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A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase

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ABSTRACT **Phenylketonuria (PKU), with its associated hyperphenylalaninemia (HPA) and mental retardation, is a classic genetic disease and the first to have an identified chemical cause of impaired cognitive development. Treatment from birth with a low phenylalanine diet largely prevents the deviant cognitive phenotype by ameliorating HPA and is recognized as one of the first effective treatments of a genetic disease. However, compliance with dietary treatment is difficult and when it is for life, as now recommended by an internationally used set of guidelines, is probably unrealistic. Herein we describe experiments on a mouse model using another modality for treatment of PKU compatible with better compliance using ancillary phenylalanine ammonia lyase (PAL, EC 4.3.1.5) to degrade phenylalanine, the harmful nutrient in PKU; in this treatment, PAL acts as a substitute for the enzyme phenylalanine monooxygenase (EC 1.14.16.1), which is deficient in PKU. PAL, a robust enzyme without need for a cofactor, converts phenylalanine to trans-cinnamic acid, a harmless metabolite. We describe (*i*) an efficient recombinant approach to produce PAL enzyme, (*ii*) testing of PAL in orthologous *N*-ethyl-*N*′-nitrosourea (ENU) mutant mouse strains with HPA, and (*iii*) proofs of principle (PAL reduces HPA)—both pharmacologic (with a clear dose–response effect vs. HPA after PAL injection) and physiologic (protected enteral PAL is significantly effective vs. HPA). These findings open another way to facilitate treatment of this classic genetic disease.**

Phenylketonuria (PKU) (1) is the prototypical human Mendelian disease (OMIM 261600) demonstrating benefits from treatment (2). PKU and a related form of less harmful hyperphenylalaninemia (HPA, termed non-PKU-HPA) result from impaired activity of phenylalanine hydroxylase (PAH; EC 1.14.16.1), the enzyme catalyzing conversion of the essential amino acid nutrient phenylalanine to tyrosine. The enzyme is responsible for disposal (by oxidative catabolism) of the majority of nutrient phenylalanine intake. The untreated PKU patient with persistent postnatal HPA is likely to experience irreversible impairment of cognitive development. Antenatal HPA, caused by transplacental transport of phenylalanine from the maternal pool to the fetus during a pregnancy in which there is maternal HPA, will harm the embryo and fetus. These disease-causing effects of PKU and maternal HPA are preventable by treatment to restore euphenylalaninemia (1).

Present treatment relies on the observation that a (semisynthetic) diet low in phenylalanine (3–5) will prevent HPA and thus the disease. Because it involves a major alteration of lifestyle, dietary treatment is difficult. Moreover, dietary therapy can be associated with deficiencies of several nutrients (1), some of which may be detrimental to brain development (6, 7). Moreover, most low phenylalanine treatment products have organoleptic properties sufficiently unsatisfactory that compliance with the treatment is compromised (8, 9). Such concerns have greater relevance now that better and longer compliance with therapy of PKU and non-PKU-HPA in all persons at risk has been recommended (10, 11).

A combination of oral enzyme therapy with phenylalanine ammonia lyase (PAL; EC 4.3.1.5) and controlled low protein diet might replace dependence on the semisynthetic diet, for treatment of PKU after infancy (10, 12). PAL is a robust autocatalytic enzyme that, unlike PAH, does not require a cofactor (13). PAL converts phenylalanine to metabolically insignificant amounts of ammonia and trans-cinnamic acid, a harmless metabolite; the latter is converted to benzoic acid and rapidly excreted in urine as hippurate (14). A preliminary report indicates that HPA is attenuated by oral administration of microencapsulated PAL in the rat with chemically induced HPA (15) and also, in preliminary studies, in naturally occurring HPA in a mouse model (16–18). Preliminary studies in human PKU patients showed analogous responses after the administration of PAL in enteric-coated gelatin capsules (19) or during use of an extracorporeal enzyme reactor (20). The human and even the animal studies were not continued because PAL was not available in sufficient amounts at reasonable cost.

