Disulfide-Bond Reshuffling in the Evolution of an Ape Placental Ribonuclease

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Disulfide bonds play important roles in the folding and stability of proteins and are evolutionarily conserved. A classic example is RNase A (also known as bovine pancreatic ribonuclease), which contains 4 conserved disulfide bonds among 8 cysteines. However, human RNase 8, a paralog of RNase A uniquely expressed in the placenta, has lost one of the conserved cysteines but gained another, when compared with RNase 8 of various monkeys and with RNase A. We here show that both the loss and gain of the cysteines in human RNase 8 occurred in the common ancestor of African great apes (humans, chimps, and gorillas) 7–13 MYA. Computational predictions suggest changes of disulfide bonding by these cysteine substitutions. Site-directed mutagenesis indicates that if the ribonucleolytic activity is essential for RNase 8's function, the gain of the cysteine must have preceded the loss. Human RNase 8 represents one of the first examples in which the presumable evolutionary change of a disulfide bond involves 1 loss and 1 gain of cysteine, instead of 2 losses or 2 gains. Our results provide the foundation for detailed analysis toward understanding the impact of disulfide-bond reshuffling on the structure, function, and evolution of proteins in general and human RNase 8 in particular.

Introduction

A disulfide bond is a covalent bond formed between the sulfur atoms of 2 nonadjacent cysteine residues that are either in the same peptide or in different peptides. Disulfide bonds are important to the folding and stability of some proteins, usually those secreted to the extracellular medium. Because of their structural importance, disulfide bonds tend to be evolutionarily conserved (Thornton 1981). In fact, cysteine is the second most conserved amino acid (after tryptophan) in protein evolution (Jones et al. 1992), and cysteines that form disulfide bonds are much more conserved than those that do not form disulfide bonds (Thornton 1981). In the event that a disulfide bond is not evolutionarily conserved, both cysteines are replaced by other amino acids (Thornton 1981; Kreisberg et al. 1995); very rarely does 1 of the 2 free cysteines remain, probably because free cysteines are potentially reactive (Thornton 1981; Kreisberg et al. 1995). The importance of cysteines and disulfide bonds to protein folding and stability is exemplified by RNase A in virtually every biochemistry textbook, after the pioneering work of the Nobel Laureate Christian Anfinsen (1973). RNase A is also known as bovine pancreatic ribonuclease. It contains 8 conserved cysteines, which form 4 disulfide bonds. Recent studies revealed details of the contribution of disulfide bonds to the folding, stability, and catalytic activity of RNase A (Laity et al. 1993; Klink et al. 2000; Ruoppolo et al. 2000). RNase A and its paralogous proteins form a large protein superfamily (Beintema and Kleineidam 1998). Analysis of the human genome sequence reveals the presence of 13 genes potentially belonging to the RNase A superfamily (Cho et al. 2005). Among them, RNases 1–8 are referred to as canonical RNases because they all share certain conserved sequence motifs (fig. 1) and have ribonucleolytic activities (Zhang, Dyer, et al. 2002), whereas RNases 9–13 lack some of these motifs and are not known to have ribonucleolytic activities (Castella et al. 2004; Cho et al. 2005; Cho and Zhang 2006). The majority of the canonical RNases of mammals, like RNase A, have 8 conserved cysteines (fig. 1). A notable exception is RNase 5 (also known as angiogenin), which has only 6 cysteines, forming 3 disulfide bonds (Strydom et al. 1985) (fig. 1). Recently, it was reported that human RNase 8 lost the sixth (C_6) of the 8 conserved cysteines but gained another (fig. 1), when compared with other canonical RNases (Zhang, Dyer, et al. 2002). The newly gained cysteine is located between the fourth and fifth conserved cysteines and is hereby referred to as C_X. A phylogenetic survey showed that the RNase 8 proteins from Old World and New World monkeys have the 8 conserved cysteines, whereas chimpanzees and gorillas share with humans the deviating pattern (Zhang, Dyer, et al. 2002). This finding indicates that the alterations of the cysteines in RNase 8 took place after the divergence of hominoids from Old World monkeys about 25 MYA, but before the separation of humans and chimps from gorillas about 7 MYA (Glazko and Nei 2003). The physiological function of RNase 8 is still unclear, although it has a ribonucleolytic activity and exclusive expression in the placenta (Zhang, Dyer, et al. 2002). In this work, we attempt to achieve 3 goals. First, we obtain a more precise date of the loss and gain of cysteines in primate RNase 8 evolution by sampling additional species. Second, we use computational modeling to investigate how the cysteine substitutions affect the disulfide bonding in RNase 8. Third, we examine the impact of the cysteine changes on the ribonucleolytic activity of the enzyme. Taking all together, we propose evolutionary scenarios of disulfide-bond reshuffling in primate RNase 8.

