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A monoclonal antibody to aromatic amino acid hydroxylases

Identification of the epitope

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PH8 monoclonal antibody has previously been shown to react with all three aromatic amino acid hydroxylases, being particularly useful for immunohistochemical staining of brain tissue [Haan, Jennings, Cuello, Nakata, Chow, Kushinsky, Brittingham & Cotton (1987) Brain Res. **426**, 19–27]. Western-blot analysis of liver extracts showed that PH8 reacted with phenylalanine hydroxylase from a wide range of vertebrate species. The epitope for antibody PH8 has been localized to the human phenylalanine hydroxylase sequence between amino acid residues 139 and 155. This highly conserved region of the aromatic amino acid hydroxylases has 11 out of 17 amino acids identical in phenylalanine hydroxylase, tyrosine hydroxylase and tryptophan hydroxylase.

INTRODUCTION

Three aromatic amino acid hydroxylases, namely phenylalanine, tyrosine and tryptophan hydroxylase, have long been postulated to be structurally related (Friedman *et al.*, 1972). Recently comparisons of the cDNA base sequences of human phenylalanine hydroxylase, rat tyrosine hydroxylase and rabbit tryptophan hydroxylase have shown extensive regions of similarity, particularly in the *C*-terminal two-thirds of the molecule (Grenett *et al.*, 1987).

We have produced a series of monoclonal antibodies which react with phenylalanine hydroxylase (Jennings *et al.*, 1986), one of which, PH8, has been shown to recognize all three aromatic amino acid hydroxylases, being particularly useful in the immunohistochemical localization of tyrosine and tryptophan hydroxylases in brain tissue sections (Haan *et al.*, 1987). PH8 has been used in the mapping of catecholaminergic axons in the dog peripheral nervous system (Harris *et al.*, 1986), and of the serotonergic neurons in the human medulla oblongata (Halliday *et al.*, 1988*a*,*b*) and in human and cat brain (Tork & Hornung, 1986; I. Tork, unpublished work). In the present paper the PH8 antibody-binding region has been localized in the known amino acid sequences of the aromatic amino acid hydroxylases.

EXPERIMENTAL

Materials

Tissues. Samples of liver from various local sources were collected on solid CO_2 and stored at −70 °C. The squid 'liver equivalent' was obtained from *Nototodarus gouldi*. Bacterial enzyme (*Chromobacterium violaceum*) was purified by Dr. H. Nakata as described by Nakata *et al.* (1979).

Methods

Preparation of phenylalanine hydroxylase. Human and rat liver phenylalanine hydroxylase was purified from fresh liver (less than 3 h between death and snap-freezing) using a monoclonal-antibody-affinity column (Choo *et al.*, 1981). The enzyme was approx. 95% pure.

Electrophoresis and immunoblotting. Preparation of liver extracts was as described by Choo & Cotton (1979). Separation of liver extract proteins was carried out in 9% (w/v) polyacrylamide gels containing 0.1% SDS, then electrophoretic transfer of these proteins from the gel on to nitrocellulose was performed as described previously (Smith *et al.*, 1984). For probing of liver extracts with the appropriate antibody, 200 μg of protein was loaded on to the gel. PH8 ascites fluid (Jennings *et al.*, 1986), diluted 1:1000, was used as the primary antibody. Detection of antigen-bound antibody on nitrocellulose was with peroxidase-conjugated anti-mouse IgG and subsequent development with chloronaphthol (Hawkes *et al.*, 1982).

Competitive e.l.i.s.a. For competitive antibody-blocking experiments, the competing peptide/protein-monoclonal-antibody mixtures were preincubated for 1 h at 4 °C before addition to microtitre plates coated with purified phenylalanine hydroxylase (100 ng/well). E.l.i.s.a. was then performed as described previously (Jennings *et al.*, 1986).

Isolation of phenylalanine hydroxylase peptides immunoreactive to monoclonal antibody PH8. Conditions for the preparation of tryptic and chymotryptic peptides of phenylalanine hydroxylase were described by Smith *et al.* (1987).

Isolation of the tryptic and chymotryptic peptides

Abbreviations used: PBS, phosphate-buffered saline (0.1 M-sodium phosphate/0.14 M-NaCl, pH 7.0; TFA, trifluoroacetic acid; r.p.h.p.l.c., reversed-phase h.p.l.c.; e.l.i.s.a., enzyme-linked immunosorbent assay.

