Recombinant human phenylalanine hydroxylase is a substrate for the ubiquitin-conjugating enzyme system

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Mammalian phenylalanine hydroxylase (PAH) catalyses the conversion of L-phenylalanine to L-tyrosine in the presence of dioxygen and tetrahydrobiopterin; it is a highly regulated enzyme. Little is known about the rates of synthesis and degradation of PAH *in vivo*. The enzyme has been reported to have a half-life of approx. 2 days in rat liver and 7–8 h in rat hepatoma cells, but the mechanism of its degradation is not known. In the present study it is shown that the tetrameric form of the recombinant

wild-type human enzyme is a substrate for the ubiquitin-conjugating enzyme system in the cytosolic fraction of rat testis. Our findings support the conclusion that multi-/poly-ubiquitination of human PAH plays a key role in the turnover of this cytosolic liver enzyme and provides a mechanism for the increased turnover observed for a number of recombinant mutant forms of the enzyme related to the metabolic disorder phenylketonuria, when expressed in eukaryotic cells.

INTRODUCTION

Vertebrates have a high level of liver phenylalanine hydroxylase (PAH; phenylalanine 4-mono-oxygenase, EC 1.14.16.1). This finding, combined with the irreversibility of the hydroxylation reaction in which L-tyrosine is formed, the inability of vertebrates to synthesize L-phenylalanine *de novo* and the necessity of maintaining a stable pool of L-phenylalanine and L-tyrosine for protein synthesis, generates a significant challenge in terms of metabolic control (reviewed in [1,2]). In addition to regulation by covalent or non-covalent changes affecting its kinetic properties (reviewed in [3]), regulation can also be affected by altering the amount of available enzyme. This has been clearly demonstrated in eukaryotic expression studies of recombinant mutant forms of the human enzyme related to the autosomal recessive disease phenylketonuria (PKU). Thus in one of the PKU mutations in exon 2 (G46S) the mutant protein has been shown to have an increased tendency to form inactive abnormal (aggregated) forms that are rapidly degraded when expressed in human embryonic kidney (A293) cells [4]. The possibility has therefore been considered that a ubiquitin (Ub)-dependent proteolytic pathway might be involved in the process [4].

The Ub/proteasome system is the main eukaryotic non-lysosomal protein degrading system (reviewed in [5,6]). The substrate selectivity of this pathway is thought to be mediated in part by members of a large family of Ub-activating/-conjugating enzymes, which catalyse the covalent attachment of Ub to protein substrates [7–10]. Whereas the Ub/proteasome system has been well characterized in liver [11–13], the specific substrates to be degraded by the system have been less well characterized [11]. The purpose of the present study is to demonstrate that one specific liver protein, i.e. the cytosolic enzyme [14] PAH, is a substrate for the Ub-conjugating enzyme system by using the cytosolic fraction of rat testis as the enzyme source and ¹²⁵I-labelled Ub as a marker. The findings also support our recent proposal of a mechanism for the rapid degradation of a PKU mutant form of human PAH (hPAH) [4].

MATERIALS AND METHODS

Materials

Bovine Ub (Sigma, St. Louis, MO, U.S.A.) was labelled with Na¹²⁵I by the chloramine-T method [15] to a specific radioactivity of 8000 c.p.m./pmol. The concentration of Ub was calculated on an *M_r* of 8500 [16]. Carrier-free Na¹²⁵I and Hyperfilm TM-β-max were obtained from Amersham (Bucks., U.K.). Urea and glycine were from Merck (Darmstadt, Germany). Tris was from Boehringer-Mannheim (Germany). Mixed-bed resin AG501-X8 (D) 20–50 mesh, agarose isoelectric focusing, Bio-Lyte 5–7 and Bio-Lyte 3–10 were obtained from Bio-Rad (Richmond, CA, U.S.A.). CHAPS, SDS and all other electrophoresis reagents were from Sigma. Ampholine pH 5–7 was from Pharmacia Biotech (Uppsala, Sweden). Recombinant hPAH was expressed as a fusion protein in the pMAL system of *Escherichia coli*, and isolated as tetrameric Δ13-hPAH (a truncated form of hPAH lacking the 13 N-terminal residues) as described previously [17,18]. The enzyme was purified to homogeneity as a homotetramer.

