. Light-chain typing with antisera to mouse light chains proved that those of PH α 1-1, PH α 2-1-1 and PH α 3-0 to be κ and that of B5-1 to be λ . However, anti-(mouse κ -chain) serum did not react with rat κ -chain from the parental myeloma, later found to be present in B5-1 antibody (see below).

In all four cases it was necessary to examine the monoclonal antibodies for the presence or absence of non-specific light chain derived from the parental myeloma line (both parental lines used produced κ -chains). For this purpose radiolabelled culture media were precipitated by using $(\text{NH}_4)_2\text{SO}_4$ and analysed by two-dimensional polyacrylamide-gel electrophoresis. The results are shown in Fig. 2, which shows that B5-1 cells are the only hybrid that still secretes parental non-specific κ -chain. Thus the three mouse monoclonal antibodies are pure except for a few minor proteins of unknown origin. These minor proteins could well be phenylalanine hydroxylase-specific light chains modified post-translationally, e.g. by degradation.

In the preliminary characterization of PH α 1-1 antibody (Cotton *et al.*, 1980) the classes of the heavy chains and light chains had not been defined. In the preliminary characterization of B5 antibody (Choo *et al.*, 1980) the κ - and λ -light chains had

Fig. 1. Fluorography of immunoprecipitates of labelled antibodies secreted by PH α 1-1 (a), PH α 2-1-1 (b) and PH α 3-0 (c) cells analysed on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

For experimental details see the text. The positions of the different immunoglobulin chains (γ and κ) are indicated. D designates the electrophoretic dye front.

been defined only on the basis of the two chains of different size seen after analysis of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis.

Immunoabsorbent affinity chromatography

Affinity columns were prepared with the different antibodies as described in the Materials and methods

section. These are respectively referred to as the PH α 1-1, PH α 2-1-1, PH α 3-0 and B5-1 columns. The ability of these columns to purify phenylalanine hydroxylase was tested by using crude monkey liver extract.

(a) *Extract with no added phenylalanine.* The following experiments were performed with an extract that was prepared in a buffer containing no phenylalanine. When this extract was applied to the different columns, with the PH α 2-1-1, PH α 3-0 and B5-1 columns 85–100% of the initial activity was adsorbed on the column; however, 17% adsorption of activity was observed with the PH α 1-1 column. This unusual property of the PH α 1-1 column was investigated further [see (b) below].

After extract application and extensive washing, the PH α 2-1-1, PH α 3-0 and B5-1 columns were eluted with pH 11.5 buffer. This resulted in approx. 50% recovery of activity from the PH α 2-1-1 and B5-1 columns, but no significant recovery and 30% of activity was observed in the two experiments in the case of the PH α 3-0 column. The eluted fractions from the three columns were separately concentrated and analysed on a sodium dodecyl sulphate/polyacrylamide (9%) gel. Fig. 3 shows the results for the concentrated samples from the PH α 2-1-1 and PH α 3-0 columns. Whereas the phenylalanine hydroxylase polypeptides in the samples from the PH α 2-1-1 column (Fig. 3a) and B5-1 column (picture not shown) represented over 80% of total protein resolved on the gel, the sample from the PH α 3-0 column carried a considerable amount (more than 50%) of contaminant protein bands. Use of 3M-ammonium thiocyanate (BDH Chemicals, Poole, Dorset, U.K.) (in 50mM-Tris/HCl buffer, pH 7.6) did not significantly improve the recovery of either the enzyme activity or the purity of the enzyme protein isolated from the PH α 3-0 column (results not shown). Attempts were made to elute enzymes bound on the PH α 2-1-1, PH α 3-0 and B5-1 immuno-adsorbent columns with column buffer containing 10mM-phenylalanine. In every case no significant recovery of enzyme activity was observed.

