The Primary Structure of Langur (*Presbytis entellus*)
Pancreatic Ribonuclease: Adaptive Features in Digestive Enzymes in Mammals

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The primary structure of pancreatic ribonuclease from langur (*Presbytis entellus*) has been determined. This sequence differs from that of human pancreatic ribonuclease at 14 (11%) of the amino acid positions. Eight of these 14 differences involve changes of charge, with the langur enzyme having five fewer positive charges than the human enzyme. The difference in charge between human and langur ribonuclease may be an adaptation to the different requirements for a nondigestive and a digestive role, respectively. A number of similarities in expression, gene duplications, and properties between mammalian ribonucleases and lysozymes have been observed, indicating similar adaptations in both enzyme systems.

Introduction

Pancreatic ribonuclease (E.C.3.1.27.5) is one of the most intensively investigated digestive enzymes in mammals (Beintema et al. 1988a). However, it differs from other digestive enzymes in having highly different levels of expression in different species (Barnard 1969; Beintema et al. 1973) and in being found in other tissues and body fluids as well (Weickmann and Glitz 1982; Morita et al. 1986; Beintema et al. 1988a), indicating that the enzyme has other physiological functions besides its role in digestion. High levels of pancreatic ribonuclease occur in ruminants, in species with ruminant-like digestion, and in several mammals with cecal digestion. Barnard (1969) proposed that these elevated levels are a response to the necessity of digesting large amounts of ribonucleic acid derived from the symbiotic microflora of the stomach or cecum of these herbivorous mammals. It even has been suggested that the digestive role of ribonuclease in ruminants is a recent evolutionary innovation arising from other roles of secretory ribonucleases (Benner and Allemann 1989). However, high levels of ribonuclease have been found in species in unrelated mammalian orders such as the marsupials (kangaroos), edentates (sloth), rodents, perissodactyls, and artiodactyls, and in all investigated mammalian species at least a trace of pancreatic ribonuclease could be demonstrated, indicating that the presence of a digestive pancreatic ribonuclease is a universal feature in mammals.

Elevated levels of lysozyme have been found in the stomach of several ruminants and species that have a ruminant-like digestion (Dobson et al. 1984) and in the intestine of species with cecal digestion (Hammer et al. 1987). These elevated levels are explained by the necessity to digest cell walls from the symbiotic bacterial microflora in these mammals. The recruitment of ribonuclease and lysozyme in the digestive systems of

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470
herbivores to hydrolyze components from the symbiotic microflora has resulted not only in adaptations in the levels of expression of these enzymes but also in convergence to similar properties in different taxa. This may be attended by extensive convergent (or parallel) changes in amino acid sequence: a large surface region (about one-third of the sequence) of pancreatic ribonucleases of pig and guinea pig B, including two glycosylation sites, is very similar in sequence as a result of parallel evolution. This has been attributed to an adaptation to cecal digestion (Beintema et al. 1977). Another example is the similarity in sequence between cow and langur stomach lysozymes as an adaptation to foregut fermentation in both mammals (Stewart et al. 1987).

Leaf-eating primates belonging to the Colobinae have a ruminant-like digestion that takes place in a large compartmentalized stomach (Bauchop and Martucci 1968). Here the isolation and amino acid sequence determination of the pancreatic ribonuclease of hanuman langur (Presbytis entellus), a Southeast Asian member of the Colobinae, is presented. This sequence is compared with that of man, a primate without foregut fermentation. Convergence to similar charge properties without additional sequence similarity has been found in several ribonucleases that have no role in microfloral digestion.

**Material and Methods**

Ribonuclease was isolated after homogenization of langur pancreas in 0.125 M sulfuric acid, ammonium sulfate fractionation between 50% and 90% saturation, gel filtration on Sephadex G-25, affinity chromatography (Wierenga et al. 1973), again gel filtration on Sephadex G-25 in dilute acid, and lyophilization. Enzyme activity was measured according to a method described by Shapira (1962).

