Expression of recombinant human phenylalanine hydroxylase as fusion protein in *Escherichia coli* circumvents proteolytic degradation by host cell proteases

Isolation and characterization of the wild-type enzyme

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Recombinant human phenylalanine hydroxylase (hPAH) was produced in high yields in *Escherichia coli* using the pET and pMAL expression vectors. In the pMAL system, hPAH was fused through the target sequences of the restriction protease factor Xa (IEGR) or enterokinase (D_4K) to the C-terminal end of the highly expressed *E. coli* maltose-binding protein (MBP). The recombinant hPAH, recovered in soluble forms, revealed a high specific activity even in crude extracts and was detected as a homogeneous band by Western-blot analysis using affinity-purified polyclonal rabbit anti-(rat PAH) antibodies. The enzyme expressed in the pET system was subject to limited proteolysis by host cell proteases and was difficult to purify with a satisfactory yield. By contrast, when expressed as a fusion protein in the pMAL system, hPAH was resistant to cleavage by host cell proteases and was conveniently purified by affinity chromatography on an amylose resin. Catalytically active tetramer–dimer (in equilibrium) forms of the fusion protein were separated from inactive, aggregated forms by size-exclusion h.p.l.c. After cleavage by restriction protease, factor Xa or enterokinase, hPAH was separated from uncleaved fusion protein, MBP and restriction proteases by hydroxylapatite or ion-exchange (DEAE) chromatography. The yield of highly purified hPAH was approx. 10 mg/l of culture. The specific activity of the isolated recombinant enzyme was high (i.e. 1440 nmol of tyrosine·min^{-1}·mg^{-1} with tetrahydrobiopterin as the cofactor) and its catalytic and physicochemical properties are essentially the same as those reported for the enzyme isolated from human liver. The recombinant enzyme, both as a fusion protein and as purified full-length hPAH, was phosphorylated *in vitro* by the catalytic subunit of cyclic AMP-dependent protein kinase. The phosphorylated form of hPAH electrophoretically displayed an apparently higher molecular mass (~ 51 kDa) than the non-phosphorylated (~ 50 kDa) form.

INTRODUCTION

Mammalian phenylalanine hydroxylase (PAH, phenylalanine 4-mono-oxygenase, EC 1.14.16.1) catalyses the conversion of L-phenylalanine into L-tyrosine in the presence of tetrahydrobiopterin and dioxygen. Phenylketonuria (PKU) is an autosomal inborn error of phenylalanine metabolism caused by the absence of, or by a severely reduced activity of, PAH. The loss of enzymic activity found in PKU patients is a result of single-base substitutions or small deletions in the PAH gene (for review, see Eisensmith and Woo, 1992). The full-length cDNA of human liver PAH (hPAH) has been cloned (Woo et al., 1983; Kwok et al., 1985) and translated to catalytically active hPAH in both eukaryotic (Ledley et al., 1985a) and prokaryotic (Ledley et al., 1987) expression systems. The purification of PAH from human liver has been reported (Woo et al., 1974; Choo et al., 1979; Yamashita et al., 1985). However, human liver is not readily available and PAH activity is very unstable and is rapidly lost after death (Friedman and Kaufman, 1973; Woolf, 1976). Thus, only scarce information on the biochemical and physicochemical properties of the human enzyme has been obtained so far. Recombinant mutant forms of hPAH have been expressed in *in vitro* eukaryotic systems (Okano et al., 1991), but without rigorous measurements of enzymic activity due to the low level of expression and instability of the enzyme. Recombinant wild-type and mutant forms of hPAH which are expressed in *Escherichia coli* (Ledley et al., 1987; Knappskog et al., 1993) undergo limited proteolysis by host-cell proteases in the selected expression systems, and only a partial purification and characterization of the recombinant wild-type enzyme has been reported (Ledley et al., 1987). A strategy that avoids some of these problems is to link the gene of interest to a second gene which is expressed well in *E. coli*, to generate a fusion protein. The *E. coli* maltose-binding protein (MBP) has been successfully used as a fusion partner (Maina et al., 1988; Lauritzen et al., 1991). In this system, the protein of interest is positioned at the C-terminal end of MBP through a target sequence for restriction protease to achieve a specific cleavage of the hybrid protein.

Abbreviations used: BH_4, (6*R*)-L-*erythro*-tetrahydrobiopterin; DTT, dithiothreitol; PAH, phenylalanine hydroxylase; hPAH, human PAH; IEF, isoelectric focusing; Δ13-hPAH, truncated form of hPAH lacking the 13 N-terminal residues; IPTG, isopropyl-thio-β-D-galactoside; 6-MPH_4, 6-methyl-5,6,7,8-tetrahydropterin; MBP, maltose-binding protein; MBP–$(pep)_{EK}$–hPAH, fusion protein containing a truncated form of MBP, a peptide linker region with the target sequence for cleavage with enterokinase and hPAH; MBP–$(pep)_{Xa}$–hPAH, fusion protein containing a truncated form of MBP, a peptide linker region with the target sequence for cleavage with factor Xa, and hPAH; PKU, phenylketonuria; PMSF, phenylmethanesulphonyl fluoride; PVDF, poly(vinylidene difluoride); LB, Luria–Bertani.

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In the present study we report on the expression of hPAH in *E. coli* using the pET and pMAL expression vectors. Expression of recombinant hPAH as a fusion protein (in the pMAL system) greatly increases the yield of enzyme and circumvents its proteolytic degradation in the *E. coli* cytoplasm reported previously (Ledley et al., 1987; Knappskog et al., 1993). The purification of the enzyme is also greatly facilitated.

MATERIALS AND METHODS

Materials

DNA primers for the PCR and sequencing were synthesized on an Applied Biosystems model 392 synthesizer (Foster City, CA, U.S.A.). *Taq* DNA polymerase was from Boehringer Mannheim (Mannheim, Germany). DNA sequencing was carried out by the Taq DyeDeoxyTerminator Cycle Sequencing Kit using an automatic DNA sequencer (model 373A from Applied Biosystems). The pET expression system was obtained from Novagen (Madison, WI, U.S.A.) and the pMAL expression system from New England Biolabs (Beverly, MA, U.S.A.). The cDNA clone of hPAH was kindly provided by Professor S. L. C. Woo (Baylor College, Texas Medical Center, TX, U.S.A.). The restriction protease factor Xa was obtained from Boehringer Mannheim and enterokinase from Biozyme Laboratories Ltd. (Blaenavon, Gwent, U.K.).

