Peptidyl-tRNA hydrolase (Pth) activity releases tRNA from the premature translation termination product peptidyl-tRNA. Two different enzymes have been reported to encode such activity, Pth present in bacteria and eukaryotes and Pth2 present in archaea and eu- karyotes. Here we report the crystallographic structure of the Homo sapiens Pth2 at a 2.0-Å resolution as well as its catalytic properties. In contrast to the structure of Escherichia coli Pth, II. sapiens Pth2 has an α/β fold with a four-stranded antiparallel β-sheet in its core surrounded by two α-helices on each side. This arrangement of secondary structure elements generates a fold not previously reported. Its catalytic efficiency is comparable with that reported for the archaeal Sulfolobus solfataricus Pth2 and higher than that of the bacterial E. coli Pth. Several lines of evidence target the active site to two close loops with highly conserved residues. This active site architecture is unrelated to that of E. coli Pth. In addition, intermolecular contacts in the crystal asymmetric unit cell suggest a likely surface for protein-protein interactions related to the Pth2-mediated apoptosis.

During the protein translation process, a significant proportion of the ribosomes that initiate mRNA readout do not reach the stop codon. Peptidyl-tRNA molecules may dissociate from the mRNA template causing a premature end of the process (1, 2). Accumulation of peptidyl-tRNAs reduces the efficiency of translation by sequestering tRNAs and impairing the initiation (3). Prokaryotic and eukaryotic cells show an enzymatic activity that releases the peptidyl moiety from the tRNA, called peptidyl-tRNA hydrolase (Pth), allowing the free tRNA and peptide to be reused in protein synthesis (4, 5). First identified in Escherichia coli, genes encoding Pth have been found in bacteria and eu karyotes but not in bacterial genomes. Pth lacks any detectable homology to solved three-dimensional structures. Sulforobus solfataricus Pth2 displays a greater catalytic efficiency toward E. coli diacetyl-lysyl-tRNA than E. coli Pth (9, 6). In addition, both enzymes differ in their mode of recognition of several tRNA features, like the 1–72-base pair mismatch in the stop codon. Peptidyl-tRNA molecules may dissociate from the ribosomes that initiate mRNA readout do not reach the stop codon. Peptidyl-tRNA molecules may dissociate from the mRNA template causing a premature end of the process (1, 2). Recent ly, a Pth-like activity has been identified in Methano- caldococcus jannaschii and characterized in Sulfolobus solfar- ticus, both archaeabacteria (8, 9). The new enzyme called Pth2 is shorter in length and lacks any significant sequence homol- ogy to Pth. It coincides with the Pfam database entry “Unchar- acterized Protein Family 0099” (UPF0099) found in archaea and eu karyotes but not in bacterial genomes. Pth2 lacks any detectable homology to solved three-dimensional structures. S. solfataricus Pth2 displays a greater catalytic efficiency toward E. coli diacetyl-lysyl-tRNA than E. coli Pth (9, 6). In addition, both enzymes differ in their mode of recognition of several tRNA features, like the 1–72-base pair mismatch in E. coli tRNAMet or the presence of a phosphorylated 5′-end.

Human Pth2, also called Bel-2 inhibitor of transcription 1 (Bit1), possesses an N-terminal signaling sequence involved in its mitochondrial localization, indicating that it may serve to maintain efficient protein translation there. To understand the structural basis of Pth2 function, we report the crystal structure of human Pth2 and the characterization of its hydrolase activity. Pth2 structural fold is unrelated to that of Pth. It belongs to the α/β class of proteins containing three layers with a twisted mixed β-sheet formed by four strands. In vitro assays demonstrate that human Pth2 is active, showing a catalytic efficiency higher than that of Pth. Analysis of the electrostatic properties of the structure, as well as the conservation of reactive residues and their position on the three-dimensional structure, delineates a putative active site.