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immunoreactive to monoclonal antibody PH8 was performed essentially as described by Smith *et al.* (1987). The peptides were separated by r.p.h.p.l.c on an Aquapore RP300 column equilibrated in 20 mM-NH_4HCO_3, pH 7.8. Portions of fractions collected over the 40 min gradient of 0–60% (v/v) acetonitrile were tested for immunoreactivity. The immunoreactive fraction was then rechromatographed on the same h.p.l.c. column, but equilibrated in 0.1% trifluoroacetic acid with a 40 min gradient of 0–60% acetonitrile. U.v.-absorbing peaks were collected and portions were tested for immunoreactivity.

The tryptic and chymotryptic peptides were then further purifed on a Bakerbond WP-C18 column equilibrated in 0.1% TFA. Elution of the peptide was carried out with a linear gradient of acetonitrile from 0 to 50% over 60 min. The immunoreactive peptide was then subjected to amino acid hydrolysis and analysis by the method of Smith *et al.* (1987).

Peptide sequence analysis. The selected peptide was subjected to automated Edman degradation, and amino acid phenylthiohydantoin derivatives were analysed as described by Simpson *et al.* (1980).

RESULTS

Reactivity of PH8 with phenylalanine hydroxylase of various species

Western blotting of liver proteins by SDS/polyacrylamide-gel electrophoresis was used to determine the cross-reactivity of PH8 with phenylalanine hydroxylase from various species ranging from squid to man (Fig. 1).

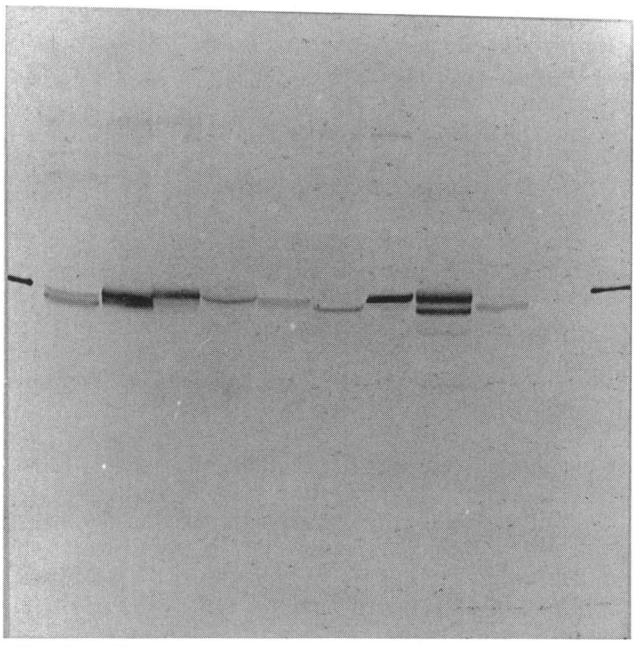

Fig. 1. Immunoblot of PH8 antibody reaction with liver extracts from various animal species

Electrophoresis and immunoblotting were carried out as described in the Experimental section. Purified rat liver phenylalanine hydroxylase of the W-allele type (Mercer *et al.*, 1984) (0.5 μg) was in lanes 1 and 12. The various liver extracts (200 μg total protein) were loaded as follows: lane 2, human; lane 3, rat; lane 4, dog; lane 5, pig; lane 6, cow; lane 7, koala; lane 8, chicken; lane 9, trout; lane 10, cane toad; lane 11, squid.

Table 1. Amino acid compositions analysis of PH8 immunoreactive tryptic and chymotryptic peptides

The values are relative and based on lysine = 1 (tryptic peptide) and leucine = 1 (chymotryptic peptide). The numbers in parentheses are the expected values for the peptide 131–155 (tryptic) and peptide 139–154 (chymotryptic) as deduced from the phenylalanine hydroxylase cDNA sequence of Kwok *et al.* (1985).

	Composition	
Amino acid	Tryptic peptide (residues 131–155)	Chymotryptic peptide (residues 139–154)
Aspartate + asparagine	3.88 (4)	2.39 (3)
Threonine	0.20 (0)	0.42 (0)
Serine	4.17 (1)	0.49 (0)
Glutamate + glutamine	2.83 (2)	1.48 (1)
Proline	1.88 (2)	1.67 (2)
Glycine	2.43 (2)	2.14 (2)
Alanine	3.00 (3)	1.51 (2)
Cysteine	0 (0)	0 (0)
Valine	0.97 (1)	0.84 (1)
Methionine	0 (0)	0 (0)
Isoleucine	1.19 (1)	0.28 (0)
Leucine	2.11 (2)	1.00 (1)
Tyrosine	1.91 (2)	0.83 (1)
Phenylalanine	1.95 (2)	0.96 (1)
Histidine	0.82 (1)	0.59 (1)
Lysine	1.00 (1)	0.94 (1)
Arginine	1.18 (1)	0.40 (0)

