Packing forces in ribonuclease crystals

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Packing in Ribonuclease A and Ribonuclease S crystals have been compared in order to determine the possible role of the precipitant on lattice contacts. Both proteins have similar tertiary structures, buth they crystallize in different space groups depending on the precipitating agent. It is found that packing differs either by the number of nearest neighbours or by the size of surface areas buried in individual contacts. Ammonium sulfate seems to promote hydrophobic interactions with interfaces similar to those found in oligomeric proteins. Organic precipitants favour electrostatic interactions.

Crystal packing; Buried surface area; Ribonuclease A; Ribonuclease S

1. INTRODUCTION

Although many proteins have been crystallized, little is known of the interactions which produce regular three-dimensional arrays of biological macromolecules. A rational approach to the growth of high quality crystals requires a better understanding of the forces that govern the association processes between large molecules. A systematic analysis of the intermolecular contacts of protein structures crystallized in different packing environments could provide some insight into the phenomenon of macromolecular crystal growth.

We have focused our purpose on the possible role of the solvent in the crystal packing forces. It is known that many kinds of precipitating agents, including salts, organic solvents or polymers, can be used to give rise to crystallization. Usually, for a given protein, only one type of precipitant is found to lead to single crystals; in some cases, like for pancreatic bovine ribonuclease, almost all types have been used successfully [1]. In order to understand the specific role played by the precipitating agent in lattice formation, the present work compares crystal properties of native ribonuclease (RNase A) and of its proteolytic modification (RNase S). Both proteins have similar tertiary structures, but they crystallize in different space groups depending on the precipitating agent.

Bovine pancreatic RNase A consists of 124 amino acid residues; when subjected to limited proteolysis using subtilisin, the resulting RNase S consists of two protein chains and the complex of these fragments is fully active [2]. Both structures have been solved in 1967 [3,4] and the resolution extended to at least 2 Å a few years later [5–7]. Comparison of the two forms have indicated very similar structures [8] except for the region of residues 17–23. Crystals of ribonuclease S are grown from ammonium sulfate and belong to the space group $P3_121$. Crystals of RNase A are grown from ethanol or *t*-butanol and belong to the space group $P2_1$. In both crystal forms, the asymmetric unit contains one protein molecule.

The comparison of crystal packing shows that, despite the similar protein structures, protein interfaces created by the packing differ in their buried surfaces and the electrostatic character of the residues involved in the contacts.

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Abbreviations: RNase A, ribonuclease A; RNase S, ribonuclease S

2. EXPERIMENTAL

Atomic coordinates at 2 Å resolution were taken from the Protein Data Bank entries 1RNS, 6RSA and atomic coordinates refined at 1.26 Å were taken from 7RSA entry.

Atomic distances between adjacent molecules were carried out with SYMVOI, a vectorized program developed in the laboratory which mostly runs on an array processor ST100 from Star Technologies (USA), installed at the Laboratoire pour l'Utilisation du Rayonnement Electromagnétique at Orsay. The vectorized version was written in Array Processor Control Language and the main procedures have been specially designed in microcode for running between 50 and 100 Megaflops on arrays up to 15400 atoms.

Molecular packing was visualized with MANOSK, a molecular graphics package written for the Evans and Sutherland PS300 graphic displays [9].

Computations of static solvent accessible surface areas were performed with ASA from A. Lesk (Cambridge) [10], using a spherical probe of 1.4 Å radius; surface areas buried in intermolecular contacts were calculated by difference.

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3. RESULTS

Bonds which are formed during the crystallization process involve protein–protein and protein–solvent–protein interactions. They consist of ion pairing, hydrogen bonds and Van der Waals contacts between surface residues.

Intermolecular bonds observed in crystal packing of RNase A and RNase S are listed in Table I. The atomic coordinates used for the calculations were 2 Å resolution for RNase S and RNase A; it was verified that refinement at 1.26 Å, in the case of RNase A, did not significantly modify the results. The list of interactions mediated by water molecules was possible only for RNase A, since water molecule positions have not been published for RNase S.