(b) *Effect of substrate (phenylalanine) on enzyme-antibody interaction.* The following chromatographic experiments were performed with a monkey liver extract that was prepared in a buffer containing 10mM-phenylalanine. Passage of the extract through any of the four columns resulted in 85–100% adsorption of initial phenylalanine hydroxylase activity on the column. When a buffer not containing phenylalanine was next used to elute the column, 50–60% of the initial enzyme activity was recovered from the PH α 1-1 column, but no significant recovery of activity was observed in the case of the other three columns. After this step, each of the four columns was eluted with pH 11.5 buffer. This resulted in a recovery of an additional very

Fig. 2. Two-dimensional electrophoretic analysis of radiolabelled culture media purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation: (a) PHa 1-1, (b) PHa 2-1-1, (c) PHa 3-0 and (d) B5-1

Only a portion of the gel (95 mm wide) is shown. Arrows indicate the position of parental light chains in each case. These were determined as described in the text.

small amount (less than 5%) of enzyme activity from the PHa 1-1 column. Results with the subsequent use of pH 11.5 buffer to elute the other three columns were the same as those described above in (a).

(c) *Other chromatographic parameters.* The enzyme bound on the PHa 1-1 column could also be eluted off by using pH 11.5 buffer (phenylalanine-free) instead of the phenylalanine-free buffer, pH 7.6. Again, only about 50% recovery of activity was achieved. Addition of 10 mM-phenylalanine to the pH 11.5 buffer did not prevent enzyme elution, nor did it significantly improve the recovery of enzyme activity.

When the characteristics of the proteins eluted from the PHa 1-1 column with the phenylalanine-free or pH 11.5 buffer were analysed on a sodium dodecyl sulphate/polyacrylamide gel, the pictures obtained (not shown) were essentially the same as that presented in Fig. 3(a), where the phenylalanine hydroxylase polypeptides were the predominant protein species and accounted for more than 80% of the total protein resolved on the gel.

A number of measures have been tried to increase the recovery of enzyme activity eluted from the PHa 1-1 column with the phenylalanine-free buffer elution procedure: (i) synthesis of immunoadsorbent with antibody that has been purified through a protein A-Sepharose column [instead of with antibody purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation] (see the Materials and methods section); (ii) variation of the ratio of the amount of antibody to CNBr-activated Sepharose gel over the range 5–0.5 mg of antibody protein/ml of gel; (iii) variation of the

concentration of NaCl present in the extract and column buffer over the range 0–1.0 M; (iv) preparation of extract in phosphate buffer instead of Tris buffer. However, none of the above measures offered significant improvements to the amount of enzyme activity that could be eluted.

A number of control columns have been used for the above study. Four columns were prepared in the standard way except for replacement of the specific antibodies with (i) albumin, (ii) serum proteins from an animal carrying a negative hybrid cell line (F3 line; see Choo *et al.*, 1980), (iii) serum proteins from an animal carrying a hybrid cell line producing a monoclonal antibody (RV4-3) that was directed against an unrelated antigen (see the Materials and methods section), or (iv) coupling buffer alone. Passage of extract (with or without 10 mM-phenylalanine added) through these columns has resulted in little or no adsorption of phenylalanine hydroxylase activity.

Species specificity

In an earlier study (Cotton *et al.*, 1980), PHa 1-1 antibody has been shown to cross-react with phenylalanine hydroxylase from monkey, human, rat and mouse liver by inhibition of activity and immunodiffusion. Cross-reactivity with enzyme from monkey, human and rat liver has also been demonstrated in the case of the B5-1 antibody (Choo *et al.*, 1980) by stimulation of activity. The ability of these antibodies as well as those of the PHa 2-1-1 and PHa 3-0 antibodies to recognize monkey liver phenylalanine hydroxylase is further established in the present study by affinity-

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of concentrated samples of phenylalanine hydroxylase purified from crude monkey liver extract by using (a) PH α 2-1-1 and (b) PH α 3-0 immunoadsorbent columns

The enzyme was eluted from the columns with a pH 11.5 buffer. Approx. 15 μ g of protein was loaded on each gel track. A very small amount (see the text) of contaminant proteins, not apparent in the photographic reproduction of the gel, was seen in the original gel track for the PH α 2-1-1 preparation. H and L designate the two molecular-weight polypeptide species of purified phenylalanine hydroxylase (Cotton *et al.*, 1980).

chromatographic experiments (see above). In the following study, the specificity of the PH α 2-1-1, PH α 3-0 and B5-1 antibodies against enzymes from human, rat and mouse liver was tested by using the antibody-Sepharose beads to test for adsorption of phenylalanine hydroxylase activity from small volumes of extract. Removal of activity was expressed as a percentage of the activity measured when control RV4-3 Sepharose beads were used. As summarized in Table 1, incubation of extracts of monkey and human livers with the PH α 2-1-1 and

Table 1. Specificity of four monoclonal antibodies against hepatic phenylalanine hydroxylase from different mammalian species

Experimental details are given in the text. + or — designates respectively that >80% or <10% of initial phenylalanine hydroxylase activity was removed from the extract after incubation of the extract with immunoadsorbent prepared from the various antibodies. Each entry in the Table has been established from experiments performed at least three times.

