‘Crystal lattice engineering,’ an approach to engineer protein crystal contacts by creating intermolecular symmetry: Crystallization and structure determination of a mutant human RNase 1 with a hydrophobic interface of leucines

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Abstract

A protein crystal lattice consists of surface contact regions, where the interactions of specific groups play a key role in stabilizing the regular arrangement of the protein molecules. In an attempt to control protein incorporation in a crystal lattice, a leucine zipper-like hydrophobic interface (comprising four leucine residues) was introduced into a helical region (helix 2) of the human pancreatic ribonuclease 1 (RNase 1) that was predicted to form a suitable crystallization interface. Although crystallization of wild-type RNase 1 has not yet been reported, the RNase 1 mutant having four leucines (4L-RNase 1) was successfully crystallized under several different conditions. The crystal structures were subsequently determined by X-ray crystallography by molecular replacement using the structure of bovine RNase A. The overall structure of 4L-RNase 1 is quite similar to that of the bovine RNase A, and the introduced leucine residues formed the designed crystal interface. To characterize the role of the introduced leucine residues in crystallization of RNase 1 further, the number of leucines was reduced to three or two (3L- and 2L-RNase 1, respectively). Both mutants crystallized and a similar hydrophobic interface as in 4L-RNase 1 was observed. A related approach to engineer crystal contacts at helix 3 of RNase 1 (N4L-RNase 1) was also evaluated. N4L-RNase 1 also successfully crystallized and formed the expected hydrophobic packing interface. These results suggest that appropriate introduction of a leucine zipper-like hydrophobic interface can promote intermolecular symmetry for more efficient protein crystallization in crystal lattice engineering efforts.

Keywords: RNase 1; crystallization; symmetry; leucine zipper

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Abbreviations: 4L-RNase 1, mutant RNase 1 with four leucine

mutations Thr24 → Leu, Gln28 → Leu, Arg31 → Leu, Arg32 → Leu; 3L-RNase 1, mutant RNase 1 with three leucine mutations, Gln28 → Leu, Arg31 → Leu, Arg32 → Leu; 2L-RNase 1, mutant RNase 1 with two leucine mutations, Arg31 → Leu and Arg32 → Leu; N4L-RNase 1, mutant RNase 1 with four leucine mutations Val52 → Leu, Asp53 → Leu, Asn56 → Leu, and Phe-59 → Leu.

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Proteins within a crystal lattice often retain full biological function and enzymatic activity; thus, protein crystals are useful not only for structure determination but also for additional applications such as drug screening or production of protein chips for proteomics studies (Ikeda et al. 2006). If a given protein can be reliably crystallized, and the packing arrangement controlled, the availability of such crystals can have a substantial impact on the biotechnology field.

Important contributions to our understanding of protein function have been made from X-ray crystallography; however, many protein structures remain unsolved because of the inability to obtain crystals. Thus, the development of novel crystallization methods is urgently needed. The inability to crystallize proteins successfully may be due in part to insufficient intermolecular site-specific interactions needed for stable lattice formation. Several reports have appeared describing the crystallization of a protein that was difficult to crystallize using standard screening methods. One example is the crystallization by complexation with a fragment derived from a monoclonal antibody (Fab fragment) (Ostermeier et al. 1995; Kuroki et al. 2002; Feese et al. 2004). Fab molecules are recognized as being able to bind not only the target molecule but also each other, thereby providing a suitable interface for crystallization. The importance of the entropic effect of polar side chains, such as lysine, arginine, or glutamate, in preventing protein molecules from crystallizing has also been reported (Derewenda 2004). Indeed, several successful protein crystallization trials have been reported that mutate lysine, arginine, or glutamate residues to alanine. It has been suggested not only that removal of polar side chains eliminates an unfavorable entropic penalty on crystallization but also that the introduction of alanine on the protein surface can provide a favorable protein-protein interface that promotes crystallization (Derewenda 2004). Moreover, we observed that the proteins having symmetry within a molecule, such as dimer protein connected by disulfide bridge (Feese et al. 2000) and by ligand (Honjo et al. 2005; Yamada et al. 2006), crystallized easily in several different conditions. Indeed, artificial introduction of intermolecular disulfide bonds promoting symmetry resulted in crystallization in the different space groups (Banatao et al. 2006). Therefore, an approach to introduce symmetry within a molecule should be useful for crystallization.