Subcloning and expression of hPAH

cDNA containing the coding sequence of hPAH was inserted into the pET and pMAL expression vectors as described (Knappskog et al., 1993; and unpublished work). To introduce the cleavage site (D_4K) for the restriction protease enterokinase the corresponding four amino acids at the factor Xa site (IEGR) and one amino acid (G) from the linker region of the pMAL [MBP–$(pep)_{Xa}$–hPAH] clone were modified by site-directed mutagenesis. The protocol described by Knappskog et al. (1993) was followed using the mutagenic oligonucleotide 5′-CAACAACCTCG**ACGA**TGA**T**TG**AT**AA**A**GTCTACTGCG-3′ (mismatch nucleotides marked in bold and underlined). Positive clones were identified by the generation of a *Taq* I restriction site and sequenced to verify the introduction of the enterokinase site (and to exclude mutations due to *Taq* DNA polymerase misincorporation).

In the pET expression system, overnight cultures of HMS174(DE3) or BL21(DE3) cells were inoculated into 12 l of Luria–Bertani medium containing 50 μg/ml of ampicillin. Cells were grown at 37 °C and the expression through T7 RNA polymerase was induced by the addition of 0.5 mM isopropyl-thio-β-D-galactoside (IPTG) or 33 mM lactose when A_{600} was about 0.8. The two inducers gave an equivalent production of hPAH. The bacteria were harvested 2 h after induction and the frozen pellets were kept at −20 °C until used. The bacteria (~ 6 l culture) were disrupted by passage through a French press (type FA-073 from SLM Instruments, Urbana, IL, U.S.A.) at 69 MPa in approx. 300 ml of medium containing 30 mM Tris/maleate, 0.2 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM EDTA, 10 mM benzamidine, 1 mM dithiothreitol (DTT) and 10% (w/v) glycerol, pH 7.4. The crude extract was then centrifuged at 98000 *g* at 4 °C for 10 min and the supernatant, referred to as the high-speed supernatant, was processed immediately or frozen at −80 °C until used.

In the pMAL expression system, overnight cultures of TB1 cells were inoculated into 1 l of LB medium containing 50 μg/ml of ampicillin. Cells were grown at 37 °C and expression of hPAH was induced by the addition of 1 mM IPTG when A_{600} was about 0.8. Unless otherwise indicated 0.2 mM ferrous ammonium sulphate was added at three time points, i.e. at the start of the culture, together with IPTG and after 3 h of induction. Bacteria were harvested 18–21 h after induction and the pellets were kept at −20 °C until used. The bacteria were resuspended in a medium containing 10 mM Tris/HCl, 0.2 M NaCl, 0.2 mM PMSF, 1 mM EDTA, 10 mM benzamidine and 10% (w/v) glycerol, pH 7.4, and disrupted as described above.

Purification of rat liver PAH

PAH was isolated from rat liver by the method (procedure II D) of Shiman et al. (1979), yielding homogeneous enzyme preparations with a typical specific activity of about 1300 nmol of tyrosine·min^{-1}·mg of protein^{-1} when assayed at pH 6.8 and 25 °C with 500 μM 6-methyl-5,6,7,8-tetrahydropterin (6-MPH_4) as the cofactor.

Purification of recombinant hPAH

Human PAH expressed in *E. coli* by the pET-vector system was partially purified from the high-speed supernatant (98000 *g*) of the cell lysate by the method of Shiman et al. (1979).

The fusion proteins expressed in *E. coli* were isolated by affinity chromatography as described by Guan et al. (1988). The cell lysates were diluted with medium containing 10 mM Tris/HCl, 0.2 M NaCl, 0.2 mM PMSF and 1 mM EDTA, pH 7.4, to reduce the protein concentration to about 3 mg/ml before application to the column (2.5 cm × 10 cm) of amylose resin (New England Biolabs, MA, U.S.A.), equilibrated with 10 mM Tris/HCl, 0.2 M NaCl and 1 mM EDTA, pH 7.4. The column was washed with about 40 bed-volumes of the equilibration buffer, and the fusion protein eluted with buffer containing 10 mM maltose. The fusion protein was further purified by size-exclusion h.p.l.c. (see below) and the tetrameric form was cleaved by restriction protease factor Xa or enterokinase (for details see the Results section). hPAH was then applied to a hydroxylapatite (Bio-Rad Laboratories, CA, U.S.A.) column (1 cm × 10 cm) equilibrated with 20 mM sodium phosphate buffer, pH 7.2, and hPAH was eluted as a homogeneous protein by a linear sodium phosphate gradient (0.2–0.4 M) at pH 7.2 and a flow rate of 1 ml/min. Purification was also obtained by ion-exchange chromatography on a DEAE-Sepharose column (Pharmacia, Uppsala, Sweden) or a Protein Pak DEAE HR column (Millipore, MA, U.S.A.). All chromatographic steps were carried out at 4 °C.

Size-exclusion chromatography

Size-exclusion chromatography was performed at 4 °C using a HiLoad Superdex 200 HR column (1.6 cm × 60 cm), prepacked from Pharmacia. The mobile phase consisted of 20 mM Na-Hepes and 0.2 M NaCl, pH 7.0, freshly prepared and degassed before use. The flow rate was 0.38 ml/min. The apparent molecular mass of the proteins was estimated from calibration curves obtained by running standard proteins: cytochrome *c* (12.5 kDa), ovalbumin (43 kDa), BSA (68 kDa), ovalbumin dimer (86 kDa), BSA dimer (136 kDa), apoferritin (440 kDa) and thyroglobulin (670 kDa). Blue dextran and ADP were used to determine the void volume (V_o = 43.2 ml) and the exclusion volume (V_T = 117.6 ml) of the column respectively. Data collection was obtained using the data acquisition software from Decision Computer International Co., Ltd. (Taipei, Taiwan), and the chromatograms were resolved into individual Gaussian peaks by using a linear least-squares fit (PeakFit program from Jandel Scientific, CA, U.S.A.).

