# Phosphorylation of recombinant human phenylalanine hydroxylase: effect on catalytic activity, substrate activation and protection against non-specific cleavage of the fusion protein by restriction protease

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The phosphorylation of human phenylalanine hydroxylase by cyclic AMP-dependent protein kinase was studied using recombinant enzyme expressed as a fusion protein in the pMAL system of *Escherichia coli*. Using the target sequence of the restriction protease enterokinase ($Asp_4$-Lys) as the linker peptide, 100% full-length human phenylalanine hydroxylase was obtained on protease cleavage. The fusion protein and human phenylalanine hydroxylase were both phosphorylated at Ser-16 with a stoichiometry of 1 mol of $P_i$/mol of subunit. The rate of phosphorylation of human phenylalanine hydroxylase was inhibited about 40% by the cofactor tetrahydrobiopterin, and this inhibition was completely prevented by the simultaneous presence of L-phenylalanine (i.e. at turnover conditions). Phosphorylated enzyme revealed a 1.6-fold higher specific activity than the non-phosphorylated enzyme form, and it also required a lower concentration of L-Phe for substrate activation. Preincubation with L-Phe increased the specific activity of phenylalanine hydroxylase 2- to 4-fold, L-Phe acting with positive cooperativity. Thus, the basic catalytic and regulatory properties of recombinant human phenylalanine hydroxylase, as well as those observed for the enzyme as a fusion protein, are similar to those previously reported for the rat liver enzyme. When the target sequence of the restriction protease factor Xa (Ile-Glu-Gly-Arg) was used as the linker between maltose-binding protein and human phenylalanine hydroxylase, cleavage of the fusion protein gave a mixture of full-length hydroxylase and a truncated form of the enzyme lacking the 13 N-terminal residues. Interestingly, phosphorylation of the fusion protein, before exposure to factor Xa, almost completely protected against secondary cleavage by this restriction protease at Arg-13 of phenylalanine hydroxylase.

## INTRODUCTION

Mammalian phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC 1.14.16.1) catalyses the conversion of L-phenylalanine (L-Phe) to L-tyrosine in the presence of (6*R*)-L-*erythro*-tetrahydrobiopterin ($BH_4$) and dioxygen. Rat liver PAH contains a unique phosphorylation site (Ser-16) which is a common acceptor for phosphorylation catalysed by the cyclic AMP-dependent protein kinase and CaM-kinase II (multifunctional calcium/calmodulin-dependent protein kinase) [1]. Phosphorylation/dephosphorylation of rat PAH represents an important mechanism for regulation of PAH activity in isolated hepatocytes [2]. Some controversy exists, however, in the literature regarding the significance of phosphorylation in other species and organs than the rat liver (for review, see [3] and [4]) which for the human enzyme can be explained by the problems with stability and lack of [5] or low stoichiometry of [6] phosphorylation. We have recently shown that recombinant wild-type full-length human PAH (hPAH) is also phosphorylated at Ser-16 *in vitro* by cyclic AMP-dependent protein kinase, both as a fusion protein and as purified full-length hPAH [7]. hPAH was fused through the target sequences of the restriction protease factor Xa (Ile-Glu-Gly-Arg) or enterokinase ($Asp_4$Lys) to the C-terminal end of the highly expressed *Escherichia coli* maltose-binding protein (MBP) purified by affinity chromatography. Enterokinase cleavage resulted in 100% full-length enzyme (hPAH), whereas factor Xa cleavage also gave a truncated form of the enzyme lacking the 13 N-terminal residues (Δ13-hPAH) due to a secondary cleavage site [7]. We report here on a detailed characterization of the effect of phosphorylation on the kinetic properties of hPAH and its expressed fusion protein as well as the substrate-directed modulations of this phosphorylation. Furthermore, it is shown that phosphorylation of the fusion protein MBP–$(pepIEGR)_{Xa}$–hPAH prevents its non-specific cleavage at Arg-13 by factor Xa.

## MATERIALS AND METHODS

### Materials

[$\gamma$-$^{32}$P]ATP (4000 Ci/mmol) was from Amersham (Amersham, Bucks., U.K.). Factor Xa was from Boehringer Mannheim (Germany). Enterokinase/enteropeptidase with a specific activity in the range of 127–174 units/$\mu$g of protein was from Biozyme Laboratories Ltd. (Gwent, U.K.). The catalytic subunit of cyclic AMP-dependent protein kinase (C subunit) was purified to homogeneity from bovine heart and was a gift from Dr. S. O. Døskeland, Department of Anatomy and Cell Biology, University of Bergen. The cofactor $BH_4$ was prepared in HCl (5 mM) due to its instability at neutral pH and stored at −20 °C in the presence of dithiothreitol (DTT).