(*i*) We used a construct of the *PAL* gene from *Rhodosporidium toruloides* (21) under the control of a high-expression promoter and expressed it in a strain of *Escherichia coli* to obtain large amounts of PAL (22). (*ii*) We used existing (23) and new strains (C.N.S., J. D. McDonald, and C.R.S. at http://www.mcgill.ca/pahdb/Pah Mouse/Mouse Pah Homologues) of the mutant *N*-ethyl-*N*′-nitrosourea (ENU)-treated mouse as orthologous models of human PKU and HPA to study enzyme substitution therapy with PAL. (*iii*) We showed that i.p. PAL injection lowers plasma phenylalanine in the mouse model (proof of pharmacological principle), and oral gavage of these mice with PAL enzyme, protected from inactivation by digestive enzymes, lowers plasma phenylalanine (proof of physiological principle). These developments point to an alternative approach to treatment of PKU, compatible with current guidelines (10, 11).

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Abbreviations: ENU, *N*-ethyl-*N*′-nitrosourea; PKU, phenylketonuria; HPA, hyperphenylalaninemia; PAL, L-phenylalanine ammonia lyase; PAH, human L-phenylalanine hydroxylase.

A Commentary on this article begins on page 1811.

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MATERIALS AND METHODS

Synthesis of Recombinant PAL. *Amplification of the PAL gene. R. toruloides* [ATCC no. 10788] was purchased from the American Type Culture Collection. Cells were grown in minimal medium containing phenylalanine as the sole carbon source (24), total RNA was extracted with hot acidic phenol (25) from a mid-logarithmic-phase culture, and mRNA was isolated with the PolyATtract mRNA isolation system (Promega). a cDNA pool was then synthesized with the RiboClone cDNA synthesis system (Promega).

Oligonucleotides (RTJP, 5′-AAGAATTCATGGCAC-CCTCGCTCGACTCGATCTCG-3′, and RT2, 5′-CCGAAT-TCTAAGCGATCTTGAGGAGGACGT-3′), synthesized on an Ecosyn D300 DNA synthesizer (Eppendorf) were designed to feature an *Eco*RI site at their 5′ end and to be homologous to 5′ and 3′ ends of the published sequence of the *R. toruloides PAL* gene (refs. 26 and 27; GenBank accession no. X51513). PCR amplification (28) was performed in 100 μl containing 100 pmol of each primer, 20 mM Tris·HCl (pH 8.0), 2 mM $MgCl_2$, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 0.1% Triton^{A}X-100, nuclease-free BSA at 10 mg/ml, all four dNTPs (each at 0.2 mM), 30 ng of cDNA as the template, and 5 units of *Pfu* DNA polymerase (Stratagene). Samples were incubated in a DNA thermal cycler (Barnstead/Thermolyne) at 95°C for 30 sec, at 50°C for 1 min, and 72°C for 3 min; repeated for 35 cycles. The PCR product was analyzed on a 1% agarose gel containing 0.6 mg of ethidium bromide per ml and subsequently cloned in pBluescript KS+ (Stratagene). Identity of the *PAL* gene was verified by sequence analysis using an AutoRead sequencing kit and an automated A.L.F. DNA sequencer (Pharmacia).

E. coli strains and plasmids. E. coli XL-1Blue was the host used for general cloning and vector construction; *E. coli* Y1091 was the host for fermentation to produce PAL. *E. coli* IBX-4, used in the animal study, was isolated from a Sprague–Dawley rat and identified by using api20E bacterial identification kit (BioMerieux, Charbonnier les Bains, France). Plasmid pBluescript was used for cloning PCR-amplified *PAL* fragments.

Construction of high-expression PAL plasmid pIBX-7. Plasmid pIBX-1 (29) was modified by site-directed mutagenesis, changing the unique *Bam*HI site to an *Eco*RI site to allow cloning of the 2.2-kb *PAL* PCR fragment; the product was then further modified, which resulted in the deletion of the *Eco*RI sites. To increase expression, an additional *tac* promoter (*Ptac*) was synthesized by PCR and cloned downstream of the existing *Ptac*. The 5′ Shine–Delgarno sequence (AGGAG) is separated from the ATG codon by a 9-nt sequence (ACAGAATTT). Kanamycin resistance for selection of *PAL*-containing cells was conferred by substituting the kanamycin resistance gene for the ampicillin resistance gene of pIBX-1. The plasmid was transformed into *E. coli* (hosts Y1091 and IBX-4) and induced with isopropyl β-D-thiogalactoside (1 mM) for expression. The expression levels are similar in both hosts when cultured in shaken flasks (data not shown).