For determination of the amino acid sequence of the protein, the disulfide groups were reduced with tributylphosphine, followed by modification with 4-vinylpyridine (Beintema et al. 1988b). The modified protein (0.5 mg) was treated with 25 mg cyanogen bromide in 50 μl 70% trifluoroacetic acid at room temperature during 20 h. Approximately 20% of the lyophilized cyanogen bromide digest was digested with 2 μg trypsin (treated with 1-1-tosylamido-2-phenylethylchloromethylketone) in 20 μl 0.2 M ammonium bicarbonate at 37°C during 2.5 h, and 0.05 mg reduced and pyridylethylated protein was digested with 5 μg Staphylococcus aureus V8 protease in 50 μl 0.2 M ammonium bicarbonate at 37°C during 20 h. Digests were lyophilized and peptides were isolated by reverse-phase high-performance liquid chromatography on a Nucleosil 10 C18 column (30 × 0.45 cm) in 0.1% trifluoroacetic acid with a linear gradient to 70% acetonitrile in 60 min. The unmodified protein and isolated peptides were submitted to automated Edman degradation in Applied Biosystems models 470A and 477A protein sequencers, respectively, equipped with on-line model PTH-analyzers.

**Results**

Approximately 0.5 mg pure ribonuclease (recovery 24%) was isolated from one single langur pancreas (8 g). On polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the purified protein exhibited one band with a mobility slightly lower than that of bovine ribonuclease A, suggesting that it is a glycoprotein with a simple carbohydrate moiety.

The amino acid sequence of langur ribonuclease is presented in figure 1, together with the elution times of the isolated peptides and with the recoveries of amino acid derivatives after automated Edman degradation. Peptides are prefixed with CN, T,
FIG. 1.—Amino acid sequence of langur (Presbytis entellus) pancreatic ribonuclease. Peptides are indicated by solid lines (--; sequenced) and dashed lines (---; not sequenced). The elution times of the peptides and the recoveries of the phenylthiohydantoin derivatives (in pmol) are indicated above and below the peptides, respectively. Peptides CN4 and CN3,4 and peptides CN5T3 and CN5TE1, respectively, were isolated as mixtures. C-terminal homoserine derivatives of cyanogen bromide peptides behaved as threonine. A plus sign (+) = present but not determined quantitatively; CHO = carbohydrate moiety.
and E for cyanogen bromide, tryptic, and \textit{Staphylococcus aureus} V8 protease peptides, respectively. The tryptic digest was contaminated with a trace of \textit{S. aureus} protease, resulting in additional cleavage behind Glu-61 (peptide CN5TE1) and Glu-111 (peptides CN6TE1 and CN6TE2).

**Discussion**

The investigated langur pancreas was found to contain 280 \( \mu \text{g} \) ribonuclease/g tissue, a value comparable to that found in ruminants and other species with ruminant-like digestion (Barnard 1969; Beintema et al. 1973). Human pancreas, on the other hand, has a low ribonuclease content (5 \( \mu \text{g} \)/g tissue), whose amino acid sequence has been determined (Beintema et al. 1984). Sequence and immunological studies (Weickmann and Glitz 1982; Morita et al. 1986; Beintema et al. 1988a) indicate that the enzyme also occurs in significant amounts in other tissues and body fluids. The presence of langur ribonuclease in other tissues and body fluids has not been investigated.

There are few ambiguities in the sequence. A very weak indication of Asn at position 34 was obtained. However, (1) the indirect evidence, from the gel electrophoresis, that the protein is glycosylated, (2) the presence of the sequence Met−Thr at positions 35-36, and (3) the very frequent occurrence of a glycosylated Asn−Met−Thr sequence at these positions in pancreatic ribonucleases indicate a glycosylated asparagine at position 34. An Asp−Asp sequence was found at positions 121-122. The presence of this sequence may explain the failure to cleave the C-terminal peptide with chymotrypsin at the Phe−Asp (120-121) bond, although this bond is the most susceptible chymotryptic cleavage site in ribonucleases having the more common Asp−Ala sequence at positions 121-122.

In figure 2 the sequences of langur and human pancreatic ribonuclease are compared. The two sequences differ at 14, or 11%, of the amino acid positions, while they differ at 25%–30% of the positions in pancreatic ribonucleases of other placental mammalian orders. Thus, even without further analysis of the data, it is clear that both primate ribonucleases are located on the same branch of the evolutionary tree of ribonucleases.