Assay of PAH activity

PAH activity was assayed at 25 °C. The standard reaction mixture performed in a final volume of 50 μl contained 0.1 M Na-Hepes, pH 7.0, 1 mg/ml of catalase, 100 μM ferrous ammonium sulphate, 5 mM DTT, 1 mM L-phenylalanine and 75 μM (6*R*)-tetrahydrobiopterin (BH_4) or 500 μM 6-methyltetrahydropterin (6-MPH_4). The enzyme (1–4 μg of hPAH) was preincubated for 2 min at 25 °C in a mixture containing buffer, L-phenylalanine and catalase. Then, Fe(II) was added and allowed to incubate for 1 min with the enzyme. The reaction was started by the addition of BH_4 (or 6-MPH_4) and DTT, and after a reaction period of 1 min was stopped by adding 50 μl of 1 % (v/v) acetic acid in ethanol. After precipitation of the protein at −20 °C and centrifugation, tyrosine was measured by h.p.l.c. and fluorimetric detection (Døskeland et al., 1984). The amount of product was linear with time and amount of enzyme added at the selected standard assay conditions.

Electrophoresis and immunoblotting

SDS/PAGE was performed at 180 V (2 h) in a 10 % (w/v) polyacrylamide gel (Laemmli, 1970), and gels were stained with Coomassie Brilliant Blue. Immunoblotting was performed using affinity-purified rabbit anti-(rat PAH) (1.6 μg/ml) as the primary antibody. The enhanced chemiluminescence (ECL) system from Amersham (U.K.) was used for the immunodetection.

High-resolution isoelectric focusing (IEF) was performed in thin-layer polyacrylamide flat gel (LKB Ampholine PAGE plate from Pharmacia) containing ampholines ranging in pH from 4.0 to 6.5. Before prefocusing, samples were filtered through 1 % (w/v) glycine (pH 7.0) pre-equilibrated Sephadex G-25. The electrofocused bands were detected by staining with Coomassie Brilliant Blue stain.

N-terminal sequence analysis

The amino acid sequence at the N-terminal region of the purified hPAH preparations was determined using an Applied Biosystems (Foster City, CA, U.S.A.) Model 477A Protein Sequencer and Model 120A PTH Analyzer after immobilization of approx. 0.6 nmol of hPAH on poly(vinylidene difluoride) (PVDF) membranes.

C-terminal sequence analysis

Automated C-terminal protein sequencing was performed at Perkin–Elmer, Applied Biosystems Division (Foster City, CA, U.S.A.) using the chemical degradation scheme as described by Boyd et al. (1992) in an Applied Biosystems Model 477A Protein Sequencer. Approximately 1.5 nmol of hPAH was immobilized on PVDF membranes. Before sequence analysis, the immobilized protein was treated *in situ* with 10 % (w/v) phenyl isocyanate (Aldrich, Milwaukee, WI, U.S.A.) in acetonitrile under basic conditions to derivatize the ϵ amino groups of the lysine molecules into stable, easily detectable phenylureas.

Phosphorylation of PAH

The phosphorylation of human and rat PAH was assayed at 30 °C, essentially as described previously (Døskeland et al., 1984). The amount of ^{32}P incorporated into PAH was determined by spotting aliquots of the incubate on phosphocellulose strips followed by liquid-scintillation counting. The phosphorylated proteins were analysed by SDS/PAGE and detected by autoradiography. In experiments where the extent of phosphorylation was followed during the cleavage of the fusion proteins by restriction proteases factor Xa or enterokinase, aliquots of the reaction mixture were taken out and protease inhibitors (1 mM EDTA, 1 mM benzamidine, 20 μg/ml leupeptin, 5 μg/ml pepstatin and 0.3 mM PMSF) were added prior to phosphorylation.

Protein measurements

Protein was measured colorimetrically (Bradford, 1976) and purified enzyme by the absorbance at 280 nm using the absorption coefficient A_{280} (1 mg·ml^{-1}·cm^{-1}) = 1.63 (i.e. ϵ_{280} = 154 mM^{-1}·cm^{-1}) for the fusion protein MBP–$(pep)_{Xa}$–hPAH. The absorption coefficient was calculated from the absorbance at 280 nm, the content of pure fusion protein estimated by amino acid analyses and the reported amino acid composition of hPAH and MBP (Kwok et al., 1985; Duplay et al., 1984). The value A_{280} (1 mg·ml^{-1}·cm^{-1}) = 1.0 was used for the purified hPAH, assuming the same absorption coefficient as for the rat enzyme (Shiman, 1980).

RESULTS

Recombinant hPAH expression in the pET-vector system

The recombinant hPAH was found to be partially degraded by host-cell proteases when expressed using the pET–hPAH construct in a HMS174(DE3) host as shown by Western immunoblot analysis. Two major bands of about 50 and 49 kDa, and several minor bands of lower molecular mass were observed on SDS/PAGE of the crude soluble extracts (Knappskog et al., 1993). Protease-deficient bacterial strains (BL21 cells) and different culture media [M9 medium with 0.1 % (w/v) casamino acids and glucose] did not decrease the proteolytic degradation of the enzyme. After 2 h of induction with 0.5 mM IPTG or 33 mM lactose, the specific activity of the crude bacterial lysates was typically 84 nmol of tyrosine·min^{-1}·mg of protein^{-1}, with 1 mM L-phenylalanine and 500 μM 6-MPH_4 as the substrates.

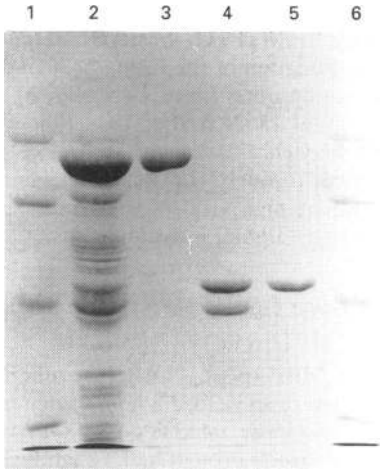

Figure 1 SDS/PAGE analysis of total cell lysate protein from *E. coli* and fractions from purification of hPAH

Lanes 1 and 6 contain low-molecular-mass standards: rabbit muscle phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), hen egg-white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21.5 kDa). Lane 2, the *E. coli* lysate 18 h after induction of recombinant hPAH by 1 mM IPTG in the pMAL system; lane 3, the 94 kDa fusion protein MBP–$(pep)_{Xa}$–hPAH isolated by affinity chromatography on the amylose resin; lane 4, the fusion protein cleaved by the restriction protease factor Xa; lane 5, hPAH isolated by hydroxylapatite chromatography.

Table 1 Expression of hPAH in *E. coli* through the pMAL-vector system

The PAH activity was measured in cell lysates (high-speed supernatant) of cultures induced by 60 mM lactose, 0.5 mM IPTG and 1 mM IPTG. The activity was measured with 1 mM phenylalanine and 0.5 mM 6-MPH_4 as the substrates. n.d., not determined. Data for expression of the fusion protein MBP–$(pep)_{Xa}$–hPAH are shown.