Fig. 1 shows that the PH8 antibody reacted with phenylalanine hydroxylase in the liver extracts of man, rat, dog, pig, ox, koala, chicken, trout and toad, but no cross-reactivity was observed with the squid liver extract, despite the presence of phenylalanine hydroxylase activity. Rabbit polyclonal antibodies against rat liver phenylalanine hydroxylase failed to cross-react with squid liver extract (results not shown). The M_r values for the subunits are similar, varying by less than 5 kDa. Some differences are observed in the number of bands, with the human, rat, dog and trout enzyme showing two bands and the pig, cow, koala, chicken and toad enzyme showing one band. The two bands shown by the human enzyme are due to differences in phosphorylation (Smith *et al.*, 1984) and in rat enzyme they are the products of allelic genes (Mercer *et al.*, 1984). The origin of the two bands in the other species is not known and has not been investigated further at this stage. In a further experiment no cross-reaction was detected between PH8 antibody and 15 μg of phenylalanine hydroxylase purified from *Chromobacterium violaceum* (results not shown)

Structural analysis of the PH8-binding region

Initial experiments demonstrated that monoclonal antibody PH8 retained antigenicity to human liver phenylalanine hydroxylase digested with trypsin (results not shown).

To isolate the tryptic peptide which retained immuno-

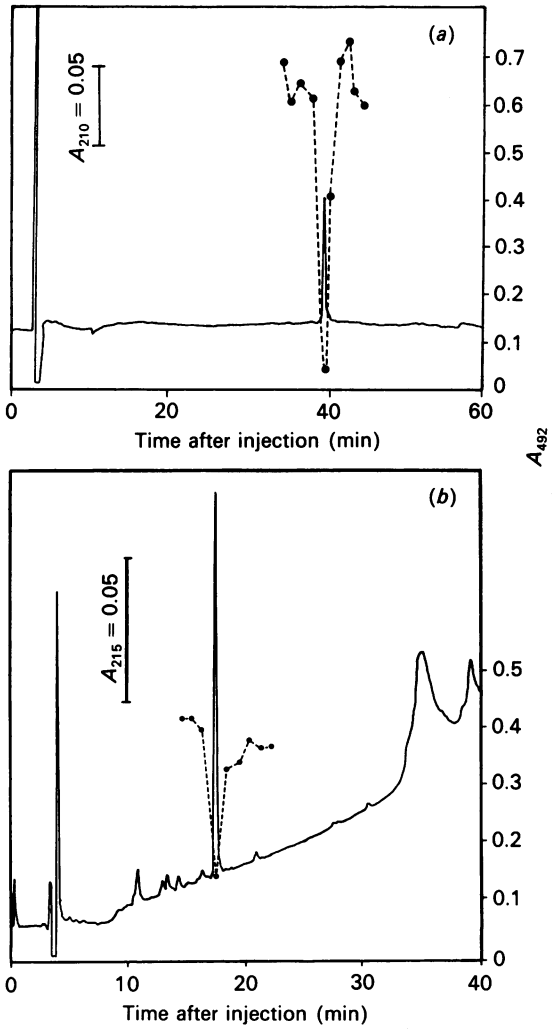

Fig. 2. Purification of PH8-immunoreactive peptides

(*a*) Tryptic peptide separation on a C18 h.p.l.c. column using a linear gradient of 0–50% acetonitrile in 0.1% TFA. Peptides were detected by absorbance at 210 nm (——). Immunoreactive peptide was identified by competitve e.l.i.s.a. and was quantified by a decrease in absorbance at 492 nm (●). (*b*) Chymotryptic peptide separation by r.p.h.p.l.c. using a 0–60% gradient of acetonitrile in 0.1% TFA. Fractions were monitored for absorbance at 215 nm (——). Immunoreactive peptide was identified by competitive e.l.i.s.a. and was quantified by a decrease in absorbance at 492 nm (●).

reactivity, the peptides (200 μg) were separated on an Aquapore h.p.l.c. column under neutral conditions as described above. The eluted fractions were tested for immunoreactivity by taking 0.1% of each fraction and subjecting them to competitive e.l.i.s.a. Only one fraction was immunoreactive, and this fraction was re-chromatographed under acidic h.p.l.c. conditions.

Subsequent purification (Fig. 2*a*) and amino acid analysis of the immunoreactive peptide (Table 1) gave a composition which was consistent with the PH8-immunoreactive tryptic peptide spanning amino acid residues 131–155 of the published human sequence (Kwok *et al.*, 1985). In addition a partial amino acid sequence was obtained:

Phe-Ala-Asn-Gln-Ile-Leu-Ser-Tyr-Gly-Ala-Glu-Leu-Asp-Ala

which also agreed with this conclusion.

