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Cloning and Expression of Human Phenylalanyl-tRNA Synthetase in *Escherichia coli:* Comparative Study of Purified Recombinant Enzymes

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Human phenylalanyl-tRNA synthetase (PheRS) was cloned and expressed in *Escherichia coli*. The cDNAs of the α and β subunits were cloned into pET-21b(+) and pET-28b(+) vectors. The 6× histidine-tagged (HT) plasmids pET-21_HTβ, pET-28_HTα, and pET-28_HTβ were constructed. Three different types of $(\alpha\beta)_2$ heterodimers of human PheRS carrying HT at the N-terminus of either of two α or β subunits or simultaneously on both of them were overproduced and purified. The heterodimeric protein with HT appended to the N-terminus of the β subunit revealed no activity in the aminoacylation reaction as opposed to those with HT on the α subunit. It is known from the structure of the *Thermus thermophilus* Phe system that the N-terminal coiled-coil domain of the α subunit is involved in the binding of cognate $tRNA^{Phe}$. Our data demonstrate that a histidine-tagged N-terminal extension appended to the α subunit does not affect the kinetic parameters of $tRNA^{Phe}$ aminoacylation. Elimination of the HT from the α subunit by thrombin cleavage leads to nonspecific splitting of the enzyme that occurs in parallel to the main reaction. In addition to the tagged proteins the properly assembled heterodimer containing intact α and β subunits free of HT was overproduced and purified. Aminoacylation activity of the overproduced human PheRS in the crude bacterial extract is two orders of magnitude higher than the corresponding activity in human placenta and the yield of the recombinant enzyme overproduced in *E. coli* is five times higher.

Phenylalanyl-tRNA synthetase (PheRS)—the largest and most complex enzyme among the 19 other members of the aaRS family has $(\alpha\beta)_2$ subunit organization and its structure was first solved for the *Thermus thermophilus* enzyme (1). It belongs to class II and in accordance with structural characteristics (the availability of the RNP-anticodon binding domain) may be separated into subclass IIc. The architecture of cytoplasmic PheRS is markedly conserved throughout evolution from prokaryotes to eukaryotes and forms heterodimers of $(\alpha\beta)_2$ type in all known species. Bacterial PheRS from *T. thermophilus* HB8 is an enzyme of 350 residues per α and 785 residues per β subunit, with no detectable sequence homology between the two subunits. The three-dimensional structures of the native enzyme complexed with cognate $tRNA^{Phe}$ and phenylalanyl-adenylate have been determined recently (1–3). The studies reveal the distinctive characteristics of PheRS subunit organization and show that bacterial heterotetramer $(\alpha\beta)_2$ consists of 22 structural domains. The $\alpha\beta$ heterodimer consists of 11 structural domains: 3 of them, coiled-coil and A1–A2, belong to the α subunit and 8, B1–B8, to the β subunit. While the α subunit domains create the catalytic module and the coiled-coil domain directly involved in aminoacylation and $tRNA^{Phe}$ binding, respectively, the β subunit is a collection of structural domains which are similar to various functional domains found in other enzymes and, thus, are likely to perform various functions in other proteins. The domains incorporated into the β subunit include a "catalytic-like" module (domains B6 and B7), "DNA binding-like" domains (B1 and B5), an "EMAP II-like" domain (B2, similar to the anticodon-binding domain of AspRS and LysRS, but free from contact with $tRNA^{Phe}$), and

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an "SH3-like" domain (B4, associated with signal transduction in a number of eukaryotic proteins) (1). The high degree of complexity of the PheRS structure suggests a puzzling evolutionary pathway of the enzyme that also may carry a large range of functions, beyond the level of the aminoacylation activity.

The structure of the PheRS-tRNA^{Phe} complex (2) offers a clearer view of the functional peculiarity of PheRS. It is the only example of tRNA binding to an aaRS molecule with $(\alpha\beta)_2$ subunit organization. It was shown that one tRNA^{Phe} molecule interacts with all four subunits of the enzyme. Thus the structure of the complex explains why the enzyme has to be a functional $(\alpha\beta)_2$ dimer. The anticodon loop of the tRNA^{Phe} is specifically recognized by the C-terminal domain of the large β subunit, which is closely similar to the RNA-binding domain of the U1A spliceosomal protein. Remarkably, *T. thermophilus* PheRS approaches the anticodon loop from the minor grove side.