The leucine-zipper sequence is known to promote formation of a stable protein-protein interaction in solution (Landschulz et al. 1988; Beck and Brodsky 1998). We hypothesized that a noncrystallizable protein might crystallize if several mutant leucine residues were aligned in an α -helix on the molecular surface to produce an intermolecular symmetry through leucine zipper-like

intermolecular hydrophobic interaction. This idea was tested using human RNase 1, a 128-residue protein whose crystallization has never been reported. RNase 1 shares 70% amino acid identity with bovine RNase A (whose structure has been solved), and therefore, the surface helical residue positions in RNase 1 can be predicted with some confidence (Fig. 1A; Avey et al. 1967; Seno et al. 1994).

Here, we report a successful crystallization and X-ray structure determination of human RNase 1 by incorporation of a leucine zipper-like hydrophobic interface on specific solvent-exposed α -helical sites. The introduced leucine residues were observed to form a hydrophobic crystal packing interface. This type of leucine mutation may be a generally applicable method to crystallize proteins that are refractory to crystallization and to also control the packing arrangement of proteins within a crystal lattice.

Results

Overall structures of leucine mutants of RNase 1

The crystal structures of mutant RNase 1 with four mutations, Thr24 $\rightarrow$ Leu, Gln28 $\rightarrow$ Leu, Arg31 $\rightarrow$ Leu, Arg32 $\rightarrow$ Leu (4L-RNase 1); three mutations, Gln28 $\rightarrow$ Leu, Arg31 $\rightarrow$ Leu, Arg32 $\rightarrow$ Leu (3L-RNase 1); and two mutations, Arg31 $\rightarrow$ Leu and Arg32 $\rightarrow$ Leu (2L-RNase 1) to helix 2; and with four leucines Val52 $\rightarrow$ Leu, Asp53 $\rightarrow$ Leu, Asn56 $\rightarrow$ Leu, and Phe-59 $\rightarrow$ Leu (N4L-RNase 1) to helix-3 were determined by X-ray crystallography to 1.7, 1.6, 1.6, and 2.0 Å resolution, respectively. The asymmetric units of the 2L-, 3L-, and 4L-RNase 1 crystals each contained two copies of two molecules (chain A and B, respectively). These two molecules were associated through the mutant leucine residues introduced into the α -helical region (helix 2) (Fig. 2A–C). The N4L-RNase 1 also contained two copies located in an asymmetric unit (Fig. 2D), with four total molecules associated by the leucines introduced into helix 3. Overall structures of these mutants were essentially identical to each other (root mean square deviation [rmsd] values among them are >1.20 Å for 123 C $^{\alpha}$ atoms). The structure of N4L-RNase 1 is also similar to the structure of bovine RNase A (Protein Data Bank [PDB]: 7RSA, rmsd values 0.84 Å for 124 C $^{\alpha}$ atoms), RNase 3 (PDB: 1DYT [Mallorqui-Fernandez et al. 2000], rmsd values 2.07 Å for 118 C $^{\alpha}$ atoms), RNase 4 (PDB: 1RNF [Terzyan et al. 1999], rmsd values 1.42 Å for 118 atoms), PM7-RNase 1 in which the first 20 amino acid residues of its N terminus were replaced by the corresponding sequence of the bovine seminal RNase and Ser50 was substituted for Pro50 (1DZA [Pous et al. 2000], rmsd values 0.92 Å for 120 C $^{\alpha}$ atoms), and Δ N7-RNase 1 that lacks 7 amino acid residues

