Experimental determination of the phosphorylation state of phenylalanine hydroxylase

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A monoclonal antibody (PH 7), which recognizes the phosphorylated form of phenylalanine hydroxylase from human liver, has been used for the analysis of the enzyme in crude cell extracts from rat. In immunoblot analyses of rat liver cell extracts, the extent of binding of PH 7 closely correlates with the phosphorylation state of phenylalanine hydroxylase, as judged by [32 P] P_i incorporation. These observations have made possible the rapid non-radioactive quantification of hormonal effects on phenylalanine hydroxylase phosphorylation state. In particular, the glucagon-dependent phosphorylation of phenylalanine hydroxylase in liver cells was investigated. Epidermal growth factor was shown to modulate this process. In addition, this technique was used to demonstrate, for the first time, that dibutyryl cyclic AMP, unlike the Ca^{2+} ionophore A23187, stimulates the phosphorylation of phenylalanine hydroxylase in isolated kidney tubules from rat.

INTRODUCTION

Phenylalanine hydroxylase [L-phenylalanine,tetrahydropteridine:oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1] catalyses the initial step of phenylalanine degradation in man and other mammals:

Enzymic activity is controlled in the short term by reversible phosphorylation (Donlon & Kaufman, 1980; Fisher & Pogson, 1984). Glucagon and α -adrenergic agents stimulate phenylalanine hydroxylation in liver cells, through the actions of cyclic AMP-dependent and Ca^{2+} /calmodulin-dependent protein kinases respectively (Doskeland *et al.*, 1984; Fisher *et al.*, 1984).

The experimental analysis of the hormonal control of the phosphorylation of phenylalanine hydroxylase typically involves the injection of [32 P] P_i into animals, or, alternatively, incubation of isolated cells in the presence of [32 P] P_i . This is followed by the specific immunoprecipitation of 32 P-labelled enzyme (Donlon & Kaufman, 1980; Fisher & Pogson, 1984). An alternative approach has been made possible since the production of a library of monoclonal antibodies generated against phenylalanine hydroxylase (Choo *et al.*, 1979; Jennings *et al.*, 1986). One of these monoclonal antibodies (PH 7) has been shown to bind specifically to the phosphorylated form of human liver phenylalanine hydroxylase (Smith *et al.*, 1987). This antibody appears to bind to a linear sequence of amino acids surrounding the single phosphoserine residue formed by cyclic AMP-dependent protein kinase action.

The present paper describes the use of antibody PH 7 for the rapid, non-radioactive, analysis of alterations in the phosphorylation state of phenylalanine hydroxylase from isolated rat liver cells and kidney tubules.

MATERIALS AND METHODS

Chemicals

Phenylmethanesulphonyl fluoride was from Sigma. Peroxidase-labelled rabbit anti-sheep IgG and peroxidase-labelled goat anti-mouse IgG were from Nordic Immunological Laboratories, Maidenhead, Berks., U.K. Chymostatin, leupeptin and pepstatin were from the Peptide Institute, Osaka, Japan. Tetraaminobiphenyl hydrochloride was from BDH. [32 P] P_i (carrier-free), [γ - 32 P]ATP (sp. radioactivity 3000 Ci/mmol), PCS scintillator cocktail and NCS solubilizer were from Amersham International, Amersham, Bucks., U.K.

All other chemicals were of the purest grade available from standard suppliers.

Animals

Male Wistar rats weighing 180–220 g were used throughout. Animals were fed *ad libitum* [Labsure Animal Diet (CRM); C. Hill Group, Poole, Dorset, U.K.].

Preparation and incubation of liver cells and kidney tubules

Liver cells were prepared as described previously (Elliott *et al.*, 1976). Cells were incubated in Krebs–Henseleit (1932) medium: experiments involving the use of [32 P] P_i were carried out in low-phosphate (0.4 mM) Krebs–Henseleit medium. In all cases, the incubation medium was supplemented with 2.5 mM- $CaCl_2$, 2% (w/v) bovine serum albumin and lactate/pyruvate (9:1; final concn. 10 mM). Incubation volumes were 2 ml throughout.

Kidney tubules were prepared essentially as described by Guder & Wieland (1971). Tubules were incubated as

Abbreviation used: EGF, epidermal growth factor.

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described above, except that incubation volumes were 0.4 ml throughout.