Materials and Methods

Comparison of Primate RNase 8 Sequences

The RNase 8 gene has only 1 coding exon, which was amplified from the genomic DNA of an orangutan (*Pongo pygmaeus*) by polymerase chain reaction (PCR) with primers 337 (5′-CTCCTAAGAGAGATGGCACCGGCC) and 338 (5′-CAAAGAGCAAGCCAGTCTGGAAACCTA). The PCR products were cloned into pCR II TA cloning vector (Invitrogen, San Diego, CA) and sequenced from both directions by the dideoxy chain termination method with an automatic sequencer. The DNA sequences of the RNase 8 genes of humans (*Homo sapiens*), chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), rhesus monkey (*Macaca mulatta*), pig-tailed macaque (*Macaca nemestrina*), baboon (*Papio hamadryas*), African green monkey (*Cercopithecus aethiops*), talapoin monkey (*Miopithecus*

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FIG. 1.—Protein sequence alignment of the 8 canonical RNases of humans. Gaps are indicated by "–." The 3 catalytic residues are indicated by arrows. The 8 conserved cysteines as well as the newly gained cysteine in RNase 8 are boxed. The starting residues of the mature proteins are bolded. The figure is modified from Fig. 1*A* of Zhang, Dyer, et al. (2002).

talapoin), tamarin (*Saguinus oedipus*), and owl monkey (*Aotus trivirgatus*) were originally reported in Zhang, Dyer, et al. (2002) and were retrieved from GenBank. The 11 primate RNase 8 gene sequences were aligned using Clustal (Thompson et al. 1994).

Recombinant RNase 8 and Its Enzymatic Activity

The mature peptide region of the human and owl monkey RNase 8 gene was subcloned into the bacterial expression vector pFLAG CTS (Kodak, New Haven, CT) and was verified by DNA sequencing. The vector adds the octapeptide DYKDDDDK (FLAG) to the carboxy terminus of the recombinant protein, which facilitates its purification and detection with M2 anti-FLAG monoclonal antibody (Sigma, St. Louis, MO) without altering ribonucleolytic activity (Rosenberg and Dyer 1995). Recombinant proteins were isolated, purified, and quantified as described (Rosenberg and Dyer 1995). The ribonuclease activity of the recombinant proteins against a standard yeast tRNA substrate was measured in 40 mM sodium phosphate buffer (pH = 7.4) at 25 °C. Purified RNase (0.1–1 pmol) was added into 0.8 ml of the aforementioned buffer with 1.42 nmol tRNA. The reaction was stopped by 0.5 ml of 20 mM lanthanum nitrate with 3% perchloric acid, and insoluble tRNA was removed by centrifugation. The amount of solubilized tRNA was determined by ultraviolet absorbance at 260 nm. The catalytic activity of the RNase was determined as the picamolar of RNA digested per second per picamolar of RNase (Rosenberg and Dyer 1995; Zhang, Dyer, et al. 2002). The average values from 3 experiments and the standard deviation of the mean are presented.

Site-Directed Mutagenesis

The QuikChange site-directed mutagenesis kit of Stratagene (La Jolla, CA) was used to generate designed mutations in the human RNase 8 gene construct following manufacturer's instructions. The mutations were confirmed by DNA sequencing. Three mutants were made. The first mutant, referred to as intermediate A, contained Gly81Cys, meaning that Gly was replaced by Cys at position 81 of the mature peptide of human RNase 8. The second mutant, referred to as intermediate B, contained Cys66His. The third mutant, referred to as node P, contained both Gly81Cys and Cys66His.

Computational Prediction of Disulfide Bonds

The amino acid sequence of the mature peptide of RNase 8 (or its mutant forms) is used to predict the disulfide bonds through the DiANNA 1.1 Web server at http://clavius.bc.edu/~clotelab/DiANNA/ and DISULFIND Web server at http://disulfind.dsi.unifi.it/.

