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The ribonuclease from an extinct bovid ruminant

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The sequence of the ribonuclease from the ancestor of swamp buffalo, river buffalo, and ox, corresponding approximately to *Pachyportax latidens*, an extinct ruminant known from the fossil record, has been reconstructed using the rule of 'maximum parsimony'. This protein and two sequences that may have been intermediates in the evolution of modern ribonuclease have been constructed in the laboratory by site-directed mutagenesis, and their properties examined.

Ribonuclease; Evolution; Parsimony; Artiodactyla; Protein engineering

1. INTRODUCTION

Recombinant DNA methods offer the prospect of preparing proteins from organisms that lived and died long ago, either by cloning DNA recovered from preserved organic material [1], or by the chemical synthesis, cloning, and expression of genes for ancient proteins whose sequences are deduced from the sequences of their modern descendants. The first method can be applied in rare cases where a sample of the organic tissue is preserved [1]. The second can be applied more generally whenever enough of descendant proteins are available, especially when these descendants are connected via a reliable evolutionary tree. We report here the first time the second approach has been used to reconstruct an ancient protein in the laboratory, a ribonuclease (RNase) from the most recent common ancestor of ox, swamp buffalo, and river buffalo [2], an organism that lived in the Pliocene [3].

RNase is an excellent system for exploring the potential of this second approach. The sequences of RNases from many ruminants are available [2], and can be used to reconstruct the sequences of the ancestral RNases using the rule of 'maximum parsimony' [4–7]. The most parsimonious sequence of an ancestral protein is defined as the one which can be transformed into the sequences of its descendants via the fewest point mutations. While a sequence reconstructed by parsimony cannot be 'proven' to have been present in an ancient organism, it can serve as a hypothesis for experimental work. If the properties of a protein with the sequence derived by parsimony are consistent with the protein's presumed role in an ancestral organism, the hypothesis is supported. Further, if the evolutionary tree is confirmed by sequences of other proteins and by the fossil record, a rather complete picture of the evolution of a family of proteins can emerge.

Further, a major role for RNase in digestion appears to have only recently evolved [8], perhaps especially adapted for ruminant digestion [9]. Digestive RNases may be descendants of proteins involved in the regulation of cell growth [10], implying that the roles of RNases have diverged significantly in recent evolution. However, before beginning an extended program to reconstruct the evolutionary history of RNases, reassurance is needed that parsimony produces plausible sequences for ancient RNases.

RNases from bovids closely related to the modern ox show structural variation at 7 positions (fig.1) [11]. The inference of the sequence of the ancestral RNases from the modern sequences of ox, swamp buffalo, and river buffalo is logically unambiguous (fig.1), being supportable both at the level of the amino acid sequence and the gene. However, it contains some interesting chemical problems. In particular, although most of the amino acid replacements occur on the surface of the protein far from the active site, that at position 35 does not.

Residue 35 is either Leu or Met. While interconversions of these two amino acids are common during divergent evolution [12], it is not appropriate to assume that the variation is without structural or functional consequence (and therefore is necessarily selectively 'neutral') [13]. In particular, in RNase from ox, the side chain of Leu 35 lies inside the packed globular pro-

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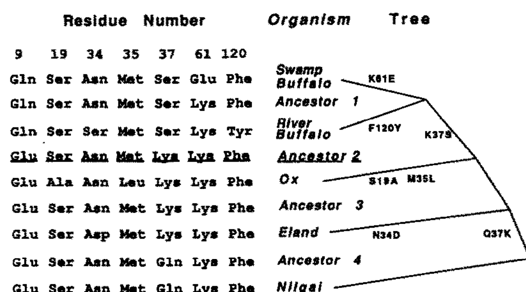

Fig.1. Sequences for ancient RNases reconstructed by parsimony. The reconstruction is based on a tree that relates the buffalos most closely (via Ancestor 1). The eland diverged prior to Ancestor 2. Unlisted amino acids are conserved within the first 3 organisms [2].

tein, forming contacts with the side chains of Arg-10, Gln-11, Met-30, Arg-33, Lys-41, and Phe-46 [14]. The contact with Lys-41 is especially significant, as many studies suggest that Lys-41 is important for catalysis [15].

The crystal structure of RNase suggests that replacing the Leu by Met might require a second point mutation to accommodate the steric requirements of the new amino acid. Indeed, in contemporary RNases, substitution of Leu by Met at position 35 is *always* accompanied by variation in either position 34 or 37. An intriguing proposal from Beintema is that variation at this position modulates the level of glycosylation in vivo [7].

However, in the sequence derived by parsimony for the ancestral protein 2 (fig.1), Leu-35 is replaced by Met *without* a compensating change either in residue 34 or in residue 37. Indeed, the only other substitution in the sequence of RNase from Ancestor 2 is at position 19, too far to influence packing at Leu/Met-35. The assignment made by parsimony can therefore be questioned on chemical grounds, and the properties of this hypothetical ancient protein are interesting.

