Phosphopeptide analysis of phenylalanine hydroxylase isolated from liver cells exposed to hormonal stimuli

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Hormonal control of the phosphorylation of phenylalanine hydroxylase was studied by using rat liver cells incubated with [32P]P. After immunoprecipitation from cell extracts, the hydroxylase was subjected to proteinase digestion and subsequent sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. V8-proteinase digestion yielded one major 32P-labelled fragment, of approx. 9 kDa. Chymotrypsin digestion gave five 32P-labelled fragments ranging from approx. 39 kDa to approx. 10 kDa. Noradrenaline (10 μM) and glucagon (0.1 μM) enhanced the 32P content of all peptide fragments uniformly. Phorbol ester, in contrast with ionophore A23187, did not stimulate enzyme phosphorylation or enhance phenylalanine metabolism in liver cells. These results are discussed in relation to the nature of the protein kinase(s) that mediate phosphorylation of phenylalanine hydroxylase in liver cells.

INTRODUCTION

Phenylalanine hydroxylase catalyses the initial step of phenylalanine degradation in the liver (Kaufman, 1971). Hormonal regulation of enzyme activity is mediated by a phosphorylation/dephosphorylation cycle. Phosphorylation by the cyclic AMP-dependent protein kinase is well characterized for the purified enzyme (Abita et al., 1976); this is the basis of glucagon stimulation of enzyme activity in vivo (Donlon & Kaufman, 1978) and in isolated liver cells (Fisher & Pogson, 1984a). Ca2+-mobilizing agents also stimulate enzyme phosphorylation in rat liver cells (Garrison, 1978; Garrison & Wagner, 1982); this can be correlated with enzyme activity changes (Fisher et al., 1984). It has been suggested that a Ca2+-calmodulin-dependent protein kinase may mediate these effects (Doskeland et al., 1984); the purified hydroxylase is a substrate for this protein kinase (Schwer & Soderling, 1983). The work of Garrison et al. (1984) suggests that the phospholipid-dependent protein kinase (protein kinase C) does not stimulate significant phosphorylation of the hydroxylase in liver cells.

The studies described in the present paper were undertaken in order to clarify the nature of the protein kinases involved in the hormonally controlled phosphorylation of phenylalanine hydroxylase.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (University of Manchester breeding colony), body wt. 180–220 g, were used throughout. Animals were fed ad libitum [Labsure Animal Diet (CRM); C. Hill Group, Poole, Dorset, U.K.].

Reagents

Reagents and radiochemicals were obtained from the sources given previously (Fisher & Pogson, 1984a).

Noradrenaline (arterenol), ionophore A23187 and PMA were from Sigma. Bovine pancreatic chymotrypsin A₄ and Staphylococcus aureus proteinase V8 were from the Boehringer Corp. (London). Monocomponent pig glucagon was a gift from Dr. W. Bromer (Eli Lilly, Indianapolis, IN. U.S.A.). All other chemicals were of the purest grade available from standard suppliers.

Preparation and incubation of liver cells

Cells were prepared as described previously (Elliott et al., 1976), except that glucose (20 mM) was added to the perfusion medium (to maintain glycerol contents). For metabolic-flux experiments, cells were incubated in Krebs–Henseleit medium (Krebs & Henseleit, 1932). For phosphorylation experiments, low-phosphate (0.4 mM-Pᵢ) Krebs–Henseleit incubation medium was used. In all cases the medium was supplemented with 2.5 mM-CaCl₂, 2% (w/v) bovine serum albumin, lactate/pyruvate (9:1; final concn. 10 mM) and glucose (10 mM). [32P]P, was added, where appropriate, to a final concentration of 100 μCi/ml. Hormones and agonists were added after 60–75 min incubation with [32P]P, at which time the 32P labelling of the hydroxylase was at a steady state (Fisher & Pogson, 1984a). Incubations (volume 2 ml) were terminated and 32P-labelled cell extracts were prepared as described previously (Fisher & Pogson, 1984a).

Immunoprecipitation of phenylalanine hydroxylase

32P-labelled hydroxylase was immunoprecipitated from cell extracts as described previously (Fisher & Pogson, 1984a). Immunoprecipitates were purified by the two-step sucrose-gradient procedure of Wicks & Su (1978) and subsequently washed twice in 0.1 M-sodium phosphate, pH 6.9.

