A monoclonal antibody to the phosphorylated form of phenylalanine hydroxylase

Definition of the phosphopeptide epitope

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Monoclonal antibody PH7 has specificity for the phosphorylated form of the human liver phenylalanine hydroxylase and negligible reactivity towards the dephosphorylated form of the native enzyme by enzyme-linked immunoassay. PH7 binds specifically to the phosphorylated form of the liver enzyme after SDS/polyacrylamide-gel electrophoresis and transfer to nitrocellulose. Competitive blocking assays have been applied in conjunction with reversed-phase h.p.l.c. of purified tryptic fragments of human liver phenylalanine hydroxylase to localize the epitope. The major immunoreactive tryptic peptide cross-reacting with PH7 had an amino acid analysis corresponding to the first 41 amino acids of the human liver phenylalanine hydroxylase sequence and included the serine residue that is thought to be the phosphorylation site. The monoclonal antibody recognized the phosphorylated form of the synthetic decapeptide corresponding to the local phosphorylation-site sequence Gly-Leu-Gly-Arg-Lys-Leu-Ser(P)-Asp-Phe-Gly, but not the dephosphodecapeptide. Thermolysin digestion of the peptide demonstrated the monoclonal antibody bound to the pentapeptide Leu-Ser(P)-Asp-Phe-Gly. Monoclonal antibody PH7 recognized the phosphodecapeptide at concentrations 10^3 -fold higher than with phenylalanine hydroxylase, compared with 10^4 – 10^7 -fold higher for other phosphopeptides and phosphoproteins. The results demonstrate that monoclonal antibody PH7 has specificity for the phosphorylated form of phenylalanine hydroxylase at the phosphorylation site.

INTRODUCTION

In mammalian liver, phenylalanine hydroxylase (phenylalanine 4-mono-oxygenase, EC 1.14.16.1) catalyses the conversion of phenylalanine into tyrosine [for reviews, see Kaufman (1976), Cotton (1977) and Goodwin (1979)]. The activity of the hepatic enzyme can be modulated by a number of effectors including phenylalanine, lysophosphatidylcholine (Fisher & Kaufman, 1972) and phosphorylation (Abita *et al.*, 1976; Donlon & Kaufman, 1977). It would be advantageous to relate the biological function of the phenylalanine hydroxylase molecule with the amino acid sequence by using monoclonal antibodies directed to single antigenic determinants (Kohler & Milstein, 1975, 1976). We have previously described the production of a library of monoclonal antibodies generated against mammalian liver phenylalanine hydroxylase to aid in the analysis of structure–function relationships (Choo *et al.*, 1979; Jennings *et al.*, 1986).

The present paper demonstrates that monoclonal antibody PH7 binds specifically to the phosphorylated form of human liver phenylalanine hydroxylase and identifies the principal part of the binding site. Monoclonal antibody PH7 binds to a linear sequence of

five amino acids containing the phosphorylation site. Moreover, monoclonal antibody PH7 binding requires the phosphoserine residue for binding to this pentapeptide.

EXPERIMENTAL PROCEDURES

Materials

Human liver phenylalanine hydroxylase was prepared (95% pure) by affinity chromatography using a monoclonal-antibody immuno-affinity column (Choo *et al.*, 1979). Procedures for the phosphorylation and enzyme dephosphorylation of affinity-purified human liver enzyme have been detailed (Smith *et al.*, 1984).

The stoichiometry of phosphorylation of the affinity-purified, phosphorylated and enzymically dephosphorylated forms of the human liver phenylalanine hydroxylase are 0.3 mol, 0.05 mol and 0.6 mol of ^{32}P /mol of human phenylalanine hydroxylase monomer respectively.

The catalytic subunit of the cyclic AMP-dependent protein kinase was prepared from bovine heart muscle (Sugden *et al.*, 1976). Methods for the preparation,

Abbreviations used: Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); e.l.i.s.a., enzyme-linked immunosorbent assay; PTH, phenylthiohydantoin.