Polyclonal antibodies against recombinant hPAH were prepared in rabbits and purified on an immunoaffinity column of Δ13-hPAH coupled to cyanogen bromide-activated Sepharose as recommended by Pharmacia Biotech. Anti-Ub serum was from Boehringer-Mannheim. Peroxidase-conjugated antibodies (goat anti-rabbit IgG) were from Bio-Rad. Mouse monoclonal anti-(20 S proteasome) was from American Research Products (Belmont, MA, U.S.A.).

Immunoblot analysis of Ub

For Western blot analysis, proteins were separated by SDS/PAGE [12.5% (w/v) gel] and blotted electrophoretically on nitrocellulose membrane (BA 85, Schleider & Schuell, Dassel, Germany). The blotted proteins were probed with anti-Ub serum at the recommended working concentration of 2 μg/ml. The secondary antibody was goat anti-rabbit IgG conjugated with

Abbreviations used: Δ13-hPAH, a truncated form of human phenylalanine hydroxylase lacking the 13 N-terminal residues; DTT, dithiothreitol; hPAH, human phenylalanine hydroxylase; PAH, phenylalanine hydroxylase; PKU, phenylketonuria; Ub, ubiquitin.

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horseradish peroxidase. Ub-protein conjugates were revealed with enhanced chemiluminescence reagents from Amersham.

Preparation of a cytosolic fraction from rat testis

Male Wistar rats of approx. 200 g body weight were obtained from Møllegaard (Ejby, Denmark). The testes were quickly excised and fine-minced in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM dithiothreitol (DTT), and homogenized (1 g of tissue to 0.5 ml of buffer) in a Potter-Elvehjem glass-Teflon homogenizer (three strokes at 720 rev./min). After centrifugation at 230 000 *g* for 20 min at 4 °C in a Beckman TL100 ultracentrifuge, the high-speed supernatant (cytosolic fraction) was aspirated with a syringe that was carefully passed through the floating layer of lipids. ATP at a final concentration of 0.5 mM was added to the crude fractions (58 mg/ml protein) as recommended [19] to stabilize the Ub-activating and Ub-conjugating enzymes, and stored at -80 °C until use.

Ubiquitination of recombinant hPAH

To examine whether Ub-hPAH conjugates are formed, a system was reconstituted *in vitro* with ¹²⁵I-labelled Ub as a substrate and the cytosolic fraction of rat testis as a rich source of Ub-activating and Ub-conjugating enzymes [20]. The formation of Ub-protein conjugates was measured at 37 °C in incubations of 0.05–0.1 ml final volume containing 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 1 mM ATP, 0.5 mM DTT, an aliquot of rat testis extract (20 µl per 100 µl assay), anti-(20 S proteasome) (4 µl per 100 µl) and protease inhibitors (1 mM benzamidine, 0.2 mM PMSF, 20 µg/ml leupeptin, 14 µg/ml pepstatin and 0.5 mg/ml soybean trypsin inhibitor). The concentration of unlabelled Ub present in the testis extract [21] was estimated to be 12–24 µM in assay. The reaction was started by the concomitant addition of Δ13-hPAH (final concentration 8 or 16 µM) and ¹²⁵I-labelled Ub (final concentration 5–13 µM). Aliquots were taken at the indicated times and quenched first with trichloroacetic acid or added directly into the Laemmli sample buffer, heated to 90 °C for 5 min and subjected to SDS/PAGE.

Immunoisolation of PAH

Affinity-purified polyclonal anti-hPAH was added to the ubiquitination assay mixture after 20 min of incubation/reaction (the ratio of antibody to hPAH was 8:1) and gently mixed for 2 h on a rotating wheel. The immunoadsorbent was Protein A-Sepharose (corresponding to 8 mg of dry beads) suspended and washed in 50 mM potassium phosphate, pH 7.0, and resuspended in the same buffer containing 10 mM EDTA, 3 mM EGTA, 0.5 mM DTT, 1 mM benzamidine, 0.2 mM PMSF, 20 µg/ml leupeptin, 14 µg/ml pepstatin and 0.5 mg/ml soybean trypsin inhibitor. For immunoisolation the beads were mixed with the samples and rocked in Eppendorf tubes for 1 h at 4 °C. The Protein A-Sepharose with bound IgG-PAH was pelleted by centrifugation at 12 000 *g* for 15 s, washed nine times with phosphate buffer containing 0.2 % Triton X-100 and finally with the same buffer without Triton X-100. The pellet was then heated (56 °C, 10 min) in sample buffer [22] and centrifuged before analysis by SDS/PAGE.