		Activity (nmol of Tyr · min^{-1} · mg of $protein^{-1}$)		
Time after induction (h)	Inducer...	Lactose (60 mM)	IPTG (0.5 mM)	IPTG (1 mM)
0		1.8	1.8	1.8
2		6.0	32.2	n.d.
3		10.5	57.7	89.0
18		n.d.	200.0	795.0

Attempts were made to purify the full-length enzyme (50 kDa band on SDS/PAGE) by the procedure of Shiman et al. (1979), including binding of phenylalanine-activated hPAH to phenyl-Sepharose in the presence of 20 mM L-phenylalanine, and eluting the enzyme by removing the amino acid and adding 3–5% (v/v) dimethylformamide or 5–20% (v/v) glycerol to the elution medium. Only a partial purification of hPAH, with a low yield (about 12%), was obtained and the specific activity ranged from 550 to 840 nmol of tyrosine · min^{-1} · mg of $protein^{-1}$ using 6-MPH_4 as the cofactor.

Recombinant hPAH expression in the pMAL-vector system

In the pMAL system the fusion protein MBP–$(pep)_{Xa}$–hPAH was expressed in high yields and obtained in soluble form (Figure 1, lane 2), with high specific activity even in crude bacterial extracts (Table 1). Lactose, at concentrations up to 60 mM, was less efficient than IPTG as an inductor in this system. The fusion protein constituted about 5% and 20% of soluble cellular proteins when the cells were harvested 3 and 18 h, respectively, after induction with 1 mM IPTG. Western-blot analysis showed that proteolytic degradation of the fusion protein did not occur even at long incubation times (results not shown), and cultures were usually harvested 18–24 h after induction with IPTG in order to increase the yield.

For hPAH fused through the target sequence (D_4K) of enterokinase, a similar high yield of catalytically active fusion protein [MBP–$(pep)_{EK}$–hPAH] was obtained.

Purification of the fusion proteins expressed in the pMAL-vector system

The purification of MBP–$(pep)_{Xa}$–hPAH by affinity chromatography on an amylose resin yielded a homogeneous preparation with a single band of approx. 94 kDa on SDS/PAGE (Figure 1, lane 3). The purified fusion protein had a slightly yellow colour which was removed as a low-molecular-mass component (not further characterized) by size-exclusion chromatography on Superdex 200 HR. At pH 7.0 and high protein concentration (i.e. 12.4 mg/ml) four peaks were resolved on this column (Figure 2a). Peak 1, near the void volume, represented inactive aggregated forms of the fusion protein, whereas the catalytically active forms eluted in three peaks of apparent molecular masses 209, 365 and 631 kDa with a relative proportion of 14%, 70% and 16% respectively. All components revealed high specific activity. At low protein concentrations the percentage of the 209 kDa

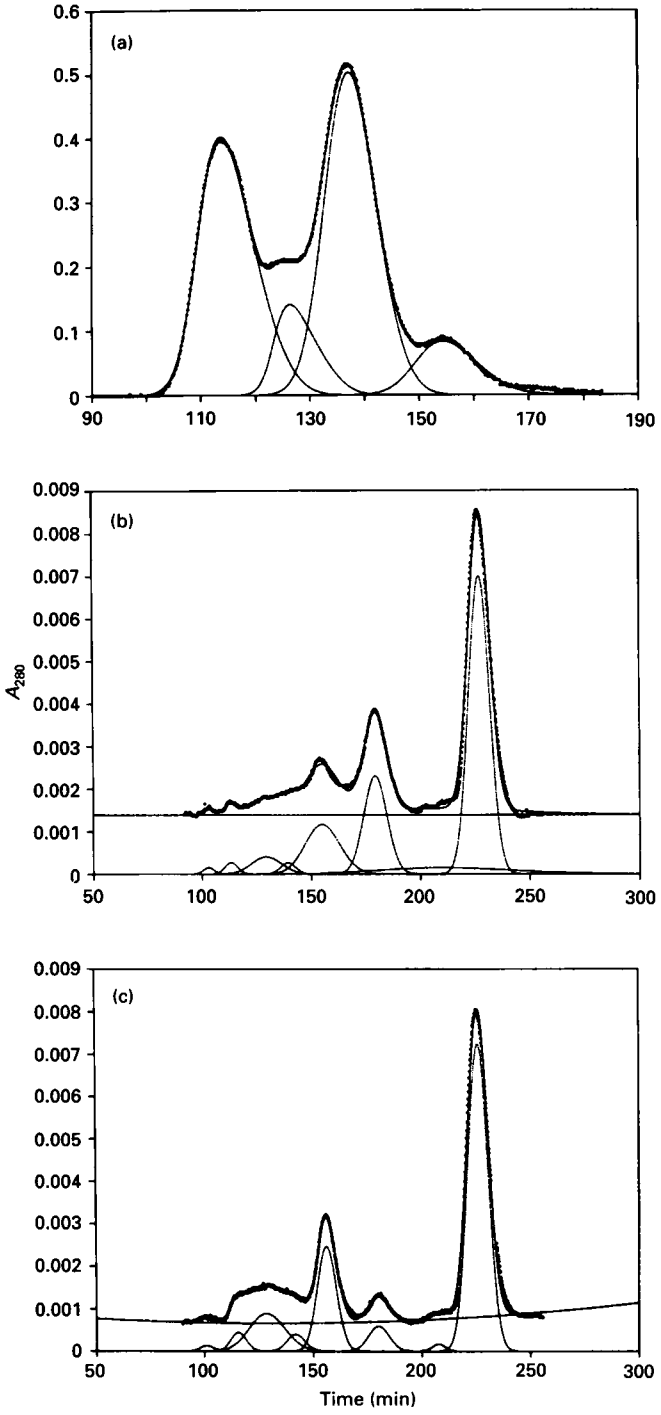

Figure 2 Size-exclusion chromatography

Chromatography on a HiLoad Superdex column (1.6 cm × 60 cm). The column was equilibrated and eluted with 20 mM Hepes and 0.2 M NaCl, pH 7.0, at a flow rate of 0.38 ml/min. Detection was at 280 nm. Data points (●) were collected every 10 s. Gaussian curves were used to fit the data points and measure peak positions. (**a**) Chromatography of the fusion protein MBP–(FX_a)–hPAH (12.4 mg). Peak positions: 113.7 min, aggregated form; 126.6 min, hexameric (631 kDa); 137.2 min, tetramer (365 kDa) and 154.4 min, dimer (209 kDa). (**b**) Chromatography of the cleaved fusion protein (225 μg). Peak positions: 155 min, tetramer of hPAH (209 kDa); 179 min, dimer of hPAH (104 kDa) and 227 min, monomer of MBP (42.5 kDa). (**c**) Chromatography of the cleaved fusion protein (225 μg) in the presence of 1 mM phenylalanine. Same peak positions as in (**b**). Note the high absorbance of MBP, due to its high content of tryptophan. The molecular-mass values of the different enzyme forms were estimated using the elution position of the standard molecular-mass markers as a reference (see the Materials and methods section).