Phosphopeptide analysis

The procedure of Cleveland et al. (1977) was used. Immunoprecipitates containing 10–20 μg of hydroxylase

Abbreviation used: PMA, 4β-phorbol 12-myristate 13-acetate.
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Table 1. Hormonal influences on phenylalanine hydroxylase phosphorylation state and phenylalanine hydroxylation flux in isolated liver cells

Total phosphate content was determined by immunoprecipitation of phenylalanine hydroxylase from ³²P-labelled liver cells, and phenylalanine hydroxylation was determined by incubation of liver cells with 0.05 mm-[⁴⁻H]phenylalanine as described in the Materials and methods section. Results are expressed as means ± s.e.m. for the numbers of different cell preparations shown in parentheses. Differences between means were assessed by Student’s t test: *P < 0.05 and **P < 0.01 (versus control).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phosphate content (mol of P/mol of subunit)</th>
<th>Hydroxylation flux (% of basal)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.21 ± 0.03 (4)</td>
<td>100 ± 4 (6)</td>
</tr>
<tr>
<td>0.1 µM-Glucagon</td>
<td>0.55 ± 0.08 (5)**</td>
<td>185 ± 7 (6)**</td>
</tr>
<tr>
<td>10 µM-Noradrenaline</td>
<td>0.32 ± 0.03 (5)*</td>
<td>139 ± 3 (6)**</td>
</tr>
<tr>
<td>0.1 µM-Glucagon + 10 µM-noradrenaline</td>
<td>0.58 ± 0.05 (4)**</td>
<td>175 ± 7 (6)**</td>
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</tbody>
</table>

protein were suspended in 0.025 ml of a buffer containing: 0.125 M-Tris/HCl, 4% (w/v) SDS, 20% (v/v) glycerol and 2.5 mM-EDTA, pH 6.8. After samples had been heated for 10 min at 100 °C, proteinase digestion was initiated by addition of 1.5 µg of S. aureus V8 proteinase or 1.5 µg of chymotrypsin. Proteolysis was allowed to proceed at room temperature for 2 h and was then terminated by addition of 0.025 ml of buffer (see above) containing 1.8% (v/v) mercaptoethanol and 0.01% (w/v) Bromophenol Blue, followed by heating to 100 °C for 10 min. Samples (0.04 ml) were then subjected to SDS/15% (w/v)-polyacrylamide-gel electrophoresis essentially as described by Laemmli (1970). Alternatively, samples were subjected to the urea/SDS/15%-(w/v) polyacrylamide-gel-electrophoresis method of Swank & Munkres (1971).

The distribution of radioactivity within gels was assessed by solubilization of 1 mm gel slices in 0.20 ml of 1.8 M-H₂O₂ at 70 °C for 5 h. Resulting solutions were counted for radioactivity with 2.0 ml of PCS scintillator cocktail. Determination of radioactivity recovered from gels indicated a yield of > 90%.

Miscellaneous methods

Metabolic flux through phenylalanine hydroxylase, in liver cells incubated with 0.05 mm-phenylalanine, was measured as described by Fisher & Pogson (1984b). In all experiments metabolic integrity was assessed by measurement of ATP content (Dickson & Pogson, 1977). The stoichiometry of ³²P incorporation into the hydroxylase was calculated as previously reported (Fisher & Pogson, 1984a). The relative molecular masses of [³²P]phosphopeptides were determined by comparison with the relative mobilities of molecular-mass standards on similar gels [standards used: catalase (60 kDa), creatine kinase (40 kDa), myoglobin (17.2 kDa), cytochrome c (11.7 kDa) and glucagon (3.5 kDa)].