Gel electrophoresis and autoradiography

Aliquots (20–40 µl) of the final supernatant obtained after immunoisolation (see above) or of the ubiquitination assay were applied to 1 or 1.5 mm polyacrylamide slab gels [10 % (w/v)] for SDS/PAGE [22]. Standard *M_r* markers used were BSA (66 000), ovalbumin (45 000), carbonic anhydrase (29 000) and trypsinogen

(24 000). The proteins were stained with Coomassie Brilliant Blue and radioactive gels were dried and autoradiographed with preflashed Kodak XAR-5 film or Hyperfilm TM-β-max.

Two-dimensional gel electrophoresis

At the end of the ubiquitination reaction (20 min) the proteins were precipitated with ice-cold trichloroacetic acid [10 % (v/v) final concentration] and kept on ice for 20 min. The pellets were washed three times with diethyl ether, dried and dissolved in lysis medium [9.5 M urea/2 % (w/v) CHAPS/1.6 % (w/v) Bio-Lyte pH 5–7/0.4 % Bio-Lyte pH 3–10/100 mM DTT] and kept at -20 °C until use. Proteins from the ubiquitination assay were resolved by two-dimensional electrophoresis [23]. The proteins contained in 50 µl of lysate were first separated by isoelectric focusing on a 0.25 cm × 14 cm cylindrical gel [4 % (w/v) acrylamide/9.5 M urea/2 % (w/v) CHAPS/3.2 % (w/v) carrier ampholytes 5–7/0.8 % carrier ampholytes 3–10] for 17 h at 1200 V (0.33 mA per gel). The gels were then equilibrated for 20 min in a buffer containing 0.06 M Tris/HCl, pH 6.8, 2 % (w/v) SDS, 100 mM DTT and 10 % (v/v) glycerol, and used immediately or frozen in ethanol cooled to -80 °C on dry ice. The proteins were further separated by SDS/PAGE (16 cm long and 1 mm thick) of 10 % (w/v) acrylamide with a 4 % (w/v) stacking gel, as described in [22]. Electrophoresis was performed at 10 °C and the current was adjusted to a starting voltage of 200 V increasing gradually to the imposed limit of 360 V. The tracking dye Bromophenol Blue reached the end of the gel after approx. 3 h. The gels containing proteins labelled with ¹²⁵I-labelled Ub were stained with Coomassie Brilliant Blue, dried on a cellophane sheet and exposed to Amersham Hyperfilm for 5–15 days.

Protein determination

Protein was determined by the Bradford dye-binding method [24] with BSA as a standard.

RESULTS AND DISCUSSION

Ubiquitination of endogenous protein substrates in the cytosolic fraction of rat testis

The rat testis expresses high levels of the non-specific Ub-activating and Ub-conjugating enzymes [20]. As expected [7,25], therefore, we found several Ub-protein conjugates in the cytosolic fraction on Western blot analysis, using affinity-purified polyclonal Ub antibodies (Figure 1A, lane 2), especially in the high-*M_r* region. With ¹²⁵I-labelled Ub, the activities of the conjugating enzymes were measured in the reconstituted assay system *in vitro* as described in the Materials and methods section, with endogenous proteins as the substrate. From Figure 1(A) (lane 3), it is seen that several cytosolic proteins were ubiquitinated, and the amount of ¹²⁵I-labelled Ub-protein conjugates accumulated as a function of time, reaching a maximum at approx. 30 min for the high-*M_r* (more than 100 000) proteins (Figure 2a). The subsequent decrease in ¹²⁵I labelling of these proteins (Figure 2a) and a concomitant increase in the level of free ¹²⁵I-labelled Ub (Figure 2e) indicates a progressive degradation of Ub conjugates even in the presence of protease inhibitors, probably by the proteasome complex also present in the rat testis cytosolic fraction [26]. Furthermore we found that the time course of the appearance of ¹²⁵I-labelled Ub-protein conjugates in the medium and low *M_r* range (Figures 2b and 2c) revealed a more stable ¹²⁵I-labelling pattern than of the proteins in the high *M_r* range.