Purification of PAL enzyme from cell extract. Frozen *E. coli* cells (Y1091; 500 g) expressing plasmid pIBX-7 were suspended in 2 liters of buffer A (30 mM Tris·HCl, pH 8.0/10 mM phenylalanine/2 mM cysteine) to which DNase I (5 mg/liter) and 5 mM $CaCl_2$ were added. The cell suspension was homogenized three times in a Rannie MiniLab 8.30H high-pressure homogenizer (APV Canada, Montreal) at 700 bar (1 bar = 100 kPa); between passages, the suspension was cooled to 12°C. The homogenate was centrifuged (14,100 × *g*) for 1 hr at 4°C, the supernatant containing PAL protein diluted 2- to 3-fold in water and loaded on a column containing 1.25 liters of Q-Sepharose Big Beads (Pharmacia). Washes were performed at a linear flow rate of 153 ml/hr with three column volumes of buffer A followed by three column volumes of buffer B (30 mM Tris·HCl, pH 8.0/10 mM phenylalanine/2 mM cysteine/1M NaCl). PAL protein is eluted with buffer B in a linear gradient (3%–100%) in six column volumes; the fractions with PAL activity were pooled. Ammonium sulfate was added slowly to the eluates (final concentration, 50%), stirred (30 min) at room temperature, and then centrifuged (14,700 × *g* for 30 min at 4°C). The final PAL protein pellet was dissolved in a minimal amount of buffer C (50 mM sodium phosphate, pH 7.5/5 mM phenylalanine/1 mM glutathione).

ENU Mice. The ENU mouse models, deficient in hepatic PAH enzyme activity, were created by treating wild-type mice (BTBR background) with the alkylating agent ENU. The original strains *Pah*$^{enu1/enu1}$(ENU1/1) and *Pah*$^{enu2/enu2}$(ENU2/2), from W. Dove and A. Shedlovsky (University of Wisconsin, Madison), were produced in Wisconsin (23, 30) and genotyped by J. D. McDonald (31). We produced the hybrid heteroallelic strain *Pah*$^{enu1/enu2}$(ENU1/2) in our facilities (see http://www.mcgill.ca/pahdb/Pah Mouse/Mouse Pah Homologues). (All procedures described below have been reviewed and approved by the Animal Care Committee, McGill University).

The homozygous mutant ENU1/1 mouse is a counterpart of human non-PKU-HPA. It has a missense mutation in the *Pah* gene [c. 364T → C in exon 3 (V106A)] (30, 31), and on breeder diet (product 8626, Teklad, Madison, WI), it has both a normal plasma phenylalanine level and normal behavior but can be made hyperphenylalaninemic under controlled conditions with a phenylalanine load [by s.c. injection or gavage of L-phenylalanine at 1.1 mg/g (body weight)]. The homozygous mutant ENU2/2 mouse is a counterpart of human PKU. It has a missense mutation [c. 835T → C in exon 7 (F263S)] (23, 31). On breeder diet, it has 10- to 20-fold elevated plasma phenylalanine and phenylketones in urine; euphenylalaninemia in this strain was achieved for these studies by placing mice on a diet free of phenylalanine (product 2826, Teklad) with ad libitum water containing L-phenylalanine (30 mg/liter), for 3 consecutive days. After establishing euphenylalaninemia, ENU2/2 animals received standardized s.c. injections of L-phenylalanine [0.1 mg/g (body weight)] to achieve reproducible HPA.

We developed the new ENU1/2 strain by crossing female ENU1/1 and male ENU2/+; all parents and offspring were typed by DNA analysis. The mutant heteroallelic animals have a normal plasma phenylalanine level on breeder diet but easily achieve a modest elevation, to levels between that of untreated ENU1/1 and ENU2/2, by s.c. injection or gavage of L-phenylalanine [1.1 mg/g (body weight)]. The ENU strains are further described on our web site (see above).