In crystals of RNase A, each molecule makes contacts with ten nearest neighbours, whereas each molecule of RNase S is in contact with only five nearest

Table I

Number and type of contacts in RNase A and RNase S crystals

Symmetry operator	No. of residues	No. of VdW contacts	No. of H bonds	No. of salt bridges	No. of solvent bridges	Buried area (Å^2)
Ribonuclease A						
A1: a translation	10	15	1	1	2	450
A2: b translation	6	16	1	–	3	320
A3: 2_1 axis + a translation	3	6	1	–	3	120
A4: 2_1 axis + 2a translation	7	31	1	1	1	420
A5: 2_1 axis + (2a + c) translation	9	25	2	–	6	350
Ribonuclease S						
S1: 2 axis	36	90	4	0		1810
S2: 2 axis + a translation	21	99	3	1		880
S3: (3_1+2) axis + a translation	18	46	3	1		1000

Van der Waals contacts: $d \leq 4$ Å; hydrogen bond: $d = 3.2$ Å and angle between donor and acceptor greater than 130°; salt bridge: $d \leq 3.5$ Å

6RSA space group $P2_1$
parameter $a = 30.30$ Å; $b = 38.35$ Å; $c = 53.70$ Å; $\beta = 106.4°$
symmetry operator: **A1** = $x+1,y,z$ and $x-1,y,z$; **A2** = $x,y+1,z$ and $x,y-1,z$; **A3** = $1-x,y+1/2,-z$ and $1-x,y-1/2,-z$; **A4** = $2-x,y+1/2,-z$ and $2-x,y-1/2,-z$; **A5** = $2-x,y+1/2,1-z$ and $2-x,y-1/2,1-z$
accessible area of isolated protein: 6894 Å^2

1RNS space group $P3_121$
parameter $a = 44.65$ Å; $b = 44.65$ Å; $c = 97.15$ Å; $\gamma = 120°$
symmetry operator: **S1** = $y,x,-z$; **S2** = $y-1,x,-z$ and $y,x+1,-z$; **S3** = $-x-1,y-x,1/3-z$ and $-x-1,y-x-1,1/3-z$
accessible area of isolated protein: 6922 Å^2

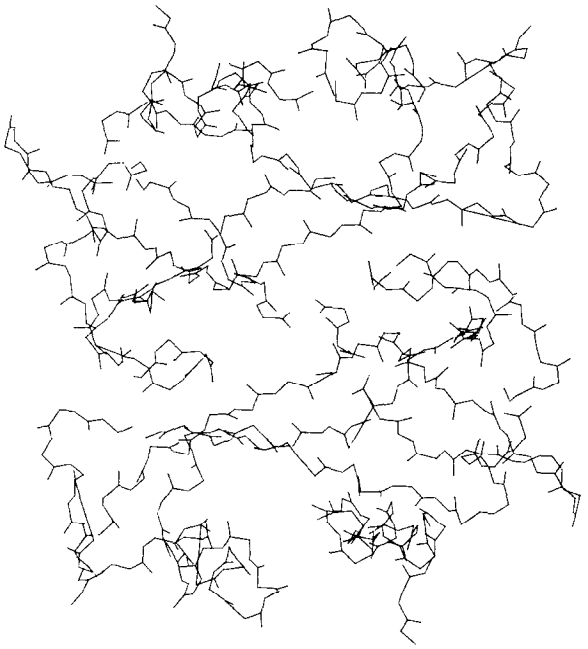

Fig. 1. Symmetric contact in RNase S crystals generated by two molecules related by the dyad axis.