**MATERIALS AND METHODS**

**Subcloning, Overexpression, and Protein Purification—**Based on a sequence alignment of homologous domains, the first 62 residues of the 179-amino acid-long human sequence do not belong to the enzyme and are likely involved in mitochondrial localization. Consequently we de- fined the human Pth2 as the 63–179 fragment. The DNA-coding frag- ment was obtained by standard PCR methods using full-length human Pth2 clone as template (GenBank™/EBI accession number AF151905) and subcloned into the NcoI/BamHI sites of the pET15b bacterial expression vector (Novagen), thus adding no tag to the protein. DNA sequencing corroborated the identity of the inserted fragment. The ligated plasmid was transformed into E. coli BL21(DE3) strain. Cells were grown at 37 °C in LB medium, and overnight protein expression at 15 °C was carried out upon addition of 0.5 mM isopropyl-1-thio-β-D- galactopyranoside. Overexpression and solubility were assessed by protein extraction with B-PER reagent (Pierce) followed by SDS-PAGE. The fragment 63–179 was highly expressed and soluble. Cell pellets...
coming from 1 liter were resuspended in 25 ml of lysis buffer (100 mM Tris pH 7.5) and sonicated three times. Taking advantage of the high isoelectric point of the protein (pI 9.3) and after centrifugation, filtered supernatant was loaded into a cationic exchange column and purified in native conditions using a gradient of NaCl by standard fast protein liquid chromatography methods. Protein eluted at a salt concentration of 250 mM. Further purification was achieved by gel filtration methods using 20 mM Tris pH 7.0 and 150 mM NaCl as running buffer. The protein eluted at its monomeric position. Purity and identity of the fragment were checked by matrix-assisted laser desorption ionization mass spectrometry techniques. Circular dichroism and differential scanning calorimetry were used to confirm the presence of secondary and tertiary structure.

**Crystallization and Structure Determination**—Crystals grew in 24 h at room temperature using sitting drop vapor diffusion techniques by mixing protein at 10 mg/ml with a solution containing 100 mM Hepes pH 7.5 with 20% (w/v) polyethylene glycol-10,000 and equilibrating against a reservoir of the same solution. For data collection, crystals were transferred into cryoprotectant solutions consisting of mother liquor containing increasing concentrations of glycerol up to 20% (v/v) and flash-frozen in liquid nitrogen. Data from native and derivative crystals were collected at 100 K at the Stanford Synchrotron Radiation Laboratory. Data were indexed, integrated, and scaled with DENZO and SCALEPACK programs (11). Phase information was obtained by multiple wavelength anomalous diffraction using NaBr-soaked crystals (12). Positions of five bromine atoms were determined, and phases were calculated to a 2.8-Å resolution applying the software SOLVE (13). After phase improvement with the program DM (14), a readily interpretable map was obtained. Based on the experimental electron density map an initial model was built with the program TURBO-FRODO and refined by simulated annealing with the CNS software package (15) against native data to a 2.0-Å resolution. Further calculations using cycles of conjugate gradient minimization and individual B-factor refinement alternated with manual model building lead to the final model. The stereochemical quality of the refined model was verified with the PROCHECK software (16).

**Enzymatic Assay**—Hexahistidine-tagged E. coli lysyl-tRNA synthetase was overexpressed in MG1655 cells and purified by nickel-nitrotriacetic acid (Qiagen) affinity chromatography as described previously (17).
Table I

Summary of crystallographic analyses and refinement statistics

|                  | Native                          | Nalc multiple wavelength anomalous diffraction | Remote |
|------------------|--------------------------------|---------------------------------------------|--------|
|                  |                               | Peak                                        | Inflection | Remote |
| Data collection* |                               | 1.0079                                      | 0.9195 | 0.8670 | 0.9199 |
| Wavelength (Å)   |                               | 2.0 (2.07–2.00)*                           | 2.8 (2.9–2.8)* | 2.8 (2.9–2.8)* | 2.8 (2.9–2.8)* |
| Resolution (Å)   |                               | 168,771                                     | 40,827 | 42,451 | 38,958 |
| Unique reflections |                             | 24,275                                      | 16,149 | 16,166 | 15,969 |
| Completeness (%) |                               | 99.7 (99.9)*                                | 99.1 (99.7)* | 99.2 (99.4)* | 98.5 (98.9)* |
| R_syn (%)        |                               | 6.6 (45.7)*                                 | 10.2 (38.3)* | 9.8 (37.3)* | 9.7 (36.9)* |
| (dof)            |                               | 27.7 (4.7)*                                 | 7.9 (2.2)* | 8.8 (2.7)* | 7.5 (1.9)* |
| Figure of merit after density modification | | 43.0–2.0 | | | |
| Refinement statistics |                             |                                            | | | |
| Resolution range (Å) |                             | 43.0–2.0 | | | |
| Unique reflections (free) |                     | 23.7 | | | |
| R_work (%)       |                               | 21.1 | | | |
| R_free (%)       |                               | 23.3 | | | |
| Number of residues |                             | 232 | | | |
| Number of solvent molecules |                       | 123 | | | |
| Average B value (Å²) |                             | 29.3 | | | |
| Protein         |                               | 36.7 | | | |
| Solvent         |                               | 0.008 | | | |
| Root mean square deviation bond lengths (Å) | | 1.40 | | | |
| Root mean square deviation angles (°) | | | | | |

* Space group, P 4 2 2 1; cell dimensions, a = b = 61.6 Å, c = 177.9 Å; molecules in asymmetric unit = 2.

