the formation of the following hydrogen bonds: O1—H = 0.938, O1···O3 = 2.807 (4), H···O3 = 1.872 Å, O1—H···O3 = 175°; O3—H1 = 0.938, O3···O2[ii] = 2.943 (4), H1···O2[ii] = 2.093 Å, O3—H1···O2[ii] = 150°; O3—H2 = 0.938, O3···N[iii] = 2.967 (5), H2···N[iii] = 2.031 Å, O3—H2···N[iii] = 175°; [where (ii) = $1-x, \frac{1}{2}+y, \frac{1}{2}-z$; (iii) = $1-x, -y, 1-z$] which stabilize the packing of the molecules in the crystal. Other contacts are of the van der Waals type.

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Structure of Ribonuclease T_1 Complexed with Zinc(II) at 1.8 Å Resolution: a $Zn^{2+}.6H_2O$.Carboxylate Clathrate

BY JIANPING DING,* HUI-WOOG CHOE, JOACHIM GRANZIN AND WOLFRAM SAENGER†

Institut für Kristallographie, Freie Universität Berlin, Takustraße 6, D-1000 Berlin 33, Germany

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Abstract

In order to study the inhibitory effect of Zn^{2+} on ribonuclease T_1 [RNase T_1; Itaya & Inoue (1982). *Biochem. J.* **207**, 357–362], the enzyme was co-crystallized with 2 m*M* Zn^{2+}, pH 5.2, from a solution containing 55% (*v*/*v*) 2-methyl-2,4-pentanediol. The crystals are orthorhombic, $P2_12_12_1$, $a = 48.71$ (1), $b = 46.51$ (1), $c = 41.14$ (1) Å, $Z = 4$, $V = 93203$ Å^3. The crystal structure was determined by molecular replacement and refined by restrained least-squares methods based on F_{hkl} for 8291 unique reflections with $F_o \geq 1\sigma(F_o)$ in the resolution range 10 to 1.8 Å and converged at a crystallographic R factor of 0.140. The Zn^{2+} is *not* bonded to the active site of RNase T_1, probably because the His40 and His92 side chains are protonated. Zn^{2+} occupies the

* Present address: CABM and Rutgers University, 679 Hoes Lanes, Piscataway, NJ 08854-5638, USA.

† Author to whom correspondence should be addressed.

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same site as Ca^{2+} in a series of crystal structures of free and nucleotide-complexed RNase T_1. It is coordinated to Asp15 carboxylate and to six water molecules forming a dodecahedron of square antiprismatic form. The $Zn^{2+}\cdots O$ distances are ~2.5 Å, suggesting that Zn^{2+} is clathrated and not coordinated, which would require distances of 2.0 Å.

Introduction

Ribonuclease (RNase) T_1 (E.C. 3.1.27.3) isolated from *Aspergillus oryzae* occurs in two isoforms containing either Lys or Gln at position 25 of the polypeptide chain. It cleaves single-stranded RNA specifically at the 3′-phosphate group of guanylic acid (Egami, Oshima & Uchida, 1980; Takahashi & Moore, 1982; Heinemann & Hahn, 1989; Pace, Heinemann, Hahn & Saenger, 1991). The hydrolysis of RNA catalyzed by RNase T_1 occurs in two steps: firstly, transesterification of RNA to yield oligonucleotides with terminal guanosine-2′,3′-cyclic phosphate; and secondly, hydrolysis of the 2′,3′-cyclic phosphate to produce terminal guanosine-3′-phosphate. The amino acids involved in this reaction are the pair His40/Glu58 which remove the proton from $O_{2'}H$ (His40 is probably involved in the positioning or activation of $O_{2'}H$) in the first step, His92 which donates a proton to the leaving $O_{5'}$ group and activates the water molecule used for hydrolysis, and Tyr38 which positions and Arg77 which positions and neutralizes the incoming negatively charged phosphate (Egami, Oshima & Uchida, 1980; Takahashi & Moore, 1982; Heinemann & Saenger, 1982; Heinemann & Hahn, 1989).