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isolation and screening of murine hybridoma lines generating monoclonal antibodies PH7, PH8 and PH9 directed against phenylalanine hydroxylase have been described (Jennings *et al.*, 1986). In all cases, ascites fluid was used as the source of monoclonal antibodies for immunochemical studies.

Reagents were purchased as follows: [γ - 32 P]ATP (sp. radioactivity 4000 c.p.m./pmol) from Amersham Corp., Sydney, Australia; Tos-Phe-CH₂Cl-treated trypsin from Worthington, Freehold, NJ, U.S.A.; h.p.l.c.-grade acetonitrile from Waters Associates, Sydney, Australia; trifluoroacetic acid and casein, phosvitin from BDH, Poole, Dorset, U.K.; nitrocellulose from Millipore, Bedford, MA, U.S.A. Water for h.p.l.c. buffer solution was deionized, glass-distilled and passed through a Millipore filtration system (0.45 μ m-pore-size filter). All other chemicals were of the highest purity commercially available.

Antibody-binding assay

Antigenic reactivity was determined by an e.l.i.s.a. method as previously described (Jennings *et al.*, 1986). Samples of antigenic preparations to be tested [0.05 ml of phosphorylated and dephosphorylated human liver phenylalanine hydroxylase/well; 2 μ g/ml of 10 mM-Tris/HCl (pH 7.4)/10 mM-phenylalanine/0.1 mM-EDTA] were coated on wells overnight at 4 °C. Binding of monoclonal antibodies was determined by colour development with peroxidase-conjugated goat anti-mouse antisera (Cappel Laboratories, Cochranville, PA, U.S.A.) and *o*-phenylenediamine (BDH). The A_{492} was recorded with a Titertek Multiskan Instrument (Flow Laboratories, North Ryde, Australia). For competitive antibody-blocking experiments, the competing peptides or proteins were pre-incubated with monoclonal antibody for 1 h at 4 °C before addition to microtitre plates coated with affinity-purified phenylalanine hydroxylase. The dilution of antibody used in competitive e.l.i.s.a. was determined as below saturation of antigenic sites. Incubation was for 1 h at 4 °C before washing, addition of enzyme conjugate, followed by substrate.

Electrophoretic transfer and immunodetection

Human liver phenylalanine hydroxylase and human liver extract were subjected to SDS/polyacrylamide-gel electrophoresis (Smith *et al.*, 1984). Electrophoretic blotting to nitrocellulose was performed by a slight modification of the method of Towbin *et al.* (1979). After transfer, the nitrocellulose sheets containing the phenylalanine hydroxylase polypeptides or liver extract polypeptides were incubated for 2 h at room temperature in 10 mM-Tris/HCl (pH 7.4)/0.15 M-NaCl containing 5% (w/v) serum albumin to prevent non-specific binding. The blots were then incubated at room temperature with either monoclonal-antibody-containing ascites fluid at a 1:200 dilution or rabbit anti-(rat phenylalanine hydroxylase) serum diluted 1:250 in 10 mM-Tris/HCl (pH 7.4)/0.15 M-NaCl, containing 5% (w/v) serum albumin. After 2 h incubation, the immunoblots were washed and incubated for 1 h at room temperature with 125 I-protein A (Amersham International) (Smith *et al.*, 1984). The blots were washed, air-dried and autoradiographed.

Preparation of tryptic fragments of human liver phenylalanine hydroxylase

Phosphorylated human liver enzyme was reduced and carboxymethylated then digested (50:1) with Tos-Phe-CH₂Cl-treated trypsin in 0.05 M-NH₄HCO₃, pH 8.0, for 6 h at 37 °C. Proteolysis was stopped by boiling the sample for 10 min. The digests were freeze-dried and then subjected to blocking e.l.i.s.a. or separation of peptides by h.p.l.c.