The crystal structure of PheRS complexed with the true intermediate, phenylalanyl-adenylate (synthesized within the native PheRS crystals soaked with phenylalanine, ATP, and $MnSO_4$) has been determined at 2.6 Å resolution (3). Analysis of three-dimensional structures of PheRS complexed with functional ligands (PheRS–tRNA^{Phe} (2), PheRS–Phe (4), and PheRS–Phe-AMP (3)) and sequences of PheRS from several species together with biochemical data (5) allow us to propose structural guidelines for phenylalanine substrate specific binding, activation, and conformational rearrangement of the 3′-terminal portion of cognate tRNA^{Phe} in the presence of phenylalanyl-adenylate in a prokaryotic Phe system. The location of the phenylalanine moiety observed in the PheRS–Phe–AMP complex, and in the PheRS structure with phenylalanine alone, shows that the free amino acid binds and holds a position close to that in phenylalanyl-adenylate. The class II conserved mode of ATP binding, and the hydrogen-bonded distance of Argα204 (or its analogs in other species) from the α-phosphate of ATP and the carbonyl oxygen of phenylalanine, is good evidence that the reactants are located in the active site of PheRS in accordance with an in-line mechanism for phenylalanine activation to occur.

Recently a novel human cDNA encoding mRNA preferentially expressed in tumorigenic human acute-phase chronic myeloid leukemia K562 cells was detected (6). This cDNA proved to encode a polypeptide chain identical to the catalytic α subunit of human cytoplasmic PheRS. This is the first example of tumor-selective and

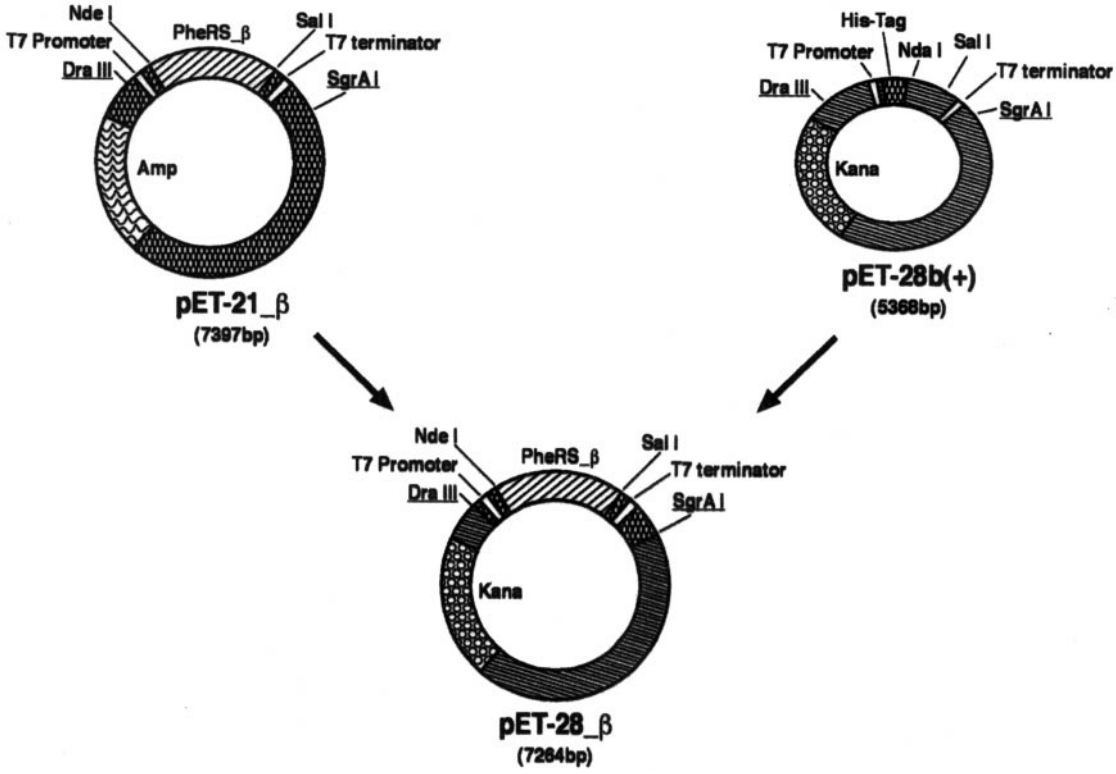

FIG. 1. Schematic representation of the plasmid pET-28__β constructed for expression of recombinant β subunit of human PheRS in *E. coli*. To obtain a plasmid that possesses a kanamycin-selectable marker and allows expression of the β subunit without HT, the β-subunit gene with the cloning/expression region of pET-21__β was subcloned into pET-28b(+) as described under Materials and Methods.