Numbers in parentheses correspond to the outer shell.

* Keeping Bijvoet pairs separate. R_syn = Σ Iᵢ − (O) Σ Iᵢ.

The trNA-charging reaction (1 ml) contained 50 msn HEPE5 (pH 7.5), 2 mM ATP, 10 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM EDTA, 60 µM lysine, 6 µM [4,5-3H]lysine (80 Ci/mol supplied by Amersham Biosciences), and E. coli trNA⁴⁰⁰ (2.4 nmol, supplied by Sigma). The mixture was equilibrated at 27 °C before the addition of lysyl-tRNA synthetase (50 nm). After 45 min the lysyl-tRNA⁴⁰⁰ reaction product was purified by phenol-chloroform and ethanol precipitation. Aminoacylated tRNA⁴⁰⁰ was then dissolved in a solution of 5 mM sodium acetate (500 µL, pH 5.5), dimethyl sulfoxide (100 µL), and acetic acid (200 µL). N-Acetylation of lysine was achieved with the addition of acetic anhydride (200 µL) followed by incubation at 0 °C for 1 h. The reaction product was precipitated with ethanol. The precipitate was redissolved in 200 mM sodium acetate (pH 5.5) containing 10 mM CuCl₂ and incubated for 1 h at 37 °C to remove unacylated lysine from tRNA⁴⁰⁰. Free amino acid was cleared by ethanol precipitation. Residual copper ions were extracted by chromatography over a column containing 300 µL of Chelex 100 resin (Sigma) in 150 mM sodium acetate (pH 5.5) (18). Prior to use in kinetic assays the substrate was ethanol-precipitated, dried at room temperature, and dissolved in sterile H₂O.

Decacylation assays (200 µL) were conducted at 27 °C in 20 mM Hepes (pH 7.5), 50 mM KCl, 20 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM EDTA, 0.5 µg/mL bovine serum albumin, 30–5000 nM diacetyl-lysyl-tRNA⁴⁰⁰, and catalytic amounts of human Pth2. Reactions were initiated with the addition of enzyme. At set time points, aliquots (36 µL) were removed and mixed with 500 µL of trichloroacetic acid (5% v/v) on ice. Precipitated tRNA was pelleted by centrifugation. A portion of the supernatant (400 µL) was then removed and mixed with scintillation fluid. Free [4,5-3H]diacetyl-lysine was quantified by liquid scintillation counting. Kinetic parameters were determined using initial rate methodology. Rates were recorded over a range of substrate concentrations. Kₘ and Vₘₐₓ values were derived from iterative non-linear fitting of the theoretical Michaelis-Menten equation to the experimental values using the Levenberg-Marquardt algorithm (19).

RESULTS AND DISCUSSION

A sequence similarity search using the human AF151905 full-length sequence as query on the non-redundant sequence data base with the Psi-Blast algorithm produced several reliable hits below a cutoff E value of 1 × e⁻20. The sequences belonged to eukaryotes and archaea organisms but not to bacteria. For the archaeal sequences the similarity extended only to the two-third C-terminal end of the query sequence suggesting that the first third of the protein in eukaryotes is a localization signal. That is the case for the human protein encoded by chromosome 17 and located in the mitochondria. The catalytic fragment (residues 63–179 of the human protein) was used to generate a multiple sequence alignment as shown in Fig. 1. This fragment coincides with the Pfam data base entry UP0099 (20). Because of its catalytic properties we will refer to it as Pth2.

The catalytic fragment (residues 63–179) was produced in E. coli as a soluble protein and purified to homogeneity behaving as a monomer in solution. Its crystal structure was solved by multiple wavelength anomalous diffraction techniques and refined to a 2.0-Å resolution (Fig. 2). The crystal contained two molecules in the asymmetric unit. The final model has an Rₜwork of 21.1% and an Rfree of 23.7% (Table I). It includes all residues of the first molecule and all but Gly-147 and Arg-148 of the second. Regarding the stereochemical quality of the model, 94% of the main chain torsion angles of non-glycine residues lie in...
the most favored regions of the Ramachandran plot and the rest in the additionally allowed regions.