Peptide separation by h.p.l.c.

Tryptic peptides of phenylalanine hydroxylase were separated by reversed-phase h.p.l.c. using either a 0.3 cm $\times$ 20 cm column of Lichrosorb C8 (5 μ m particle size; Merck; part no. 50322-1) or a 0.46 cm $\times$ 25 cm column of Aquapore RP 300 (10 μ m particle size; Brownlee Laboratories, Santa Clara, CA, U.S.A.; part no. C03-10A). Details of the separation conditions are given in the Results section.

Peptide sequence analysis

Selected peptides were subjected to automated Edman degradation and PTH-amino acids were analysed as described by Simpson *et al.* (1980).

Peptide synthesis

The synthetic peptides Gly-Leu-Gly-Arg-Lys-Leu-Ser-Asp-Phe-Gly (peptide A) and Leu-Arg-Arg-Ala-Ser-Leu-Gly (peptide B) were assembled using the method of Hodges & Merrifield (1975) on an Applied Biosystems synthesizer, model 430A. The peptide amides were cleaved by HF treatment and purified by ion-exchange chromatography on sulphopropyl-Sephadex as described previously (Kemp, 1979). Peptide purity was 99% as judged by analytical reversed-phase h.p.l.c. The amino acid composition of peptide A determined after acid hydrolysis (5.7 M-HCl for 24 h at 110 °C) was as follows: Gly_{2.90} Leu_{2.05} Arg_{1.00} Lys_{1.04} Ser_{1.05} Asp_{1.02} Phe_{0.96}. The amino acid composition of peptide B was as previously described (Kemp, 1979).

Phosphorylation of the synthetic peptides was carried out as described previously (Smith *et al.*, 1984). Stoichiometric phosphorylation was confirmed by h.p.l.c. analysis and high-voltage paper electrophoresis at pH 1.9. Partially hydrolysed phosphopeptide (5.7 M-HCl, 2 h, 40 °C) contained phosphoserine as assessed by high-voltage paper electrophoresis at pH 1.9 (Smith *et al.*, 1984).

Competitive e.l.i.s.a. was performed on the peptides as described above.

Proteolytic cleavage of the synthetic peptide

The phosphorylated synthetic peptide A was subjected to digestion with trypsin and thermolysin. Digestion was performed in 100 mM-NH₄HCO₃, pH 8.0 at 37 °C with an enzyme/substrate ratio of 1:1 (w/w) for 4 h.

The proteolytic digests were then separated by reversed-phase h.p.l.c. using the Brownlee RP300 column equilibrated in 0.1% trifluoroacetic acid as described in the Experimental procedures section. The u.v. profile was observed at 215 nm and the h.p.l.c. run was screened by competition e.l.i.s.a. to detect immuno-reactive peptides.

RESULTS

Specificity of monoclonal antibody PH7 for phosphorylated human liver phenylalanine hydroxylase

Initial characterization of the monoclonal antibody PH7-defined antigenic determinant using an e.l.i.s.a. technique demonstrated apparent specificity of monoclonal antibody PH7 for human liver phenylalanine hydroxylase after phosphorylation with cyclic AMP-dependent protein kinase (Fig. 1a). The binding was dependent on the phosphorylation state, since there was negligible reactivity with the dephosphorylated form of the human liver enzyme (Fig. 1b). Previous testing of two

Fig. 1. Determination of monoclonal antibody binding phosphorylated human liver phenylalanine hydroxylase

Titration of monoclonal antibody binding to preparations of phosphorylated (a) and enzymically dephosphorylated human liver phenylalanine hydroxylase (b) was determined as described in the Experimental procedures section. Bound antibody was proportional to A_{492} . Monoclonal antibodies tested were PH7 (●, ○), PH8 (■, □) and PH9 (▲, △).

monoclonal antibodies PH8 and PH9 against phenylalanine hydroxylase has demonstrated that these antibodies do not compete with PH7 binding to phenylalanine hydroxylase (Jennings *et al.*, 1986). As controls, the two monoclonal antibodies, PH8 and PH9 demonstrated equivalent cross-reactivity with both phosphorylated and enzymically dephosphorylated forms of the liver enzyme. Monoclonal antibodies PH7 and PH8 were used at a 1:200 000 dilution, whereas PH9 was used at a 1:50 000 dilution in the e.l.i.s.a. to give below saturation point in the competition.