Protocols to Study the Effect of PAL Enzyme on Phenotype. *i.p. administration of recombinant PAL (for proof of pharmacological principle).* Studies were done on mice, younger than 1 year of age (all three strains). Each animal served as its own control; a sham (saline) injection was given in the first week, followed by the PAL injection 1 week later (same day of the week and time of day). Efficacy of i.p. PAL was measured by change in the plasma phenylalanine between the first and second week; animals (n = 5 per trial) received 2, 20, and 100 units of PAL. ENU1/1 and ENU1/2 animals were fasted overnight before the experiment. Food was reintroduced after PAL administration. During the first week, 0.2 ml of saline i.p. was followed directly by an L-phenylalanine challenge [1.1 mg/g (body weight) by gavage] for the ENU1/1 and ENU1/2 mice; tail blood samples taken at zero min and 1, 2, 3, and 24 hr after the phenylalanine challenge. For the second-week protocol, PAL (replacing saline) was diluted to the dosage required with 0.1M Tris·HCl (pH 8.5).

Gavage of recombinant PAL (for proof of physiological principle). Studies were done in mice younger than 1 year of age. Each animal served as its own control and efficacy of PAL treatment was measured by change in plasma phenylalanine. We used two different PAL preparations: (*i*) recombinant PAL inside *E. coli* cells (IBX-4) and (*ii*) unprotected PAL (purified from Y1091 *E. coli* cells) in solution with aprotinin

(protease inhibitor). Saline gavage was used as the control treatment. Plasma phenylalanine levels were adjusted in ENU2/2 animals ($n = 4$) to achieve euphenylalaninemia. In the first week, the animals received a phenylalanine load [0.1 mg/g (body weight) by s.c. injection] on day 4, followed at 1 hr and 2 hr by gavage of saline bicarbonate (6 mg, to neutralize gastric acidity). Tail blood samples were taken before treatment (at time zero minus 5 min), and five times at hourly intervals after the phenylalanine challenge. In the second week, the gavage contained (*i*) 25 units of PAL (as induced recombinant *E. coli* cells, OD_{500}) or (*ii*) 200 units of PAL, in combination with 10 mg of aprotinin in the sodium bicarbonate buffer.

***In Vitro* Assays of PAL Effect.** We measured efficacy of the PAL preparation *in vitro* by analyzing its effect on a solution containing 4 mM phenylalanine (initial concentration) at 37°C and pH 8.5. We compared (*i*) the individual effects of nonrecombinant *E. coli* cells ($OD_{600} = 50$), chymotrypsin (100 mg/ml), and mouse intestinal fluid (diluted 1:10 with Tris buffer at pH 8.5) with (*ii*) the effect of naked recombinant PAL either alone or in the presence of chymotrypsin (100 mg/ml) or of intestinal fluid (1:10 dilution with Tris buffer at pH 8.5) and (*iii*) the effect of *E. coli* cells ($OD_{600} = 50$) expressing PAL (5 units) alone or in the presence of chymotrypsin (100 mg/ml) or mouse intestinal fluid (1:10 dilution with Tris buffer at pH 8.5).

Analytical. *Plasma phenylalanine concentration.* We collected blood from tail into heparinized tubes, extracted plasma, and measured phenylalanine by HPLC (Beckman System Gold, DABS amino acid analysis kit).

DNA Analysis. Animals were genotyped, as described (31), for the Pah^{enu2} mutation. We developed a method to detect the Pah^{enu1} mutation that eliminates a recognition site for the restriction enzyme *Taq*I. We used PCR, with primers 5′-GAGAATGAGATCAACCTGACA-3′ and 5′-TGTCTCGGGAAAGCTCATCG-3′, to amplify a 169-bp segment of exon 3 (mouse *Pah*) from blood spots collected on Guthrie cards. The product subjected to *Taq*I digestion yields a distribution of fragments with a distinct banding pattern for each of three possible genotypes: $Pah^{+/+}$ generates two fragments (148 bp and 21bp), $Pah^{enu1/+}$ generates three (169 bp, 148 bp, and 21bp), and $Pah^{enu1/enu1}$ generates one fragment (169 bp).