Results

Time of the Cysteine Substitutions in RNase 8

To date the loss of C_6 and gain of C_X in primate RNase 8 and to determine which event occurred first, we sequenced the RNase 8 gene from the orangutan *P. pygmaeus* and compared it with the available RNase 8 sequences from various primates. The comparison showed the presence of C_6 and absence of C_X in the orangutan as well as in all Old World and New World monkeys, but the absence of C_6 and presence of C_X in all African great apes (humans, chimps, and gorillas) (fig. 2). Thus, both the loss of C_6 and gain of C_X took place in the common ancestor of African great apes since its separation from the orangutan (bolded branch in fig. 3). African great apes and orangutans diverged around 13 MYA, and gorillas diverged from the common ancestor of humans and chimps about 7 MYA (Glazko and Nei 2003). Hence, the loss and gain of the cysteines occurred in a short window between 7 and 13 MYA. Because there are no extant species that diverged from African great apes within the above time window, it is not possible to further narrow the time window of the cysteine substitutions. Nor is it possible to use phylogenetic information to infer the temporal order of the loss and gain of the cysteines. It has been reported that RNase 8 was independently pseudogenized

FIG. 2.—Nucleotide sequences of 11 primate RNase 8 genes. Identical nucleotide sequence to human RNase 8 is indicated by dots, whereas gaps are indicated by "-." The conceptually translated amino acid sequence of human RNase 8 is shown in bold, with the signal peptide underlined. Potential pseudogenes bear asterisks after the organism name. These pseudogenes have mutations at structurally/functionally critical sites or have deletions that either interrupt the open reading frame or drastically alter the encoded amino acid sequence distal to the deletion (Zhang, Dyer, et al. 2002). The positions of the 2 cysteine changes are indicated by arrows, with the amino acids shown next to the nucleotides. The orangutan sequence is newly determined in this work (GenBank accession number: EF100699) and all other sequences were from Zhang, Dyer, et al. (2002).

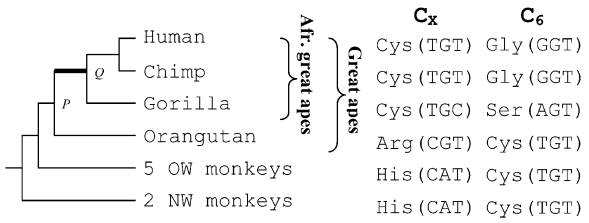

FIG. 3.—Two cysteine substitutions in the evolution of primate RNase 8. The phylogeny depicts the well-established relationships of the primate species analyzed in this work. The 2 cysteine substitutions took place on the bolded branch. The codon and amino acid residue at the 2 cysteine substitution positions are shown next to the phylogeny. OW, Old World; NW, New World.

multiple times in primate evolution (Zhang, Dyer, et al. 2002). Indeed, figure 2 shows that in 6 of the 11 primates, RNase 8 is likely a pseudogene because of the lack of an open reading frame or substitutions at catalytically or structurally important positions. However, the pseudogenizations do not affect our phylogenetic analysis and conclusion, because the nucleotide substitutions at C_6 and C_X can be reliably inferred even in pseudogenes (fig. 2). Interestingly, the gain of C_X apparently required 2 nucleotide substitutions, one from C to T at the first-codon position and the other from A to G at the second-codon position (fig. 3). Given the orangutan codon of CGT at the C_X position, it is likely that the substitution at the second-codon position preceded that at the first position. In other words, 2 amino acid substitutions, from His to Arg (in the common ancestor of great apes) and then to Cys (in the common ancestor of African great apes), took place at the C_X position (fig. 3). The loss of C_6, however, was due to a change of T to A or G at the first-codon position, which occurred in the common ancestor of African great apes (fig. 3).