Binding of monoclonal PH7 to phosphorylated human liver phenylalanine hydroxylase transferred to nitrocellulose

Since the binding of monoclonal antibody PH7 to human phenylalanine hydroxylase might be sensitive to change in the native protein structure, we decided to find out whether the immunoreactivity of the antigenic determinant was abolished by SDS treatment. Phosphorylated, dephosphorylated and affinity-purified human liver enzymes were separated by SDS/polyacrylamide-gel electrophoresis, transferred to nitrocellulose and subjected to immunodetection using the monoclonal antibodies PH7 and PH9. Amido Black staining of the nitrocellulose after electrophoretic blotting indicated

Fig. 2. PH7 specifically binds phosphorylated human liver phenylalanine hydroxylase on nitrocellulose

Phosphorylated, enzymically dephosphorylated and affinity-purified human liver phenylalanine hydroxylase were electrophoresed on SDS/polyacrylamide gel (9%), under reducing conditions, before electroblotting on to nitrocellulose. For immunodetection, nitrocellulose strips with 5 μ g of appropriate phosphorylated (lane 1) or dephosphorylated (lane 2) antigen and 10 μ g of affinity-purified (lane 3) antigen were incubated with monoclonal antibodies and bound antibody detected with 125 I-protein A. The arrows indicate the positions of the phosphorylated (M_r 50 000) and dephosphorylated (M_r 49 000) forms of human liver phenylalanine hydroxylase. (a) PH7; (b) PH9.

the same amount of phosphorylated and dephosphorylated forms of enzyme were transferred.

It has been previously demonstrated that the dephosphorylated form of phenylalanine hydroxylase has an apparent M_r of 49000 and can be converted into the M_r -50000 phosphorylated form by phosphorylation in the presence of cyclic AMP-dependent protein kinase (Smith *et al.*, 1984). As indicated in Fig. 2, monoclonal antibody PH7 demonstrated specificity for the phosphorylated form (Fig. 2a, lanes 1 and 3), with no detectable cross-reactivity with the dephosphorylated enzyme (Fig. 2a, lanes 2 and 3). By contrast, monoclonal antibody PH9 demonstrated equivalent cross-reactivity with both forms of the enzyme (Fig. 2b). A similar result was obtained when monoclonal antibody PH7 was used to probe nitrocellulose filters containing reduced and carboxymethylated human liver phenylalanine hydroxylase (results not shown).

These results indicate that the sequence-specific rather than conformational nature of the antigenic determinant was recognized by monoclonal antibody PH7, since there was no modification in immunoreactivity on incubation with denaturing or chemical agents.

Characterization of PH7-immunoreactive peptide

In initial experiments designed to localize the monoclonal-antibody-PH7 antigenic site, carboxymethylated phosphorylated human liver phenylalanine hydroxylase was digested with trypsin at 37 °C and the mixture of tryptic fragments subjected to competitive blocking immunoassays. The results obtained indicated that antigenicity was retained after digestion with trypsin (results not shown).

Fig. 3. Immunoreactivity and ^{32}P radioactivity of peptides isolated by reversed-phase h.p.l.c.