Abbreviations used: $BH_4$, (6*R*)-L-*erythro*-tetrahydrobiopterin; C subunit, the catalytic subunit of cyclic AMP-dependent protein kinase; DTT, dithiothreitol; PAH, phenylalanine hydroxylase; hPAH, full-length human phenylalanine hydroxylase; 6-$MPH_4$, 6-methyl-5,6,7,8-tetrahydropterin; MBP, maltose-binding protein; MBP–$(pepD_4K)_{EK}$–hPAH, fusion protein containing a truncated form of MBP, a peptide linker region with the target sequence for cleavage with enterokinase, and human PAH; MBP–$(pepIEGR)_{Xa}$–hPAH, fusion protein containing a truncated form of MBP, a peptide linker region with the target sequence for cleavage with factor Xa, and human PAH; Δ13-hPAH, a truncated form of the enzyme lacking the 13 N-terminal residues; $[S]_{0.5}$, substrate concentration required for half-maximal activation.

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### Production and isolation of recombinant hPAH

The fusion proteins MBP–$(pepIEGR)_{Xa}$–hPAH and MBP–$(pepD_4K)_{EK}$–hPAH were expressed in the pMAL system in *E. coli* and purified as described [7]. The specific activity of the purified MBP–$(pepD_4K)_{EK}$–hPAH, when assayed with 200 $\mu$M 6-methyl-5,6,7,8-tetrahydropterin (6-$MPH_4$) was about 1850 nmol of tyrosine $\cdot$ $min^{-1}$ $\cdot$ $mg^{-1}$ and, when assayed with 75 $\mu$M $BH_4$, was about 1660 nmol of tyrosine $\cdot$ $min^{-1}$ $\cdot$ $mg^{-1}$ for the phenylalanine-preincubated enzyme (3 min at 25 °C with 1 mM Phe). The fusion proteins were cleaved by their respective restriction proteases, i.e. enterokinase [8] or factor Xa [9]. The cleavage reaction was carried out in 10 mM Tris/HCl, 0.2 M NaCl, 10 mM maltose at pH 7.4 and at a protein concentration of 1–2 mg/ml. Cleavage with factor Xa was performed at 0 °C or 25 °C as indicated in the text at a protease to substrate ratio of 1:90 (w/w). Cleavage with enterokinase was performed at 25 °C and at a ratio of 1:50 (w/w). When the time course of the proteolysis was studied, the reaction was stopped by the addition of a mixture of protease inhibitors (1 mM EDTA, 1 mM benzamidine, 20 $\mu$g/ml leupeptin, 5 $\mu$g/ml pepstatin supplemented with 0.3 mM PMSF, all in 15 mM Na-Hepes, pH 7.5). Full-length hPAH derived from MBP–$(pepD_4K)_{EK}$–hPAH (tetrameric form) by proteolysis with enterokinase was, at completion of the cleavage reaction, isolated by ion-exchange chromatography or by size-exclusion chromatography [7]. The purified enzyme was homogeneous by analysis on SDS/PAGE.

### Assay of PAH activity

PAH activity was assayed at 25 °C in a medium containing 15 mM Na-Hepes, pH 7.0, 1600 units/ml of catalase, 3 mM DTT, 0.1 mM Fe(II), 1 mM L-Phe and cofactor (75 $\mu$M $BH_4$ or 200 $\mu$M 6-$MPH_4$). After preincubation with L-Phe (3 min at 25 °C), the reaction was initiated, unless otherwise stated, by the addition of the cofactor with DTT, conducted for 1 or 2 min and processed as described [10]. When the potency of L-Phe as an activator of the non-phosphorylated and phosphorylated hPAH was studied (see below), modifications were as follows. Non-phosphorylated and phosphorylated hPAH (2.5 $\mu$M each) were preincubated for 5 min at 25 °C with various concentrations of L-Phe before assay of catalytic activity [1 mM L-Phe, 75 $\mu$M $BH_4$, 0.2 mM Fe(II)]. The concentration of L-Phe required for half-maximal activation ($[S]_{0.5}$) and Hill coefficient ($h$) were calculated by non-linear regression analysis [11].

### Assay of cyclic AMP-dependent phosphorylation

The standard protein kinase reaction mixture contained 15 mM Na-Hepes (pH 7.0), 0.1 mM ethylene glycol bis($\beta$-amino ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 0.03 mM EDTA, 3 mM DTT, 10 mM magnesium acetate, 60 $\mu$M [$\gamma$-$^{32}$P]ATP and C subunit. The concentrations of kinase and hydroxylase are given in the main text or the legends to the Figures. Unless otherwise indicated, the incubation contained BSA (0.5 mg/ml). The amount of $^{32}$P transferred to substrate was determined by spotting aliquots of the incubate on phosphocellulose strips [12]. On a preparative scale, fully phosphorylated hPAH was prepared by incubation of 10–20 $\mu$M enzyme for 30 min at 30 °C under phosphorylating conditions with 70 nM C subunit and 60–150 $\mu$M ATP. When non-phosphorylated hydroxylase was required for comparison, controls were obtained by incubating the enzyme in the absence of added C subunit under otherwise identical conditions. When required, the preparations were diluted in a 15 mM Na-Hepes buffer, pH 7.0, containing 0.05% BSA (w/v) before assay of PAH activity.