Previous chemical and biochemical studies showed that several metal ions and especially Zn^{2+} inhibit RNase T_1 catalytic activity (Uchida & Egami, 1971). Later, steady-state kinetic studies indicated that the inhibitory action of Zn^{2+} on RNase T_1 catalysis is neither clearly competitive nor non-competitive or uncompetitive and was referred to as 'fully mixed inhibition' (Itaya & Inoue, 1982). The data suggested that the Zn^{2+} coordinates to the active site and facilitates the binding of substrate to form a catalytically inactive ternary complex RNase $T_1.Zn^{2+}$.substrate. The location of Zn^{2+} was thought to be near His40 and or His92 of the RNase T_1 molecule. The present investigation on the structure of the RNase T_1*Zn^{2+} complex was initiated to find out whether the binding site of Zn^{2+} is at the catalytic site as proposed by Itaya & Inoue (1982) or at the calcium binding site. The latter site is at Asp15 carboxylate as shown by the crystal structures of free RNase T_1 complexed with Ca^{2+} (Martinez-Oyanedel, Choe, Heinemann & Saenger, 1991), and of RNase T_1 complexed with vanadate/Ca^{2+} (Kostrewa, Choe, Heinemann & Saenger, 1989), with 2′-AMP (adenosine 2′-phosphate)/Ca^{2+} (Ding, Koellner, Grunert & Saenger, 1991), with 2Guo (guanosine)$_2$/Ca^{2+} (Lenz, Cordes, Heinemann & Saenger, 1991), with 2′,5′-GpG (guanylyl-2′,5′-guanosine)/Ca^{2+} (Koepke, Maslowska, Heinemann & Saenger, 1989), and with 2′-GMP (guanosine 2′-phosphate)/Ca^{2+} (the Ca^{2+} was interpreted as water) (Arni, Heinemann, Tokuoka & Saenger, 1988); for binding studies see Pace & Grimsley (1988).

Materials and methods

Co-crystallization and X-ray diffraction data collection

Recombinant Lys25-RNase T_1 (hereafter RNase T_1) was expressed using the pA_2T_1 vector system and purified as described previously (Quaas, McKeown, Stanssens, Frank, Blöcker & Hahn, 1988). The RNase T_1*Zn^{2+} complex was crystallized at room temperature using the sitting-drop vapour-diffusion technique. 10 μl protein solution (20 mg ml^{-1} was mixed with 10 μl reservoir solution containing 55% (v/v) MPD (2-methyl-2,4-pentanediol), 20 mM sodium acetate and 2 mM zinc acetate pH 5.2, and equilibrated against 10 ml reservoir solution. The crystals obtained are colorless transparent prisms with dimensions of 0.3 × 0.4 × 0.8 mm. The habit is the same as that of the needle-shaped clusters commonly observed for crystals of RNase T_1 complexed with Ca^{2+} and with or without nucleotides, but the shape differs largely in the c direction. Crystals of the RNase T_1*Zn^{2+} complex can also be grown by mixing 10 μl protein solution (20 mg/ml) with 10 μl of a solution containing 55% MPD, 20 mM Tris.-HCl, 2 mM $ZnCl_2$ at pH 7.5 and equilibrating against the latter; however, these crystals are mostly twinned and too small to use for X-ray studies.

A crystal of dimensions 0.5 × 0.2 × 0.2 mm was mounted in a glass capillary with a drop of mother liquor. The X-ray diffraction measurements were performed on a Delft Instruments Turbo-CAD-4 diffractometer at room temperature, using Ni-filtered Cu $K\alpha$ radiation ($\lambda = 1.5418$ Å) from a rotating-anode X-ray generator (45 kV, 99 mA; 3 × 0.3 mm^2 focus). As for the other RNase T_1 complexes, the crystals belong to the orthorhombic space group $P2_12_12_1$. The cell dimensions $a = 48.71$ (1), $b = 46.51$ (1), $c = 41.14$ (1) Å, were obtained by least-squares refinement against well-determined settings of 76 reflections ($16 < 2\theta < 25°$). Diffraction data of 9459 reflections were measured in the ω–2θ scan mode for one asymmetric unit ($h_{max} = 27$, $k_{max} = 25$, $l_{max} = 22$) to $2\theta_{max} = 51°$ in four overlapping shells, corresponding to a nominal resolution of 1.8 Å. Of the 8580 unique reflections in the resolution range

10.0–1.8 Å [$R_{merge}(F^2) = 0.03$], 8291 reflections have $F_o \geq 1\sigma(F_o)$, 7922 have $F_o \geq 2\sigma(F_o)$ and 7852 have $F_o \geq 3\sigma(F_o)$. The completeness of the data set [$F_o \geq 1\sigma(F_o)$] for the 1.8 Å sphere is 95%, and in the 2.0–1.8 Å shell it is 91%. The intensities were corrected for crystal decay which did not exceed 16%, for Lorentz and polarization effects, and for absorption (with min. and max. transmission of 0.8 and 0.98) (North, Phillips & Mathews, 1968).