Human Pth2 is 116 residues and folds into a α/β structure with a twisted mixed β-sheet formed by four strands surrounded by two α-helices on each side (Fig. 3). The mixed β-sheet is arranged in the order β2-β1-β4-β3, with β4 being antiparallel to the rest. Browsing of the SCOP data base (20) indicates that this particular arrangement has not been observed in other proteins. The closest fold is the thioredoxin family where the antiparallel strand is β3 and occupies the same internal position as β4 in Pth2. The Pth2 fold differs from that of Pth. E. coli Pth (Protein Data Bank code 2PTH) is 193 residues long and shows a hydrolase-like fold with a mixed β-sheet of five strands (order 2-1-3-5-4) in its core with strand 4 antiparallel to the rest (6).

It has been reported that human Pth2, also called Bit1, is a multifunctional protein (21) that mediates apoptosis via an interaction with a transcriptional co-repressor.² The contacts observed between molecules in the crystal asymmetric unit suggest a likely area for such protein-protein interaction. The two molecules are related by a 2-fold noncrystallographic symmetry axis where the main contacts are through the α1 α-helix of molecule A with that of molecule B and the loop between strand 3 and 4 (β3-β4 loop) of molecule A with the loop between helix 2 and strand 2 (α2-β2 loop) of molecule B (Fig. 4).

Because the fold is unlike that of any other known hydrolase, it was important to establish the hydrolytic activity of human Pth2. Substrate kinetics was analyzed using purified diacetyl-lysyl-tRNA from E. coli. Initial reaction rates were determined at different substrate concentrations. Rate data were best fit to the theoretical Michaelis-Menten equation with $k_{\text{cat}} = 3.6 \pm 0.2 \text{ s}^{-1}$ and $K_{m} = 0.22 \pm 0.06 \mu\text{M}$. Thus, E. coli-derived diacetyl-lysyl-tRNA represents an efficient substrate for human Pth2. The catalytic efficiency ($k_{\text{cat}}/K_{m} = 5.9 \mu\text{M}^{-1} \text{s}^{-1}$) for the human enzyme is also comparable with that of the archaea S. solfataricus Pth2 (9), although both are higher than that reported for E. coli Pth (Table II) (6). Most likely Pth2s recognize more extensively the tRNA structure than Pths accounting for the lower $K_{m}$ of Pth2s.

Both enzymes (Pth and Pth2) are carboxylic ester hydrolases acting as monomers on N-substituted aminoacyl-tRNA and water to produce N-substituted amino acid plus the free tRNA (EC 3.1.1.29) (20). The sequence hallmark for α/β hydrolase fold enzymes, like the E. coli carboxylesterase BioH (Protein Data Bank code 1M33; EC 3.1.1.11) (22), is the conserved catalytic triad formed by a nucleophile (serine), an acid (aspartic acid), and a basic (histidine) residue. Analysis of the E. coli Pth catalytic activity showed that multiple residues are implicated in the recognition of the extended substrate, including Lys-105 and Arg-133 for 5′-tRNA recognition and Asn-68 and Asn-114 for 3′-tRNA and peptide backbone recognition (7).

As expected for molecules interacting with nucleic acids, Pth
and Pth2 show a high isoelectric point (9.1 and 9.3, respectively) and a strong electrostatic polarization (23). An electrostatic potential representation of the human Pth2 structure (Fig. 5) showed a sidedness of electropositive density (blue) along the $\beta_1$-$\alpha_1$ and $\beta_3$-$\beta_4$ loop (top) as well as along the $\alpha_1$-helix (left), indicating a potential surface for tRNA recognition.

Analysis of the multiple sequence alignment for Pth2s (Fig. 1) shows several residues either strictly or highly conserved between species from archaea to eukaryotes, indicative of a functional or structural role in the protein. Of these residues, those highly exposed to the solvent and in close spatial proximity in the three-dimensional structure and with an appropriate reactive side chain are prime candidates to be involved in catalytic activities. From these observations we propose the $\beta_1$-$\alpha_1$ loop residues Arg-72, Asp-74, and Lys-81 and the $\beta_3$-$\beta_4$ loop residues Asp-145, Arg-148, Thr-149, Gln-150, and Ser-155 as the candidates for forming the active site of the enzyme (Fig. 6). In particular, Asp-145 in our refined model is capable of forming hydrogen bonds with the side chains of Lys-81, Arg-148, and Ser-155. Moreover, three well ordered water molecules are in its close vicinity.

Taken together, this work describes the first structure of a Pth2. Functionally, this enzyme resembles a Pth. However, its amino acid length and sequence, structural fold, and catalytic efficiency are different. We conclude, therefore, that the structure of human Pth2 illustrates a new type of peptidyl-tRNA hydrolase. The availability of this three-dimensional structure and the proposed localization of the active site should be helpful to design experiments aimed at probing the basis of specificity in this new family.

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