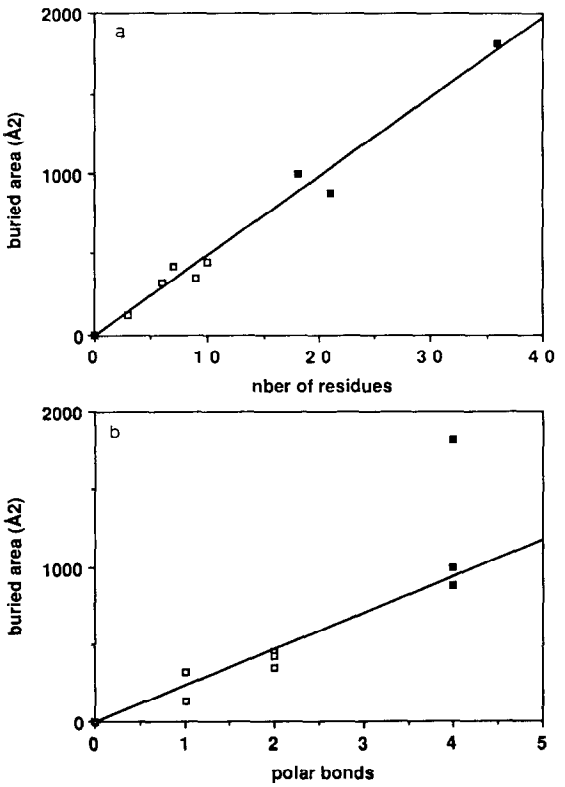

Fig. 2. (a) Buried surface area as a function of the number of residues involved (□, RNase A; ■, RNase S). For each contact, the total accessible surface areas of two protein molecules in isolation and as part of a dimer of two neighbouring molecules were determined; the area of the protein buried in the interface is the difference between these two numbers; for one contact, it is the sum of the two individual interface areas. (b) Buried surface area as a function of the number of polar bonds (salt bridges and hydrogen bonds); □, RNase A; ■, RNase S. The point which deviates from the straight line represents the symmetric contact about the 2-fold axis in RNase S.

neighbours; the odd number comes from the 2-fold symmetry character of one of the interfaces (Fig. 1). The size of the domains in contact varies in a very large range. Fig. 2a shows that, for all the contacts, areas of buried surfaces remain proportional to the number of residues involved, with about 50 Å^2 per residue. The areas of buried surfaces vary between 120 and 450 Å^2 for RNase A, and from 880 to 1810 Å^2 for RNase S; the largest buried surface, around 2000 Å^2, is found in the symmetric contact of RNase S obtained from two molecules related by the dyad axis. For the reference molecule of RNase A, the total loss of accessible area due to packing is 1880 Å^2, which is only about 25% of the total solvent accessible surface of 6894 Å^2. In the case of RNase S, the total loss of accessible area due to packing is higher and represents 40% of the total solvent accessible surface.

The electrostatic interactions (ionic pairs or hydrogen bonds) are reported in Table II. There are a few salt bridges, never more than one per contact. The number of hydrogen bonds between two neighboring molecules in the crystal is slightly higher and four direct contacts between peptide atoms can be observed: three for RNase A and only one for RNase S. When compared to buried surfaces, it appears that, for all contacts except the symmetric contact in RNase S, there is about one electrostatic interaction per 250 Å^2 (Fig. 2b).

Table II

Hydrogen bonds and ionic pairs involved in contacts

Symmetry operator	Residues involved	Distance (Å)
Ribonuclease A ($P2_1$)		
A1	O Lys 66---N_{d2} Asn 34	3.2
	N_z Lys 61---O_{e1} Glu 9	*3.4*
A2	N_{e2} Gln 55---O Gly 88	3.2
A3	N Lys 1---O Lys 37	2.8
A4	N Thr 70---O Asp 38	3.2
	O_{e1} Glu 111---NH_1 Arg 39	*3.3*
A5	N Ser 23---O Ser 15	2.9
	N_{d2} Asn 24---O_g Ser 18	2.7
Ribonuclease S ($P3_121$)		
S1	N Ala 64---O Glu 86	2.9
	O Glu 86---N Ala 64	2.9
	O_g Ser 123---O Val 124	3.1
	O Val 124---O_g Ser 123	3.1
S2	*O_{d1} Asp 14---N_z Lys 91*	*3.2*
	O_g Ser 16---O Tyr 92	2.9
	O Ala 19---O_{d1} Asn 94	2.5
	O_{x1} Ala 20---N Cys 95	3.1
S3	O Lys 1---O_{e2} Glu 49	2.5
	NH_2 Arg 10---O_{d1} Asp 53	*2.5*
	N_e Arg 33---O_g Ser 59	2.5
	NH_2 Arg 33---O Ser 59	3.1