Chymotrypsin digestion of phenylalanine hydroxylase was also performed followed by successive h.p.l.c. separations under neutral and acidic conditions as detailed under 'Methods' in the Experimental section. The PH8 immunoreactive peptide was identified (Fig. 2*b*) and subjected to amino acid hydrolysis and analysis. The composition of the PH8-immunoreactive chymotryptic peptide corresponded to the sequence from residue 139 to residue 154 (Table 1) as published by Kwok *et al.* (1985). Thus the PH8 epitope is located within the sequence from residue 139 to residue 154 (Fig. 3), which is the overlap sequence between the tryptic and chymotryptic immunoreactive peptides.

DISCUSSION

A monoclonal antibody to monkey liver phenylalanine hydroxylase, PH8, isolated in a previous study (Jennings *et al.*, 1986) and later shown to react with human and rat brain tyrosine hydroxylase and tryptophan hydroxylase (Haan *et al.*, 1987) has now been characterized more fully.

Hsieh & Berry (1979) have shown that livers from a wide range of vertebrates have phenylalanine hydroxylase activity. In the present study PH8 antibody reacted with liver phenylalanine hydroxylase from a wide range of vertebrates and thus demonstrated a structural homology within the PH8-binding site in vertebrate phenylalanine hydroxylases. In addition it raises the possibility of the PH8 antibody being useful in the characterization of the aromatic amino acid hydroxylases of a wide range of

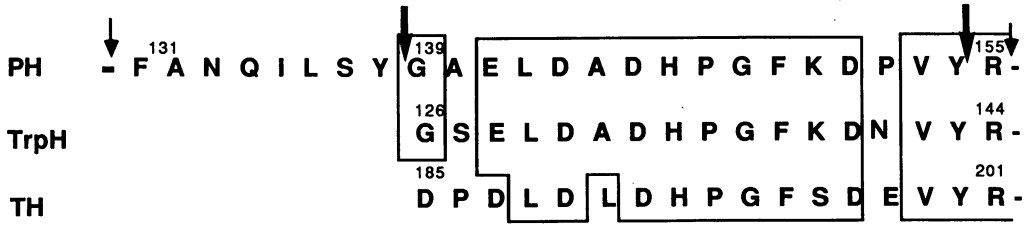

Fig. 3. Sequences of PH8-immunoreactive tryptic and chymotryptic peptides

The ↓ and ⇓ arrows indicate tryptic and chymotryptic cleavage points respectively. Values above the sequences are amino acid residue numbers in the total sequence of human phenylalanine hydroxylase (PH), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TrpH) as described by Grenet *et al.* (1987). Boxed areas show areas of identity within the sequences of the three hydroxylases.

species by both immunohistochemical and immunopurification techniques.

Using tryptic and chymotryptic digestion of phenylalanine hydroxylase we have localized the PH8-antibody-binding region to residues 139–154 of the phenylalanine hydroxylase sequence (Fig. 3). This region shows a high degree of identity between human phenylalanine hydroxylase, rat tyrosine hydroxylase and rabbit tryptophan hydroxylase, with 11 out of 17 amino acids identical. Quantitative comparison between the reaction of PH8 antibody with phenylalanine hydroxylase and proteolytic digests of phenylalanine hydroxylase in a competitive e.l.i.s.a. showed that the digests were in fact more efficient in binding to the PH8 antibody (results not shown). This could be due to the denaturation of the tertiary structure of the protein after proteolytic digestion, leading to the epitope being more accessible to the antibody. Therefore we are unable to draw a firm conclusion as to whether the entire PH8 epitope is contained in the proteolytic digests, although both digests when separated on h.p.l.c. contained only one immunoreactive fragment.

Our previous study (Jennings *et al.*, 1986) showed that PH8 has no effect on phenylalanine hydroxylase enzyme activity and that PH8 antibody binding is unaffected by activation of phenylalanine hydroxylase by phenylalanine, lysophosphatidylcholine and phosphorylation. Thus although the PH8-antibody-binding region is highly conserved in the aromatic amino-acid hydroxylases, it appears not to be involved in enzyme function, but may have an important structural function.

These results emphasize the potential of a monoclonal antibody (PH8) raised against an abundant and easily purified protein (phenylalanine hydroxylase) to permit the analysis of related, rare and difficult-to-purify proteins (tyrosine hydroxylase and tryptophan hydroxylase) from a wide range of species. In particular the PH8 antibody is proving to be important in the mapping of serotonergic neurons, where the only antibody previously available was made to the unstable product, 5-hydroxytryptamine (serotonin), of the tryptophan hydroxylase enzyme reaction (Consolazione *et al.*, 1984). This compound disappears after death, making staining of serotonergic neurons with this antibody impossible, but the PH8 epitope remains intact.

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