Table 2 Purification of recombinant fusion protein of hPAH expressed in *E. coli* through the pMAL-vector system

The activity was measured with 1 mM phenylalanine and 75 μM BH_4 at 25 °C. The apparent molecular mass was 365 kDa for the tetrameric form and 209 kDa for the dimeric form (Figure 2a). Data for the purification of the fusion protein MBP–$(pep)_{Xa}$–hPAH are shown.

Step	Total protein (mg)	Specific activity (nmol · min^{-1} · mg^{-1})	Yield (%)	Purification (fold)
1. High-speed supernatant	599	298	100	1.0
2. Amylose resin	59	1187	43	4.0
3. Size-exclusion chromatography				
Tetrameric form	24.8	1283	18.6	4.3
Dimeric form	3.4	424	0.8	–

component increased, indicating an equilibrium between the three forms. The fusion protein MBP–$(pep)_{EK}$–hPAH was successfully isolated following the same procedure as for MBP–$(pep)_{Xa}$–hPAH.

Table 2 shows the result of a typical purification experiment, yielding 27.1 mg of the fusion protein MBP–$(pep)_{Xa}$–hPAH with a specific activity of 1283 nmol of tyrosine · min^{-1} · mg^{-1} (tetrameric form) and 424 nmol of tyrosine · min^{-1} · mg^{-1} (dimeric form). The hexameric form of the fusion protein (apparent molecular mass 631 kDa) was not isolated due to its incomplete separation from the aggregated and tetrameric forms (Figure 2a).

Cleavage of the fusion proteins and the phosphorylation of recombinant hPAH by cyclic AMP-dependent protein kinase

The non-aggregated form(s) of the fusion protein MBP–$(pep)_{Xa}$–hPAH was very efficiently cleaved by the restriction protease factor Xa at pH 7.0 (Figure 1, lane 4). The extent of cleavage of the tetrameric form of the fusion protein was measured by SDS/PAGE (Figure 1, lane 4 and Figure 3, left *y*-axis). In addition to the expected specific restriction site (IEGR) a secondary cleavage site (GLGR) was observed on N-terminal sequence analysis of the purified hPAH (for details see below), indicating that the cleavage occurred between the residues Arg-13 and Lys-14 in the hPAH sequence. Full-length hPAH could not be separated from the enzyme lacking the 13 N-terminal residues (Δ13-hPAH) by the standard conditions of SDS/PAGE (Figure 1, lane 4). The proportion of full-length hPAH after cleavage could, however, be determined by measuring the phosphorylation of the enzyme by cyclic AMP-dependent protein kinase. As shown for rat and human liver PAH (Abita et al., 1976; Døskeland et al., 1984; Smith et al., 1984), the recombinant hPAH obtained from the pMAL expression system also incorporated phosphate at Ser-16 when incubated with [γ-^{32}P]ATP and the catalytic subunit of cyclic AMP-dependent protein kinase. The uncleaved fusion protein MBP–$(pep)_{Xa}$–hPAH incorporated up to 0.97 mol of phosphate per mol of subunit (Figure 3a). The incorporation of phosphate progressively decreased following cleavage of the fusion protein by factor Xa (Figure 3a, right *y*-axis legend). The stoichiometry of phosphorylation corresponded to the proportion of full-length hPAH as determined by N-terminal sequence analyses of the further purified enzyme, confirming that only full-length hPAH was phosphorylated. The recovery of full-length hPAH varied depending on the reaction temperature and time (Figure 3a), as well as the protein:protease ratio. Thus, the cleavage of the fusion protein (at 2 mg/ml) was approx. 95% within 2 h at 0 °C in the presence of 22 μg/ml of factor Xa (Figure 1, lane 4 and Figure 3a), with a recovery of about 50% full-length hPAH.

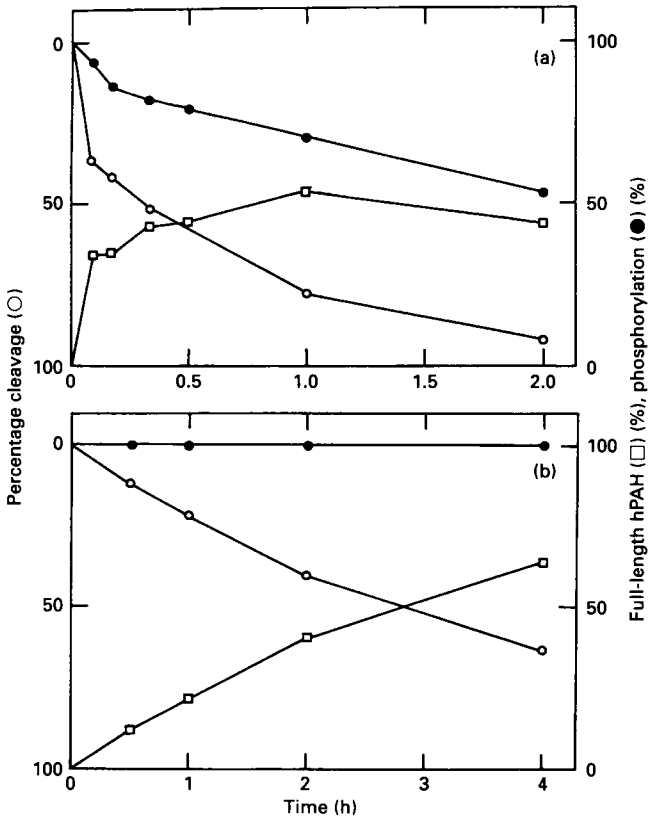

Figure 3 Time course for the cleavage of the fusion proteins by restriction proteases

(**a**) Cleavage of MBP–$(pep)_{Xa}$–hPAH by factor Xa at 0 °C (ratio of protease:protein = 1:90), and (**b**) cleavage of MBP–$(pep)_{EK}$–hPAH by enterokinase at 25 °C (ratio 1:50). At different time points during proteolysis aliquots of the reaction mixture were taken out and protease inhibitors were added (see the Materials and methods section). Samples were then phosphorylated (30 min with 67 nM C-subunit of the cyclic AMP-dependent protein kinase) and analysed by SDS/PAGE. The percentage cleavage (○) was estimated by densitometric scanning of the Coomassie Blue-stained gels, and the percentage phosphorylation (●) from the amount of [γ-^{32}P]ATP incorporated per mol of subunit. The percentage full-length hPAH (□) was estimated from the mobility shift of the phosphorylated hPAH on SDS/PAGE (see Figure 5).