### Interaction with phenyl-Sepharose

An aliquot (150 $\mu$g) of MBP–$(pepD_4K)_{EK}$–hPAH was incubated at 25 °C for 20 min in a medium containing 0.03 M Tris/HCl (pH 7.25) and 0.2 M KCl with 0.02 M L-Phe to allow for activation by phenylalanine [13]. The same amount of fusion protein was also incubated under similar conditions except that the medium did not contain L-Phe. The preparations were cooled to 0 °C and applied to a small batch of either phenyl-Sepharose CL-4B or Sepharose 2B equilibrated, at 0 °C, with the respective incubation media. After 20 min equilibration (on a rotary wheel), the supernatant was removed and the gel washed twice with the incubation buffer. The eluting buffer used was 0.03 M Tris/HCl (pH 7.25) containing 15% (v/v) glycerol.

### Other methods

Protein was determined by using the absorption coefficient $A_{280nm}$ (1 mg $\cdot$ $ml^{-1}$) 1.63 for the fusion proteins and $A_{280nm}$ (1 mg $\cdot$ $ml^{-1}$) 1.0 for the purified hPAH [7]. SDS/PAGE was performed according to the method of Laemmli [14], using 10% (w/v) polyacrylamide gels.

## RESULTS AND DISCUSSION

### Phosphorylation of recombinant hPAH and its expressed fusion proteins

The fusion proteins, MBP–$(pepD_4K)_{EK}$–hPAH and MBP–$(pepIEGR)_{Xa}$–hPAH, and hPAH incorporated up to 1.0 mol of

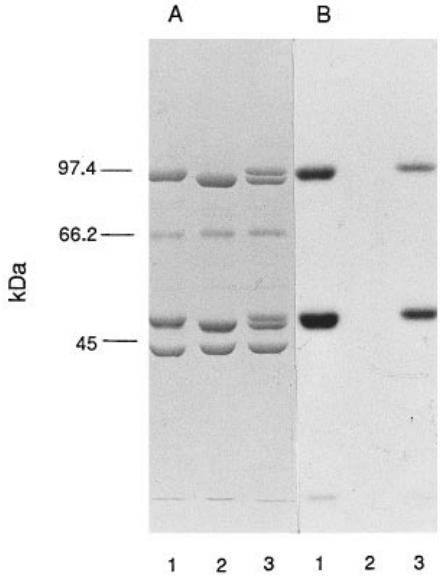


**Figure 1 Effect of phosphorylation on the electrophoretic mobility of recombinant hPAH as fusion protein and enterokinase-cleaved fusion protein**

Panel A: Lanes 1–3, SDS/PAGE of purified recombinant fusion protein MBP–$(pepD_4K)_{EK}$–hPAH partially cleaved by enterokinase (lane 1, phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase; lane 2, non-phosphorylated enzyme; lane 3, mixture of phosphorylated and non-phosphorylated enzymes). Panel B: Lanes 1–3, autoradiogram of lanes 1–3 in panel A. The fusion protein was cleaved by enterokinase (enterokinase/fusion protein, 1:50; at 25 °C for 4 h at pH 7.4) and phosphorylated (70 nM C subunit; 60 $\mu$M [$\gamma$-$^{32}$P]ATP; at 30 °C for 40 min). The major bands corresponded to the subunits of the fusion protein (94 kDa and 93 kDa for phosphorylated and non-phosphorylated forms respectively) and to those of the derived cleavage products, hPAH (51 kDa for phospho-PAH; 50 kDa for non-phospho-PAH) and MBP (43 kDa). BSA (66.2 kDa) was present in all reactions as an internal marker and stabilizing agent. The positions of standard molecular-mass markers are indicated on the left: phosphorylase *b* (97.4 kDa) and ovalbumin (45 kDa).

**Table 1 Regulatory properties of recombinant hPAH compared with those of PAH isolated from human and rat liver**

Abbreviation: n.d., not determined.