Tryptic peptides of reduced, carboxymethylated and phosphorylated human liver phenylalanine hydroxylase (200 μg , 4.0 nmol) were separated on an RP-8 Lichrosorb column (0.3 cm $\times$ 20 cm) using a 40 min gradient from 8 to 60% (v/v) acetonitrile in 0.1% trifluoroacetic acid at room temperature. Fractions (1 ml each) were collected and 0.1 ml aliquots of each fraction were assayed for immunoreactive peaks in competitive-blocking assays and ^{32}P radioactivity as described in the Experimental procedures section. ---, ^{32}P radioactivity; —, A_{492} .

Fig. 4. H.p.l.c. purification of the PH7-cross-reactive peptide

(a) A tryptic digest of carboxymethylated, phosphorylated human phenylalanine hydroxylase (200 μg , 4 nmol) was separated on a 0.46 cm $\times$ 25 cm Aquapore RP300 (10 μ) column. The column was developed at room temperature with a gradient of from 8 to 60% acetonitrile in 20 mM- NH_4HCO_3 , pH 7.8, over 40 min. The gradient was started at the time of injection. The flow rate was 1 ml/min, and 1 ml fractions were collected. The black bars at 19 and 21 min define positions of fractions T1 and T2 respectively, as discussed in the text. (b) Fraction T1 ($t = 19$ min) from (a) was rechromatographed using the same conditions as in (a), except that 0.1% trifluoroacetic acid was used in place of NH_4HCO_3 in the mobile phase. The black bar at 27 min defines the position of fraction T1-7, the immunoreactive component.

Table 1. Amino acid composition of peptides of human liver phenylalanine hydroxylase (PH)

Amino acid	Composition (residues/molecule of protein)				
	Tryptic peptide T1-7	Expected residues PH 1-41	Peptide A (starting material)	Peptides generated from digestion of synthetic peptide A	
				Tryptin digest	Thermolysin digest
Asp	4.54 (5)	6	1	1.09	1.03
Thr	2.30 (2)	2			
Ser	4.08 (4)	5	1	0.79	0.51
Glu	5.86 (6)	5			0.08
Pro	2.78 (3)	1			
Gly	5.86 (6)	4	3	0.97	0.79
Ala	4.20 (4)	2			
Val	1.68 (2)	1			
Met	0.6 (0-1)				
Ile	1.88 (2)	3			
Leu	4.02 (4)	5	2	1.00	1.16
Tyr	1.34 (1)	2			
Phe	1.62 (2)	2	1	1.07	1.00
His	0.64 (1)				
Lys	2.12 (2)	2	1	1.00	
Arg	1.84 (2)	1	1		

^{32}P -labelled tryptic peptides were separated by reversed-phase h.p.l.c. on a Lichrosorb column (Fig. 3). The effluent fractions were monitored by competitive-blocking immunoassay and counting of radioactivity to detect ^{32}P -labelled tryptic peptides. A significant proportion of the applied PH7 immunoreactivity was recovered as a major peak eluted at 35 min and a smaller peak eluted at 32 min (Fig. 3). Coincident with the elution of the immunoreactive peaks was the emergence of two peaks of ^{32}P radioactivity (Fig. 3). There was also a large peak of radioactivity at the start of the h.p.l.c. run which coincides with unincorporated [^{32}P]ATP.

Purification of immunoreactive peptide

A tryptic digest of reduced, carboxymethylated phenylalanine hydroxylase was fractionated by reversed-phase h.p.l.c. on a 0.46 cm $\times$ 25 cm Aquapore RP 300 column using a linear gradient of acetonitrile at pH 8.0 (Fig. 4a). Two peaks of immunoreactivity (cf. Fig. 3) were detected, a major one at 19 min (T1) and a lesser one at 21 min (T2). The major peak of immunoreactivity was further fractionated on the same column with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (Fig. 4b). At least seven distinct components could be identified, one of which eluted at 27 min (T1-7) and cross-reacted strongly with PH7.