RESULTS

Table 1 shows the effect of exposure to hormones on the phosphorylation state of phenylalanine hydroxylase and phenylalanine hydroxylation flux in isolated liver cells. The increases observed in phosphate content and metabolic flux in response to glucagon or noradrenaline are similar to those previously reported (Fisher et al., 1984). The combination of maximally effective concentrations of glucagon plus noradrenaline produces non-additive phosphorylation and flux changes; non-additivity of flux changes has been previously reported (Santana et al., 1985).
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Fig. 2. SDS/polyacrylamide-gel electrophoresis of chymotrypsin-treated immunoprecipitates

Immunoprecipitates, containing $^{32}$P-labelled phenylalanine hydroxylase from liver cells exposed to various hormonal stimuli (a, control; b, 10 $\mu$M-noradrenaline; c, 0.1 $\mu$M-glucagon), were treated with chymotrypsin as described in the Materials and methods section. Zero radioactivity is indicated by the position of scale marks on the ordinates. The positions of the dye front and the undegraded phenylalanine hydroxylase subunit are indicated by arrows. These gels are representative of those obtained from three different cell preparations.

Figs. 1 and 2 respectively show the distributions of $^{32}$P in V8-proteinase- and chymotrypsin-digested immunoprecipitates subjected to SDS/polyacrylamide-gel electrophoresis. V8-proteinase digestion produced a single major $^{32}$P phosphopeptide of approx. 9 kDa after 2 h digestion. Chymotrypsin digestion produced five $^{32}$P phosphopeptides of approx. 39, 30, 22, 14 and 10 kDa. In each case there remained a variable amount of undegraded hydroxylase protein, which served as an internal mass marker. Overnight digestion with either proteinase did not result in the appearance of any new low-mass $^{32}$P phosphopeptides. A peak of radioactivity was consistently observed at the position of the dye front; this varied between cell preparations, but was unaffected by hormonal treatment of cells or proteinase digestion of immunoprecipitates. This radioactivity co-migrated with $^{32}$Pi, and its appearance was substantially diminished by performing electrophoresis in phosphate-based media (as used in urea/SDS/polyacrylamide-gel-electrophoresis experiments; see Fig. 3). These observations suggest that the dye-front-associated radioactivity was $^{32}$Pi, and therefore did not interfere with interpretation of proteinase-digestion data.

Fig. 3. Urea/SDS/polyacrylamide-gel electrophoresis of V8-proteinase-treated immunoprecipitates

Immunoprecipitates, containing $^{32}$P-labelled phenylalanine hydroxylase from liver cells exposed to various hormonal stimuli (a, control; b, 10 $\mu$M-noradrenaline; c, 0.1 $\mu$M-glucagon), were treated with V8-proteinase as described in the Materials and methods section. Zero radioactivity is indicated by the position of scale marks on the ordinates. The positions of the dye front and the undegraded phenylalanine hydroxylase subunit are indicated by arrows. These gels are representative of those obtained from a single cell preparation.
during SDS/polyacrylamide-gel electrophoresis if the amount of [32P] in cell incubations was substantially increased; in this case peptides of approx. 10, 8 and 6 kDa were found (results not shown). This heterogeneity may reflect exoproteinase degradation of a primary [32P]phosphopeptide; exposure of cells to noradrenaline or glucagon enhanced radioactivity, approximately equally, into each peak of the triplet.

The effects of the calcium ionophore A23187 (2.5 μM) and of PMA (1 μg/ml), on hydroxylase phosphorylation were also assessed. A23187 enhanced the total phosphate content [basal, 0.16 ± 0.02, +2.5 μM-A23187, 0.32 ± 0.04 (mol of P/mol of subunit); means ± s.e.m. for three different cell preparations; P < 0.05] as previously reported (Garrison, 1978; Fisher et al., 1984) without qualitatively altering the [32P]phosphopeptide patterns generated by V8-proteinase or chymotrypsin digestion. Phorbol ester, however, neither significantly increased the total phosphate content of the hydroxylase [basal, 0.16 ± 0.02, PMA (1 μg/ml), 0.19 ± 0.02 (mol of P/mol of subunit); means ± s.e.m. for three different cell preparations] nor stimulated metabolic flux from 0.050 mm-phenylalanine through the hydroxylase [control, 4.87 ± 0.44, PMA (1 μg/ml) 4.60 ± 0.35 (nmol/h per mg); means ± s.e.m. for three different cell preparations]. Experiments in which purified hydroxylase was incubated with protein kinase C under the conditions described by Wolf et al. (1984) proved negative; no significant [32P] was transferred from [γ-32P] ATP to the hydroxylase (results not shown). These observations are in good agreement with the findings of Garrison et al. (1984), who showed that A23187, but not phorbol esters, stimulated phosphorylation of a phosphopeptide, resolved by SDS/polyacrylamide-gel electrophoresis of liver cell extracts and identified as the hydroxylase subunit.