| Effects of phosphorylation | Recombinant hPAH | | Human liver PAH | Rat liver PAH |
|---|---|---|---|---|
| | Fusion protein | hPAH | | |
| Stoichiometry of phosphorylation | 1.0[a,b] | 1.0[a,b] | 0.2–0.67[c] | 0.7–1.0[d] |
| Apparent molecular mass of subunit on SDS/PAGE | | | | |
| Non-phosphorylated | 93 kDa[a] | 50 kDa[a,b] | 49 kDa[c] | 51 kDa[e] |
| Phosphorylated | 94 kDa[a] | 51 kDa[a,b] | 50 kDa[c] | 51 kDa[e] |
| Ligand effects on phosphorylation rate | | | | |
| Decrease by $BH_4$ | 25%[a] | 40%[a] | n.d. | 50%[f], 80%[g] |
| Reversal by L-Phe | +[a] | +[a] | n.d. | +[f] |
| Increase by L-Phe | 0%[a] | 0%[a] | n.d. | 200%[f], 20–50%[g] |
| Change in specific activity by phosphorylation | | | | |
| Fold increase with $BH_4$ | 1.6[a] | 1.6[a] | n.d. | 4–6[f] |
| Fold increase with 6-$MPH_4$ | 1.1[a] | n.d. | n.d. | 1[h] |
| Apparent $[S]_{0.5}$ for L-Phe activation ($\mu$M) | | | | |
| Non-phosphorylated | 22[a] | 50[a] | n.d. | 51[f] |
| Phosphorylated | 13[a] | 26[a] | n.d. | 29[f] |
| Hill coefficient for L-Phe activation | | | | |
| Non-phosphorylated | 2.2[a] | 2.4[a] | n.d. | 2.6[f] |
| Phosphorylated | 2.5[a] | –[i] | n.d. | 3[f] |
| Apparent $K_m$ for $BH_4$ ($\mu$M)[j] | | | | |
| Non-phosphorylated | 31[b] | 34[a], 29[b] | n.d. | 21[l], 20[m], 18[n], 29[o] |
| Phosphorylated | n.d. | 35[a] | n.d. | n.d. |
| Activation by L-Phe preincubation only | | | | |
| Change in specific activity | | | | |
| Fold increase with $BH_4$ | 4.5[a] | 2.1[a] | n.d. | 25–30[k] |
| Fold increase with 6-$MPH_4$ | 1.8[a] | n.d. | n.d. | n.d. |
| Effects on adsorption to phenyl-Sepharose | –[a] | n.d. | +[p] | +[q,r] |

Data were obtained from references as follows: [a]this paper, [b][7], [c][6], [d][15], [e][1], [f][16], [g][17], [h][18], [k][4], [l][19], [m][20], [n][21], [o][22], [p][5], [q][13], [r][23]. [i]The algorithm of non-linear regression analyses used to determine *h* did not converge. [j]The assay included preincubation with L-Phe.

phosphate per mol of enzyme subunit when phosphorylated *in vitro* by cyclic AMP-dependent protein kinase, as previously reported [7]. As seen by SDS/PAGE, it was not only hPAH generated by protease cleavage that revealed a slight decrease in the electrophoretic mobility [7], but also the expressed fusion protein (Figure 1). Thus, the apparent molecular mass increased from 93 kDa to 94 kDa and from 50 kDa to 51 kDa on phosphorylation of MBP–$(pepD_4K)_{EK}$–hPAH fusion protein and hPAH respectively (Table 1). A similar change was also observed for the MBP–$(pepIEGR)_{Xa}$–hPAH fusion protein (see Figure 4 below). A complete shift in electrophoretic mobility of the subunits correlated with a stoichiometry of 1 mol of phosphate/mol of subunit. The site of phosphorylation of hPAH seems to be Ser-16 (Figure 2) [7], a highly conserved seryl residue in mammalian species [1,6,10,24,25].

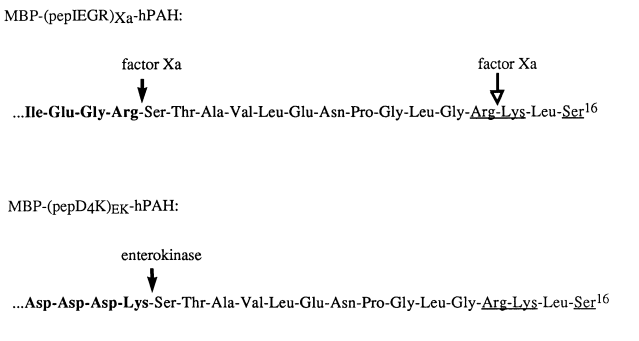


**Figure 2 The specific and non-specific sites of cleavage of the fusion proteins by restriction proteases**

The numbering of the amino acid sequence denotes the initiating methionine as 1 in order to correlate with the numbering of human PAH. Ser-16 is the serine residue phosphorylated by cyclic AMP-dependent protein kinase. The consensus sequence for cyclic AMP-dependent protein kinase is underlined. The amino acid residues of the recognition sites are indicated in bold face. The arrows indicate specific (closed arrow) and unspecific (open arrow) cleavage points for protease, respectively.

## Substrate-directed modulation of phosphorylation of hPAH

Our previous studies on rat PAH have revealed that L-Phe and $BH_4$, at physiologically relevant concentrations, modulate the phosphorylation of Ser-16 by a stimulation and an inhibition, respectively, of the reaction rate [16]. Experimental evidence has been presented that this substrate-directed regulation has physiological significance [2]. Considerable controversy exists, however, in the literature regarding the functional and regulatory significance of phosphorylation of PAH in other species than the rat.