cell cycle stage- and differentiation-dependent expression of an aaRS family representative in mammalian cells. Our studies on cloning and sequencing of human PheRS (7) demonstrate that the α subunit contains 508 amino acids and β subunit contains 589 amino acids. The identification of human PheRS as a member of the class II synthetases is based on the discovery of three signature motifs in the catalytic α subunit. Multiple sequence alignment clearly outlines regions in the α subunit involved in formation of the active site framework and thus confirms that the human α subunit is indeed catalytic with general characteristic features of class II. It also provides a way to delineate the human PheRS domain structure and to display the conservation of the internal domains of the enzyme. Knowledge of the three-dimensional structure of *T. thermophilus* PheRS coupled with these results enables the prediction that the topology of the $(\alpha\beta)_2$ subunit communication in human PheRS will be mostly the same as in the reported structure (1) of the *T. thermophilus* heterodimer. This conclusion stems from the high degree of similarity of characteristic residues located in signature motifs of the α and β subunits, which are known to participate in the formation of the interface core of PheRS.

Northern blot hybridization analysis from malignant and normal human tissues allows assessment of the relative expression levels of the α and β subunits independently, in view of the additional cellular role proposed for the β subunit in tumorigenic events (6, 7). It was shown that the levels of mRNA corresponding to the α and β subunits were remarkably similar in all cell types and tissues examined, thus indicating the implication not only of the α subunit but of the entire $(\alpha\beta)_2$ heterodimer in tumorigenic events.

Primary structure analysis reveals also that the length of the polypeptide chains of eukaryotic aaRSs is significantly higher than that of the homologous prokaryotic enzymes. Numerous observations show that elongation of the chains mostly occurs at the N- or C-terminal extremities of the subunits, rather than by insertion into the catalytic domain region. The human catalytic α subunit, as it appears (7), obeys this rule of thumb, whereas the β subunit turns out to be ~200 residues shorter than respective prokaryotic analogs. More specifically, human PheRS demonstrates the absence of the anticodon-binding domain in the C-terminal sequence of eukaryotic β subunit. From the structure of *T. thermophilus* PheRS and its complexes with functional ligands it is clear that the β subunit has not been directly implicated in the aminoacylation reaction and exerts control over the recognition and binding mode of cognate $tRNA^{Phe}$. The major observation which follows from these results (7) is that the modes of binding and of recognition of cognate $tRNA^{Phe}$ are different in prokaryotes and eukaryotes.

TABLE 1

Human PheRS Expression Clones and Vectors

Clone name	Vector name	Selectable marker	His-tag/thrombin cleavage site
PheRS__α__HTβ	pET-21__α	Amp	No
	pET-28__HTβ	Kana	Yes
PheRS__HTα__β	pET-21__β	Amp	No
	pET-28__HTα	Kana	Yes
PheRS__HTα__HTβ	pET-21__HTβ	Amp	Yes
	pET-28__HTα	Kana	Yes
PheRS	pET-21__α	Amp	No
	pET-28__β	Kana	No

These sharp biological distinctions prompted us to investigate the structural bases of the superspecificity and the discrimination mechanism of human PheRS. To accomplish these ends, the initial objectives were (a) to overproduce human PheRS in large amounts and (b) to work out the protocol of the enzyme purification for its subsequent crystallization and determination of the three-dimensional structure.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Esherichia coli strain BLR(DE3) (Novagen, Germany) was grown in Luria Bertani (LB) or 2YT medium. For construction of expression plasmids the vectors pET-21b(+) and pET-28b(+) (Novagen) were used.

Enzymes and Reagents

Restriction enzymes *Nde*I, *Sal*I, *Dra*III, and *SgrA*I were obtained from New England BioLabs (U.S.A.). T4 DNA ligase was from Fermentas (Lithuania). Protein molecular weight markers (in the range of 7–204 kDa) were purchased from Bio-Rad (Israel). Thrombin was from Sigma–Aldrich (Israel). All other reagents were of the highest purity available.

Plasmid Constructions

To guarantee coexpression and coexistence of two individual plasmids in one bacterial cell they need to have distinct origins of replication and selectable distinctive markers. For this purpose we used two expression vectors, pET-21b(+) and pET-28b(+) (with ampicillin and kanamycin resistance, respectively). The cDNAs of the α and β subunits of human cytoplasmic PheRS were cloned and sequenced as described previously (7). The 6× histidine-tagged (HT) plasmids pET-28__HTα and pET-28__HTβ were constructed by inserting the cDNA of the corresponding subunit (as an *Nde*I/*Sal*I fragment) into the pET-28b(+) vector (Fig. 1). The plasmids pET-21__α and pET-21__β were prepared in a similar way by using the expression vector pET-21b(+).