Salt bridges are in italics

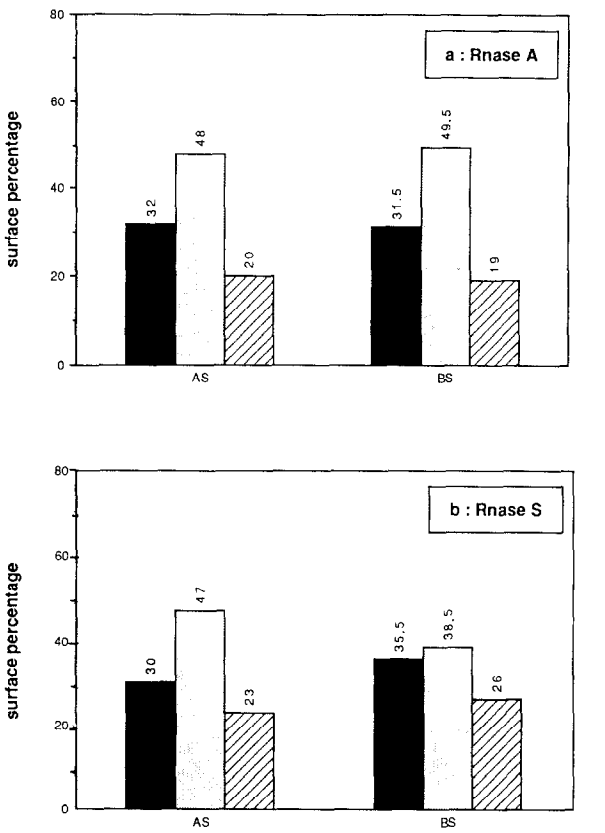

Fig. 3. Polarity of contacts: amino acid composition of accessible surface of isolated protein (AS) and of surface buried in crystal contacts (BS) for RNase A (a) and RNase S (b). ■, non-polar residues (Ala, Gly, Ile, Leu, Met, Phe, Pro, Val); □, polar residues (Asn, Cys, Gln, His, Thr, Tyr, Ser); ▨, charged residues (Arg, Asp, Glu, Lys).

The chemical nature of the groups involved in contacts is analysed in Fig. 3, which details the relative contributions of polar and non-polar amino acid side chains to the accessible surface of the isolated molecules and to the interfaces in crystal packing. For crystals of RNase A, surfaces involved in contacts do not differ from the rest of the molecule surface; interfaces generated by RNase S packing are enriched in non-polar groups, yet they contain a great number of charged groups.

4. DISCUSSION

RNases A and S are globular proteins with an ellipsoidal shape. Whereas the refined structures of these two molecules have been obtained from different types of crystals, grown from different precipitating agents, the folding of the main chain is very similar [1].

The physicochemical properties of the diverse solvents used for crystallization of ribonuclease differ essentially in their polarity. Monoclinic crystals of RNase A belonging to space group $P2_1$ can be grown from aqueous solutions of various organic liquids (50%

ethanol, 55% 2-methyl-2,4-pentane-diol or 43% 2-methyl-2-propanol) at a pH between 5 and 7; there are two molecules in the unit cell and the solvent content is around 50% [1]. The trigonal crystals $P3_121$ of RNase S, called Y form, are grown at room temperature from 40% saturated ammonium sulfate, 3 M CsCl and 0.1 M acetate buffer (pH 6.1). There are six molecules per unit cell; the solvent content is 40% [4]. RNase A and RNase S have been crystallized isomorphously at pH 9 from $(NH_4)_2SO_4$/CsCl in the trigonal space group $P3_121$ [11]. A comparison of these forms having identical crystal lattice contacts could be of interest, but the results have not been published yet.

For all contacts except one, Fig. 2b indicates a contribution of electrostatic interactions proportional to buried surface areas in a manner similar to subunit interfaces in oligomeric proteins [12]. Therefore, the more spectacular difference between packing of both RNase molecules is the amount of surface buried in individual contacts. The buried surface areas appear to be related to the solvent content in the crystal and to depend on the polarity of the precipitating agent: the diverse values are less than 500 $Å^2$ in the presence of organic solvent and greater than 800 $Å^2$ in the presence of salt.

Association can be described as transfer of surface atoms from solvent to interior; therefore buried surfaces may be related to hydrophobic interactions with an energy of 25 $cal \cdot mol^{-1}$ per $Å^2$, usually [13]. The large area interfaces observed for RNase S reflect strong hydrophobic interactions; the hydrophobic contribution to the free energy of contact formation varies from 20 $kcal \cdot mol^{-1}$ to 45 $kcal \cdot mol^{-1}$ per contact. It is known that salt solutions favour such hydrophobic interactions. The analysis of polarity of amino acids involved in contacts reveals that, compared to surface of the whole protein, interfaces in RNase S crystals are enriched in hydrophobic (Leu, Ile, Ala, Val), but also in charged (Asp, Arg) side chains. On the contrary, the composition of interfaces in crystals of RNase A does not differ significantly from that of the molecule surface.