Figure 1. Amino acid sequence alignment of human pancreatic RNase 1, its variants (4L-, 3L-, 2L-, and N4L-RNase 1), and bovine pancreatic RNase A. Only residues different from RNase 1 are shown. Each extra methionine residue at the -1 position of the recombinant proteins is indicated in italics. The α -helices and β -strands are displayed as labeled dark squares and light arrows above the sequences. Secondary structure elements determined by crystal structures are also indicated in the sequences with shaded background. Secondary structure elements in RNase A are those indicated in the data of PDB accession code 7RSA.

of the N terminus (1E21 [Pous et al. 2001], rmsd values 1.28 Å for 119 C α atoms). In these structural comparison, each chain A was used as representative structure by assuming that the structures in asymmetric units are similar.

Packing interfaces of the leucine mutants in the crystals

The molecules in the asymmetric unit of 4L-RNase 1 are confirmed to be associated by the hydrophobic interface created by leucine mutation. Three leucines, Leu28, Leu31, and Leu32 in 4L-RNase 1, are involved in this packing, in which Leu28 is surrounded by Leu32 as well as Leu28 and Leu32 from a separate molecule within the same asymmetric unit (Fig. 3). However, Leu24 in 4L-RNase 1 seems not to be involved in the interface between molecules A and B (Fig. 3A). To identify the key residue for this hydrophobic packing, back-mutation was performed to reduce the number of leucines (3L- and 2L-RNase 1). Both 3L- and 2L-RNase 1 were successfully crystallized, and the crystal structures were determined. Similar molecular associations were seen in the asymmetric unit of the 3L- and 2L-RNase 1. The same space group and similar crystallization condition of 3L-RNase 1 to those of 4L-RNase 1 indicate that three residues at 28, 31, and 32 are important for creation of a crystal packing interface. The crystal packing of 2L-RNase 1 was similar to that of 4L- and 3L-RNase 1. The interface seems to be created mainly by Leu32 only. Although the relative packing between molecules in the 4L-, 3L-, and 2L-RNase 1 were similar, the side chain interactions of leucine residues exhibited slight differences as shown in Figure 3A–C.

The crystal packing of N4L-RNase 1 having leucines in helix 3 was quite different from that of other mutants having leucines in helix 2 (Fig. 2D). Four N4L-RNase 1 molecules from two adjacent asymmetric units are associated through the newly introduced leucine hydrophobic interface (Fig. 3D). Three leucines at positions 52, 53, and 56 in helix 3 of RNase 1 participate in this association, and the leucine introduced at position 59 only participates in creating the interface between the molecules A and B in an asymmetric unit.

Effect of leucine incorporation on the properties of RNase 1

The introduction of hydrophobic leucine residues may result in the alteration of some molecular characteristics of human RNase 1. Several properties of wild-type and leucine-containing RNase 1 mutants are summarized in Table 1. The molecular surface hydrophobicity of these proteins was compared by their retention on hydrophobic high-performance liquid chromatography (HPLC). Wild-type, 2L-, 3L-, 4L-, and N4L-RNase 1 were eluted at 20.1, 33.2, 42.2, 50.4, and 37.6 min, respectively (Table 1), after the increase in the number of leucine in RNase 1. The hydrophobicity of N4L-RNase 1 having four leucines was intermediate between those of 2L- and 3L-RNase 1.

The enzymatic activities of the wild-type, 4L-, 3L-, 2L-, and N4L-RNase 1 using yeast RNA as a substrate are summarized in Table 1. The specific activities of the 4L-, 3L-, 2L-, and N4L-RNase 1 were almost similar to that of the wild-type RNase 1; thus, the leucine incorporation did not affect the enzymatic activity of RNase 1.

Figure 2. Packing of the asymmetric unit(s). (A) 4L-, (B) 3L-, (C) 2L-, and (D) N4L-RNase 1. Introduced leucine residues (Leu24, Leu28, Leu31, and Leu32 in A–C, and Leu52, Leu53, and Leu56 in D) are depicted with stick models. The figures were made with Web Lab Viewer (Accelrys).