TABLE 2

Purification of the Overproduced PheRS__HTα__β from 55 g of *E. coli* BLR(DE3) + pET-28__HTα + pET-21__β

	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude extract	1010	1860	1.8		100
$(NH_4)_2SO_4$ precipitation (35–55% saturation)	544	1710	3.1	1.7	92
Ni–NTA-agarose	25	1626	65	35	87
Heparin–Sepharose CL-6B	9.8	1417	144	78	76
Sephadex G-200	6.7	1233	184	100	66

To obtain pET-21__HTβ and pET-28__β, the cloning/expression regions with the β-subunit coding sequence were cleaved from vectors pET-21__β and pET-28__HTβ with endonucleases *Dra*III and *SgrA*I. The gel-purified DNA fragments were inserted into vectors pET-21b(+) and pET-28b(+) (double digested with *Dra*III and *SgrA*I) by ligation. The pET-21__HTβ vector carrying ampicillin resistance allows expression of the HT β subunit; the pET-28__β vector carrying kanamycin resistance allows expression of the intact β subunit.

The clones that express heterodimers with HT appended to the α subunit (PheRS__HTα__β) or to the β subunit (PheRS__α__HTβ) or to both subunits (PheRS__HTα__HTβ) and the wild-type PheRS (i.e., without the HT) were obtained by cotransforming pairs of vectors, pET-21__β + pET-28__HTα, pET-21__α + pET-28__HTβ, pET-21__HTβ + pET-28__HTα, and pET-21__α + pET-28__β, respectively, in competent *E. coli* BLR(DE3) using electroporation.

Purification of Recombinant PheRS

For overexpression of human PheRS with HT appended to the N-terminus of the α subunit (PheRS__HTα__β), *E. coli* strain BLR(DE3) with the expression plasmids pET28__HTα and pET21__β were grown at 37°C in LB in the presence of 30 μg/ml kanamycin, 50 μg/ml ampicillin, and 12.5 μg/ml tetracycline. Precultures were grown overnight and used for inoculation of the main culture (10 L) at a volume ratio 1:50. The bacteria were grown until the culture reached an OD_{600} of 0.6. Expression of PheRS__HTα__β was induced by 1 mM IPTG, and growth was continued for 15 h at 25°C. About 55 g of wet cells was harvested by centrifugation and resuspended in 50 ml of buffer A (25 mM Tris–HCl, pH 7.9) containing 10 mM 2-mercaptoethanol, 0.4 M NaCl, 10% (v/v) glycerol, and protease inhibitor cocktail set III (Calbiochem, U.S.A.). The cells were lysed by french press. The homogenate was centrifuged at 50,000 rpm for 1 h at 4°C to remove the cell debris. The supernatant was sonicated. Protein expression level was determined by testing PheRS aminoacylation activity (as described below) in the crude extract. The PheRS was salted out by ammonium sulfate in the 35–55% saturation range. The precipitate was recovered by centrifugation and dissolved in 15 ml of buffer A1 (buffer A + 100 mM NaCl). The protein solution was added to Ni–NTA-agarose resin (Qiagen, U.S.A.; volume 15 ml) equilibrated with buffer A1 and mixed gently by shaking at 4°C for 2 h. The protein solution–Ni–NTA-agarose mixture was loaded onto a column. After being washed (flow rate 70 ml/h) with 20 ml buffer A1, 100 ml 0.3 M NaCl in buffer P (PBS × 2, 10% (v/v) glycerol), and 50 ml buffer P, the column was developed stepwise with 5, 10, and 200 mM imidazole, 50–100 ml of each in buffer P. The fractions containing PheRS__HTα__β were pooled; the protein was precipitated with 55% saturated ammonium sulfate, dissolved in a minimal volume of buffer B1 (50 mM NaCl in buffer B: 20 mM Tris–HCl, pH 8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol), and dialyzed twice against 0.5 L buffer B1. The supernatant after centrifugation was loaded on a heparin–Sepharose CL-6B column (Pharmacia Biotech, U.S.A.; volume 15 ml) equilibrated with buffer B1. After being washed (flow rate 40 ml/h) with 25 ml buffer B1, the column was developed with 100 ml 120 mM NaCl in buffer B followed by a 300-ml linear gradient from 120 to 400 mM NaCl in buffer B. The fractions containing PheRS__HTα were pooled and the protein was precipitated as above. Final purification was achieved by gel filtration on a column (0.9 × 45 cm) of superfine Sephadex G-200 (Amersham Pharmacia Biotech) by developing (flow rate 2 ml/h) the column with 40 mM Tris–HCl, pH 8.0, 200 mM NaCl, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 5% glycerol. The protein fractions of each chromatography were analyzed for PheRS activity in the aminoacylation reaction and by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were stained with Coomassie Blue R-250.

