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Human pancreatic ribonuclease

Deletion of the carboxyl-terminal EDST extension enhances ribonuclease activity and thermostability

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Mammalian ribonucleases constitute one of the fastest evolving protein families in nature. The addition of a four-residue carboxyl-terminal tail: Glu-Asp-Ser-Thr (EDST) in human pancreatic ribonuclease (HPR) in comparison with bovine pancreatic RNase (RNase A) could have adaptive significance in humans. We have cloned and expressed human pancreatic ribonuclease in *Escherichia coli* to probe the influence of the four-residue extension and neighboring C-terminal residues on the biochemical properties of the enzyme. Removal of the C-terminal extension from HPR yielded an enzyme, HPR-(1–124)-peptide, with enhanced ability to cleave poly(C). HPR-(1–124)-peptide also exhibited a steep increase in thermal stability mimicking that known for RNase A. Wild-type HPR had significantly low thermal stability compared to RNase A. The study identifies the C-terminal boundary in the human pancreatic ribonuclease required for efficient catalysis.

Keywords: ribonuclease; enzyme; protein engineering; secondary structure; RNA.

Mammalian ribonucleases (RNases) constitute a ubiquitous superfamily of proteins with a high level of structural and functional divergence. Although the most obvious function of these enzymes is to degrade RNA, there is increasing evidence that they participate in many additional and more subtle biological processes apparently unrelated to routine RNA metabolism, earning them the designation RISBASEs (RNases with special biological actions; D'Alessio, 1993). Human pancreatic ribonuclease (HPR), unlike its bovine analog (RNase A), has been considered to be vestigeal in the non-ruminant human host where it is not required for the salvage of phosphorus from intestinal microflora (Barnard, 1969). Although HPR is present in very small amounts (10 μg/g tissue as compared to 1200 μg RNase A/g bovine pancreas) (Barnard, 1969), the faithful retention of an enzyme for an apparently redundant function is intriguing.

Compared to RNase A, HPR contains a higher net proportion of basic residues (Sorrentino et al., 1992) and possesses substantial activity against double-stranded RNA (Bardon' et al., 1976; Sorrentino and Libonati, 1994). While rigorously retaining ribonucleolytically essential Lys and His residues, HPR differs from RNase A, in the presence of a C-terminal extension of four residues, Glu-Asp-Ser-Thr (EDST) beyond the terminal Val124 of RNase A. Although the carboxyl-terminal extension is known to arise as a result of a single base TAG→GAG mutation in the first termination codon in the open reading frame of HPR, the biological significance of the tetrapeptide tail is not known. The consequences of these differences could have relevance to the *in vivo* function of HPR and would be interesting to evaluate in that light. The presence of almost identical C-terminal extensions in pancreatic RNases from a host of non-ruminating mammals (Beintema et al., 1986) could be of significance in this respect.

We have cloned and expressed the HPR gene in *Escherichia coli* with an aim to analyze the role of the tetrapeptide extension and associated C-terminal residues in the enzymatic activity of HPR. The study reveals that the tetrapeptide C-terminal extension is not necessary for ribonuclease activity; presence of additional residues beyond Asp121, implicated in catalysis, are detrimental to enzymatic activity.

EXPERIMENTAL PROCEDURES

Preparation of plasmid constructs for full-length and mutant HPRs. Mature HPR is a 128-amino-acid protein (Seno et al., 1994). The intronless 384-bp HPR gene was amplified from genomic DNA isolated from human blood by a 30-cycle hot-start PCR. Primers, H1 (GTCCAGCCTTCCGCTAGCAAG-GAATCCCGG) and H2 (GCTCTGAATTCAGGTAGAGTCCT-CCACAGA), were designed to introduce an *Nhe*I restriction site on the 5′ end and an *Eco*RI site at the 3′ end, respectively, of the gene. The purified *Nhe*I+*Eco*RI-restricted HPR DNA was inserted into pAP 110, digested with the same enzymes; pAP 110 is a bacteriophage T7 promoter-based *E. coli* expression vector, containing a multiple cloning site, *E. coli* and phage origin of replication and T7 transcription terminator. The resulting plasmid, designated as pHPR, has the 384-bp DNA encoding HPR under the T7 promoter.