Disulfide-Bond Reshuffling in RNase 8 Evolution: DiANNA Analysis

How did the loss of C_6 and gain of C_X affect the disulfide bonds in RNase 8? We used the computational tool DiANNA (Ferre and Clote 2005b) to predict the disulfide bonds in RNase 8 based on its primary sequence. DiANNA prediction involves 3 steps. First, PSIPRED is run to predict the protein's secondary structure and PSIBlast is run against the nonredundant SwissProt to generate a multiple sequence alignment of the input sequence and its relatives. The predicted secondary structure and the alignment are used to train a neural network. Second, cysteine oxidation state is predicted and each pair of cysteines is assigned a likelihood of forming a disulfide bond through the diresidue neural network. Finally, Rothberg's implementation of Gabow's maximum weighted matching algorithm is applied to diresidue neural network scores to produce the final connectivity prediction (Ferre and Clote 2005b). There are 3 species (orangutan, talapoin, and owl monkey, see fig. 2) that have functional RNase 8 genes whose products contain the 8 conserved cysteines (i.e., with C_6 but without C_X). As expected, the DiANNA-predicted disulfide bonds of RNase 8 for each of these 3 species are identical to those of RNase A, with the 4 disulfide bonds being C_1–C_6, C_2–C_7, C_3–C_8, and C_4–C_5. Hence, it can be safely inferred that the disulfide bonds in the common ancestor of great apes (node P in fig. 3, before the cysteine substitutions) are also identical to those of RNase A (fig. 4).

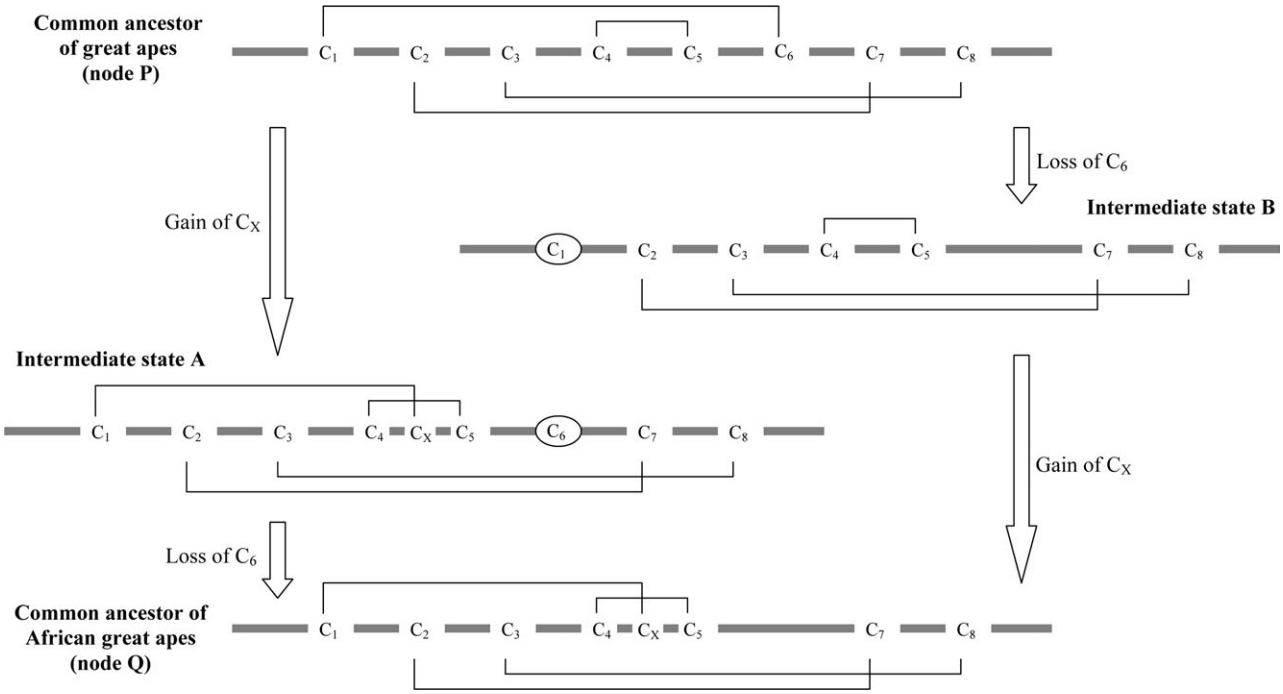

FIG. 4.—Disulfide bonds in human RNase 8 and its variants, predicted by DiANNA. The thick gray line represents the primary sequence of the protein, with the N terminus located at the left-hand side. Cysteine residues are indicted by the letter C and disulfide bonds are shown by solid-line bridges below or above the primary sequence. Free cysteines are circled. On the left side of the figure is the first evolutionary scenario, where the gain of C_X preceded the loss of C_6. On the right side of the figure is the second evolutionary scenario where the loss of C_6 preceded the gain of C_X.