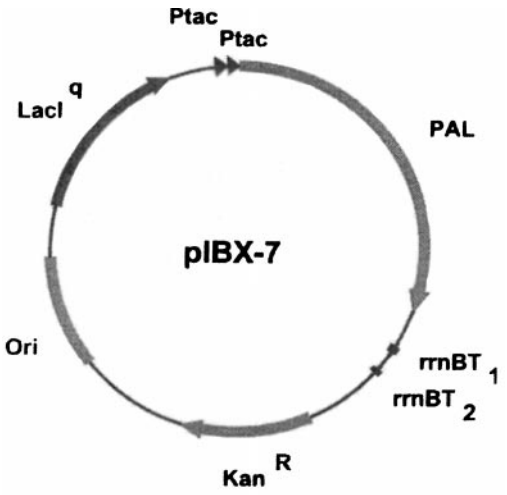

FIG. 1. *PAL* gene from yeast *R. toruloides* was cloned in the expression vector pIBX-7 where transcription is controlled by the strong inducible *tac* promoter and terminated by the rRNA transcription terminator sequences rrnBT1 and rrnBT2. *LacI*^{q} represses the *tac* promoter, and hence isopropyl β-D-thiogalactoside is required to release it from the promoter. The kanamycin resistance gene (Kan^R) is included in the construct to allow selection of cells containing the plasmid.

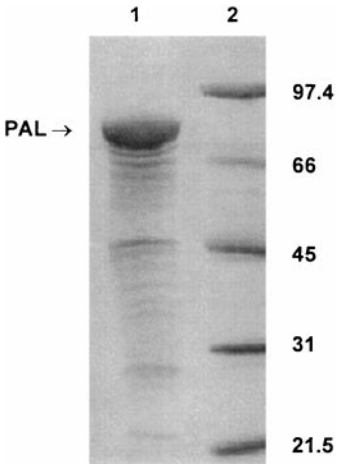

FIG. 2. Purified PAL enzyme (5 μg) separated on 4–15% gradient SDS/PAGE. Molecular mass markers in kDa are to the right. Lanes: 1, sample of PAL with ~20% impurities indicated by additional bands; 2, low-range molecular mass standards (Bio-Rad).

RESULTS

Synthesis and Purification of PAL. We obtained PAL by expressing the pIBX-7 construct (Fig. 1) in *E. coli*, followed by purification on a Q-Sepharose column. The product is a yeast (*R. toruloides*) PAL enzyme, at 80% purity (Fig. 2). The yield in our present system is 100–150 units/g of *E. coli* cells, with a K_m of 250 μmol/liter, at specific activity of 2.2–3.0 units/mg of PAL protein; 1 unit of PAL deaminates 1.0 mmol of L-phenylalanine to *trans*-cinnamate (and NH_3) per min at pH 8.5 and 30°C.

Effect of PAL in ENU Mice. We used the ENU mouse orthologues of human PKU and non-PKU-HPA to obtain proof of pharmacologic and physiologic principles by demonstrating efficacy of PAL enzyme against the hyperphenylalaninemic phenotype.

Pilot Study. PKU mice (ENU 2/2; $n = 12$) on regular diet were treated with PAL (2, 20, or 100 units) by i.p. injection without any additional manipulations. Within 3 hr, the postinjection value for blood phenylalanine had fallen (range, 0–984 μmol/liter) from the pretreatment value (range, 389–2,012 μmol/liter). These preliminary observations demonstrated both an apparent treatment response and troublesome inter-

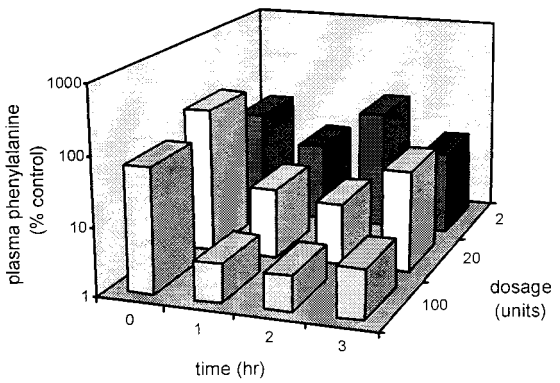

FIG. 3. Injection i.p. of recombinant PAL enzyme reduces plasma phenylalanine in the ENU2/2 mouse (*y* axis is logarithmic scale) over time (*x* axis) ($P < 0.05$). Reduction of plasma phenylalanine by PAL shows a dose–response relationship (*z* axis). Data are normalized to the control (sham-treated) values for each animal at each point. Data depicted are the average of five paired series. The range of control (100%) values was 390–2,013 μmol/liter for animals receiving 2 units of PAL, 572–1,488 μmol/liter for animals receiving 20 units, and 504–1,474 μmol/liter for animals receiving 100 units.