Longer incubation times, higher temperature and lower protein:protease ratios favoured the unspecific cleavage and recovery of Δ13-hPAH.

The fusion protein MBP–$(pep)_{EK}$–hPAH was specifically cleaved by enterokinase at its target sequence (D_4K). Only full-length hPAH resulted from this cleavage, as demonstrated by N-

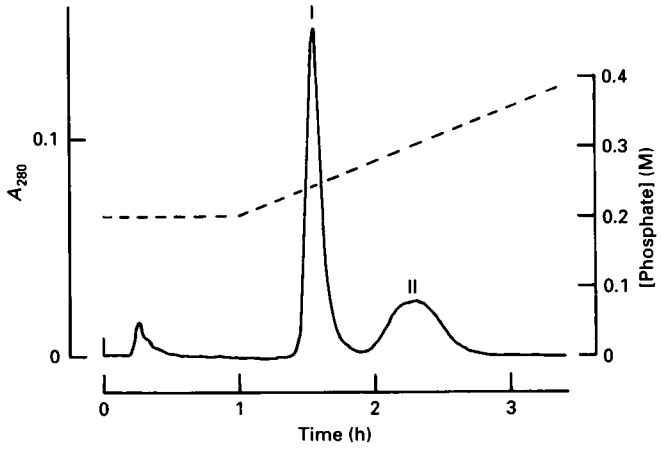

Figure 4 Hydroxylapatite chromatography of the fusion protein MBP–$(pep)_{Xa}$–hPAH after cleavage by restriction protease factor Xa

The cleaved fusion protein (dissolved in 10 mM Tris/HCl, 0.2 M NaCl, pH 7.4) was adsorbed to the column and eluted by a linear sodium phosphate gradient (0.2–0.4 M) at pH 7.2 and at a flow rate of 1 ml/min (dotted line). Peaks I and II represent MBP and hPAH, respectively.

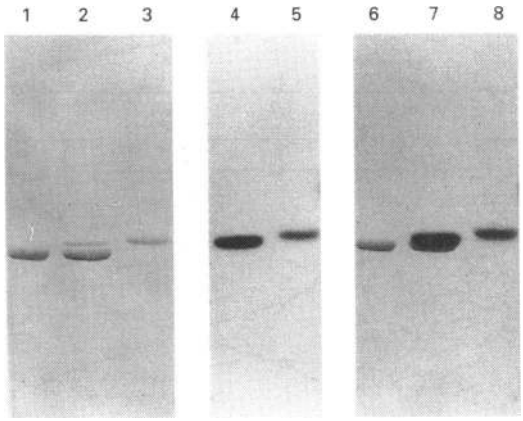

Figure 5 Phosphorylation of recombinant hPAH by the catalytic subunit of cyclic AMP-dependent protein kinase

Lane 1, SDS/PAGE of purified recombinant hPAH (mixture of full-length and Δ13-hPAH), non-phosphorylated; lane 2, same as in lane 1 after incorporation of ~ 0.1 mol of phosphate per mol of subunit; lane 3, rat liver PAH after incorporation of ~ 0.09 mol of phosphate per mol of subunit; lanes 4 and 5, autoradiogram of the SDS/PAGE gel shown in lanes 2 and 3. Lanes 6–8, the autoradiogram in lanes 4 and 5 overlayed the Coomassie Brilliant Blue-stained SDS/PAGE gel in lanes 1–3.

terminal sequence analysis of the further purified hPAH and a high stoichiometry of phosphorylation at Ser-16 (see below and Figure 3b). This proteolytic cleavage was slower than that of MBP–$(pep)_{Xa}$–hPAH by factor Xa (Figure 3a).

Purification of recombinant hPAH

After cleavage of the fusion proteins by either factor Xa or enterokinase, hPAH was further purified at large scale either by hydroxylapatite chromatography (Figure 4) or by chromatography on a Protein-Pak DEAE HR column (results not shown). The pure enzyme was dialysed against 20 mM Na-Hepes/0.15 M NaCl, pH 7.0, and concentrated by ultrafiltration. The yield of

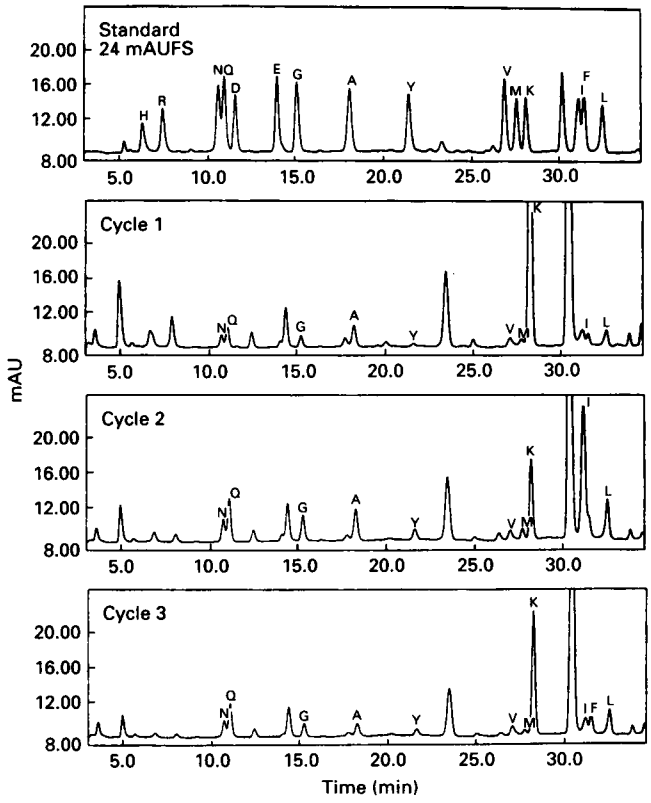

Figure 6 Chromatograms showing the result of the automated C-terminal protein sequencing performed using the chemical degradation scheme