From Figure 3(A) it is seen that the rate of phosphorylation of hPAH by C subunit was not affected by the presence of a saturating concentration (5 mM) of L-Phe in the phosphorylation assay. By contrast, the rate of phosphorylation decreased by about 40% in the presence of 50 $\mu$M $BH_4$. The initial rate of phosphorylation was inhibited to a similar degree after preincubation of the enzyme with $BH_4$ at the final concentration of

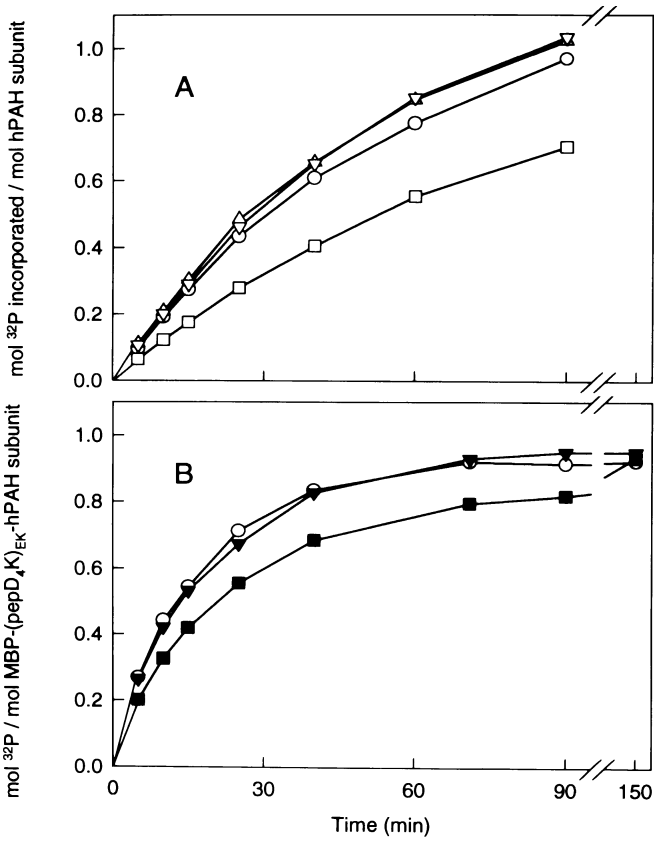


**Figure 3 The effect of L-phenylalanine and/or $BH_4$ on the time-course of phosphorylation of recombinant hPAH by C subunit of cyclic AMP-dependent protein kinase**

hPAH (panel A) and MBP–(pepD$_4$K)$_{EK}$–hPAH (panel B) were phosphorylated {10 $\mu$M hPAH; 5 nM C subunit (panel A)/25 nM C subunit (panel B); 60 $\mu$M [$\gamma$-$^{32}$P]ATP; at 30 °C} in the absence of any added ligand (○), in the presence of 50 $\mu$M $BH_4$ (□), of 100 $\mu$M $BH_4$ (■) or of 5 mM L-phenylalanine (△) and in the presence of both ligands, i.e. 5 mM L-Phe + 50 $\mu$M $BH_4$ (▽) or 5 mM L-Phe + 100 $\mu$M $BH_4$ (▼).

100 or 150 $\mu$M. When the enzyme was preincubated with both $BH_4$ and phenylalanine, the rate of incorporation of phosphate was identical to that observed in the absence of added ligands (Figure 3A). Thus, the inhibitory effect observed with added cofactor was prevented by L-phenylalanine. The ligands did not affect the extent of phosphorylation since the hydroxylase incorporated maximally 1 mol of phosphate/mol of subunit in the presence as well as in the absence of added ligands. Phosphorylation studies such as effect of ligands on the rate and extent of phosphorylation were also performed with the fusion proteins MBP–(pepIEGR)$_{Xa}$–hPAH and MBP–(pepD$_4$K)$_{EK}$–hPAH (Figure 3B) as substrates and the results obtained were similar to those described above for the isolated hPAH.

The substrate-directed modulations of the phosphorylation rate of rat PAH have been interpreted in terms of conformational changes of the enzyme induced by the ligands, as indicated by their effects on the intrinsic fluorescence of highly conserved tryptophan residues [22,26] and limited proteolysis catalysed by chymotrypsin [27]. Due to the sequence homology between the rat and the human enzyme [25], a finding of a similar modulation of phosphorylation of Ser-16 in hPAH was indeed expected. Our results imply that the binding of cofactor affects the accessibility of the kinase to Ser-16 and that the rate of phosphorylation may be considered a sensitive conformational probe.