The inclusion of protease inhibitors in the standard assay was found to result in an increased recovery of ¹²⁵I-labelled Ub-protein conjugates by reducing their rate of degradation by prote-

Figure 1 Ubiquitination of recombinant hPAH *in vitro*

(A) Immunodetection of Ub-protein conjugates in the cytosolic fraction of rat testis. The proteins in the cytosolic fraction of rat testis were resolved by SDS/PAGE [12.5% (w/v) gel], stained with Coomassie Brilliant Blue (lane 1) or analysed for Ub-protein conjugates by Western blot analysis using affinity-purified anti-Ub as the primary antibody (lane 2). Lane 3, ubiquitination of proteins with ^{125}I -labelled Ub as a marker; for standard incubation conditions see the Materials and methods section. The lane shows ^{125}I -labelled Ub-protein conjugates formed during incubation for 20 min in the absence of hPAH resolved by SDS/PAGE in 10% (w/v) gel and subjected to autoradiography. The regions I to V in the gel shown in lane 3 correspond to proteins with M_r values above 100 000 (region I), from 100 000 to 58 000 (region II), from 58 000 to 37 000 (region III), from 37 000 to 26 000 (region IV) and below 26 000 (region V). (B) Immunodetection of ^{125}I -labelled Ub-PAH conjugates. Ubiquitination was performed as in lane 3 of (A), in the absence (lanes 4 and 6) or presence (lanes 5 and 7) of PAH. After 20 min of incubation, hPAH was immunoprecipitated (see the Materials and methods section) and analysed by SDS/PAGE [10% (w/v) gel]. Lanes 4 and 5 represent Coomassie Brilliant Blue-stained gels, and lanes 6 and 7 are autoradiograms of lanes 4 and 5. The protein subunits with an apparent M_r of 52 000 (lanes 4 and 5) correspond to that of heavy-chain IgG. The arrow indicates the position of $\Delta 13$ -hPAH (M_r 50 000 for the subunit).

asomes and by protecting against the inactivation of Ub [27,28]. When the assay was performed in the absence of added MgATP^{2-} , the formation of Ub-protein conjugates was decreased (results not shown), as expected from the ATP dependence of the reaction [29].

Ubiquitination of recombinant hPAH

Owing to the presence of several endogenous substrates for the Ub-conjugating enzyme system of rat testis (Figures 1A and 2), ^{125}I -labelled Ub was routinely used in our studies to see whether hPAH forms Ub conjugates in a cell-free system. However, owing to the high background labelling of endogenous proteins in the high- M_r region (Figures 1A and 2a) direct analysis of the reaction products by SDS/PAGE could not be performed.

However, on immunoprecipitation of hPAH based on affinity-purified polyclonal hPAH antibodies, SDS/PAGE (Figure 1B, lane 7) revealed several distinct ^{125}I -labelled Ub-protein conjugates of $M_r > 50 000$ (the M_r of the hPAH subunit) as well as a less distinct labelling pattern in the very high- M_r region. Because the control experiments, i.e. with no hPAH present in the assay mixture, recovered no detectable ^{125}I -labelled proteins (Figure 1B, lane 6), it can be concluded that a clean immunoprecipitation of hPAH was achieved. The results therefore indicate that hPAH is mono- and multi-/poly-ubiquitinated in our assay system *in vitro*. Additional support for this conclusion was obtained by

Figure 2 Time course of the ubiquitination of rat testis cytosolic proteins in the absence and presence of hPAH

Ubiquitination was performed by the standard assay procedure in the absence (○) or presence (▲) of 16 μM hPAH (see the Materials and methods section). At the indicated time points the reaction was stopped and the ^{125}I -labelled Ub-protein conjugates were analysed by SDS/PAGE. Gel regions I–V, as indicated in lane 3 of Figure 1(A), were cut out and the radioactivity counted by a γ -counter. Quantitative analyses for the radioactivity of the five regions are given in (a) to (e) respectively.

two-dimensional electrophoresis of the reaction mixture without immunoprecipitation. It should be particularly noted that our purified recombinant hPAH revealed a microheterogeneity of the subunit (Figure 3A), with a pattern similar to that previously reported for the enzyme as isolated from human liver [30–33]. The four or five species of the recombinant hPAH observed had the same M_r (approx. 50 000), but differed slightly in pI, and they are most probably explained by a non-enzymic deamidation, a very common post-translational modification of cellular proteins [34]. When the profile of ^{125}I -labelled proteins including hPAH was analysed by two-dimensional electrophoresis (Figure 3B), compared with the Coomassie Blue-stained two-dimensional gels of a similar sample (Figure 3A) and with the pattern of ^{125}I -labelled endogenous proteins in the cytosolic fraction (Figure 3C), it is evident that hPAH generates several ^{125}I -labelled Ub-protein conjugates.