The average molecular weight of the wild-type, 4L-, and 3L-RNase 1 was determined by static light scattering. The average molecular weights of 4L- and 3L-RNase 1 were 24,230 Da and 20,050 Da, respectively; these values are slightly larger than that of the wild-type RNase 1 (19,600 Da), suggesting weak association between the RNase 1 mutants. The stability of the wild-type and RNase 1 mutants was determined by GdmCl denaturation

as shown in Table 2. The leucine incorporation into RNase 1 resulted in a slight decrease in stability, but it is not proportional to the number of leucines incorporated. From these observations, the characteristics of the 4L-, 3L-, and 2L-RNase 1 were concluded to be similar to that of the wild-type RNase, that is, mostly monomeric in solution, similarly enzymatically active, and thermodynamically as stable as wild-type RNase 1.

Figure 3. Packing regions in α 2- or α 3-helices are enlarged. (A) 4L-, (B) 3L-, (C) 2L-, and (D) N4L-RNase 1. Introduced leucine residues are depicted with stick models. The figures were made with Web Lab Viewer (Accelrys).

Discussion

It is known that protein interfaces tend to be conserved when a given protein crystallizes in more than one space group (Zhang and Matthews 1994). This observation suggests that there may be a key interface that promotes the crystallization of a protein. Although it is difficult to predict such interfaces, it may be possible to introduce such an interface by protein engineering. The “leucine zipper” is known to be a hydrophobic packing interface between α -helices that are involved in DNA recognition. Thus, our approach was to introduce a leucine zipper-like sequence into the helical region of RNase 1 to investigate the effect of the introduction of this molecular interface on the crystallization and packing of RNase 1.

The RNase A contains three α -helices (α 1, α 2, α 3; Fig. 1). To incorporate a leucine zipper-like structure, our

attention was directed to four residues, Thr24, Gln28, Arg31, and Arg32 of the second α -helix (α 2) in RNase 1, corresponding to residues Asn24, Gln28, Lys31, and Ser32 in RNase A, because they are well exposed to solvent. Thus, RNase 1 variant 4L-RNase 1 (Thr24 $\rightarrow$ Leu, Gln28 $\rightarrow$ Leu, Arg31 $\rightarrow$ Leu, Arg32 $\rightarrow$ Leu) in helix 2 (Fig. 1) was designed as potentially having a leucine zipper-like hydrophobic packing interface. To characterize further the role of the number of introduced leucine residues in the crystallization of RNase 1, a three-leucine incorporation Gln28 $\rightarrow$ Leu, Arg31 $\rightarrow$ Leu, Arg32 $\rightarrow$ Leu (3L-RNase 1) and a two-leucine incorporation Arg31 $\rightarrow$ Leu, Arg32 $\rightarrow$ Leu (2L-RNase 1) into the same helix 2 were designed.

The leucine incorporation into helix 2 of RNase 1 (4L-RNase 1) resulted in successful crystallization. Two RNase 1 mutants were located in an asymmetric unit and are associated through the hydrophobic interface created by leucines introduced into helix 2 (Fig. 3A–C; Scheme 1). Although the only mutant having a complete leucine zipper sequence is the 4L-RNase 1, other leucine mutants (3L- and 2L-RNase 1) not having the complete leucine zipper sequence were also crystallized successfully. From the structure analysis of these leucine mutants, the hydrophobic interface was created mainly by the leucines at positions 28, 31, and 32 (Fig. 3A–C) and leucine at position 24 did not show any interaction with other RNase 1. Thus, leucine incorporation may successfully promote crystallization of a protein if an appropriate helical region is available.