**Table 2 Effect of activation by phosphorylation and L-phenylalanine preincubation on phenylalanine hydroxylase activity of tetrameric fusion protein**

Non-phosphorylated hydroxylase MBP-(pepD$_4$K)$_{EK}$-hPAH was obtained by incubating the enzyme under phosphorylating conditions, but exempt of C subunit. The preparations were also phosphorylated and assayed as indicated in the Materials and methods section. The reaction was initiated by the addition of either enzyme or cofactor. In the latter case, the enzyme was L-Phe-preincubated. To determine the effect of phosphorylation on the specific activity, a short incubation time (1 min) was used in order to reduce activation of enzyme by L-Phe during assay.

| Enzyme form | Specific activity (nmol · min$^{-1}$ · mg$^{-1}$) | | Relative specific activity | Fold increase in activity with cofactor | |
|---|---|---|---|---|---|
| | $BH_4$ | 6-$MPH_4$ | $BH_4$/6-$MPH_4$ | $BH_4$ | 6-$MPH_4$ |
| Non-phosphorylated | 377 | 1046 | 0.36 | 1.0 | 1.0 |
| Phosphorylated | 610 | 1126 | 0.54 | 1.6 | 1.1 |
| Non-phosphorylated and L-Phe-preincubated | 1699 | 1946 | 0.87 | 4.5 | 1.9 |
| Phosphorylated and L-Phe-preincubated | 1627 | 1770 | 0.91 | 4.3 | 1.7 |

As shown above, the binding of L-Phe to hPAH did not affect the rate of phosphorylation, at least in the absence of added cofactor, suggesting that there was apparently no change in the conformation of the enzyme around the phosphorylation site. For rat liver PAH, it is well documentated in the literature [4] that a significant conformational change occurs following the binding of L-Phe. This characteristic has been successfully exploited in the purification of rat liver PAH by hydrophobic affinity chromatography on phenyl-Sepharose [13], and later in the purification of PAH from other species [5,28]. Thus, preincubation with phenylalanine induced a conformation of rat PAH with increased hydrophobic surface properties. In the present study (results not shown), it was observed that hPAH as fusion protein, even without preincubation with L-Phe (20 mM L-Phe, 20 min, 25 °C) was tightly bound to the hydrophobic gel matrix of phenyl-Sepharose from which it could not be eluted by the conventional procedure described by Shiman et al. for rat PAH [13]. This finding is compatible with the tendency of recombinant hPAH and its expressed fusion proteins to form aggregates [7,29], as well as the lack of any effect of L-Phe on the rate of phosphorylation of hPAH (Figure 3).

## Effect of phosphorylation on the catalytic properties of recombinant hPAH

As shown above, the kinetics and stoichiometry of phosphorylation of Ser-16 were similar for the fusion protein and the isolated hPAH. Furthermore, phosphorylation was found to have no significant effect on their catalytic activity when determined under the standard assay conditions (Table 2). Only when the activity was assayed without preincubation with L-Phe, and with $BH_4$ as the cofactor, was a 1.6- to 1.9-fold increase in specific activity observed on phosphorylation (Table 2). The effect was qualitatively similar to that previously reported for the rat liver enzyme where phosphorylation of Ser-16 resulted in a 4- to 6-fold increase in its catalytic activity [1,16,30]. A similar qualitative difference was also observed between the rat and the

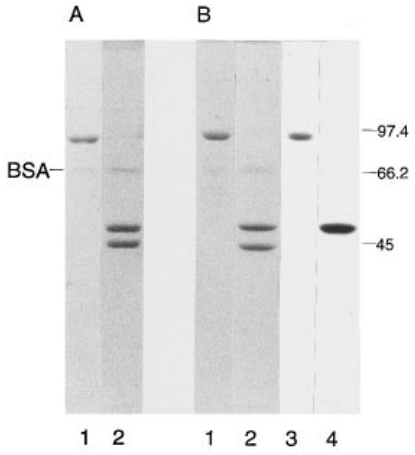


**Figure 4 Effect of phosphorylation on the proteolytic cleavage of MBP–(pepIEGR)$_{Xa}$–hPAH fusion protein by factor Xa**

The fusion protein was either non-phosphorylated (A) or 100% $^{32}$P-phosphorylated by 70 nM C subunit for 40 min at 30 °C (B). Both fusion proteins were completely cleaved by factor Xa for 15 h at 0 °C and a factor Xa/fusion protein ratio of 1:90. Lanes 1 and 2 (in A and B) represent Coomassie Brilliant Blue SDS/PAGE gels of the non-cleaved and cleaved fusion protein respectively, and lanes 3 and 4 are autoradiograms of lanes 1 and 2 in (B). The cleaved fusion protein of (A), lane 2 was not phosphorylated and the protein subunit with apparent molecular mass of 50 kDa corresponds to that of the non-phosphorylatable proteolysed form Δ13-hPAH. The protein subunit with apparent molecular mass of 51 kDa (B, lane 2) corresponds to that of the full-length phosphorylated hPAH.