(**a**) Amino acid standards. (**b**) K as first residue. (**c**) I as the second residue. (**d**) K as the third residue. Sequencing was performed as described in the Materials and methods section and the absorbance was monitored at 254 nm. AUFS, absorption units full scale.

highly purified hPAH was approx. 10 mg/l of culture with a specific activity of 1440 and 2320 nmol of tyrosine·min^{-1}·mg^{-1} with BH_4 and 6-MPH_4 as the cofactor respectively, after freezing/storage in liquid nitrogen. hPAH was also successfully separated from MBP by high-performance size-exclusion chromatography on Superdex 200 HR (Figure 2b), which revealed an equilibrium between the tetrameric and dimeric forms. Preincubation of the cleaved fusion protein with 1 mM phenylalanine for 30 min at 4 °C and chromatography also in the presence of 1 mM phenylalanine increased the recovery of tetrameric and aggregated forms of hPAH relative to the dimeric form (Figure 2c). The oligomeric structure was similar for the cleaved fusion protein obtained either by factor Xa or enterokinase.

As previously found for the human liver enzyme (Cotton et al., 1988), the electrophoretic mobility of hPAH on SDS/PAGE was slightly faster than that of the rat enzyme (Figure 5, lanes 1 and 3). High-resolution IEF gels gave only a single band with a p*I* of about 5.0 (figure not shown).

The amino acid sequence of hPAH contained an N-terminal sequence as predicted from the cDNA sequence (i.e. NH_2-STAVLENPG...), and when the fusion protein was cleaved by factor Xa a variable proportion of the additional sequence (NH_2-KLSDFGQET...) was also obtained due to the unspecific cleavage.

The C-terminal sequence data further confirmed the isolation of a full-length recombinant hPAH. The C-terminal sequence

Table 3 Some catalytic and physicochemical properties of recombinant hPAH compared with those of PAH isolated from human and rat liver

Abbreviation: n.d., not determined.

Property	Recombinant hPAH	Human liver PAH	Rat liver PAH
Activation by phenylalanine	+	n.d.	+[a]
pH optimum with 6-MPH_4	~ 7.0	7.0[b]	7.0[a]
Molecular mass			
Non-phosphorylated	~ 50 kDa	~ 49 kDa[c]	~ 51 kDa[d]
Phosphorylated	~ 51 kDa	~ 50 kDa[c]	~ 51 kDa[d]
pI	5.0	5.0–5.3[e]	5.2–5.5[f]
Quaternary structure	Tetramer/dimer, higher oligomers, aggregates	Tetramer/dimer[g], aggregates[b]	Tetramer/dimer[f]

[a] Kaufman (1993). [b] Woo et al. (1974). [c] Smith et al. (1984). [d] This paper. [e] Petruschka et al. (1990). [f] Døskeland et al. (1982). [g] Woolf (1976).

Table 4 Michaelis constants for recombinant hPAH

The apparent K_m values for L-phenylalanine (L-Phe), (6*R*)-L-*erythro*-tetrahydrobiopterin (BH_4) and 6-MPH_4 were determined for the tetrameric form of the purified fusion protein MBP–$(pep)_{Xa}$–hPAH (365 kDa; Figure 2a), the fusion protein after cleavage by factor Xa and hPAH purified from the cleaved fusion protein by hydroxylapatite chromatography (Figure 4). Standard assay conditions and variable concentrations of L-Phe (0–5 mM), BH_4 (0–100 μM) and 6-MPH_4 (0–500 μM) were used. The cofactor used in the assay is given in the parentheses. The numbers represent the K_m values ± S.E.M. calculated by non-linear regression analysis. Abbreviation: n.d., not determined.

	Apparent K_m			
Enzyme	L-Phe (BH_4) (μM)	L-Phe (6-MPH_4) (μM)	BH_4 (μM)	6-MPH_4 (μM)
Fusion protein	194 ± 8	504 ± 11	31 ± 2	73 ± 3
Cleaved fusion protein	236 ± 11	n.d.	25 ± 1	n.d.
Purified hPAH	175 ± 22	382 ± 15	29 ± 3	88 ± 3

obtained for the enzyme isolated through the cleavage of both MBP–$(pep)_{Xa}$–hPAH and MBP–$(pep)_{EK}$–hPAH fusion proteins ... LQKIK-COOH was the one expected from the cDNA sequence. Figure 6 shows the chromatograms for the amino acid standards and the first three residues.

Kinetic and physicochemical properties of recombinant hPAH

Some kinetic and physicochemical properties of the purified recombinant hPAH are summarized in Table 3 and compared with those reported for PAH isolated from human and rat liver. The apparent Michaelis constants for L-Phe, BH_4 and 6-MPH_4, determined for the fusion protein MBP–$(pep)_{Xa}$–hPAH, the cleaved fusion protein and the purified hPAH (mixture of full-length and Δ13-hPAH), are given in Table 4. The kinetic properties of hPAH isolated from MBP–$(pep)_{EK}$–hPAH (only full-length hPAH) were similar to those shown in this Table and its phosphorylation resulted in a slight decrease in the apparent K_m for BH_4 (to about 18 μM) and a 1.2-fold increase in the apparent $V_{max.}$. All enzyme preparations were found to be activated to a variable extent by preincubation with 1 mM L-phenylalanine, when the activity was measured with the natural cofactor BH_4: the uncleaved fusion proteins > cleaved fusion proteins > purified hPAH. Moreover, Fe(II) (added as ferrous ammonium sulphate) increased the activity by about 30% in enzyme preparations from bacterial cultures grown in the absence of iron, but only about 10% in enzyme preparations obtained from cultures grown with 0.2 mM ferrous ammonium sulphate, with half-maximal activation at 12 μM at pH 6.8. The pH optimum for the activity of purified hPAH was about 7.0 as reported for human liver enzyme (Table 3) and for the partially purified recombinant human enzyme from *E. coli* (Ledley et al., 1987).