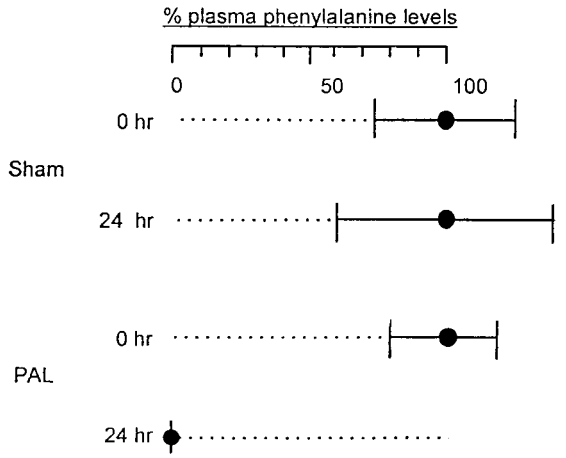

FIG. 4. A single i.p. injection of recombinant PAL enzyme (100 units at zero time), reduces plasma phenylalanine level in ENU1/1 mice by 95% at 24 hr ($P < 0.02$) relative to sham-treated controls. Data for five paired series (mean ± 1 SD) are normalized to paired control values to accommodate inter- and intraindividual variation. The range of control values (100% at zero time) was 41–528 μmol/liter; every animal showed a response to PAL.

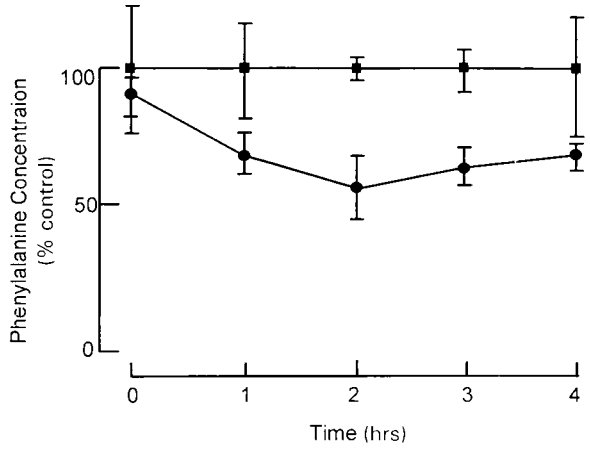

FIG. 5. Plasma phenylalanine levels in ENU2/2 mice after oral administration (25 units per mouse) of induced recombinant *E. coli* cells expressing PAL: 31% reduction within 1 hr ($P < 0.04$) and 44% reduction in 2 hr ($P < 0.004$). Data are normalized to control values (mean ± 1 SD). ■, Sham; ●, PAL enclosed in *E. coli*. The range of control values (100%) was 425–800 μmol/liter; every animal showed a response to PAL.

and intra-individual variation. Accordingly, we controlled for the latter by adopting the protocols described above.

Proof of pharmacological principle. A single i.p. injection of PAL enzyme significantly lowered plasma phenylalanine in PKU mice (ENU2/2; $P < 0.05$) and showed a dose-response effect (Fig. 3); at each point, data are normalized to the control (sham-treated) values for each animal to accommodate inter- and intra-individual variation. The non-PKU-HPA mice (ENU1/1) and the heteroallelic HPA stain (ENU1/2) also responded to PAL treatment (data not shown). The effect of a single i.p. PAL injection in ENU1/1 mouse model persisted for 24 hr ($P < 0.02$; Fig. 4); ENU1/2 and ENU2/2 mice both had similar 24-hr responses (data not shown).

Proof of physiological principle. To demonstrate the effect of orally administered PAL on plasma phenylalanine levels required protease-resistant PAL formulations. Recombinant PAL enzyme activity was protected against inactivation by gastric acidity and intestinal digestive enzymes by retaining and shielding it in the *E. coli* cells where it was synthesized. We used these cells in the absence of a different form of PAL protection.