Structure determination and refinement

Since this crystal structure is practically isomorphous with the structure of free RNase T_1 complexed with Ca^{2+} ($a = 48.73$, $b = 46.39$, $c = 41.10$ Å) (Martinez-Oyanedel, Choe, Heinemann & Saenger, 1991), the coordinates of the 777 protein atoms served as the starting model. The model was oriented as a rigid body in the unit cell of the RNase T_1*Zn^{2+} complex using the program *CORELS* (Sussman, Holbrook, Church & Kim, 1977; Sussman, 1985) with reflection data in the resolution range 10 to 3 Å. After ten refinement cycles, the R factor converged at 0.30 with a correlation coefficient of 0.87. The structure was further refined by least-squares methods with stereochemical restraints in the fast Fourier transformation version *PROFFT* (Finzel, 1987; Sheriff, 1987) of the program *PROLSQ* (Hendrickson, 1985; Hendrickson & Konnert, 1980) based on 8291 F_{hkl} with $F_o \geq 1\sigma(F_o)$ in the resolution range 10–1.8 Å. During refinement, the X-ray diffraction data were weighted globally *versus* stereochemical terms using pseudo-standard deviations for structure-factor amplitudes. In the final stages of refinement, the weights for the temperature-factor restraints were reduced relative to the values proposed originally (Hendrickson, 1985), according to suggestions by Yu, Karplus & Hendrickson (1985). The refinement was guided by omit electron density maps and ($F_o - F_c$) or ($2F_o - F_c$) difference maps using the computer-graphics program *FRODO* (Jones, 1978, 1985), Version E4.4 (Hubbard & Evans, 1985).

After several cycles of refinement, the difference electron density maps with coefficients ($F_o - F_c$) and $2F_o - F_c$) clearly indicated a dense spherical peak at the Ca^{2+} binding site close to the Asp15 carboxylate (Koepke, Maslowska, Heinemann & Saenger, 1989; Martinez-Oyanedel, Choe, Heinemann & Saenger, 1991) and was interpreted as a Zn^{2+} ion. Spherically shaped residual electron density in the ($F_o - F_c$) difference maps was interpreted as a water O atom if its position was within 3.5 Å and had reasonable hydrogen-bonding contacts with N or O atoms of amino acids or the Zn^{2+} ion, or other water O atoms. Almost all peaks higher than 0.3 e Å^{-3} in the ($F_o - F_c$) difference density maps could be assigned as solvent sites. The water molecule positions were refined with unit occupancies following Kundrot & Richards (1987). The refinement converged at a crystallographic R value of 0.140 for 8291 observed reflections $\geq 1\sigma(F_o)$ between 10 and 1.8 Å resolution. The mean error in atomic positions was estimated by the method of Luzzati (1952) to be 0.15 Å. A summary of refinement and weighting details is given in Table 1.

Table 1. *Statistics of refinement procedure*

Data used in refinement	
Starting model	777 protein atoms from free RNase T_1
Program for model building	*CORELS*
Initial R factor	0.30
Min. resolution (Å)	10
Max. resolution (Å)	1.8
Structure amplitudes	$F_o \geq 1\sigma(F_o)$
No. of reflections used	8291
No. of refinement cycles	250
Final R factor*	0.140
Mean error in atomic positions	0.15
R.m.s. electron density of final $F_o - F_c$ map (e Å^{-3})	0.081
Final non-H atomic set	
RNase T_1	777 atoms
Zn^{2+}	1 atom
H_2O	104 atoms
Statistics of final structure†	
Restraints information	
Bond distance (Å)	0.024 (0.020)
Angle distance (Å)	0.057 (0.050)
Planar 1–4 distance (Å)	0.070 (0.050)
Plane restraints information	
R.m.s. δ (Å)	0.014 (0.015)
Chiral center restraints	
R.m.s. δ (Å^3)	0.244 (0.150)
Non-bonded contact restraints	
Single-torsion contacts (Å)	0.133 (0.150)
Multiple-torsion contacts (Å)	0.137 (0.150)
Possible ($X \cdots Y$) hydrogen bonds (Å)	0.128 (0.150)
Conformational torsion angles	
Planar (°)	2.4 (3.0)
Staggered (°)	13.7 (15.0)
Orthonormal (°)	25.6 (20.0)
Isotropic thermal factor restraints	
Main-chain bonds (Å^2)	2.110 (2.000)
Main-chain angles (Å^2)	2.769 (3.000)
Side-chain bonds (Å^2)	4.876 (4.500)
Side-chain angles (Å^2)	6.627 (6.000)
Hydrogen bond (Å^2)	11.212 (15.000)

* This is a weighted R factor with $\sigma_F \simeq 0.5(||F_{obs}| - |F_{calc}||)$.
† Target restraints in parentheses.