For overexpression of PheRS HT appended to the N-terminus of the β subunit (PheRS__α__HTβ), *E. coli* strain BLR(DE3) with the expression plasmids pET28__HTβ and pET21__α were grown and induced as described above. The protein was salted out by ammonium sulfate in the 35–55% saturation range and subsequently purified by Ni-affinity chromatography.

The clone that expresses wild-type PheRS (free from HT at the N-terminus of both subunits) was grown in

2YT medium in the presence of 30 μg/ml kanamycin, 50 μg/ml ampicillin, and 12.5 μg/ml tetracycline. Precultures were grown overnight and used for inoculation of the main culture (10 L) at a volume ratio 1:50. The bacteria were grown at 37°C to OD_{600} of 0.85. Then expression was induced by adding 1 mM IPTG and the culture was allowed to continue growing at 25°C for 15 h. About 75 g of wet cells was harvested and lysed as described above. PheRS was salted out by ammonium sulfate in the 40–60% saturation range. The precipitate was recovered by centrifugation and dissolved in 70 ml buffer C (15 mM potassium phosphate, pH 8.5, containing 1 mM EDTA and 10 mM 2-mercaptoethanol). The protein was dialyzed twice against buffer C (1 L) and loaded on a DEAE-Sepharose (Amersham Pharmacia Biotech) column (180 ml) equilibrated with buffer C. The column was developed with 450 ml 25 mM potassium phosphate, pH 8.5, followed by a 1-L linear gradient from 25 mM potassium phosphate, pH 8.5, to 300 mM potassium phosphate, pH 7.0 (flow rate 100 ml/h). All buffers contained 1 mM EDTA and 10 mM 2-mercaptoethanol. The fractions of PheRS were pooled and dialyzed twice against 1 L buffer B1. Further purification by chromatography on a heparin–Sepharose and by gel filtration was achieved as described for the PheRS__HTα__β protein.

The final PheRS preparations were stored at −20°C in 50 mM Tris–HCl (pH 8.0) containing 200 mM NaCl, 40% (v/v) glycerol, 5 mM dithiothreitol, and 5 mM 2-mercaptoethanol.

Probing of Human PheRS by Dynamic Light Scattering (DLS)

The recombinant PheRS was dialyzed against 40 mM Tris–HCl, pH 8.0, containing 100 mM NaCl, 7 mM $MgCl_2$, 5 mM dithiothreitol, 5 mM 2-mercaptoethanol, and 1 mM EDTA. The DLS measurements were performed at 4 and 20°C using DynaPro (Protein Solutions, U.S.A.) with the protein concentration in the sample at 0.4 mg/ml. Control samples of *T. thermophilus* PheRS were prepared and measured in a similar way.

Aminoacylation Kinetics

PheRS activity was determined in the tRNA aminoacylation reaction by a filter binding assay; the reaction mixture contained 50 mM Tris–HCl, pH 8.0, 20 mM KCl, 30 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 μg/ml BSA, 5 mM ATP, 10 μM L-[^{14}C]phenylalanine or L-[^{3}H]phenylalanine (475 m Ci/mmol and 54 Ci/mmol, respectively; Amersham Pharmacia Biotech), 140 μM yeast $tRNA^{bulk}$ (Boehringer Mannheim Biochemica, Germany). The reaction was started by addition of a protein amount which causes within the first 2 min a linear increase in phenylalanylation. One unit of PheRS activity was defined as the amount of enzyme which