Eight of the amino acid replacements between both primate ribonucleases involve changes of charge (fig. 2). The net charge of human ribonuclease is +6 and that of langur ribonuclease is only +1 (when the five unvaried histidines are excluded). In previous studies (Libonati et al. 1976) we have demonstrated that strongly positively-charged ribonucleases are considerably more active on double-stranded RNA than are ribonucleases having a smaller excess of positive charges. We do not know whether the activity of these ribonucleases on double-stranded RNA has any function, but it may be an antiviral defense mechanism. The ribonucleases with the largest excess of positive charge are the pancreatic ribonucleases from man and whale and the bovine and brain ribonuclease (Beintema et al. 1988c). These enzymes have no or a minor function in the digestion of microfloral RNA from the stomach or cecum. However, there is preliminary evidence for foregut fermentation in baleen whales (Herwig et al. 1984). Thus, the difference in charge between human and langur ribonuclease may be an adaptation to the different requirements for a nondigestive and a digestive role, respectively. (Since all isolated langur ribonuclease was used for the sequence determination, no studies of the enzymic properties were performed.)

There are few unique amino acid replacements between langur and human pancreatic ribonuclease. Most have been observed earlier in other pancreatic ribonucleases.
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Fig. 2.—Amino acid sequences of langur and human pancreatic ribonucleases. Only the residues of human ribonuclease that differ from langur ribonuclease are shown. Above and below the sequences the positively and negatively charged residues are indicated with plus (+) and minus (−) symbols, respectively. There are five histidine residues, at identical positions (12, 48, 80, 105, and 115), in both proteins.
Positions involving amino acid replacements between charged and noncharged residues belong to the most varied ones. Evolutionary trees show that ribonucleases with a high excess of positive charges evolve from less positively charged ones by a rather random pattern of replacements, with relatively more changes to—rather than from—positive charges.

If ribonucleases expressed in the pancreas and in other tissues have different functions and have different structural adaptations to these separate functions, the use of a single gene product in a species may be considered as a compromise between different requirements. In man, the same ribonuclease gene product has been found in several tissues and body fluids, although large differences in organ-specific glycosylation have been observed (Beintema et al. 1988a). In the genome of rodents such as mouse and rat, only one or, possibly, two ribonuclease genes have been demonstrated by hybridization (Schüller et al. 1990). The situation is, however, different in ruminants. There are at least three closely related ribonuclease genes in the bovine genome (Confalone et al. 1987). Three homologous bovine secretory ribonucleases have been isolated and sequenced (Beintema et al. 1988c). These are the enzyme from the pancreas and the already mentioned basic enzymes produced in the seminal vesicles and in the brain. These ribonucleases are products of gene duplications that occurred rather recently, in the ancestors of the ruminants after divergence from the other artiodactyls (Beintema et al. 1988c). Thus, in ruminants, different ribonucleases occur with structural adaptations to the different structural requirements in different tissues.

A similar observation has been made on the number of lysozyme genes in different mammalian species. Generally, only one or a few lysozyme genes are found, but in ruminants as many as 8–10 genes have been demonstrated (Irwin et al. 1989). Several of them, with a digestive function, are expressed in the stomach, while others are found elsewhere and have a more general antibacterial function.

There are many similarities, in a number of features, between ribonuclease and lysozyme:

1. There are high levels of expression of ribonuclease in the pancreas (Barnard 1969; Beintema et al. 1973) and of lysozyme in the stomach (Dobson et al. 1984) in ruminants and in species with ruminant-like digestion.
2. The enzymes are usually expressed both in the digestive glands and in other tissues. However, in ancestral ruminants gene duplications occurred, leading to different enzyme species expressed in digestive glands and in other tissues of their descendants (Beintema et al. 1988c; Irwin et al. 1989).
3. The enzymes with a function in digestion are less basic than their nondigestive counterparts. However, this feature has a different explanation with both enzymes: in lysozyme the less basic ones have a lower pH optimum of enzymic activity as an adaptation to the low pH of the stomach (Jollès et al. 1989). Ribonucleases act in the intestine and do not need a low pH optimum. Here the large excess of positive charges may serve to degrade double-stranded RNAs (Libonati et al. 1976) and to combat viral infections in tissues other than the pancreas.
4. These adaptations have led to a stable system in ruminants and have slowed down evolutionary rates in them, both for pancreatic ribonuclease (Beintema et al. 1977) and for stomach lysozyme (Jollès et al. 1989).

Both ribonuclease and lysozyme have been recruited as a digestive enzyme in ruminants and in species with ruminant-like digestion, and adaptive structural and functional changes and gene duplications have led to a versatile system for both enzymes.
in the ruminants. Since separate mammalian taxa underwent similar adaptive changes of their digestive system, enzymes involved in this process have turned out to exhibit a rich repertoire of examples of convergent and parallel evolution.

Sequence Availability

The sequence has been deposited in NBRF Protein Data Library under accession number A33083.

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LITERATURE CITED


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