Results and discussion

Description of the complex RNase T_1*Zn^{2+}

The final atomic coordinate set contains 777 protein atoms, 1 zinc ion and 104 water molecules in fully occupied positions. The electron density for all amino acids is clearly defined except for the side chain of Asp98 which has no electron density at all. The atomic numbering is 105 for Zn^{2+} and 106–209 for water molecules, with increasing number indicating increasing temperature factors in the range 3.4 to 61.6 Å^2. In the final coordinate set, three distances

deviate between 5σ and 9σ from ideality. The atomic coordinates and temperature factors of the RNase T_1*Zn^{2+} complex are deposited in the Brookhaven Protein Data Bank.†

For the analysis of hydrogen-bonding interactions, the positions of protein H atoms covalently bonded to sp^2-hybridized N atoms were calculated with the program *MOLEDT* (Biosym Technologies, 1988). The criterion for possible hydrogen-bonding interactions used in this paper is: donor–acceptor (D—A) distances ≤ 3.5 Å, hydrogen-acceptor distances ≤ 2.8 Å and angles D—H$\cdots A \geq 90°$.

The structure of the complex formed between RNase T_1 and Zn^{2+} is very similar to the structure of guanosine-free RNase T_1 complexed with Ca^{2+} (Martinez-Oyanedel, Choe, Heinemann & Saenger, 1991). The Zn^{2+} cation is *not* located at the catalytic site but in the same position as Ca^{2+} in the calcium-containing RNase T_1 complexes (Kostrewa, Choe, Heinemann & Saenger, 1989; Ding, Koellner, Grunert & Saenger, 1991; Martinez-Oyanedel, Choe, Heinemann & Saenger, 1991; Lenz, Cordes, Heinemann & Saenger, 1991), see Fig. 1 and below.

The conformation of the RNase T_1 molecule in the present guanosine-free Zn^{2+} complex is comparable to that in the Ca^{2+}, vanadate*Ca^{2+} and 2′-AMP*Ca^{2+} complexes, with r.m.s. deviations for main-chain/side-chain/and all atoms being, respectively, 0.09/0.40/0.28 Å; 0.14/0.85/0.59 Å; 0.14/0.95/0.066 Å. If RNase T_1 in the present Zn^{2+} complex is compared with structures of RNase T_1 complexed with guanosine as in 2′-GMP*Ca^{2+} and 2′,5′-GpG*Ca^{2+}, the r.m.s. deviations are significantly higher, ~0.46, ~1.29, ~0.95 Å respectively.

† Atomic coordinates and structure factors have been deposited with the Protein Data Bank, Brookhaven National Laboratory (Reference: 8RNT, R8RNTSF), and are available in machine-readable form from the Protein Data Bank at Brookhaven. The data have also been deposited with the British Library Document Supply Centre as Supplementary Publication No. SUP 37058 (as microfiche). Free copies may be obtained through The Technical Editor, International Union of Crystallography, 5 Abbey Square, Chester CH1 2HU, England.

These differences in r.m.s. deviations indicate that small conformational changes occur in RNase T_1 upon Guo binding. The major differences between free RNase T_1 complexed with Zn^{2+} and with Ca^{2+} are that Glu31 and Asn83 engage in different intermolecular contacts and are oriented differently in the Zn^{2+} complex, and that Lys41, Val78 and Asn99, which are disordered and adopt two conformations in the free RNase T_1*Ca^{2+} complex, are ordered in the present structure. As observed for the RNase T_1 molecule in the other crystal structures, the amino acids involved in secondary and tertiary structure hydrogen bonding feature smaller temperature factors (averages for main-chain and side-chain atoms are 6 and 8 Å respectively) than those in the loop regions, especially in the two base recognition loops, amino acids 47–52 and amino acids 96–99 (averages are 17 and 24 Å for main-chain and side-chain atoms), which are on the surface of the protein [see Fig. 8 of Arni, Heinemann, Tokuoka & Saenger (1988) and Fig. 3 of Kostrewa, Choe, Heinemann & Saenger (1989)].