In all experiments, metabolic integrity was assessed by measurement of cellular ATP content (Dickson & Pogson, 1977).

Cell suspensions were incubated with [32 P]P_i at a final radiochemical concentration of approx. 100 μ Ci/ml. Hormones and other effectors were added after 60–90 min, by which time both the [γ - 32 P]ATP specific radioactivity and the [32 P]phosphate content of phenylalanine hydroxylase had attained a steady state (Fisher & Pogson, 1984). Incubations were terminated by dilution with a 4-fold excess of ice-cold incubation medium without P_i and albumin. Cells were separated from medium by centrifugation at 500 *g* for 30 s at room temperature and then resuspended in a buffer containing 250 mM-sucrose, 20 mM-Tris/HCl, 2 mM-sodium phosphate, 5 mM-EDTA, 100 mM-NaF, 0.5 mM-phenylmethanesulphonyl fluoride, leupeptin (20 μ g/ml), pepstatin (2 μ g/ml) and chymostatin (2 μ g/ml), pH 7.4. Cells were broken by three cycles of freezing in liquid N₂ and thawing in water at 37 °C. The final extracts were centrifuged at 12000 *g* for 2 min at room temperature before immunoprecipitation of 32 P-labelled phenylalanine hydroxylase.

Quantification of the phosphate content of phenylalanine hydroxylase by analysis of 32 P labelling

32 P-labelled phenylalanine hydroxylase was immunoprecipitated from cell extracts as described previously (Fisher & Pogson, 1984). Immunoprecipitates were resuspended in 0.05 ml of a buffer containing 12 mM-sodium phosphate, 1% (w/v) sodium deoxycholate, 1% (w/v) SDS and 0.1% (v/v) Triton X-100, pH 7.4. After centrifugation at 12000 *g* for 5 min through a two-step sucrose gradient (0.15 ml of buffer containing 1.0 M-sucrose under 0.2 ml of buffer containing 0.5 M-sucrose), precipitates were resuspended in 0.05 ml of buffer and re-centrifuged (12000 *g* for 2 min at room temperature). This washing procedure was repeated once, then immunoprecipitates were solubilized in NCS before counting for radioactivity in 2 ml of scintillation cocktail. Alternatively, after washing as described above, the immunoprecipitates were subjected to SDS/polyacrylamide-gel electrophoresis essentially as described by Laemmli (1970). The distribution of radioactivity within gels was assessed by autoradiography using Fuji (RX) X-ray film and intensifying screens.

The stoichiometry of phosphate incorporation was obtained from the 32 P content of immunoprecipitates and the specific radioactivity of cellular [γ - 32 P]ATP assessed as described by Hawkins *et al.* (1983).

Quantification of the phosphate content of phenylalanine hydroxylase by immunoblot analysis

Cell extracts prepared as described above, in the absence of [32 P]P_i, were treated with trichloroacetic acid (final concn. 10% w/v). Insoluble material was isolated by centrifugation at 12000 *g* for 2 min and then resuspended in 3:1 (v/v) mixture of 10 mM-sodium phosphate buffer, pH 7.2, and saturated Tris solution. These samples were subjected to SDS/polyacrylamide-gel electrophoresis and then transferred to nitrocellulose filters (Towbin *et al.*, 1979). Immunoblotting was performed with a sheep antiserum to rat liver phenylalanine hydroxylase (Fisher & Pogson, 1984) or

PH 7 monoclonal antibody (Smith *et al.*, 1987). Antibody binding was revealed by incubation with 20 μ l of either peroxidase-labelled rabbit anti-sheep IgG or peroxidase-labelled goat anti-mouse IgG, with subsequent addition of 25 mg of tetra-aminobiphenyl hydrochloride and 0.05 ml of 30% (w/v) H₂O₂ in a final volume of 50 ml.

Samples of each cell extract were probed with both PH 7 antibody and the polyclonal antibody so that the ratio of phosphorylated enzyme to total enzyme could be established. Hydroxylase protein was quantified by densitometric scanning of immunoblots at 500 nm with a Zeiss chromatographic scanner. A linear relationship between protein load and absorption was verified for each blot.