**DISCUSSION**

Under the conditions of the experiments presented here, noradrenaline acts via an α-adrenergic mechanism and does not cause a significant rise in cellular cyclic AMP (Santana et al., 1985). Distinct (i.e. cyclic AMP-dependent and cyclic AMP-independent) protein kinases must therefore recognize the hydroxylase as a substrate in the intact liver cell. The non-additivity of phosphorylation responses to maximally effective concentrations of glucagon and noradrenaline indicates either that phosphorylation by one agent (glucagon) inhibits phosphorylation, at a distinct site, by the other agent (noradrenaline), or that hormonally controlled phosphorylation of the hydroxylase subunit is directed to a unique residue. Glucagon and noradrenaline produce qualitatively similar chymotrypsin and V8-proteinase phosphopeptide patterns. On the basis of the urea/SDS/polyacrylamide-gel-electrophoresis analysis, it is clear that the site(s) of phosphorylation lie within a phosphopeptide of approx. 5–6 kDa. Only by more extensive digestion and sequencing studies will the identity or otherwise of the site(s) of phosphorylation be confirmed.

The cyclic AMP-dependent protein kinase, stimulated by exposure of liver cells to glucagon, is well characterized. Although the identity of the protein kinase(s) stimulated by α-adrenergic agents is less clear, two Ca2+-dependent protein kinases can be discounted as candidates for mediation of the α-adrenergic phosphorylation of phenylalanine hydroxylase. As demonstrated by Garrison et al. (1984) and in the present paper, phorbol esters do not stimulate hydroxylase phosphorylation by the phospholipid-dependent protein kinase C either in the intact cell or in vitro. Garrison et al. (1984) have also provided evidence that the Ca2+-dependent phosphorylase kinase is not involved in hydroxylase phosphorylation (there being no diminution of Ca2+-dependent phosphorylation of the hydroxylase in liver cells from gsd/gsd rats, which lack this enzymic activity). In contrast, the purified hydroxylase is a substrate for the liver Ca2+-calmodulin-dependent protein kinase (see the Introduction), which is present in livers of gsd/gsd rats (Schworer et al., 1983).

The characteristics of hydroxylase phosphorylation by this protein kinase are similar to those of hydroxylase phosphorylated by α-adrenergic agents in liver cells. There is a direct correlation between the extent of phosphorylation and enzyme activity (Doskeland et al., 1984; Fisher & Pogson, 1985), and [32P]phosphopeptide maps are similar to those generated by cyclic AMP-dependent phosphorylation (Doskeland et al., 1984; the present work). Using pepsin- and trypsin-generated phosphopeptides, Doskeland et al. (1984) were unable to distinguish cyclic AMP- and Ca2+-calmodulin-dependent phosphorylation patterns, and concluded that both protein kinases phosphorylate the same site on the hydroxylase polypeptide.

The data reported in the present paper are therefore consistent with a role for the Ca2+-calmodulin-dependent protein kinase in the phosphorylation of phenylalanine hydroxylase in liver cells. It is interesting to compare the present findings with those obtained with another, evolutionarily related, aromatic amino acid hydroxylase. Tyrosine hydroxylase from adrenal medulla is sensitive to both cyclic AMP-dependent and Ca2+-dependent phosphorylation (Niggli et al., 1984), but only the cyclic AMP-dependent phosphorylation is directly related to changes in enzyme activity. Ca2+-calmodulin-dependent protein kinase stimulates phosphorylation of another distinct site and is not associated with any change in activity (Vulliet et al., 1984). More recently Vulliet et al. (1985) have shown that tyrosine hydroxylase, in contrast with phenylalanine hydroxylase, is an excellent substrate for protein kinase C-dependent phosphorylation. This phosphorylation is suggested to occur at the cyclic AMP-dependent site of phosphorylation and not the site uniquely phosphorylated by the Ca2+-calmodulin-dependent protein kinase.

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**REFERENCES**


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