Antibody	Monkey	Human	Rat	Mouse
PH α 1-1	+	+*	+*	+*
PH α 2-1-1	+	+	—	—
PH α 3-0	+	+†	—	—
B5-1	+	+	+	+
RV4-3	—	—	—	—

* This result was derived from an earlier study (Cotton *et al.*, 1980) where positive cross-reaction between antibody and phenylalanine hydroxylase was established by enzyme inhibition assay.

† Incubation of PH α 3-0 immunoadsorbent with human liver extract consistently resulted in only approx. 50% removal of enzyme activity from the extract.

PH α 3-0 immunoadsorbents has resulted in the removal of a significant amount of the initial phenylalanine hydroxylase activity present in the extracts. No detectable loss of enzyme activity was, however, observed when these two immunoadsorbents were incubated with extracts of rat and mouse liver. This result suggests that the PH α 2-1-1 and PH α 3-0 antibodies are able to cross-react with phenylalanine hydroxylase from monkey and human sources, but not with that from rat and mouse.

Table 1 also indicates that B5-1 antibody cross-reacted with phenylalanine hydroxylase from monkey, human, rat and mouse liver extracts.

Summary of results

The different properties of the four monoclonal antibodies described in the present study are summarized in Table 2.

Discussion

By using the method of myeloma-spleen-cell fusion, we have isolated four hybrid cell lines that produce monoclonal antibodies against mammalian phenylalanine hydroxylase. These antibodies exhibit a number of different properties, which are summarized under Table 2. The existence of such an array of functionally different antibodies to phenylalanine hydroxylase has not been demonstrated in the past, as earlier studies have used unfractionated antisera that contained a heterogeneous population of antibodies.

Table 2. Summary of the properties of four different monoclonal antibodies to phenylalanine hydroxylase

See the text for detailed presentation and discussion of these results. Each result represents that drawn from the numbers of experiments shown in parentheses. N.D., Not done.

Antibody	Immunoglobulin chains*		Effect on enzyme activity†	Species specificity‡				Immunoadsorbent affinity chromatography				
								Extract with no added phenylalanine		Extract with 10 mM-phenylalanine added		
Heavy	Light		Monkey	Human	Rat	Mouse	Removal	pH 11.5 elution	Removal§	Phenylalanine-free elution	pH 11.5 elution	
PHA 1-1	γ ₃	κ	62% inhibition (12)	+	+	+	+	17% (2)	N.D.	(a) 92% (3) (b) 90% (1)	46% (3) N.D.	3% (2)¶ 58% (1) 51% (1)
PHA 2-1-1	γ _{2a}	κ	11% inhibition (10)	+	+	—	—	97% (5)	50% (5)	95% (1)	0% (1)	0% (1)
PHA 3-0	γ _{2a}	κ	No effect (5)	+	+	—	—	93% (5)	0% (3) 22% (2)	100% (1)	0% (1)	Enzyme protein detected
B5-1	γ ₁	λ, κ	92% stimulation (5)	+	+	+	+	90% (2)	42% (2)	92% (2)	0% (2)	N.D.

* Class of immunoglobulin chains produced by hybrid cells.

† Effect of hybrid culture supernatant on monkey liver phenylalanine hydroxylase activity.

‡ Species of mammalian phenylalanine hydroxylase recognized by the antibody.

§ Removal (or adsorption) of initial enzyme activity on the affinity column: the value was obtained by subtracting the activity that passed straight through the column from that which was initially applied.

|| The amount (expressed as a percentage of that initially applied on the column) of enzyme activity recovered from the column after elution with pH 11.5 buffer or phenylalanine-free buffer.

¶ pH 11.5 buffer was applied to this column after the phenylalanine-free elution step.