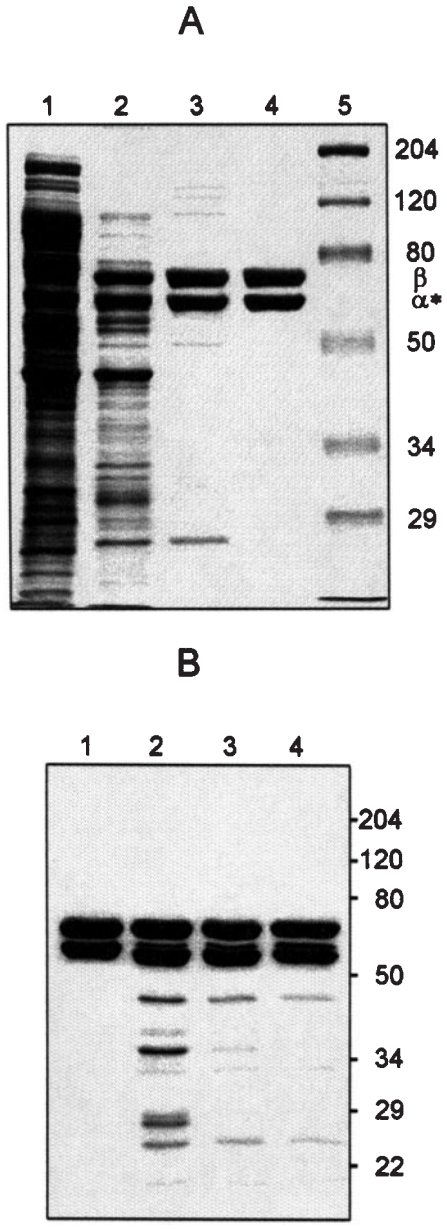

FIG. 2. SDS–PAGE analysis of overproduced PheRS with the histidine-tagged α subunit (PheRS__HTα__β). (A) Coomassie-blue-stained 10% SDS–polyacrylamide gel illustrating the purity of the enzyme after different steps: lane 1, ammonium sulfate precipitation (15 μg was loaded on the gel); lane 2, nickel chelate chromatography (7 μg); lane 3, heparin–Sepharose chromatography (5 μg); lane 4, gel filtration (4 μg). Lane 5 represents a mixture of marker proteins; PheRS subunits (α* indicates the histidine-tagged α subunit) and molecular weights of the markers are indicated on the right side of the gel. (B) Cleavage of PheRS__HTα__β with thrombin. The enzyme (0.6 mg/ml) was incubated with thrombin (15 U/ml) at 25°C for 2 h in the absence (lane 2) or presence of specific ligands: 100 μM phenylalaninyladenylate (lane 3) or 5 mM ATP, 10 mM $MgCl_2$, and 30 μM L-phenylalanine (lane 4). Control lane 1 represents PheRS__HTα__β incubated under identical conditions in the absence of thrombin. Positions and molecular weights of protein markers are indicated on the right of the gel.

catalyzes attachment of 1 nmol [^{14}C]phenylalanine to the tRNA in 1 min at 25°C. The protein concentration was determined by ultraviolet absorption at 228.5 and 234 nm, with BSA used as a standard (8). Apparent kinetic parameters (V_{max} and K_m) for human $tRNA^{Phe}$ (isolated from human placenta as described (9)) were determined from initial rate data (in the concentration range 0.1–1 μM) fitted to the Michaelis–Menten equation by the computer program Origin 4.1; standard errors did not exceed 10%.

Cleavage of the HT PheRS with Thrombin

To cleave HT at the N-terminus of α or β subunits the recombinant HT PheRS (PheRS__HTα__β or PheRS__α__HTβ) was incubated for 1–10 h at 25°C with thrombin taken in the range of concentrations from 10 to 30 units/ml in 50 mM Tris–HCl, pH 8.0, containing 150 mM NaCl, 2.5 mM $CaCl_2$, and 10 mM 2-mercaptoethanol. The concentration of the PheRS varied in the range 0.5–2 mg/ml. In protection experiments the reaction mixture contained 100 μM phenylalaninyladenylate (the COO group of the amino acid is replaced by CH2O (10)) or 5 mM ATP, 10 mM $MgCl_2$, and 30 μM L-phenylalanine. The cleavage products were analyzed by SDS–PAGE.

RESULTS AND DISCUSSION

Molecular Cloning and Expression of Human PheRS

The cDNA of the α and β subunits of human PheRS was cloned in the pET-system vectors (Table 1). Simultaneous production of both subunits using pairs of plasmids (pET-21__β and pET-28__HTα, pET-21__α and pET-28__HTβ, pET-21__HTβ and pET-28__HTα, pET-21__α and pET-28__β) with two different selectable markers in the same bacterial cell was done to ensure the coexpression of α and β subunits in one cell and their assembling of the functional $(\alpha\beta)_2$ heterodimer of human PheRS. The wild-type PheRS and three types of proteins that carry a histidine tag with the thrombin cleavage site were overexpressed in *E. coli.* strain BLR(DE3) (see Table 1). The expression was carried out under the control of the strong bacteriophage T7 transcription and translation signals upstream of the α- and β-subunit coding regions. The expression was induced by the addition of 1 mM IPTG.

Purification of Overproduced Human PheRS

The pET-28b(+) vector carrying an N-terminal HT and a thrombin cleavage site was used to facilitate purification of the overproduced human PheRS in *E. coli*. Two types of properly assembled $(\alpha\beta)_2$ heterodimers of human PheRS tagged with HT at the N-terminus of the α or β subunit, respectively, were overproduced and then purified by affinity chromatography on a nickel–NTA column. It turned out that after this column PheRS__α__HTβ reveals no activity in the aminoacylation reaction as opposed to the PheRS__HTα__β; the latter was subjected to further purification by two chromatographies on heparin–Sepharose and Sephadex G-200. The purification procedure is summarized in Table 2. The final preparation of PheRS__HTα__β was homogeneous as evidenced by PAGE under denaturing conditions (Fig. 2A).