Leucine incorporation into another helix in RNase 1 was also tested. Four positions, 52, 53, 56, and 59 in helix 3, were chosen to incorporate leucines. The crystallization of N4L-RNase 1 resulted in a different space group from other leucine mutants. The crystal packing of the N4L-RNase 1 was created by four molecules through the hydrophobic interaction of leucines introduced into helix 3 (Figs. 2D, 3D). This finding suggests that leucine incorporation can control crystal packing of a protein in several different ways. There are two known examples of a change in crystal packing for human RNase 1. These are PM7 RNase 1, in which the first 7 amino acid residues of its N terminus were replaced by the corresponding

Table 1. Summary of the characteristics of the wild type, 4L-, 3L-, 2L-, and N4L-RNase 1

	Wild type	4L	3L	2L	N4L
Enzymatic activity (%)	100	103	100	102	104
Hydrophobicity ^a					
Elution time (min)	20.1	50.4	42.2	33.2	37.6
Molecular mass [Da]	19600	24230	20050	n.d.	n.d.

(n.d.) Not determined.

^aHydrophobicity of each mutant protein was evaluated by retention time on a TSK-gel Ether-5PW hydrophobic column.

Table 2. Thermodynamic parameters for GdmCl-induced unfolding of the wild type, 4L-, 3L-, 2L-, and N4L-RNase 1

Stability	$C_{1/2}$ [M]	m	ΔG_{H_2O} [kJ/mol]
Wild type	1.71	13.4	22.8
4L-RNase 1	1.64	12.8	21.1
3L-RNase 1	1.49	13.2	19.7
2L-RNase 1	1.82	11.9	21.6
N4L-RNase 1	1.37	15.6	21.4

sequence of the bovine seminal RNase and Ser50 was substituted for Pro50 (Pous et al. 2000), and $\Delta N7$ RNase 1, in which 7 amino acid residues of the N terminus were truncated (Pous et al. 2001). Both PM7 and $\Delta N7$ showed quite different packing at helix 2 (Scheme 1).

In the crystal packing of PM7 (Pous et al. 2000), the N-terminal sequence derived from bovine seminal RNase (same sequence as that of bovine RNase A except for the mutation of Ser3 to Thr) resulted in similar crystal packing to that of bovine RNase A located in the adjacent cell. After the replacement, the interface comprising of the two regions, N-terminal region (Lys1–Lys7) of RNase 1 and the loop region of the neighboring RNase 1 (Cys65–Gln69), are common in both PM7 and bovine RNase A, suggesting the reason for PM7 to create a similar crystal contact to bovine RNase A. This type of packing may be predicted because of more chance to conserve crystal packing of the original molecule.

The deletion of 7 N-terminal residues ($\Delta N7$) resulted in the creation of a new crystal contact with a symmetry-

related molecule as shown in Scheme 1. These interactions were completely different from those seen in the structures of 4L-, 3L-, 2L-, and PM7-RNase 1. The crystal packing of these mutants seems to be difficult to predict a priori.

The fact that introduced leucines predominantly associate with themselves suggests the possibilities not only to help crystallization but also to control the intermolecular crystal packing of a protein. In addition, the packing may be predicted if the tertiary structure of the protein has already been determined. The crystal structures of drug target molecules are used for fundamental information in drug design. But the hydrophobic active site of the protein (enzyme) is often blocked by the other molecules. The leucine incorporation may further control the crystal packing of the protein to observe the specific site of the structure excluding the effect of crystal packing.

In conclusion, we have demonstrated the successful engineering of a crystal packing interface within a protein by introducing leucine residues into specific sites on the surface. Introduced leucine residues provide a new interface for crystallization through hydrophobic interaction. By substitution of at most four residues (in the case of 4L- and N4L-RNase 1), uncrystallized full-length human RNase 1 was crystallized and the tertiary structure was determined. This leucine-substitution method, combined with secondary structure prediction methods (Edwards and Cottage 2003), opens the way for crystallization and structural analysis of numerous uncrystallized proteins and permits engineering of the crystal lattice of the protein for further biological applications (e.g., effective drug

Scheme 1. The control of the crystal packing by amino acid mutations. (1) Replacement of N-terminal region with bovine RNase A. (2) Seven-residue truncation of N-terminal region. (3) Leucine incorporation to helix 3. (4) Leucine incorporation to helix 2.

screening, protein chip for proteomics, and new materials made from functional proteins) in the near future.