On the basis of their effects on phenylalanine hydroxylase activity, the antibodies can be classified as strongly inhibiting (PH α 1-1), moderately inhibiting (PH α 2-1-1), stimulating (B5-1) and null (PH α 3-0) antibodies. Although the mechanisms responsible for the different effects these antibodies have on the enzyme molecule are not known, it appears likely that the antibodies react with different antigenic determinants present on the phenylalanine hydroxylase molecule. This is also partly borne out by the fact that two of the four antibodies (PH α 2-1-1 and PH α 3-0) failed to recognize an antigenic determinant on rat liver phenylalanine hydroxylase.

The PH α 1-1 antibody displays the interesting property of requiring the presence of phenylalanine for maximum interaction with the enzyme. This feature was not fully described in an earlier study (Cotton *et al.*, 1980), where the experiments were performed with crude extracts or purified enzymes that had been prepared in a buffer containing phenylalanine. In the present study, the requirement for phenylalanine is clearly demonstrated by the affinity-chromatographic experiments, where the presence of 10 mM-phenylalanine in the tissue extract becomes a prerequisite for complete adsorption of phenylalanine hydroxylase on the immunoabsorbent column. This property is intrinsic to the antibody and is not a function of the coupling process on to the Sepharose gel. Previously it was shown that extensive immunoprecipitation of enzyme occurred when PH α 1-1 antibody was incubated with crude extracts containing added phenylalanine (Cotton *et al.*, 1980). In the present study, when a similar experiment was performed with extracts prepared in a phenylalanine-free buffer, a very poor immunoprecipitation reaction was observed (results not shown), which was analogous to the results obtained in the antibody affinity-chromatographic experiments.

The fact that phenylalanine hydroxylase bound on the PH α 1-1 immunoabsorbent could be eluted off with a phenylalanine-free buffer suggests a reversible conformational change in the enzyme. Other workers have reported that phenylalanine played a role in the activation of phenylalanine hydroxylase (Nielson, 1969; Tourian, 1971; Shiman & Gray, 1980), and that the activation is accompanied by a conformational change (Shiman *et al.*, 1979). Alternatively, it is possible that phenylalanine forms part of the binding site for this antibody. As phenylalanine does not appear to interfere with the binding of the other three antibodies to the enzyme, the phenylalanine-binding site may not be included in the binding site of these three antibodies.

The affinity-chromatographic studies have indicated that the immunoabsorbent columns are useful for rapid isolation of the enzyme protein from crude extract. Further, they should be useful for the

isolation of the different peptides carrying the various antigenic determinants.

Currently, the reason for the recovery of only about 50% of the initial enzyme activity applied to the PH α 1-1, PH α 2-1-1 and B5-1 antibody columns is not clear. Similarly, the cause of the total or partial loss of initial enzyme activity from the PH α 3-0 column as well as the presence of a substantial amount of contaminant or possibly altered proteins in the pH 11.5 elution fractions from this column is unknown.

Earlier experiments with rabbit antisera have indicated that phenylalanine hydroxylase from monkey and rat showed only partial antigenic identity (as shown by 'spurring' of precipitin lines in immunodiffusion) (Choo & Cotton, 1979). It is therefore not surprising that we have isolated two monoclonal antibodies (PH α 2-1-1 and PH α 3-0) that are directed against antigenic determinant(s) unique to the enzyme from monkey (and human) but that is not present on the enzyme from rat (and mouse).

It is possible that monoclonal antibodies may be used to create models of human disease by their specific inhibition of a normal function. This appears not to be possible with our four monoclonal antibodies, as the serum phenylalanine/tyrosine ratio is not significantly different from normal in animals carrying each of the four tumours (D. Kirby, K. Fowler & R. G. H. Cotton, unpublished work). We assume that these antibodies cannot reach the phenylalanine hydroxylase in the liver cells.

It may be seen that, while this study has defined a number of interesting properties of monoclonal antibodies that may be useful for the study of phenylalanine hydroxylase, it has, at the same time, posed some interesting questions that should form the basis for further investigation. Also, it can be seen that, in general terms, monoclonal antibodies will be useful in the study of structure-function relationships of enzymes. However, of the small number of monoclonal antibodies that have been made to enzymes so far, only one study has reported monoclonal antibodies that affect catalytic activity (Frackleton & Rotman, 1980).

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