The amino acid composition of peptide T1-7 is given in Table 1. It corresponds closely to residues 1-41 of the reported amino acid sequence of phenylalanine hydroxylase (Kwok *et al.*, 1985). Amino-acid-sequence determination gave no detectable PTH-amino acid, indicating that the *N*-terminus of the peptide was blocked, which is consistent with the reported *N*-terminal residue acetylserine (Kwok *et al.*, 1985).

Attempts to obtain a small immunoreactive peptide with an unblocked *N*-terminus by subdigestion of T1-7 with various enzymes were unsuccessful.

Immunoreactive test of synthetic peptides

The peptide homologous with the phosphopeptide isolated from rat liver phenylalanine hydroxylase (Wretborn *et al.*, 1980) was synthesized. Monoclonal antibody PH7 has been previously shown to cross-react with rat liver phenylalanine hydroxylase (Jennings *et al.*,

Fig. 5. Determination of ability of monoclonal antibody PH7 to bind to the synthetic peptide

Titration of phosphorylated (closed symbols) and unphosphorylated (open symbols) synthetic peptide with monoclonal antibody PH7 (●, ○) and two control monoclonal antibodies PH8 (■, □) and PH9 (▲, △).

Table 2. Amounts of peptide/protein and P_i required to give inhibition of competitive e.l.i.s.a.

Peptide/protein	Amount	P_i	Inhibition (%)
Phosphopeptide A [Gly-Leu-Gly-Arg-Lys-Leu-Ser(P)-Asp-Phe-Gly]	75 pmol	75 pmol	50
Phosphopeptide B [Leu-Arg-Arg-Ala-Ser(P)-Leu-Gly]	0.77 nmol	0.77 nmol	50
Phosvitin	5.7 nmol	96 nmol	50
Casein	6.5 nmol	33 nmol	33
Affinity-purified phenylalanine hydroxylase (2 phosphate groups/tetramer)	0.02 pmol	0.04 pmol	50

1986). This peptide sequence corresponds to residues 10–19 of the reported human phenylalanine hydroxylase sequence (Kwok *et al.*, 1985). The decapeptide synthesized was a substrate for the kinase, as phosphoserine was isolated by cleavage (Smith *et al.*, 1984) and behaved similarly to the phenylalanine hydroxylase in that the antibody PH7 recognized this peptide only in the phosphorylated state (Fig. 5). Two control monoclonal antibodies PH8 and PH9 were used to test the specificity of PH7 binding to the phosphopeptide. PH8 and PH9 did not react with either the synthetic peptide or the phosphopeptide.

Phosphoserine itself did not compete with the phosphorylated synthetic peptide for binding to the PH7 monoclonal antibody (results not shown).

Proteolytic cleavage of synthetic peptide

After digestion of the phosphorylated synthetic peptide A with trypsin and thermolysin, two new peptides were eluted from the h.p.l.c. column. Both the tryptic peptide and that from the thermolysin digest

retained immunoreactivity to the monoclonal antibody. Amino acid analysis was performed on the new immunoreactive peptides (for results, see Table 1). This shows that the antibody still recognizes the peptides Lys-Leu-Ser(P)-Asp-Phe-Gly and Leu-Ser(P)-Asp-Phe-Gly.

Cross-reactivity of monoclonal antibody PH7 with other phosphoproteins and phosphopeptides

Several known phosphoproteins were tested to demonstrate the specificity of monoclonal antibody PH7. In the competitive e.l.i.s.a., 75 pmol of phosphorylated synthetic peptide A gave 50% competition of monoclonal antibody PH7 (dilution 1:100000) binding to phenylalanine hydroxylase (Fig. 5 and Table 2). By contrast, casein and phosvitin, both containing multiple bound phosphate, required 33 nmol and 96 nmol of P_i respectively for 50% competition (Table 2).