We next predicted the disulfide bonds in human and chimp RNase 8 as the gorilla RNase 8 is a pseudogene (see fig. 2). We found that the disulfide bonds are identical between humans and chimps. Presumably, the common ancestor of African great apes (node Q in fig. 3) also had these disulfide bonds (fig. 4). Compared with node P, 3 disulfide bonds (C_2–C_7, C_3–C_8, and C_4–C_5) remain unchanged for node Q. However, C_1–C_6 is replaced by C_1–C_X in node Q due to the loss of C_6 and gain of C_X.

Because the temporal order of the loss of C_6 and gain of C_X is unknown, there are 2 possible evolutionary scenarios. In the first scenario, the gain of C_X preceded the loss of C_6. We predicted the disulfide bonds in a human RNase 8 mutant with both C_X and C_6, representing the intermediate state in this evolutionary scenario (see intermediate state A in fig. 4). This intermediate state has the same disulfide bonds as those in human and chimp RNase 8, with the extra C_6 being a free cysteine. In the second scenario, the loss of C_6 preceded the gain of C_X. We predicted the disulfide bonds in a human RNase 8 mutant with neither C_X nor C_6, representing the intermediate state in the second evolutionary scenario (see Intermediate state B in fig. 4). Not unexpectedly, the ancestral C_1–C_6 is now broken owing to the loss of C_6. As a result, C_1 becomes a free cysteine.

Disulfide-Bond Reshuffling in RNase 8 Evolution: DISULFIND Analysis

Because computational predictions of disulfide bonds are not always correct, we tried 2 other commonly used prediction algorithms, DiPro (Cheng et al. 2006) and DISULFIND (Vullo and Frasconi 2004). DiPro uses machine-learning methods to predict whether a given protein chain contains intrachain disulfide bonds and uses recursive neural networks to predict the bonding probabilities of each pair of cysteines in the chain. However, we found that DiPro (http://contact.ics.uci.edu/bridge.html) predicted only 3 disulfide bonds in typical members of the RNase superfamily such as RNase A and RNase 2, in which the presence of 4 disulfide bonds have been experimentally demonstrated. Thus, DiPro is unreliable, at least for the RNases. DISULFIND predicts disulfide bonds using recursive neural networks and evolutionary information. We found that it correctly predicted the 4 conserved disulfide bonds for Old World and New World monkey RNase 8 as well as that of node P (fig. 5). Its prediction for human and chimp RNase 8, however, was slightly different from the DiANNA prediction. That is, DISULFIND predicted C_1–C_5 and C_4–C_X instead of C_1–C_X and C_4–C_5, resulting in 2 altered disulfide bonds in human RNase 8, compared with the 4 conserved disulfide bonds in canonical RNases (fig. 5). DISULFIND and DiANNA also made different predictions for intermediate states A and B (fig. 5). DISULFIND predicted that intermediate A and node P have the same disulfide bonds, indicating that the addition of C_X in intermediate A did not alter disulfide bonding. For intermediate B, DISULFIND predicted C_1–C_3 with C_8 being unpaired, whereas DiANNA predicted C_3–C_8 with C_1 being unpaired.

Impact of the Cysteine Substitutions on the Ribonucleolytic Activity of RNase 8

It is interesting to know whether the loss of C_6 and gain of C_X in RNase 8 have affected its catalytic activity. We cloned the human RNase 8 gene into a bacterial expression vector, expressed the gene, purified the recombinant protein, and examined its ribonucleolytic activity against yeast tRNAs in a standard assay. Using site-directed mutagenesis, we also constructed 3 mutant forms of human RNase 8 and examined their activities. The first mutant has both C_6 and C_X, representing the intermediate state A in the first evolutionary scenario described above (figs. 4 and 5). The second mutant has neither C_6 nor C_X, representing the intermediate state B in the second evolutionary scenario (figs. 4 and 5). The third mutant has C_6 but not C_X, representing the ancestral protein at node P (figs. 4 and 5). We found that the mutant protein representing node P and the intermediate protein A both have slightly lower activities than that of human RNase 8 (fig. 6). The activity of the intermediate protein B is over 300-fold lower than that of human RNase 8 (fig. 6). In fact, our RNase assay cannot reject the null hypothesis of no activity in protein B ($P > 0.1$, t-test). If we trust the DiANNA prediction of disulfide bonds (fig. 4), the above results suggest that the C_1–C_X bond may substitute the C_1–C_6 bond without substantively reducing the RNase activity. However, removing the C_1–C_6 bond without adding a substitute leads to an almost complete loss of the RNase activity. If the DISULFIND prediction is correct, our results suggest that replacing C_1–C_6 and C_4–C_5 by C_1–C_5 and C_4–C_X does not change the RNase activity much, but the loss of C_3–C_8 and C_1–C_6 dramatically reduces the enzyme activity.