PCR was used to obtain genes encoding mutant HPR derivatives. pHPR was used as the template for constructing plasmids containing DNA encoding the mutants. Since all mutations were made at the C-terminus, a common 5′ primer, XUP (ACTCAC-TATAGGGAGACCAC) was designed using the vector sequence

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Abbreviations. HPR, human pancreatic ribonuclease; ds, double-stranded; RNase A, bovine pancreatic ribonuclease.

Enzyme. Pancreatic ribonuclease (EC 3.1.27.5).

at the 5′ end of the T7 promoter sequence in pHPR. Primer H3 (GGTAGGAATTCAGACAGAAGCATCAA) was used to construct plasmid pHPR(1−124) lacking four C-terminal codons and primer H4 (AGCAGCCGAATTCAATCAAAGTGGACTGGCAC), for plasmid pHPR(1−121), lacking seven C-terminal codons of HPR. PCR products were purified and digested with *Xba*I and *Eco*RI, and ligated into pVex 11 that had been cleaved with the same enzymes; pVex 11 is a T7 bacteriophage promoter based vector similar to pAP 110. It is a high-copy-number plasmid and is essentially different from pAP 110 only with respect to the multiple cloning site. The sequence at the beginning of the genes was 5′-ATG GCT AGC AAG GAA...-3′, introducing two additional residues, Ala and Ser, at the amino terminus of all proteins. The additional Ala and Ser residues at the N-termini of proteins arise from the *Nhe*I site (GCT AGC), which is immediately downstream of the *Nde*I site (CAT ATG) in the vectors that provides the translation start. The underlined sequences represent the first two codons of HPR. Inserts were fully sequenced to confirm correct integration of DNAs and the presence of the desired mutations (Sanger et al., 1977).

Expression and purification of recombinant HPR and its mutants. Initial screening for expression was performed in Luria-Bertani medium; 5 ml of the medium containing 100 μg/ml sodium ampicillin was inoculated with a single colony of *E. coli* BL21(λDE3) containing the recombinant plasmid and grown at 37°C in a gyratory shaker at 225 rpm. Expression of the recombinant protein was induced at an absorbance at 600 nm of 1.0 with 1 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested 90 min after induction and the periplasm was separated from spheroplast by cold osmotic shock. The separated fractions were electrophoresed on a 12.5% SDS/polyacrylamide gel and visualized by Coomassie blue staining. HPR and its mutant forms were found to be present entirely in the insoluble form as inclusion bodies. Mutant proteins were prepared from 1-l shake-flask cultures of *E. coli* BL21(λDE3) grown in superbroth medium containing 100 μg/ml sodium ampicillin. Bacterial inclusion bodies containing the heterologous protein were prepared and recombinant proteins processed as described (Buchner et al., 1992). Protein was isolated from the inclusion bodies by denaturation in guanidine hydrochloride and renaturation in a buffer containing arginine and oxidized glutathione. Renatured protein was dialyzed against 20 mM Tris/HCl pH 7.4 and loaded onto an 8-ml S-Sepharose column (Pharmacia Biotech AB) at 4°C equilibrated with 20 mM Tris/HCl pH 7.4. The protein was eluted from the column with a linear gradient of 0−1 M NaCl and fractions containing protein of the desired size, as detected by 12.5% SDS/PAGE, were pooled. Protein from the S-Sepharose column was further purified on a Spherogel TSK column (3000 SW, Beckman). Fractions containing monomeric protein were pooled, quantitated (Bradford, 1976) and stored frozen at −70°C for further experiments.