Other methods

Phenylalanine hydroxylase was purified from rat liver essentially as described by Shiman *et al.* (1979). Purified enzyme (approx. 2.0 μ g) was phosphorylated in a cyclic AMP-dependent phosphorylation system containing 5 mM-magnesium acetate, 10 mM-dithiothreitol, 10 mM-NaF and 12 mM-potassium phosphate, pH 6.5, with 70 μ M-ATP and catalytic subunit of cyclic AMP-dependent protein kinase [6 units (pmol/min)] for 1 h at 30 °C.

RESULTS AND DISCUSSION

Specificity of PH 7 for phosphorylated phenylalanine hydroxylase from rat liver

Previous work has indicated that the PH 7 monoclonal antibody is specifically immunoreactive with the phosphorylated form of phenylalanine hydroxylase from human liver (Smith *et al.*, 1987). Fig. 1 shows that enzyme purified from rat liver, by a method which yields

Fig. 1. Immunoblot analysis of phenylalanine hydroxylase purified from rat liver

Phenylalanine hydroxylase was purified from rat liver essentially as described by Shiman *et al.* (1979). Samples of enzyme (approx. 2.0 μ g) were subjected to SDS/polyacrylamide-gel electrophoresis and then transferred to nitrocellulose and probed with PH 7 (lanes 1 and 2) or polyclonal antibody (lanes 3 and 4) as described in the Materials and methods section. Lanes 1 and 3, no preincubation of enzyme. Lanes 2 and 4, enzyme preincubated in a cyclic AMP-dependent phosphorylation system.

Fig. 2. Correlation between $[^{32}\text{P}]$ phosphate incorporation into phenylalanine hydroxylase and relative intensity of staining of immunoblots of liver cell extracts probed with PH 7 antibody

Liver cells were incubated in the presence of various effectors of cyclic AMP metabolism and Ca^{2+} mobilization as described in the Materials and methods section. The phosphorylation state of phenylalanine hydroxylase was then determined by analysis of $[^{32}\text{P}]$ phosphate incorporation or, alternatively, by PH 7 immunoblot analysis as described in the Materials and methods section. Results are the means $\pm$ S.E.M. for at least three different liver cell preparations.

dephosphorylated enzyme (Parniak *et al.*, 1981), does not show significant immunoreactivity with PH 7 antibody. It was only after the enzyme was phosphorylated by incubation in the presence of a cyclic AMP-dependent protein kinase phosphorylation system that PH 7 specific immunoreactivity was observed. As controls, the monoclonal antibody PH 8 (Jennings *et al.*, 1986) (results not shown) and a polyclonal antibody (Fisher & Pogson, 1984) (see Fig. 1) demonstrated equivalent cross-reactivity, in immunoblot analyses, with both phosphorylated and unphosphorylated forms of the rat liver enzyme. Fig. 1 shows that there was little discernible difference between the extent of migration of the phosphorylated and the dephosphorylated forms of the rat liver enzyme subunit on SDS/polyacrylamide-gel electrophoresis. This confirms previous observations and contrasts with the behaviour of the phosphorylated and dephosphorylated forms of the enzyme from human liver (Smith *et al.*, 1987).

In addition to recognition of the purified enzyme, subjected to phosphorylation *in vitro*, PH 7 antibody also recognized the phosphorylated enzyme in crude liver cell extracts (see Fig. 3a).

Other workers have indicated that immunoblot analyses of rat phenylalanine hydroxylase sometimes reveal the presence of forms of the hydroxylase subunit

with different M_r (Mercer *et al.*, 1986). These different subunits have been termed W and L forms, which appear to be allelic in nature and are unrelated to the phosphorylation status of the hydroxylase polypeptide. In the Wistar strain of rat only the W form of the enzyme has been reported to be expressed (Mercer *et al.*, 1984). In the present work, however, we did identify cell extracts from certain Wistar rats which appeared to express hydroxylase subunits analogous to both the W and L subunits (A. K. Green & M. J. Fisher, unpublished work). Quantitative assessments of phosphorylation status, described in the present paper, were based on immunoblots which showed only a single hydroxylase subunit (analogous to the W type).

Use of PH 7 in the quantitative analysis of the phosphorylation status of phenylalanine hydroxylase in isolated liver cells

Considerable data exist concerning the impact of hormones and other effectors on the control of the phosphorylation state of phenylalanine hydroxylase in isolated liver cells (Pogson *et al.*, 1986; Kaufman, 1986). In such studies the phosphorylation state of phenylalanine hydroxylase was determined from the extent of incorporation of $[^{32}\text{P}]\text{P}_i$ into the enzyme. It was therefore of interest to compare estimates of phosphorylation status obtained by this radioactive method and by an immunoblotting technique based on the use of the PH 7 antibody.