We also examined the ribonucleolytic activity of owl monkey RNase 8 and found it to be 7.4 times that of human RNase 8 (fig. 6). Although the human and owl monkey RNase 8 proteins differ at the C_X and C_6 positions, these differences are not the cause of their divergence in the ribonucleolytic activity because the mutant RNase 8 at node P has a catalytic activity comparable to that of human RNase 8 despite having the same residues at the 2 positions as owl monkey RNase 8 has.

Discussion

Disulfide bonds in proteins are evolutionarily conserved. Rarely does an existing bond disappear or a new bond appear during evolution. In the event when an existing disulfide bond is lost, both cysteines are replaced by other amino acids. Similarly, when a new disulfide bond emerges in evolution, 2 new cysteine residues are required. Primate RNase 8 is probably the first reported case where the change of a disulfide bond involves the loss of 1 cysteine and the gain of another. Our phylogenetic analysis showed that both the loss of C_6 and gain of C_X occurred in a relatively short evolutionary time between 7 and 13 MYA in the common ancestor of African great apes since its separation from orangutans. If the gain of C_X preceded the loss of C_6, the intermediate state A has 9 cysteines. Our RNase assay showed that A has a catalytic activity comparable to that of human RNase 8. Alternatively, if the loss of C_6 preceded the gain of C_X, the intermediate state B has only 7 cysteines and has virtually no catalytic activity. Thus, if the ribonucleolytic activity is important to the physiological function of RNase 8, the second evolutionary scenario cannot be true and the first must be true. However, it is currently unknown