The phosphorylated synthetic peptide B, Lys-Arg-Arg-Ala-Ser(P)-Leu-Gly, corresponding to the local phosphorylation-site sequence in liver pyruvate kinase was synthesized and tested for competitive inhibition of PH7 binding (Table 2). The pyruvate kinase phosphopeptide, a substrate for cyclic AMP-dependent protein kinase, did not give significant competition with monoclonal antibody PH7. At 50% inhibition the phosphopeptide concentration was 10 times greater than that for the phosphopeptide corresponding to the phenylalanine hydroxylase phosphopeptide.

When human liver extract (500 μ g) was separated by SDS/polyacrylamide-gel electrophoresis and tested by immunoblotting with PH7, only the band corresponding to the phosphorylated human liver phenylalanine hydroxylase was detected (Fig. 6, lane 1). Monoclonal antibody PH9 demonstrated equivalent cross-reactivity with both forms of phenylalanine hydroxylase (Fig. 6, lane 2). Moreover, the same result was obtained when the human liver extract was acid-precipitated immediately after homogenization (results not shown).

DISCUSSION

The epitope of antibody PH7 has been localized to the phosphodecapeptide Gly-Leu-Gly-Arg-Lys-Leu-Ser(P)-Asp-Phe-Gly and some binding has been observed with the phosphopentapeptide Leu-Ser(P)-Asp-Phe-Gly. That the phosphate is essential for binding the peptide and human liver phenylalanine hydroxylase is indicated by the fact that neither the dephosphodecapeptide nor the dephosphoenzyme binds to PH7. The antibody PH7

Fig. 6. Immunoreactivity of monoclonal antibody PH7 with liver proteins

Human liver extracts before and after precipitation with trichloroacetic acid were electrophoresed on SDS/polyacrylamide gel (9%) under reducing conditions before immunoblotting on to nitrocellulose. Monoclonal antibodies PH7 (lane 1) and PH9 (lane 2) were used for immunodetection of bound antigen.

binds to the phosphodecapeptide at concentrations 10^3 -fold higher than does the parent protein. The difference in binding may suggest other structural features are important. Phosphoserine is not able to displace the phosphorylated enzyme from PH7. This is supported by the observation that the antibody reacts only weakly with the phosphoproteins casein and phosvitin. The phosphodecapeptide Gly-Leu-Gly-Arg-Lys-Leu-Ser(P)-Asp-Phe-Gly and the phosphopeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly, corresponding to the local phosphorylation site of liver pyruvate kinase, are both phosphorylated by cyclic AMP-dependent protein kinase. However, the pyruvate kinase phosphopeptide reacts weakly with PH7, suggesting a specific PH7-binding epitope on human liver phenylalanine hydroxylase. When fresh and trichloroacetic acid-precipitated human liver extracts were probed with PH7, the monoclonal antibody did not react with human liver extract phosphoproteins other than phosphorylated phenylalanine hydroxylase, even after trichloroacetic acid precipitation of the liver extract to prevent phosphatase action (Fig. 6).

The amino acid composition of the 41-residue peptide (Table 1) appears to differ slightly from that indicated by the sequence data (Kwok *et al.*, 1985). The reason for the inconsistent amino acid data is not clear. One possible explanation is that there may be some error in the nucleotide sequence data. However, the fact that this peptide is blocked at the *N*-terminus is consistent with it being the *N*-terminal peptide.

Previous workers have made polyclonal antisera which distinguish phosphorylated and dephosphorylated forms of proteins (Nairn *et al.*, 1982). A monoclonal antibody reactive with acetylcholine receptor is thought to include phosphate in its epitope because of its reactivity with the phosphoprotein phosvitin (Pizzighella *et al.*, 1983). A similar requirement for a phosphorylated serine in the epitope appears to exist with monoclonal antibody PH7. A monoclonal antibody to phosphotyrosine has been used to study proteins phosphorylated on the tyrosine residue (Frackelton *et al.*, 1984).

The present results suggest that it may be possible to construct monoclonal antibodies specific for hypothetical phosphoproteins not purified but for which there is only DNA-sequence-derived information.

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