The conjugates differ in M_r , as expected from SDS/PAGE (Figure 1B), as well as in pI, as expected from the microheterogeneity in terms of pI of the enzyme as isolated (Figure 3A). Most of the radioactive spots occur near or above the

Figure 3 Multi-/poly-ubiquitination of hPAH as revealed by two-dimensional electrophoresis

Ubiquitination was performed for 20 min by the standard assay procedure in the absence or presence of 8 μ M hPAH, and then quenched by the addition of trichloroacetic acid (for preparation of samples and methods for two-dimensional electrophoresis see the Materials and methods section). (A) Coomassie Brilliant Blue-stained proteins from the reaction mixture containing hPAH. (B, C) Autoradiograms of two-dimensional electrophoresis of the reaction mixture in the presence (B) or absence (C) of hPAH. Arrow-heads in (A) mark the multiple molecular forms of PAH. Braces mark the 125 I-labelled Ub-PAH conjugates. Ub is identified by an arrow in (B). Abbreviation: IEF, isoelectric focusing.

parent protein. The apparent M_r of the conjugates resulting from the covalent addition of one or several Ub molecules to the protein do not always correspond to defined molecular species of PAH. The anomalous migration on SDS/PAGE of the conjugates is not surprising and has been reported for other proteins (for example, see [35]). Thus the addition of only one phosphate group to the hPAH (at Ser-16) is sufficient to confer an apparent lower mobility of the protein [17,18].

Figure 2 shows the time course of the ubiquitination of endogenous proteins in four M_r regions (I and IV in Figure 1A) and the effect of added 16 μ M hPAH. The overall incorporation of radioactivity in proteins was at maximum labelling estimated to be approx. 25% of the total radioactivity of 125 I-labelled Ub (Figure 2e). From Figures 2(a) to 2(e) it is seen that the degree of ubiquitination of total proteins during the initial 30 min is

decreased in the presence of added hPAH whereas the overall labelling of proteins in the high- M_r regions (Figures 2a to 2c) revealed a less pronounced decline with time. This finding may indicate that hPAH functions as a competitive substrate and that Ub-hPAH conjugates are degraded more slowly than some of the endogenous Ub-protein conjugates.

Conclusions

The 26 S proteasome (multicatalytic proteinase complex) is a large multimeric complex (M_r approx. 1 700 000) that is found in the nucleus and cytoplasm of eukaryotic cells [36,37]. It plays a major role in the Ub-dependent non-lysosomal pathways of protein degradation in an ATP-dependent fashion *in vitro*, notably of abnormal, short-lived and regulatory proteins [37,38]. In this pathway, large multi-ubiquitinated proteins are reported to be preferentially degraded [39]. Although the enzymes of the Ub-proteasome pathway are expressed at high levels in hepatocytes, and several proteins are ubiquitinated, the formation of Ub conjugates with specific liver proteins has not yet been studied [11]. Little is known about the rates of synthesis and degradation of PAH *in vivo* [1]. The enzyme has been reported to have a half-life of 7–8 h in rat hepatoma cells [40] and about 2 days in rat liver [41], but the mechanism of its degradation is not known. In the present study we have found that recombinant hPAH is poly-/multi-ubiquitinated, supporting the conclusion that the turnover of PAH occurs by a Ub-dependent proteolytic pathway. This mechanism has particular significance for the turnover of mutant forms of hPAH. Thus many cases of PKU are caused by mutations that interfere with the normal folding and interaction of the 50 000- M_r subunit to form oligomers (tetramer-dimer) in an equilibrium characteristic of the native catalytically active form of wild-type hPAH, leading to an increased rate of degradation of hPAH molecules [4,42]. Thus when the recombinant mutant enzymes are expressed in eukaryotic cells, a characteristic finding is a low level of mutant enzyme protein at normal cellular mRNA levels, compatible with an increased protein breakdown. Further studies are in progress to study the degradation of the wild-type and mutant forms of hPAH catalysed by the proteasome system both *in vitro* and *in vivo*.

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