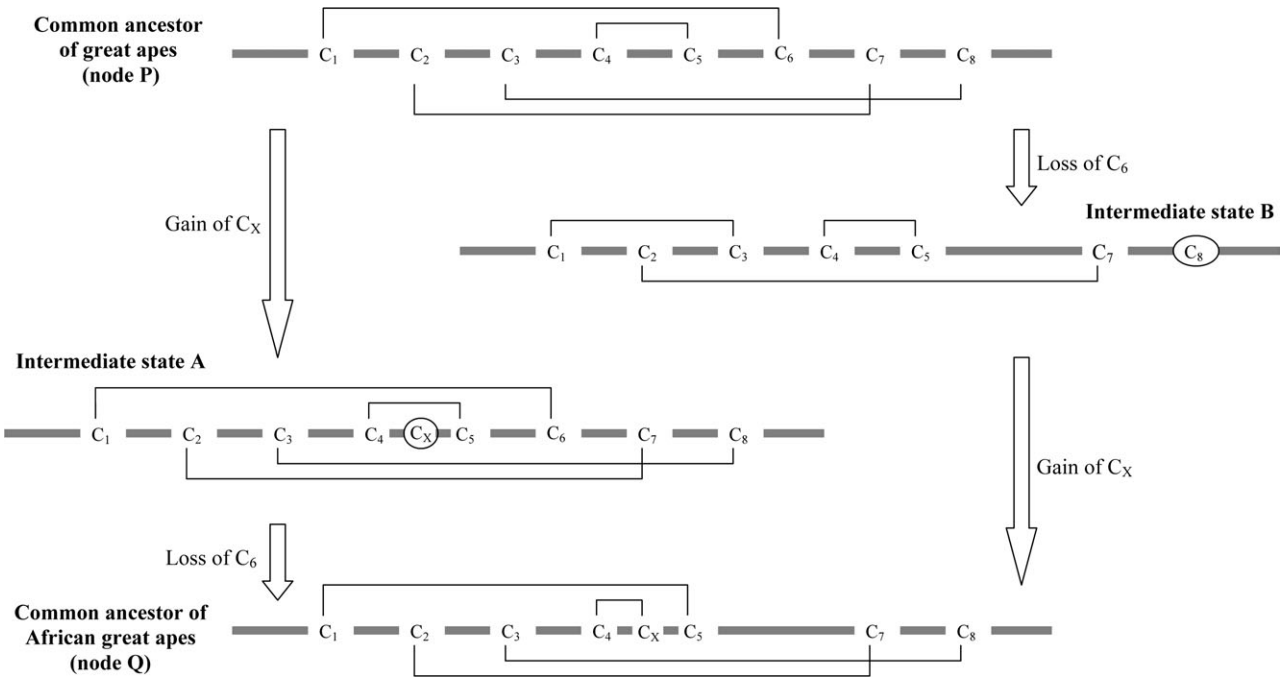

FIG. 5.—Disulfide bonds in human RNase 8 and its variants, predicted by DISULFIND. The thick gray line represents the primary sequence of the protein, with the N terminus located at the left-hand side. Cysteine residues are indicted by the letter C, and disulfide bonds are shown by solid-line bridges below or above the primary sequence. Free cysteines are circled. On the left side of the figure is the first evolutionary scenario where the gain of C_X preceded the loss of C_6. On the right side of the figure is the second evolutionary scenario where the loss of C_6 preceded the gain of C_X.

whether the physiological function of RNase 8 requires the ribonucleolytic activity, although it seems unlikely that a specific catalytic activity in a natural protein would be physiologically irrelevant.

We used 2 computational methods to predict disulfide bonds in RNase 8. DiANNA predicted that A has 4 disulfide bonds that are identical to those in human RNase 8, whereas B has only 3 disulfide bonds, with C_1 being a free cysteine (fig. 4). DISULFIND, however, predicted that A has the 4 conserved disulfide bonds as in canonical RNases, whereas B has 2 conserved and a new disulfide bond, with C_8 being a free cysteine. Although it is difficult to judge which computational prediction is more likely to be correct, we note that both are consistent with our knowledge of the RNase A superfamily in mammals because all canonical RNases with 4 disulfide bonds have appreciable levels of ribonucleolytic activity (Zhang, Dyer, et al. 2002), whereas RNase 5, which has only 3 disulfide bonds (lacking C_4–C_5), has virtually no RNase activity (Shapiro et al. 1986). Outside mammals, however, there are known RNases with 3 disulfide bonds but with relatively high RNase activities (Nitto et al. 2006). Interestingly, the double mutant of human RNase 8 that represents the ancestral protein at node P has a comparable ribonucleolytic activity with human RNase 8, suggesting that the 2 cysteine substitutions together did not substantively change the catalytic activity. However, here we only used yeast tRNAs as the substrate in the RNase assay. It is possible that the cysteine substitutions may have changed the catalytic activity of RNase 8 in degrading other RNA substrates.

The intermediate state A has the extra C_X, in addition to the 8 conserved cysteines. DISULFIND predicted that it has the same disulfide bonds as the ancestral protein of node P. However, DiANNA predicted the breakage of the conserved C_1–C_6 bond and the formation of the new C_1–C_X bond. It is unclear why C_1 tends to form a disulfide bond with C_X rather than its original partner C_6. Although a final set of disulfide bonds are predicted in DiANNA, it also generates scores for all possible disulfide bonds. For protein A, the score for the C_1–C_X pair (0.0119) is only slightly higher than that for C_1–C_6 (0.0104). Hence, in reality, some A molecules may form the C_1–C_X bond, whereas others form

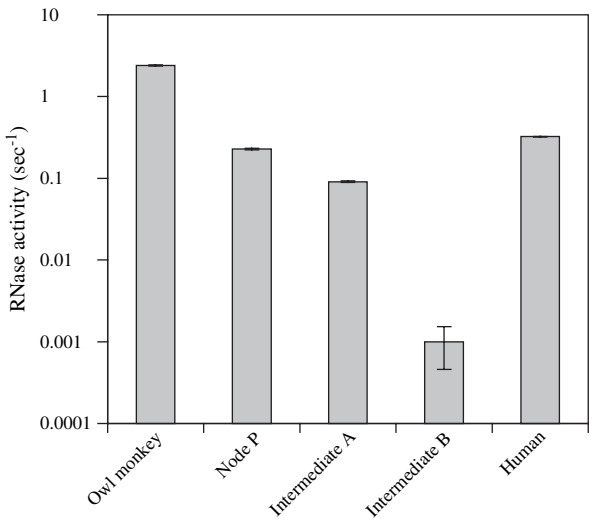

FIG. 6.—RNase activity of recombinant RNase 8 proteins against yeast tRNA. Error bar shows 1 standard deviation of the mean.

the C_1–C_6 bond. In fact, if we assume that human RNase 8 and RNase A share a similar 3-dimensional structure, the C_1–C_X bond may be difficult to form because of a long distance between the 2 cysteines. We stress that computational prediction of disulfide bonds, although relatively well developed, still generates errors (Vullo and Frasconi 2004; Ferre and Clote 2005a; Chen et al. 2006). Thus, our predictions here should be experimentally verified in the future.