2. MATERIALS AND METHODS

Genes coding for the ancient proteins were prepared by 'modular mutagenesis' [16]. Each protein was expressed as a fusion protein, yielding a product lacking the amino-terminal formylmethionine [17]. Two of the evolutionary intermediates were also expressed directly behind the lambda promoter [18], yielding identical proteins except an N-terminal formylmethionine. The genes for all RNases were sequenced in their respective expression vectors. The expressed proteins were purified to homogeneity using procedures described previously [17,18]. All gave single bands in polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. Single peaks (to within limit of UV detection at 280 and 220 nm) were also observed by HPLC on a sizing column under 'naturing' conditions (LKB G3000SW in 0.2 M sodium phosphate, pH 6.47); aggregates of RNase (prepared by lyophilization from 50% acetic acid) are known to be stable in this buffer [19]. To demonstrate that the mutant RNases had not undergone post-translational modification, they were converted to octa-*S*-sulfo derivatives [20], and subjected to isoelectric focusing (Ampholine 3.5–9.5 and 4.0–7.0, LKB prepoured IEF gel kit). Carbamoylated (8 M urea, 10 mM DTT, 10 mM Tris, pH 8.0, 37°C, 30 min) and 67-desamino RNases were standards of possible products of post-translational modification or artifacts of purification. Single bands were again observed, with the isoelectric points expected for the octa-sulfo derivatives of RNase A or the appropriate mutant.

The catalytic activities of the modern and mutant RNases were measured using 5′-(3′-phosphouridylyladenine) (UpA) as a substrate [21]. Assays were run at 25°C in acetate buffer (100 mM sodium acetate, pH 5.0). All enzymes were subjected to multiple kinetic analyses (more than 7 complete sets of kinetic data per enzyme). The stability of the RNases (expressed as a fusion protein) to tryptic digestion at various temperatures was determined by the method of Lang and Schmidt [22], which measures the loss of enzymatic activity upon incubation with trypsin following preincubation from 20–70°C.

Table 1

Kinetic behavior of ancient ribonucleases

	k_{cat} (s^{-1})	K_M (mM)	
Modern ox	1459 ± 50	0.211 ± 0.020	
A19S (no Met)	2055 ± 495	0.250 ± 0.041	
A19S (with Met)	1906 ± 301	0.229 ± 0.038	
L35M (no Met)	2003 ± 107	0.208 ± 0.018	
L35M (with Met)	1602 ± 102	0.169 ± 0.018	
A19S/L35M (no M)	1944 ± 136	0.203 ± 0.027	(Ancestor 2)

3. RESULTS AND DISCUSSION

Three variants of RNase A were prepared (table 1), one containing a Leu35Met mutation, one containing an Ala19Ser mutation, and the third containing both mutations, Leu35Met and Ala19Ser, the sequence of Ancestral RNase 2 deduced by parsimony. The behaviors of RNases expressed with and without a terminal formylmethionine residue were only insignificantly different.

The kinetic behaviors of the ancient RNases were found to be essentially identical to those of modern RNase. For example, the k_{cat} and K_M of modern RNase and the mutants were, within experimental error, the same (table 1). Similarly, there was no difference (outside of experimental error) in the apparent sensitivity of RNase to tryptic digestion at a range of temperatures (fig.2).

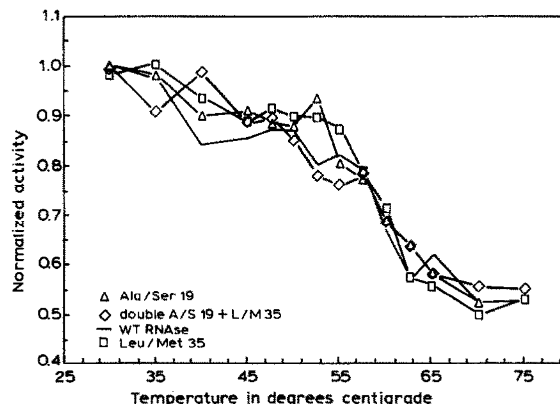

Fig.2. Stability curves for native RNase and the mutants prepared here, obtained by the method of Lang and Schmidt [22] in sodium acetate buffer (0.1 M, 0.1 M NaCl, pH 5.0).

While these results suggest that the recent evolution of RNase is behaviorally neutral, we must emphasize that no experiment can either rigorously establish or rigorously exclude the possibility that this structural evolution is adaptively neutral. However, because the 'ancient' RNase displays both reasonable catalytic and physical properties, these results strengthen the notion that parsimony has provided a correct ancestral structure in this case. This should encourage continued examination of ancestral proteins reconstructed by parsimony.

As this is the first time an ancient protein has been reconstructed by parsimony (hybrids between modern proteins, of course, are often made by mutagenesis, but ask fundamentally different questions), it is appropriate to ask which *fossil* organisms most nearly approximate the ancestral organism presumed here. Two extinct genera from the Pliocene, *Proamphibos* and *Ugandax*, appear to correspond to the most ancient members of (respectively) the *Bubalus* (water buffalo), and *Syncerus* (African buffalo) branches of the Bovini tree *following* their divergence, while *Leptobos* may be ancestral to *Bos* (cattle, bison) [2,23,24]. Prior to these three lineages, both in the geological strata and in many of its structural characteristics, is *Pachyportax*, known from the Upper Miocene in South Asia. Thus, a plausible candidate for the fossil species with the closest correspondence to the common ancestor of these lineages is *Pachyportax* [23]. While caution must be exercised in equating an evolutionary branch point (the organisms defined by parsimony) and fossil species (which are believed to be generally not at branch points), the approximation of *Pachyportax* and the organism that contained one of the RNases prepared here does not appear to be a serious error.

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