Materials and Methods

Materials

The wild-type RNase 1 was a recombinant protein expressed in MM294(DE3)/pLysS *Escherichia coli* and purified as described previously (Futami et al. 1995). Deoxyribonuclease-I (DNaseI) was purchased from Sigma. All other chemicals were of highest grade and used without any further purification.

Plasmid construction and protein expression

Site-directed mutagenesis and expression of recombinant wild-type and mutant RNase 1, as inclusion bodies in *E. coli*, was performed using previously described methods (Futami et al. 1995, 2000a, b). The gene coding for the mutants containing four mutations, Thr24 → Leu, Gln28 → Leu, Arg31 → Leu, Arg32 → Leu (4L-RNase 1), three mutations, Gln28 → Leu, Arg31 → Leu, Arg32 → Leu (3L-RNase 1), and two mutations, Arg31 → Leu and Arg32 → Leu (2L-RNase 1) to helix 2, and the mutant containing four leucines Val52 → Leu, Asp53 → Leu, Asn56 → Leu, and Phe-59 → Leu (N4L-RNase 1) to helix 3 were constructed using the Quick Change Site-Directed mutagenesis kit (Stratagene) and RNase 1 expression plasmid pBO26 (Futami et al. 1995).

Refolding and purification of RNase 1 proteins

Refolding and purification of the wild-type, 4L-, 3L-, 2L-, and N4L-RNase 1 followed previously described methods (Futami et al. 1995, 2000a, b). Briefly, inclusion bodies of mutant RNase 1 were solubilized in 6 M guanidinium chloride (GdmCl) in the presence of 2-mercaptoethanol, diluted to a final protein concentration of 100 µg/mL with a redox buffer [10 mM Tris-HCl at pH 8.5, containing 0.5 mM oxidized glutathione, 2 mM reduced glutathione/2-mercaptoethanol, 30%(v/v) glycerol, and 0.4 M GdmCl], and then incubated for 36 h at 4°C. After removal of insoluble material by centrifugation, the pH was adjusted to 5.0 with acetic acid, and the supernatant was dialyzed against water. The refolded protein was concentrated by adsorption to a column of 10 mL of CM-Toyopearl 650C (Tosoh), followed by elution with 0.4 M NaCl at pH 5.0. The eluted protein was further purified by ion-exchange chromatography on an open column of CM-Toyopearl 650M (Tosoh; 13 × 680 mm) eluted with a linear gradient of 0–0.8 M NaCl in 50 mM sodium acetate buffer at pH 5.0. The fractions containing pure mutant RNase 1 were pooled and precipitated with saturated ammonium sulfate. The protein precipitate was subsequently dissolved in a minimal volume of water, exhaustively dialyzed against water, and then lyophilized. Approximately 8 mg of RNase 1 mutant was purified from 1 L of cultivation.

Enzymatic activity assay of the wild-type and mutant RNases

Enzymatic activities of the wild-type, 4L-, 3L-, 2L-, and N4L-RNase 1 were determined using yeast RNA (Sigma, type IV) as a substrate, as previously described (Futami et al. 1995).

Guanidinium chloride-induced unfolding of wild-type and mutant RNases

GdmCl-induced unfolding of the wild-type, 4L-, 3L-, 2L-, and N4L-RNase 1 (20 µM) was carried out at 32°C in the presence of the various concentrations of GdmCl (Katayama Chemicals) in 50 mM sodium acetate buffer at pH 5.4. The unfolding was monitored by measuring the change in absorbance at 287 nm using a 1-cm light path quartz cell.

Hydrophobicity of the wild-type and mutant RNases

The molecular surface hydrophobicity of mutant proteins was evaluated by retention time on a TSK-gel Ether-5PW hydrophobic HPLC column (7.5 mmID × 75 mm) with a linear gradient elution from 2 M to 0 M ammonium sulfate in 100 mM phosphate buffer at pH 7.0, with a flow rate of 0.5 mL/min over 60 min.