To remove the terminal HT, which usually does not favor formation of good quality crystals (11), we cleaved the enzyme with thrombin. Serious efforts made to obtain homogeneous enzyme preparation after the cleavage (by variation of the cleavage conditions and following purification) failed, because of nonspecific splitting of the enzyme, which occurs in parallel to the main reaction (Fig. 2B, lane 2). The nonspecific proteolysis is accompanied by a loss of the synthetase activity: under the conditions resulting in the complete cleavage of the N-terminal extension, a 50% loss of the activity was observed. This suggests that the nonspecific degradation of the enzyme occurs in the active site area. Indeed, addition of the phenylalaninyladenylate (see above) or two substrates (phenylalanine and Mg^{2+}-ATP) to the cleavage reaction mixture (see Fig. 2B, lanes 3 and 4, respectively) substantially inhibits the process of nonspecific proteolysis. By this is meant also that binding of the specific substrates (or their analogs) provides protection of the PheRS active site region against thrombin.

The inability to remove specifically the HT from the recombinant PheRS__HTα__β prompted us to overproduce PheRS with an intact N-terminus of the α subunit.

TABLE 3

Purification of the Overproduced PheRS from 75 g of *E. coli* BLR(DE3) + pET-21__α + pET-28__β

	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude extract	2435	4560	1.9		100
$(NH_4)_2SO_4$ precipitation (40–60% saturation)	1120	3920	3.5	1.8	86
DEAE-Sepharose	56	2862	51	27	62
Heparin–Sepharose CL-6B	12.2	2060	169	90	45
Sephadex G-200	9.5	1834	193	103	40

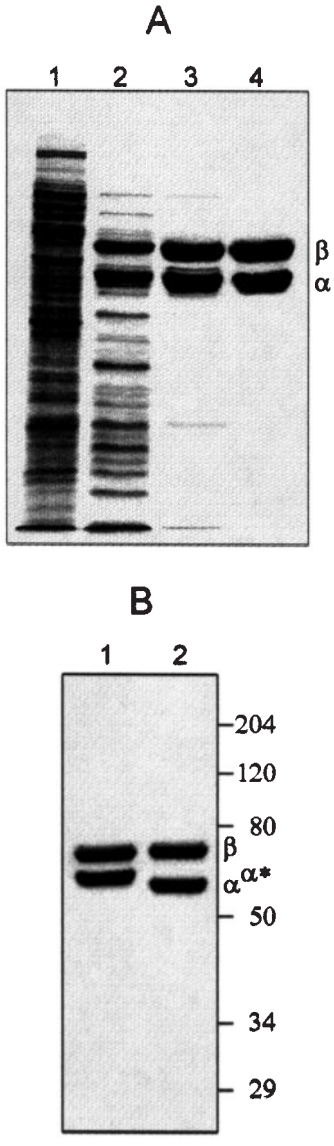

FIG. 3. SDS–PAGE analysis of the recombinant human PheRS. (A) The preparations of the overproduced enzyme purified stepwise by ammonium sulfate precipitation (lane 1; 10 μg was loaded on the gel), DEAE-Sepharose chromatography (lane 2; 7.5 μg), heparin–Sepharose chromatography (lane 3; 5.5 μg), and gel filtration (lane 4; 5 μg) are shown. (B) Relative migration of the subunits of the recombinant PheRS__HTα__β (lane 1) and PheRS (lane 2) in 10% SDS–polyacrylamide gel is shown. 4 μg of each protein sample was loaded on the gel. The subunits and positions of molecular weight markers are indicated on the right; the histidine-tagged α subunit is marked with an asterisk.

The enzyme was overproduced and purified in accordance with the procedures summarized in Table 3. To increase the level of overproduction, we varied the conditions of cell growing and induction. At the selected conditions (2YT medium, for other details see Materials and Methods) the amount of the overproduced protein per 1 g of cells was 1.8 times higher in comparison with that of PheRS__HTα__β. The purification step by Ni-chelate affinity chromatography used for the HT proteins was substituted by chromatography on DEAE-Sepharose; all other steps remained invariant. Use of anion-exchange chromatography results in a 1.6-fold lower yield of the overproduced PheRS compared to its HT counterpart. The developed procedure yielded an enzyme of high purity as shown by SDS–PAGE analysis (Fig. 3A). Comparing our data with those described for the PheRS isolated from human placenta (12) we conclude that the PheRS activity of the overproduced protein in crude extract is two orders of magnitude higher than the corresponding activity in human placenta and the yield of the recombinant human PheRS overproduced in *E. coli* is five times higher. Thus, coexpression of the α and β subunits of human PheRS gives rise to the formation of an active heterotetrameric enzyme in amounts appropriate for further structural investigations.