Assay for enzymatic activity. RNase activity was assayed as described (Bond, 1988). Briefly, 40 μg RNA was incubated at 37°C with enzyme in a reaction buffer that consisted of 100 mM Tris/HCl pH 7.5, 10 mM EDTA and 0.01% acetylated BSA. The reaction was terminated after 60 min with 1 vol. cold 1.16 M perchloric acid and 5.9 mM uranyl acetate and chilled on ice for 15 min. Unreacted high-molecular-mass RNA was removed by centrifugation at 15000×*g* for 10 min and absorbance of supernatants at 260 nm was measured in a Varian DMS 100S ultraviolet/visible spectrophotometer after 1:4 dilution into distilled water. The absorbance of the blank reaction containing 0.01% acetylated BSA in place of enzyme was subtracted from all the readings. Michaelis constants (K_m) with poly(C) substrate were determined for the enzymes from double-reciprocal plots of initial reaction velocities.

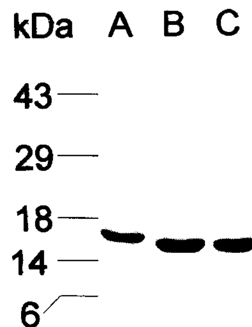

Fig. 1. Purification of recombinant HPR and mutant analogs used in the study. The genes encoding HPR and the indicated mutants were expressed in *E. coli* BL21(λDE3) and purified. Aliquots of 5 μg protein were then subjected to SDS/PAGE using 12.5% resolving gels and stained with Coomassie brilliant blue R 250. The position of molecular mass markers is shown on the left. Lane A, wild-type HPR; lane B, HPR-(1−124)-peptide; lane C, HPR-(1−121)-peptide.

Assay for thermal stability. Full-length and mutant proteins in 0.01% acetylated BSA were heated to 80°C for 40 min. in a dry-heat bath and RNase activity was assayed with poly(C) as described. Residual RNase activity was calculated with the activity of untreated enzymes taken as 100%.

Circular dichroic measurements. CD spectra were recorded at room temperature using a Jasco J 720 (Easton) dichrograph equipped with a thermostated cell holder. A cell of 1 cm optical path length was used to record spectra of proteins in the far-ultraviolet region (250−190 nm) at a protein concentration of 100 μg/3 ml in 10 mM sodium phosphate pH 7. Spectra were acquired at a scan speed of 50 nm/min with a sensitivity of 50 mdeg and response time of 1 s. The sample compartment was purged with nitrogen and spectra were averaged over five scans. The solvent dichroic absorbance contribution was subtracted using the Jasco software. Secondary structures were calculated from the Jasco secondary structure estimation program based on reference parameters of Yang et al. (1986). The thermal stability of wild-type HPR and HPR-(1−124)-peptide was evaluated by monitoring CD spectra in the far-ultraviolet region at temperatures of 20, 37, 50, 65 and 80°C. Results are presented as mean residue ellipticity values [θ], based on the amino acid mean residue mass of 110 Da. Bovine RNase A was included as a control.

RESULTS

High-yield purification of recombinant human pancreatic ribonuclease. We have expressed the HPR gene using a T7 promoter-based *E. coli* expression vector and report here an essentially two-step purification scheme that results in high yields of pure protein. The overexpressed protein was isolated from inclusion bodies, solubilized in guanidine · HCl and renatured *in vitro* using a glutathione-based refolding buffer. Recombinant HPR was subsequently purified to homogeneity by successive cation-exchange and gel-filtration chromatography. Purified recombinant HPR migrated with an apparent molecular mass of 16 kDa on a 12.5% SDS/PAGE (Fig. 1). Typical final yields of purified protein were in the range of 30−50 mg/l. Mutant forms of HPR were also cloned in the T7 promoter-based *E. coli* expression vector. All proteins were localized in inclusion bodies in bacteria, and could be purified in similar fashion to homogeneity with high yields (Fig. 1).