Gel filtration and light scattering analysis

Gel filtration of the wild-type and the 4L- and 3L-RNase 1 was performed using a Superdex 200 HR 10/300 column (Amersham Pharmacia Biotech AB) equilibrated with 20 mM of sodium phosphate buffer at pH 7.0, containing 200 mM of NaCl. Light scattering analysis of the wild type and 3L- and 4L-RNase 1 mutants was performed using a mini-DAWN spectrometer (Wyatt Technologies) equipped with the above-described gel filtration column. Approximately 50 µg of RNase 1 was injected onto a Superdex 200 column equilibrated with 20 mM sodium phosphate buffer at pH 7.0, containing 200 mM NaCl at a flow rate of 0.5 mL/min. The elution of the complex was monitored by both the refractive index and UV-absorption.

Crystallization

Crystallization of the wild-type, 4L-, 3L-, 2L-, and N4L-RNase 1 was screened at 20°C by the hanging-drop vapor-diffusion method using Crystal Screen 1 and 2 crystallization screening kits (Hampton Research). Drops (2 µL), containing 1 µL of 15–20 mg/mL of freeze-dried protein in pure water and 1 µL of reservoir solution, were equilibrated against 1 mL of the reservoir solution. Crystal formation was evaluated after one week.

Although the wild-type RNase 1 setups did not produce any crystals, both 4L- and 3L-RNase 1 crystallized within one week as either hexagonal bipyramidal crystals (with typical dimensions of 0.3 mm × 0.3 mm × 0.4 mm) from 2.0 M ammonium sulfate (crystal-1) or as an octahedral crystal form (with dimensions of 0.3 mm × 0.3 mm × 0.3 mm) from 0.1 M sodium acetate at pH 4.6, containing 0.1 M cadmium chloride and 30% polyethylene glycol 400 (PEG400) (crystal-2). The 4L- and 3L-RNase 1 were also crystallized as blade-like microcrystals from three other conditions: 0.1 M sodium cacodylate at pH 6.5, containing 0.2 M ammonium sulfate and 30% PEG8000; 0.2 M ammonium sulfate containing 30% PEG8000; and 0.2 M ammonium sulfate containing 30% PEG4000. The 2L-RNase 1 was crystallized with a petal-like morphology (with dimensions of 0.1 mm × 0.1 mm × 0.3 mm) from 0.1 M sodium acetate at pH 4.6, containing 0.2 M ammonium sulfate and 25% PEG4000 (crystal-3). The N4L-RNase 1 crystallized with a prismatic morphology (with dimensions of 0.1 mm × 0.1 mm × 0.3 mm).

Table 3. Data collection, processing, and refinement statistics for 4L-, 3L-, 2L-, and N4L-RNase 1

Crystal	4L-RNase 1	3L-RNase 1	2L-RNase 1	N4L-RNase 1
Data statistics				
X-ray source				
Space group	$I4_1$	$I4_1$	$P4_12_12$	$P6_122$
Unit cell dimensions (Å)	$a = b = 93.5$, $c = 94.3$	$a = b = 93.4$, $c = 93.0$	$a = b = 97.5$, $c = 53.2$	$a = b = 98.5$, $c = 112.1$
Resolution (Å)	1.7	1.6	1.6	2.0
Completeness (%)	99.9 (100.0)	99.7 (100.0)	99.8 (99.6)	99.1 (91.9)
$I/\sigma I$	34.6 (6.3)	27.1 (4.8)	29.3 (3.4)	35.5 (1.9)
No. of observed reflections	632,265	509,822	431,141	407,073
No. of unique reflections	44,558	52,471	34,457	22,154
R_{merge} (%) ^a	8.5 (44.6)	6.0 (50.9)	9.2 (55.4)	8.2 (49.6)
Redundancy	14.2 (13.1)	9.7 (8.3)	12.5 (12.3)	18.4 (6.3)
Refinement statistics				
No. of molecules per asymmetric unit	2	2	2	2
Resolution range used for refinement (Å)	20–1.7	20–1.6	20–1.6	20–2.0
No. of reflections in working set	40,014	46,934	30,863	19,814
No. of reflections in test set	2242	2663	1737	1123
No. of protein atoms included in refinement	2014	2012	2014	2008
No. of solvent molecules	400	401	403	154
No. of cadmium ions	10	10	-	-
No. of cadmium ions	15	15	-	-
No. of sulfate ions	-	-	-	12
No. of glycerols	-	-	-	6
R factor/ R_{free} ^b	0.192/0.210	0.195/0.212	0.188/0.215	0.209/0.257
Average B factors (Å ²)	37.6	36.2	34.1	47.9
Rmsd from target values				
Bond length (Å)	0.018	0.010	0.010	0.033
Bond angle (°)	1.52	1.39	1.33	2.41
Ramachandran plot statistics				
Most favored regions (%)	91.2	91.7	89.9	85.7
Additionally allowed regions (%)	8.8	8.3	10.1	12.5