Fig. 2 shows the correlation between $[^{32}\text{P}]\text{P}_i$ incorporation into phenylalanine hydroxylase and the intensity of staining of immunoblots of liver cell extracts probed with PH 7 antibody. Alterations in the phosphorylation state of the hydroxylase were brought about by incubation of liver cells with a range of effectors which alter cyclic AMP concentration or mobilize intracellular Ca^{2+} . Fig. 2 shows that, over the range of phosphorylation states investigated (approx. 0.20–0.60 mol of phosphate/mol of enzyme subunit), there was a good correlation ($r = 0.91$) between PH 7 antibody binding and phosphorylation state as determined by $[^{32}\text{P}]\text{P}_i$ incorporation. It should be pointed out that even under basal, unstimulated, incubation conditions liver cell phenylalanine hydroxylase contains approx. 0.20 mol of phosphate/mol of enzyme subunit. Unphosphorylated enzyme appears to represent only a very minor component of total enzyme in the intact liver cell under physiological conditions (Kaufman, 1986). Figs. 3(a) and 3(b) show the results of PH 7 immunoblot analyses of the effect of a range of glucagon concentrations on liver cell phenylalanine hydroxylase phosphorylation state. From Fig. 3(b), a half-maximal stimulation of phosphorylation was obtained at a glucagon concentration of approx. 0.2 nM, which is similar to previous estimates obtained from ^{32}P -incorporation experiments and phenylalanine-hydroxylation flux assays (Fisher & Pogson, 1984).

The usefulness of PH 7-based immunoblot analyses for the estimation of hormonally stimulated alterations in liver cell phenylalanine hydroxylase phosphorylation state is illustrated in Table 1. Previous work has indicated that EGF (epidermal growth factor) can, like insulin, counteract the effect of glucagon on the activity of liver cell phenylalanine hydroxylase (Fisher & Dickson, 1988). Insulin has been shown to exert this effect through a diminution in the extent of phosphorylation of the enzyme (Fisher *et al.*, 1987). It may therefore be expected

Fig. 3. Relationship between the relative intensity of staining of immunoblots of liver cell extracts probed with PH 7 antibody and the concentration of glucagon in liver cell incubations

Liver cells were incubated in the presence of the indicated concentration of glucagon (lane 0: no hormone) and then the phosphorylation state of phenylalanine hydroxylase was determined by PH 7 immunoblot analysis as described in the Materials and methods section. The results are presented for a typical experiment (a) and as means $\pm$ S.E.M. for four different liver cell preparations (b).

Table 1. Impact of EGF on glucagon-stimulated phosphorylation of phenylalanine hydroxylase in isolated liver cells

Liver cells were incubated in the presence of the indicated effector(s), and the phosphorylation state of phenylalanine hydroxylase was determined by PH 7 immunoblot analysis as described in the Materials and methods section. In addition, phenylalanine hydroxylation flux was determined essentially as described in Fisher & Pogson (1984). Results are the means $\pm$ S.E.M. for at least three different liver cell preparations. The significance of differences between means was assessed by Student's *t* test: * *P* (glucagon only versus glucagon plus EGF) < 0.05 .

Condition	Phosphorylation state (% of control)	Hydroxylation flux (% of control)
No additions	100	100
0.1 μ M-glucagon	195 $\pm$ 8	180 $\pm$ 7
10 nM-EGF	106 $\pm$ 12	102 $\pm$ 2
0.1 μ M-glucagon + 10 nM-EGF	150 $\pm$ 10*	147 $\pm$ 4*

that EGF action is also associated with a decrease in the phosphorylation state of phenylalanine hydroxylase. This was confirmed by PH 7 immunoblot analyses, which revealed that EGF caused a significant decrease in the glucagon-stimulated phosphorylation of phenylalanine hydroxylase (see Table 1). This decrease in phosphorylation correlated well with the effect of EGF on phenylalanine hydroxylation flux in isolated liver cells, as shown in Table 1. The mechanism underlying the effect of EGF, and indeed that of insulin, on the phosphorylation state of phenylalanine hydroxylase remains to be established. However, the ability of both these effectors to diminish glucagon-stimulated increases in liver cell cyclic AMP concentrations may be a significant factor (Bosch *et al.*, 1986).