Deletion of C-terminal EDST tail and ASVEDST enhances ribonuclease activity. The influence of the EDST extension in

Table 1. Comparative catalytic activity of HPR mutants. Ribonuclease activity of recombinant HPR and its derivatives was assessed using natural and synthetic RNA substrates. Values represent average of duplicate determinations with individual values not differing from each other by more than 10%. Amount of enzyme used/40 µg substrate = 12.5−100 ng for poly(A) · poly(U), 0.0625−0.5 ng for poly(C) and 0.125−1 ng for yeast tRNA.

Enzyme	Activity on		
	poly(C)	yeast tRNA	poly(A) · poly(U)
	ΔA_{260} · min^{-1} · mg protein^{-1}		
HPR (full-length)	214667	19067	330
HPR-(1−124)-peptide	1253333	30400	1270
HPR-(1−121)-peptide	467200	24600	1350

catalysis was studied with a mutant, HPR-(1−124)-peptide lacking the four C-terminal amino acids. This peptide exhibited enhanced ribonuclease activity as compared to HPR with all substrates tested (Table 1). Marked differences in RNase activities of HPR-(1−124)-peptide were observed with the favored model substrate poly(C) and the double-stranded substrate, poly(A) · poly(U). Changes in activity profiles with yeast tRNA were less dramatic with only marginal increases in activity. Thus, while HPR-(1−124)-peptide was almost 4 and 6 times as active as the wild-type enzyme on poly(C) and poly(A) · poly(U) respectively, it was only 1.6 times more active on yeast tRNA (Table 1).

HPR-(1−121)-peptide, devoid of the seven carboxyl-terminal residues ASVEDST, also exhibited enhanced activity with all substrates tested (Table 1). As seen with the HPR-(1−124)-peptide, increase in activity was more pronounced with the favored substrate poly(C) and the duplex substrate poly(A) · poly(U), being respectively, 2 and 4 times more on these substrates. This mutant was only marginally (1.3 times) more active on yeast tRNA substrate (Table 1). Deletions beyond D121 resulted in complete inactivation of HPR (data not shown).

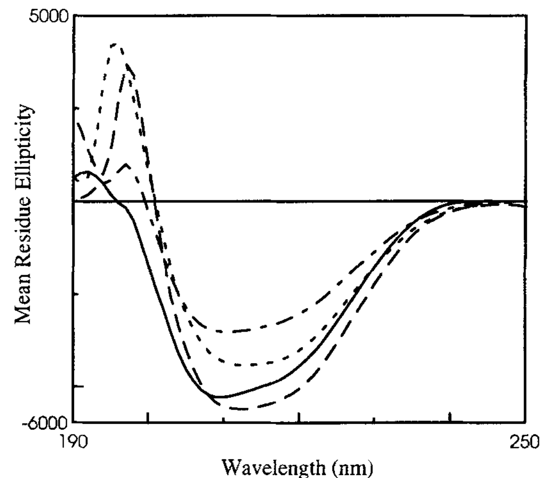

Fig. 2. CD spectra of HPR and mutant proteins. Far-ultraviolet spectra of recombinant proteins were recorded at 25°C; units are deg · cm^2 · dmol^{-1}. Data was averaged over five scans. Bovine RNase A was included as a control. (– – –) HPR; (——) RNase A; (· · · · ·) HPR-(1−124)-peptide; (— · —) HPR-(1−121)-peptide.

Michaelis constants (K_m) for enzymes were derived from double-reciprocal plots of initial rates of reactions using poly(C) substrate. In 100 mM Tris/HCl pH 7.5, the K_m values of the substrate, poly(C), were marginally higher for the HPR-(1−124)-peptide (1145 µM) and HPR-(1−121)-peptide (920 µM) mutants as compared with the wild-type enzyme (881 µM). Removal of acidic residues (E, D) in the tetrapeptide extension may alter local charge distributions and facilitate release of cleaved ribonucleotides as an increase in K_m is reflected in the lowered affinity of substrate binding.