Recombinant PAL enzyme protected by the cell wall and membrane of *E. coli* resists inactivation by proteolytic intestinal enzymes *in vitro*; otherwise activity of naked enzyme is abolished (data not shown). PAL enclosed in *E. coli* cells was shown to reduce phenylalanine content of the *in vitro* solution (Table 1). When given by oral gavage, recombinant PAL (25 units) expressed in an *E. coli* strain isolated from Sprague–Dawley rat lowered plasma phenylalanine in ENU2/2 mice by 31% in 1 hr ($P < 0.04$) and 44% in 2 hr ($P < 0.0004$) (Fig. 5).

Other experiments *in vivo*, conducted with aprotinin (protease inhibitor) and recombinant PAL enzyme purified from *E. coli* (Y1091), gave further proof of physiological principle. This formulation resists inactivation by chymotrypsin and mouse intestinal fluid *in vitro* (data not shown). Oral gavage of naked PAL (200 units) combined with aprotinin (protease inhibitor) lowered plasma phenylalanine in ENU2/2 mice: by 50% in 1 hr ($P < 0.017$) and 54% in 2 hr ($P < 0.023$) (Fig. 6).

DISCUSSION

We describe a method to produce recombinant PAL (Fig. 1) that may enable the use of this enzyme to degrade excess phenylalanine in PKU where the normal pathway for its disposal is impaired. Why would this alternative be useful if, as is generally assumed, early treatment of PKU by a semisynthetic low phenylalanine diet is one of the success stories of medical genetics (1, 2)? The answers lie in the incidence of PKU, our expectations for its treatment, and the anticipated "prevalence" of patient treatment years.

The combined incidence of PKU and non-PKU-HPA is on the order of 10^{-4} live births in populations of European descent. Current evidence reveals similar rates in Asian-Oriental and Arabic populations (1). New guidelines (10, 11) for the treatment of HPA have appeared so that residual imperfections in outcome (1) can be overcome and expectations met; the guidelines advise treatment to restore blood phenylalanine levels as near normal as possible, as early as possible, and for as long as possible, perhaps for a lifetime. In this context, the "prevalence" for patient treatment years takes on new meaning when it involves the difficult existing modes of treatment. The predicted "prevalence" in patient treatment years for a population of 10^8 persons, assuming 50 years of treatment per patient, would be 500,000 patient treatment years in half a century.

Table 1. Change in phenylalanine content *in vitro* solutions under various treatments

Experimental conditions	Treatment	Decrease in phenylalanine, % control†
Control	Nonrecombinant *E. coli* cells	0
	Chymotrypsin	0
	Intestinal fluid	0
PAL	In induced recombinant *E. coli* cells	76
	In induced recombinant *E. coli* cells + chymotrypsin in medium	72
	In induced recombinant *E. coli* cells + intestinal fluid in medium	66

*Treatments were for 1 h followed by measurement of L-phenylalanine content.
†Initial concentration, 4 mM of L-phenylalanine.

PKU is a multifactorial disease; mutation in the *PAH* gene and dietary exposure to the essential nutrient amino acid L-phenylalanine are equally necessary causes of the mutant phenotype (1). Treatment of HPA is feasible because the dietary experience can be purposefully modified, and the low phenylalanine diet has been its mainstay.

Untreated PKU is a disease at three phenotypic levels. At the proximal (enzyme) level, many different mutations in the *PAH* gene impair PAH integrity and function (32). Because PAH enzyme is the principal determinant of phenylalanine homeostasis *in vivo* (1), its impairment leads to HPA, which is the intermediate (metabolic) level of the variant phenotype. Impaired cognitive development and neurophysiological function is the distal (clinical phenotype); phenylalanine is the neurotoxic molecule (1), and hence the rationale to restore euphenylalaninemia.

Three modalities for treatment exist in theory or in practice: gene therapy, enzyme therapy, and diet therapy. The first has its appeal but is only at the experimental stage (33) (also C. Harding, personal communication) and unlikely to be put into practice unless the alternatives fail. The third method (low phenylalanine diet therapy) was inaugurated in the 1950s (3–5) and has achieved its primary goal: it has prevented mental retardation in the adequately treated patient (1, 34). However, dietary treatment of PKU and HPA is difficult; it involves rigorous compliance, a major alteration in lifestyle, and use of treatment products that have unusual organoleptic qualities. Moreover, measurements of cognitive and neurophysiologic outcomes show subtle deficits (IQ scores are 0.5 SD below normal), and there are lacunae in neuropsychological and neurophysiological performance. Accordingly, there is growing interest in the second form of treatment (enzyme therapy).