The bovine pancreatic ribonuclease (i.e., the famous RNase A) gene was duplicated twice in the common ancestor of ruminants (Beintema and Kleineidam 1998; Breukelman et al. 2001; Zhang 2003). Interestingly, 1 resultant duplicate, named seminal RNase, gained 2 cysteines between C_1 and C_2. Because the 2 new cysteines are adjacent to each other in the primary sequence, they do not form a disulfide bond. Rather, they form 2 intermolecular disulfide bonds between 2 seminal RNase peptides, thus creating a homodimer (D'Alessio et al. 1972). It is possible that the altered cysteines make RNase 8 a homodimer, as in seminal RNase. Because DiANNA and DISULFIND do not predict intermolecular disulfide bonds, we conducted a Western analysis of recombinant human RNase 8, owl monkey RNase 8, and the 3 RNase 8 mutants in nonreducing conditions. The estimated molecular weights indicated that all these RNase 8 proteins are monomers (supplementary fig. S1, Supplementary Material online).

Human RNase 8 is uniquely expressed in the placenta, although its physiological function is unclear (Zhang, Dyer, et al. 2002). The closest relative to RNase 8 is RNase 7, and the gene duplication that gave rise to this gene pair likely occurred shortly before the divergence of Old World and New World monkeys within primates (Zhang, Dyer, et al. 2002; Cho and Zhang 2006). RNase 7 is expressed in several tissues including the kidney, spleen, heart, skeletal muscle, and skin, but not in the placenta (Harder and Schroder 2002; Zhang et al. 2003). Interestingly, although human RNase 7 has a potent antibacterial activity against multiple bacteria (Harder and Schroder 2002; Zhang et al. 2003), human RNase 8 lacks such an activity (Zhang, Dyer, et al. 2002; but see Rudolph et al. 2006). This functional dissimilarity is unsurprising because members of the RNase A superfamily are known to have a diverse array of biochemical activities and functions ranging from digestion to host-defense and angiogenesis (Barnard 1969; Fett et al. 1985; Rosenberg and Dyer 1995; Domachowske et al. 1998; Harder and Schroder 2002; Zhang J, Zhang YP, et al. 2002; Hooper et al. 2003; Zhang et al. 2003; Zhang 2006). Previous studies of RNase 3 (also known as the eosinophil cationic protein) showed that the key molecular determinant of the antibacterial activity is not the ribonucleolytic activity (Rosenberg 1995), but positively charged amino acid residues (Young et al. 1986; Zhang et al. 1998; Carreras et al. 2003). In fact, all RNases known to have antibacterial activities have a large number of positively charged amino acids (Zhang et al. 2003). Thus, we believe that the disulfide-bond reshuffling is unlikely the reason why human RNase 8 lacks the antibacterial activity.

An interesting phenomenon about primate RNase 8 is its independent pseudogenizations in multiple lineages, although there are no signs of duplication of the RNase 8 gene in any of the primates examined here and elsewhere (Zhang, Dyer, et al. 2002). Of the 11 primates examined, only 5 (humans, chimp, orangutan, talapoin, and owl monkey) appear to have functional RNase 8. However, these 5 species belong to all major lineages of higher primates (hominoids, Old World monkeys, and New World monkeys). This phylogenetic distribution suggests that at present the function of RNase 8 is needed only in a limited number of species, which nevertheless are scattered throughout higher primates. The RNase 8 pseudogenes identified all have a relatively intact open reading frame, suggesting that even in these lineages RNase 8 had been functional till relatively recently. It is intriguing to consider potential alterations of RNase 8 function by disulfide-bond reshuffling. Because RNase 8 is a paralog of RNase A, the classic model for studying the role of disulfide bonds in protein folding and stability, our findings provide the foundation for future detailed structural and functional analyses that may reveal the effect of disulfide-bond reshuffling on protein evolution.

Supplementary Material

Supplementary figure S1 is available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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