Secondary structure analysis of HPR and mutants by CD. Far-ultraviolet CD spectra of HPR recorded at 25°C showed negative ellipticities at 208, 217 and 222 nm and strong positive peaks in the region of 190 and 195 nm indicating that the protein possesses both α-helix and β-strand structure (Fig. 2). The α-

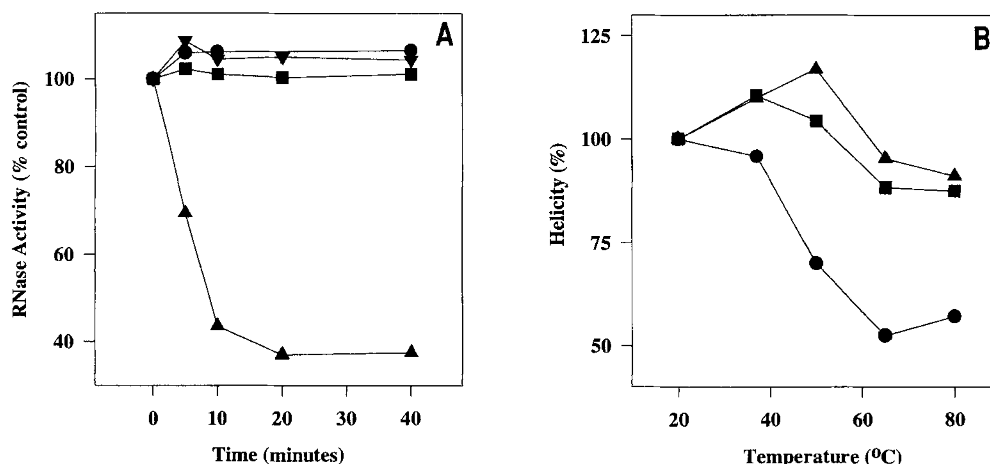

Fig. 3. (A) Effect of thermal denaturation on the catalytic activity of HPR and its mutants and (B) change in helicity of proteins with increasing temperature. (A) Proteins were heated at 80°C and ribonuclease activity with poly(C) was assayed at various time points. Residual ribonuclease activity is indicated as a percentage of activity at time zero. Bovine RNase A was used as a control. (▲) Full-length HPR; (●) HPR-(1−124)-peptide; (■) HPR-(1−121)-peptide; (▼) RNase A. (B) Far-ultraviolet spectra of proteins were recorded as a function of increasing temperature. Helicity was calculated from the mean residue ellipticity according to Morrisett et al. (1973) using the equation: % helicity = ($[\theta]_{222}$−3000/−36000−3000)×100. (●) HPR; (■) HPR-(1−124)-peptide; (▲) RNase A.

helical content of HPR (17.45%), calculated according to Yang et al. (1986) appears to be higher than that of RNase A (10.60%). Estimates of secondary structure elements for RNase A were in close agreement with reported values. The helical content of HPR was observed to decrease successively upon deletion of four and seven C-terminal residues. This is in keeping with structure predictions by the method of Chou and Fasman (1974) which reveal that HPR possesses an additional helical region at the carboxyl terminus encompassing the eight terminal residues DASVEDST (data not shown).

Deletion of C-terminal EDST enhances thermal stability. The HPR-(1−124)-peptide lacking C-terminal residues EDST was found to be more stable than the full-length protein towards heat denaturation. While the wild-type enzyme lost upto 60% of its activity when heated at 80°C for 40 min, HPR-(1−124)-peptide retained full activity at the end of 40 min. A similar effect was seen with the HPR-(1−121)-peptide lacking seven carboxyl-terminus residues ASVEDST. The thermal stability profiles of the mutant constructs are comparable to those of RNase A, which is known to be stable at high temperatures (Fig. 3A).