Characterization of the Overproduced Enzymes

The unmodified β and α subunits from the recombinant proteins PheRS__HTα__β and PheRS migrate in SDS–PAGE with apparent molecular weight (M_r) of 67 $\pm$ 1 and 57 $\pm$ 1 kDa, respectively, being evaluated relative to marker proteins (see Figs. 2A and 3B). The M_r values agree well with those derived from the sequences (66 and 57 kDa for the β and α subunits, respectively (7)). An increased size (59 $\pm$ 1 kDa) of the HT–α subunit (marked as α* in Figs. 2A and 3B) is due to the 20-amino-acid N-terminal extension.

The recombinant PheRS and PheRS__HTα__β are closely similar in their values of specific activity, which are 1.5 times higher than that of PheRS isolated from human placenta (measured under identical conditions) (12), while PheRS__α__HTβ shows no activity in the aminoacylation reaction. The activity of this enzyme

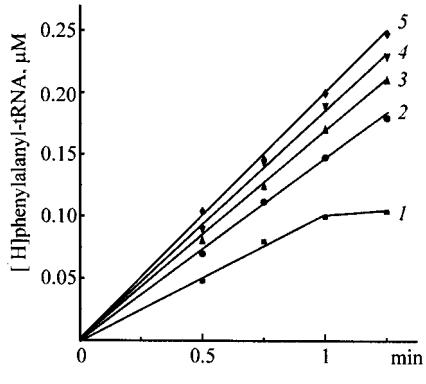

FIG. 4. Rates of aminoacylation with PheRS__HTα__β for human $tRNA^{Phe}$. Concentrations of the tRNA were 0.10 μM similar to 1.0 μM(*1*), 0.20 μM similar to 1.0 μM(*2*), 0.40 μM similar to 1.0 μM(*3*), 0.60 μM similar to 1.0 μM(*4*), and 1.0 μM (*5*). The enzyme concentration was 1.2 μg/ml.

TABLE 4

Kinetic Parameters of Aminoacylation of Human $tRNA^{Phe}$ with PheRS__HTα__β or PheRS

Enzyme	K_m (μM)	V_{max} (nmol/min/mg)
PheRS__HTα__β	0.11	184
PheRS	0.10	193

was not restored after thrombin cleavage resulting in deletion of the 17-amino-acid extension of the N-terminus. These data suggest that the presence of 3 additional amino acids at the N-terminus of the β subunit prevents aminoacylation from occurring. As follows from the structure of *T. thermophilus* PheRS complexed with cognate $tRNA^{Phe}$ (2), the N-terminal residues of the β subunit (namely NH_3^+-terminus and Argβ2) participate in the binding of the $tRNA^{Phe}$ acceptor end and exert an effect on aminoacylation reaction.

The X-ray study of *T. thermophilus* PheRS-$tRNA^{Phe}$ complex (2) revealed that the N-terminal domain of the α subunit is also involved in the binding of $tRNA^{Phe}$. Thus it was of interest to examine how the α-subunit N-terminal extension (namely HT) affects the kinetic parameters of $tRNA^{Phe}$ aminoacylation in human Phe system. The kinetic constants (K_m and V_{max}) for $tRNA^{Phe}$ were determined from initial rates of aminoacylation with two recombinant synthetases, PheRS__HTα__β (Fig. 4) and PheRS (not shown), at variable concentrations of the substrate. The two enzymes reveal closely similar values of K_m and V_{max} (Table 4); the K_m values fall within the same range as for human $tRNA^{Phe}$ aminoacylated with PheRS isolated from human placenta (13). Thus, we can conclude that the presence of the N-terminal HT extension in the α subunit does not disturb significantly the interaction of human PheRS with tRNA.

We probed the recombinant PheRS by DLS. The observed monomodal distribution of the size of molecules (data not shown) indicates homogeneity of the assembled oligomeric protein. The data of DLS measurements are summarized in Table 5. For comparison we used PheRS from *T. thermophilus*, whose three-dimensional structure is known (1). The two enzymes are very similar in the values of R_h and M_r determined at two different temperatures. The data strongly suggest that human PheRS resembles the *T. thermophilus* synthetase in its overall three-dimensional architecture.

TABLE 5

Comparison of Human PheRS with *T. thermophilus* PheRS by DLS Measurement

	R_h (nm)		M_r (kDa)	
Enzyme	4°C	20°C	4°C	20°C
Human PheRS	7.08	6.74	334	297
T. thermophilus PheRS	7.18	6.74	345	297

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