Analysis of the phosphorylation state of phenylalanine hydroxylase in isolated kidney tubules

Although the major site of phenylalanine hydroxylase activity is the liver, there is evidence for an appreciable enzyme activity in the kidney cortex (McGee *et al.*, 1972). There appears to be a basic similarity between the enzyme from these sources; however, there are indications that the regulation of the enzyme may show some tissue specificity (Rao & Kaufman, 1986). Although the role of reversible phosphorylation in the control of the hepatic enzyme is now well established (see the Introduction), little is known about the contribution, if any, of this process to the regulation of the renal enzyme. Possible alterations in the phosphorylation state of the enzyme from isolated kidney tubules were investigated by PH 7 immunoblotting techniques. Fig. 4(a) shows the effect of incubation of isolated kidney tubules with either dibutyryl cyclic AMP (0.1 mM) or A23187 (1 μ M) on the subsequent probing of tubule-cell extracts with PH 7 antibody. It is clear, from this and other analyses, that dibutyryl cyclic AMP caused a significant stimulation of hydroxylase phosphorylation [0.1 mM-dibutyryl cyclic AMP, 298 $\pm$ 40% ($P < 0.05$); 1 μ M-A23187, 117 $\pm$ 27% of control: means $\pm$ S.E.M. for four independent tubule

Fig. 4. Phosphorylation of phenylalanine hydroxylase in isolated kidney tubule extracts

Kidney tubules were incubated in the presence of the indicated effector and [32 P] P_i , where appropriate, as described in the Materials and methods section. Extracts were then subjected to PH 7 immunoblot analysis (a) or autoradiographic analysis (b). Results are from a typical experiment. Lanes: L, liver cell extract; 1, 100 μ M-dibutyl cAMP; 2, 1 μ M- Ca^{2+} ionophore A23187; 3, no additions.

preparations). These alterations in phosphorylation state were confirmed by autoradiographic analysis of [32 P]phosphate incorporation (Fig. 4b). To the best of our knowledge this is the first report of cyclic AMP-stimulated phosphorylation of kidney phenylalanine hydroxylase. Other workers have been unable to demonstrate cyclic AMP-stimulated phosphorylation of enzyme purified from kidney (Rao & Kaufman, 1986).

On the basis of the present data, it appears that the Ca^{2+} ionophore A23187 does not cause a significant stimulation of phenylalanine hydroxylase phosphorylation in isolated tubules (A23187 did, however, stimulate gluconeogenesis from lactate in isolated kidney tubule preparations; results not shown). This contrasts with the observation that A23187 and α -adrenergic agents caused a significant enhancement of phenylalanine hydroxylase phosphorylation in isolated liver cells (Fisher *et al.*, 1984). The reason for this difference in the behaviour of the renal and hepatic enzyme remains to be elucidated. The possible correlation between changes in phosphorylation state and renal enzyme activity also remains to be established. There are some indications that the relationship between the state of activation of the hydroxylase and its phosphate content may not be entirely straightforward (Rao & Kaufman, 1986). Our

own work (Askin *et al.*, 1989) has also indicated that the relationship between cyclic AMP-induced alterations in phosphorylation state and phenylalanine hydroxylation in isolated kidney tubules is less marked than in the hepatic system.

General conclusions

The present paper describes the use of the monoclonal antibody PH 7 for the rapid non-radioactive analysis of alterations in the phosphorylation state of phenylalanine hydroxylase in crude cell extracts from a variety of tissues. The estimates of enzyme phosphorylation obtained from this approach are comparable with those obtained from traditional approaches involving analysis of [32 P] P_i incorporation into the enzyme. The present approach makes possible the rapid quantification of hormonal effects on phenylalanine hydroxylase phosphorylation state. This method has been used to confirm the cyclic AMP-dependent phosphorylation of the liver cell enzyme and also the modulation of this process by EGF. In addition, it has revealed, for the first time, the cyclic AMP-dependent phosphorylation of the enzyme from kidney tubules. However, the possible hormonal control of this phenomenon, and its physiological significance, remain to be established.

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