Enzyme therapy could be done by replacement (of PAH enzyme) or by substitution (with another enzyme to degrade excess phenylalanine). Replacement of PAH requires the intact multienzyme complex for catalytic hydroxylating activity (1); it could be done best perhaps only by hepatic transplantation and this approach has not been pursued. But if the PAL enzyme could be administered by mouth to the PKU patient, it would have a certain appeal; therapy with enzymes protected from inactivation is feasible for the treatment of metabolic conditions (35, 36).We propose that PAL will "substitute" for deficient hepatic PAH activity and degrade phenylalanine in the PAH-deficient organism. To test this hypothesis, we used the induced mutant (ENU) mouse orthologues of human PKU and non-PKU-HPA (23). We first demonstrated proof of pharmacologic principal: given by injection, PAL acts *in vivo* to lower ambient blood phenylalanine levels (Fig. 3). We then demonstrated proof of physiologic principal; PAL placed in the intestinal lumen acts *in vivo* to suppress HPA (Figs. 5 and 6). The latter is a significant finding that capitalizes on three prior concepts and observations: (*i*) Amino acids are in equilibrium between various compartments of body fluids (37, 38) and ultimately in equilibrium with the intestinal lumen (39), so that treatment of intestinal lumen phenylalanine will affect all body pools. (*ii*) PAL placed in the intestinal lumen will modify phenylalanine content of body fluids in the whole animal (15, 18). (*iii*) PAL placed in the intestinal lumen will act on both the dietary phenylalanine and the endogenous run out of free phenylalanine from its bound pools (40). Accordingly, the appropriate dosage and schedule (to avoid under- or overtreatment) of oral PAL, perhaps in combination with a controlled and modestly low protein diet, should control the phenylalanine pool size without need of the drastic restriction of dietary phenylalanine as now practiced and requiring artificial diets. Ancillary treatment of PKU with PAL and prudent protein intake would become analogous to treatment of diabetes mellitus with insulin, with an additional feature—the enteral route would avoid problems with immune recognition of PAL.

Until now, the cost of PAL has prohibited any consideration of therapy; even animal studies were curtailed. The recombinant enzyme we describe herein may avoid this constraint and has enabled our investigations. The relatively low specific activity of the recombinant PAL product and relative inefficiency at pH 7.0 (R.H., unpublished data) may be offset by the long contact time between enzyme and substrate during passage through small and large intestine. The formulation currently under development is focused on completely protecting the PAL enzyme against a protease environment.

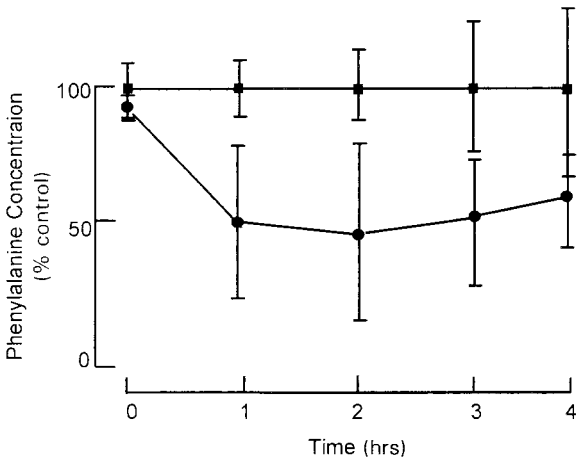

FIG. 6. Plasma phenylalanine levels in ENU2/2 mice after combined oral administration of naked PAL enzyme (200 units per mouse) and protease inhibitor (aprotinin): 50% reduction within 1 hr ($P < 0.017$) and 54% in 2 hr ($P < 0.023$). Data are normalized to control values (mean ± 1 SD). ■, Sham; ●, unprotected PAL plus aprotinin. The range of control values (100%) was 458–1,051 μmol/liter; every animal showed a response to PAL.

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