The interface found in the symmetric contact of RNase S obtained from two molecules related by the dyad axis appears as a continuation of β-pleated sheets with the 2-fold axis perpendicular to the sheet plane; such continuations across interfaces are common in aggregates of subunits and of structural domains [14]. This contact is the one which involves the largest buried surface, around 2000 $Å^2$ and the corresponding dimer has a total accessible surface area which is related to its molecular weight similarly to oligomer proteins [15]. An intermolecular contact, identical in most respects, has been found in the C2 monoclinic crystals of RNase B crystallized from poly(ethylene glycol) 4000 [16] and in the $P2_12_12_1$ crystals of a derivative of RNase A crystallized from ammonium sulfate [17]. It suggests that this dimer could be a precursor in the crystallization process when salts or polymers are used as precipitant agents.

In the presence of ammonium sulfate, i.e. at high ionic strength, the mean charge of the molecule does not seem to play an important role in the crystallization process, since crystals of similar packing are obtained for pH values varying from 6.1 to 9.0 [11]. The isoelectric point of RNase A is 9.6. As the pH decreases, the N-terminal and four histidine residues become more positively charged and, probably, the protein carries its own partial complement of counterions. It is known that this protein may bind specific anions (phosphate, sulfate or chloride ions) in its active site; this leads to a lower apparent isoelectric point, since it compensates part of the positive charge due to His-12 and His-119 located in the active site [18].

Ethanol, with a low dielectric constant, may enhance electrostatic (polar and dipolar) interactions. Small-angle neutron scattering studies from RNase A in alcohol solvents indicate no water associated with the protein [19]. On the other hand, a single 2-methyl-2-propanol binding site has been located in crystals of RNase A during the course of the high-resolution refinement [20]. Moreover, a combination of X-ray and neutron diffracation has permitted the location of many water molecules adjacent to the protein surface and it appears that a number of intermolecular bridges are also provided by water molecules [8]. Nevertheless, since Coulombic attractions and repulsions have the longest range of all the elementary atomic interactions, electrostatic forces may play an important role in absence of salts, whatever the composition of the solvent in the vicinity of the molecule. The highly refined structure of RNase A has revealed discrete multiple conformations of some charged amino acid residues distributed over the surface of the molecule. For example, Lys-61 has multiple conformers and only one of them forms a salt bridge with the side chain of Glu-9 of another molecule [21]. Therefore, the network of closest electrostatic interactions, in the case of RNase A, occurs between molecules that are related by the 2_1-fold axis of symmetry; it involves two types of contacts (numbers 4 and 5 in Table II). The first one includes a salt bridge between Glu-111 and Arg-39 and a hydrogen bond; the second exhibits two intermolecular hydrogen bonds involving serine and asparagine residues. Neutron experiments have shown that the amide hydrogen of Ser-18 is in fact protected from exchange [22]. Such strong electrostatic interactions suggest the formation of molecular chains along the 2_1-fold axis in the early steps of the crystallization process and emphasize the role played by the low dielectric constant of the crystallization agent. Similarly, the presence of aggregated chains in crystals of lysozyme and cytochrome c' has been proposed as a precursor to crystals [23].

By looking at the properties of interactions in crystals, we have been able to analyse the role played by the precipitating agent in the intermolecular association process. In the case of ribonuclease, the use of high salt concentrations seems to favour large buried surface areas and crystallization seems to be driven by hydrophobic interactions; the corresponding numerous Van der Waals contacts involve a complementarity of surfaces which can be adjusted by translational diffusion along the protein surface only after the protein molecules have collided. On the other hand, organic solvents enhance strong electrostatic interactions; since coulombic forces are long range interactions, our observations suggest a preorientation in a direction parallel to the dyad axis of protein molecules before they come into contact. In order to better understand this process, a study of the electrostatic field in crystals of RNase A is under investigation.

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