Evaluation of thermal stability of HPR mutants by CD. Thermal unfolding of HPR, HPR-(1−124)-peptide and RNase A was evaluated by monitoring changes in CD spectra in the far-ultraviolet range (250−190 nm). Marked deviation in absorption pattern of HPR at 50°C in comparison to HPR-(1−124)-peptide and RNase A indicates loss of considerable secondary structure. HPR underwent upto 30% and 50% decrease in helical content at 50°C and 65°C, respectively, whereas the helical contents of HPR-(1−124)-peptide or RNase A were not drastically affected upto 65°C (Fig. 3B). This suggests that unfolding occurs earlier or at a faster rate in HPR than in HPR-(1−124)-peptide or RNase A.

DISCUSSION

The T7-promoter based *E. coli* expression system was used to obtain high-level expression of human pancreatic ribonuclease. Earlier, HPR has been expressed in mammalian cells (Russo et al., 1993) and, more recently in *E. coli*, cloned downstream of the T7 promoter (Futami et al., 1995). However, the investigators have employed a complicated down-stream processing protocol to purify the enzyme from *E. coli* and obtain low levels of the purified product, 1.1−1.2 mg/l. Analysis of CD spectra of HPR indicated a similar overall organization of backbone structure as RNase A, albeit with a higher helical content. These observations suggest that the renaturation process employed in this study for purification of recombinant HPR yields correctly folded protein. Further, the presence of two N-terminal residues, Ala and Ser, introduced as a consequence of the strategy used for cloning do not appear to be detrimental to the conformation or the enzymatic activity of the protein.

This study reveals that the EDST extension is not essential for ribonuclease activity of HPR. It appears, however, to modulate the biochemical behavior of the enzyme. Thus, removal of the tetrapeptide extension enhanced enzymatic activity over that of the wild-type enzyme. This mutant has a carboxyl terminus that is identical to that present in RNase A. CD spectral analysis predicts similar distribution of secondary structural elements in HPR-(1−124)-peptide and RNase A and the increase in activity observed with HPR-(1−124)-peptide reflects a shift towards a structure more like RNase A. Presence of additional C-terminal residues beyond Asp121 in HPR are detrimental to RNase activity.

In addition, compared to RNase A, wild-type HPR was found to be a thermolabile ribonuclease. HPR-(1−124)-peptide, in contrast, exhibited enhanced thermostability mimicking that observed in RNase A; HPR-(1−121)-peptide also exhibited enhanced thermal stability of the same order. The presence of negatively charged residues, Glu125 and Asp126, near the carboxyl terminus of HPR may lead to destabilization of the C-terminal helix according to the helix dipole model (Shoemaker et al., 1985) and contribute to decreased stability of the wild-type protein. In addition, these extra residues may hinder efficient folding in the β-sheet region encompassing residues 105−124 known in RNase A to contain a primary chain folding initiation site (Beals et al., 1991). Beals et al. (1991) have further reported stabilization of a 20-amino-acid peptide consisting of the RNase A cluster 105−124 (HIVACEGNPYVPVHFDASV), which was identified as a nucleation site, as a result of intramolecular hydrophobic interactions. The hydrophobic interactions observed in this stretch were found to be sufficient to direct the partial folding of the peptide. Deletion of four C-terminal residues from HPR would eliminate the restrictive/destabilizing forces and enable the carboxyl terminus chain-folding initiation site to function optimally and lead to a folded structure that resembles that of RNase A.

An extended carboxyl-terminus, similar to that present in HPR, has been observed in pancreatic RNases from a number of mammalian orders (Beintema et al., 1986). Curiously, these are present only in mammals with a non-ruminant habit. An EDST-like extension, for instance, is found in pancreatic RNase from langur (*P. entellus*) and sloth (*C. hoffmanii* and *B. infuscatus*), whereas some of its degenerate forms: EVST, GPST and EPST are found respectively in horse (*E. caballus*), porcupine (*H. cristata*), and guinea pig (*C. porcellus*) and capybara (*H. hydrochaeris*). Although a definitive role for the retention of the pancreatic ribonuclease in the human host has not been found, it is possible that HPR plays a role in mammalian physiology in addition to RNA digestion; the presence of the tetrapeptide extension may be of significance in this respect.

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