Numbers in parentheses are for the highest resolution shell.

^a $R_{\text{merge}} = \sum |I_h - \langle I_h \rangle| / \sum \langle I_h \rangle$, where $\langle I_h \rangle$ is the average intensity of reflection h and their symmetry-related reflections.

^b $R = R_{\text{free}} = \sum ||F_o| - |\bar{F}_c|| / \sum |F_o|$ calculated for the reflections of the working and test (5%) sets, respectively.

from 0.1 M MES at pH 6.5 containing 1.0 M ammonium sulfate (crystal-4).

Data collection and processing

X-ray diffraction data were collected at 100K at the BL38B1 beamline of the SPring-8 synchrotron (Mikazukicho) using crystal-1 and -2 for 4L- and 3L-RNase1, crystal-3 for 2L-RNase 1, and crystal-4 for N4L-RNase 1. Diffraction data were processed with HKL2000 (Otwinowski and Minor 1997).

The unit cell dimensions of crystal-1 were too large for structure analysis (space group unidentified, tentative cell dimensions: $a = 163.9$ Å, $b = 94.2$ Å, $c = 451.4$ Å), the crystal-2, -3, and -4 were pursued for further analysis. Crystal-2 of 3L- and 4L-RNase 1 both belonged to space group $I4_1$ with similar unit cell dimensions ($a, b = 93.4$ Å, $c = 93.0$ Å for 3L-RNase 1, and $a, b = 93.5$ Å, $c = 94.3$ Å for 4L-RNase 1). Crystal-3 of 2L-RNase 1 belonged to space group $P4_12_12$ with unit cell dimensions of $a, b = 97.5$, $c = 53.2$ Å. Each contained two RNase 1 monomers in the respective asym-

metric units. Crystal-4 of N4L-RNase 1 belonged to the space group $P6_122$ with unit cell dimensions of $a, b = 98.5$, $c = 112.1$ Å. The data collection and processing statistics are listed in Table 3.

Molecular replacement and refinement of X-ray structures

The crystal structures of 4L-, 3L-, 2L-, and N4L-RNase 1 were independently solved by molecular replacement using CNS v1.0 (Brunger et al. 1998) with the coordinates of bovine RNase A (PDB accession code 7RSA) (Wlodawer et al. 1988) as the search model. The structural models were built using XtalView (McRee 1992), and refinement was accomplished using CNS v1.0 followed by REFMAC (Murshudov et al. 1997). Ramachandran plot parameters were calculated by PROCHECK (Laskowski et al. 1993). The statistics of data collection and refinement are listed in Table 3. Atomic coordinates and structure factors have been deposited into the PDB (accession codes 2E0M for 4L-RNase 1, 2E0L for 3L-RNase 1, 2E0J for 2L-RNase 1, and 2E0O for N4L-RNase 1).

Structural comparison between the RNase 1 mutants and their homologs were performed using the program MATRAS (Kawabata 2003).

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