Stoichiometry of phosphorylation of recombinant hPAH

hPAH obtained by cleavage of the fusion protein MBP–$(pep)_{Xa}$–hPAH by factor Xa, representing a mixture of full-length and Δ13-hPAH, incorporates at maximum about 0.5 mol of phosphate per mol of subunit (Figure 3a). hPAH obtained by cleavage of the fusion protein MBP–$(pep)_{EK}$–hPAH by enterokinase (only full-length hPAH) incorporated up to 0.97 mol of phosphate per mol of subunit (Figure 3b). As previously found for the enzyme isolated from human and monkey livers (Smith et al., 1984), phosphorylation of the recombinant hPAH was accompanied by a change in the electrophoretic mobility on SDS/PAGE (Figure 5), the phosphorylated enzyme giving a slightly lower electrophoretic mobility (~ 51 kDa) than the non-phosphorylated enzyme (~ 50 kDa). It is interesting to note that the phosphorylated form of hPAH co-migrates with the rat liver enzyme the electrophoretic mobility of which is not altered by phosphorylation (Figure 5).

DISCUSSION

Expression and purification of recombinant hPAH

In the present study, the pMAL expression vector has been used successfully to produce catalytically active hPAH at high levels in *E. coli*. In agreement with our previous observations (Knappskog et al., 1993) it was not possible to avoid limited proteolysis of the enzyme using the pET–hPAH construct in the HMS174(DE3) or BL21(DE3) hosts, and purification of homogeneous, full-length hPAH was unsuccessful. By contrast, expression in the pMAL system in the TB1 host circumvented the proteolytic degradation of hPAH by host-cell proteases, and purification of the enzyme was also greatly facilitated. Thus, recombinant hPAH was most conveniently purified in the fusion protein form. The yield of catalytically active fusion protein was high (approx. 20%) and after cleavage of the fusion protein with

the restriction proteases, pure preparations of recombinant hPAH were obtained either by chromatographic separation on hydroxylapatite or on Protein-Pak DEAE HR, in both cases with a high yield ($\sim$ 10 mg/l of culture medium).

Characteristics of recombinant hPAH

The purified recombinant hPAH revealed kinetic and physicochemical properties similar to those previously reported for the enzyme isolated from human liver (Table 3), including the activation by phenylalanine, the pH optimum for activity (approx. 7.0), subunit molecular mass (approx. 50 kDa) for the non-phosphorylated form, pI (approx. 5.0), subunit oligomerization (tetramer/dimer in equilibrium and aggregated forms) and the phosphorylation by cyclic AMP-dependent protein kinase. The apparent Michaelis constants for Phe, BH_4 and 6-MPH_4 (Table 4) were similar to those reported for PAH activity in human liver and for partially purified recombinant hPAH (Ledley et al., 1987). It is interesting to note that the non-cleaved and the cleaved fusion protein MBP–$(pep)_{Xa}$–hPAH had apparent K_m values comparable with those of the purified hPAH, indicating that the accessibility of the substrates to the active site is not affected by the fusion partner (MBP). The recombinant hPAH was activated to a variable extent by L-phenylalanine, i.e. the uncleaved fusion proteins > cleaved fusion proteins > purified hPAH, in line with the reported effects of purification of the rat liver enzyme on the degree of substrate activation (for review, see Kaufman, 1993).

PAH isolated from human liver has been reported to represent a mixture of dimeric, tetrameric and aggregated forms (Woo et al., 1974; Choo et al., 1979; Yamashita et al., 1985). By gel-permeation chromatography we have found that the active form of recombinant hPAH is a mixture of tetramers and dimers, in equilibrium. As previously described for the bovine (Døskeland et al., 1982) and rat liver (Parniak, 1990) enzymes, this equilibrium is shifted towards the tetrameric form by incubation with phenylalanine.

Both the fusion proteins and the purified hPAH obtained after cleavage of the fusion protein MBP–$(pep)_{EK}$–hPAH by enterokinase (full-length hPAH) are phosphorylated by cyclic AMP-dependent protein kinase with a high stoichiometry, i.e. about 1 mol of phosphate per mol of subunit. Although Abita et al. (1983) failed to incorporate phosphate in their enzyme preparation obtained from human liver, Smith et al. (1984) found that purified hPAH, treated with alkaline phosphatase, incorporated up to 0.67 mol of phosphate per mol of subunit. The enzyme was phosphorylated at a single serine residue, changing the apparent molecular mass from approx. 49 kDa to approx. 50 kDa. By immunoblotting it was also found that both forms are present in cytosolic extracts of human and monkey liver, indicating that the enzyme is partially phosphorylated in both species (Smith et al., 1984; Cotton et al., 1988; Chestkov et al., 1992). Our preparations of recombinant hPAH also undergo this change in electrophoretic mobility following phosphorylation. The mobility shift is in the opposite direction of that expected for the introduction of an additional negative charge, and is most likely explained by a change in conformation of the SDS-denatured state of the enzyme subunit. Full phosphorylation of hPAH also resulted in a small activation of the enzyme. Moreover, our N-terminal sequence analyses also suggest that the site of phosphorylation in the human enzyme is Ser-16, similar to rat PAH (Wretborn et al., 1980; Ledley et al., 1985b), since the truncated enzyme form (Δ13-hPAH), obtained by the unspecific cleavage by factor Xa between Arg-13 and Lys-14, failed to incorporate any phosphate. Arg-13 is part of the established recognition site for the cyclic AMP-dependent protein kinase and the sequence at residues 13 to 21 is identical for the rat and the human enzymes (Kwok et al., 1985). Thus, phosphorylation can be used as a valuable probe to check the integrity of the N-terminal sequence of the human enzyme.

Strategy for expression of mutant forms of hPAH

A general problem faced with in the characterization of mutant forms of hPAH leading to PKU and hyperphenylalaninaemias, has so far been the expression of the full-length mutant enzymes in sufficient quantities. Thus, the limited proteolysis observed in the pET expression system for wild-type hPAH was even more pronounced in some of the mutant forms (P. M. Knappskog and T. Flatmark, unpublished work). Our preliminary studies have shown that expression in the pMAL system greatly facilitates the production of recombinant mutant forms. Their detailed structural and functional properties can now be studied and compared with those of the wild-type enzyme.

Mr. Ali J. Sepulveda Muñoz is thanked for expert technical assistance. We are grateful to Professor S. L. C. Woo for supplying the hPAH cDNA clone, Professor S. Elsayed for the N-terminal amino acid sequence analyses and Pau-Miau Yuan and Victoria L. Boyd at Perkin–Elmer, AB Division for the C-terminal sequence analysis. This study was supported by grants from the Research Council of Norway (A.M., P.M.K. and T.F.) as well as by grants (T.F.) from The Nordic Insulin Foundation, The Nansen Fund, The Blix Family Fund for the Advancement of Medical Research, The Norwegian Council on Cardiovascular Diseases and the Norwegian Cancer Society.

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Received 14 July 1994/24 October 1994; accepted 8 November 1994