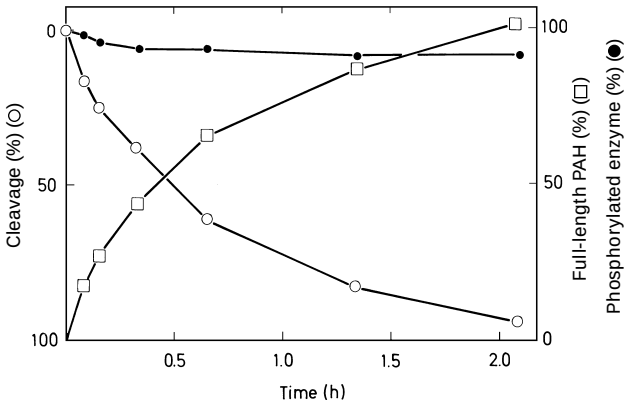


**Figure 5 Time-course for the cleavage of phosphorylated fusion protein by factor Xa**

The fusion protein was phosphorylated (70 nM C subunit; 30 °C; 40 min) and cleaved by factor Xa (factor Xa/fusion protein ratio 1:90; 25 °C; pH 7.0). At different time points during proteolysis, aliquots of the reaction mixture were analysed by SDS/PAGE. The percentage cleavage and the percentage of phosphorylated hPAH derived from phospho[MBP–(pepIEGR)$_{Xa}$–hPAH] were estimated from the autoradiogram by scanning at 633 nm using the LKB Ultroscan XL laser densitometer. The percentage of [$^{32}$P]phosphate incorporated in the enzymes (fusion protein and/or recombinant hPAH) was determined by the filter method described in [12].

human enzyme in terms of activation by preincubation with L-Phe, which was only 2- to 4-fold for hPAH (Table 2).

From Table 2 it is also seen that phosphorylation increased the relative specific activity of the non-L-Phe-preincubated enzyme, with $BH_4$ versus 6-$MPH_4$, as previously reported for the rat liver enzyme [18] and used as a criterion of activation [31]. Furthermore, as observed for the rat liver enzyme [16], the potency of L-Phe as an activator of hPAH increased on phosphorylation. Thus, phospho-hPAH required a lower concentration of L-Phe, 26 $\mu$M versus 50 $\mu$M for the non-phosphorylated form, to obtain half-maximal substrate activation, but with no significant change in the positive cooperativity of L-Phe binding (Table 1).

## Phosphorylation protects against limited proteolysis of hPAH by factor Xa

As previously reported [7], it was found that cleavage of the fusion protein MBP–(pepIEGR)$_{Xa}$–hPAH by the restriction protease factor Xa resulted in a truncated form (Δ13-hPAH) of the enzyme (Figure 2), which is not a substrate for the cyclic AMP-dependent protein kinase. Indeed, autoradiography of products derived from complete cleavage (Figure 4, panel A, lane 2) and submitted to phosphorylating conditions did not reveal any bands corresponding to hPAH, confirming that no phosphate was incorporated into the enzyme (results not shown). Unspecific cleavage by factor Xa has also been reported for cleavage of other fusion proteins [32–34]. Due to the proximity of Arg-13 and Ser-16 in the fusion protein MBP–(pepIEGR)$_{Xa}$–hPAH (Figure 2), it was of interest to test whether phosphorylation has any effect on the unspecific cleavage by factor Xa. On the basis of SDS/PAGE analysis it was found that phosphorylation had no effect on the rate of cleavage at the specific Ile-Glu-Gly-Arg site (results not shown). By contrast, full phosphorylation of the fusion protein completely protected the enzyme from the secondary cleavage at the site (Figure 4). The intensity of the $^{32}$P-labelled band correlated with the intensity of the stained 51 kDa band and was dependent on the percentage of specific cleavage (Figure 5).

Using phosphorylation as a probe, it is likely that the binding of the substrates to hPAH results in conformational changes similar to those proposed for the rat liver enzyme [16]. The effect of ligand binding was therefore also studied in the cleavage reaction (see above). It was found, however, that the presence of 5 mM L-Phe and/or 75 $\mu$M $BH_4$ in the reaction mixture did not affect the secondary cleavage of hPAH by factor Xa (results not shown).

## Conclusions

Recombinant hPAH expressed as a fusion protein in *E. coli* and cleaved by restriction protease results in a homogeneous enzyme which shares a number of catalytic and regulatory properties reported for the rat liver enzyme, including the effects of phosphorylation (Table 1). The stoichiometry of phosphorylation at a conserved serine residue (Ser-16) is the same for both species of the enzyme, and the modulation of the rate of phosphorylation by the two substrates ($BH_4$ and L-Phe) is qualitatively very similar. For both enzyme forms phosphorylation results in a comparable increase in specific activity as well as an increase in the affinity for L-Phe. Thus, our studies on the recombinant hPAH *in vitro* have shown that there is an interplay between substrate (L-Phe) activation and phosphorylation of the enzyme, as previously reported for the rat liver enzyme [1]. To what extent these mechanisms operate in humans *in vivo* remains to be seen.

It should also be noted that the fusion protein of hPAH revealed similar catalytic and regulatory properties as purified hPAH (Table 1). This is of considerable practical value in studies on mutant forms of the enzyme in patients with phenylketonuria and hyperphenylalaninaemia since some mutant forms are unstable as isolated enzymes and their catalytic properties can only be studied as fusion proteins [29].

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## REFERENCES

1 Døskeland, A. P., Schworer, C. M., Døskeland, S. O., Chrisman, T. D., Soderling, T. R., Corbin, J. and Flatmark, T. (1984) Eur. J. Biochem. **145**, 31–37
2 Døskeland, A. P., Vintermyr, O. K., Flatmark, T., Cotton, R. G. H. and Døskeland, S. O. (1992) Eur. J. Biochem. **206**, 161–170
3 Kaufman, S. (1987) in The Enzymes (Boyer, P. D. and Krebs, E. G., eds.), vol. 18, pp. 217–282, Academic Press, London
4 Kaufman, S. (1993) Adv. Enzymol. **67**, 77–264
5 Abita, J.-P., Blandin-Savoya, F. and Rey, F. (1987) Methods Enzymol. **142**, 27–35
6 Smith, S. C., Kemp, B. E., McAdam, W. J., Mercer, J. F. B. and Cotton, R. G. H. (1984) J. Biol. Chem. **259**, 11284–11289
7 Martínez, A., Knappskog, P. M., Olafsdottir, S., Døskeland, A. P., Eiken, H. G., Svebak, R. M., Bozzini, M. L., Apold, J. and Flatmark, T. (1995) Biochem. J. **306**, 589–597
8 Hopp, T. L., Prickett, K. S., Price, V. L., Libby, R. T., March, C. J., Cerretti, D. P., Urdal, D. L. and Conlon, P. J. (1988) Bio/Technology **6**, 1204–1210
9 Nagai, K. and Thøgersen, H. C. (1987) Methods Enzymol. **153**, 461–491
10 Døskeland, A. P., Døskeland, S. O. and Flatmark, T. (1987) Methods Enzymol. **142**, 35–44
11 Knack, I. and Röhm, K. H. (1981) Hoppe-Seyler's Z Physiol. Chem. **362**, 1119–1130
12 Roskoski, R. (1983) Methods Enzymol. **99**, 3–6
13 Shiman, R., Gray, D. and Pater, A. (1979) J. Biol. Chem. **254**, 11300–11306
14 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
15 Abita, J. P., Milstien, S., Chang, N. and Kaufman, S. (1976) J. Biol. Chem. **251**, 5310–5314
16 Døskeland, A. P., Døskeland, S. O., Øgreid, D. and Flatmark, T. (1984) J. Biol. Chem. **259**, 11242–11248
17 Phillips, R. S. and Kaufman, S. (1984) J. Biol. Chem. **259**, 2474–2479
18 Donlon, J. and Kaufman, S. (1980) J. Biol. Chem. **255**, 2146–2152
19 Bailey, S. W. and Ayling, J. E. (1983) Biochemistry **22**, 1790–1798
20 Abita, J.-P., Parniak, M. and Kaufman, S. (1984) J. Biol. Chem. **259**, 14560–14566
21 Døskeland, A. P., Haavik, J., Flatmark, T. and Døskeland, S. O. (1987) Biochem. J. **242**, 867–874
22 Haavik, J., Døskeland, A. P. and Flatmark, T. (1986) Eur. J. Biochem. **160**, 1–8
23 Parniak, M., Hasegawa, H., Wilgus, H. and Kaufman, S. (1981) Biochem. Biophys. Res. Commun. **99**, 707–714
24 Smith, S. C., McAdam W. J., Kemp, B. E., Morgan, F. J. and Cotton, R. G. H. (1987) Biochem. J. **244**, 625–631
25 Ledley, F. D., Grenett, H. E., Dunbar, B. S. and Woo, S. C. L. (1990) Biochem. J. **267**, 399–406
26 Phillips, R. S., Parniak, M. A. and Kaufman, S. (1984) Biochemistry **23**, 3836–3842
27 Iwaki, M., Phillips, R. S. and Kaufman, S. (1986) J. Biol. Chem. **261**, 2051–2056
28 Døskeland, A. P., Ljones, T., Skotland, T. and Flatmark, T. (1982) Neurochem. Res. **7**, 407–421
29 Eiken, H. G., Knappskog, P. M., Apold, J. and Flatmark, T. (1995) Human Mut. **6**, in the press
30 Citron, B. A., Davis, M. D. and Kaufman, S. (1994) Biochem. Biophys. Res. Commun. **198**, 174–180
31 Kaufman, S. (1986) Adv. Enz. Regul. **25**, 37–64
32 Nagai, K. and Thøgersen, H. C. (1984) Nature (London) **309**, 810–812
33 Gardella, T., Rubin, D., Abou-Samra, A., Keutmann, H. T., Potts, J. T., Kronenberg, H. M. and Nussbaum, S. R. (1990) J. Biol. Chem. **265**, 15854–15859
34 Wearne, S. J. (1990) FEBS Lett. **263**, 23–26

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