The side chain of Val78 had been found disordered in all RNase T_1 crystal structures where the guanine recognition site was 'empty', but it was ordered when the enzyme was complexed with Guo, 2′-GMP or 2′,5′-GpG. This correlation was of interest (Ding, Koellner, Grunert & Saenger, 1991; Martinez-Oyanedel, Choe, Heinemann & Saenger, 1991) since the Val side chain is of a hydrophobic character and is located in a hydrophobic environment in the interior of the RNase T_1 structure; consequently, it should not be disordered (Smith, Hendrickson, Honzatko & Sheriff, 1986). The ordered Val78 in the present structure, which also has an 'empty' guanine recognition site, suggests that the structural correlation between the guanine recognition site and Val78 is not as simple as previously thought.

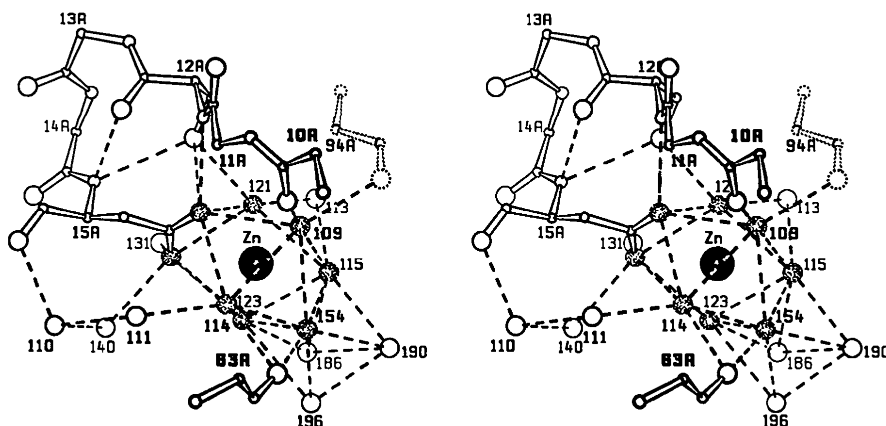

Fig. 1. Stereoview of the structure of the zinc binding site of the RNase T_1*Zn^{2+} complex. The zinc ion, water O atoms and C_α atoms are labeled. Only those side-chain atoms which are involved in cation binding are shown. Gly94 is symmetry related with $(-x+\frac{1}{2}, -y, z+\frac{1}{2})$. Drawn with *SCHAKAL*88 (Keller, 1988).

Crystal packing of RNase T_1

Table 2 gives possible intermolecular hydrogen-bonding contacts in the crystal lattice of the RNase T_1*Zn^{2+} structure. Compared with the other published crystal structures of RNase T_1 complexes, which all crystallized in the presence of Ca^{2+} in space group $P2_12_12_1$ with nearly isomorphous unit cells, the molecular packing schemes are the same, with similar close contacts between equivalent surface regions of the RNase T_1 molecules. Most of the molecular hydrogen bonds are comparable in these crystal structures, but a few peripheral residues engage in unique hydrogen bonds to amino residues of neighboring molecules, such as Asn43 $N_{\delta2}\cdots$O Asn83. This interaction is only observed in the present structure, see the corresponding tables in the Ca^{2+}-containing complexes of RNase T_1*2′-GMP (Arni, Heinemann, Maslowska, Tokuoka & Saenger, 1987), RNase T_1*vanadate (Kostrewa, Choe, Heinemann & Saenger, 1989), RNase T_1*2′,5′-GpG (Koepke, Maslowska, Heinemann & Saenger, 1989), RNase T_1*(Guo)$_2$ (Lenz, Cordes, Heinemann & Saenger, 1991), RNase T_1*2′-AMP (Ding, Koellner, Grunert & Saenger, 1991), and free RNase T_1 (Martinez-Oyanedel, Choe, Heinemann & Saenger, 1991).

Table 2. *Possible intermolecular hydrogen bonds* (≤3.5 Å)

Residue···Residue	$D\cdots A$ (Å)	$H\cdots A$ (Å)	D—H···A (°)	Symm*	T_x	T_y	T_z
Ala1 N···O His92	3.0			2	0	0	0
Ala1 N···O Ala95	3.0			2	0	0	0
Ala1 O···N Gly94	3.5	2.7	135	2	0	0	0
Asn9 $O_{\delta1}\cdots O_{\epsilon1}$ Glu31†	3.3			3	1	−1	0
Asn9 $O_{\delta1}\cdots O_{\epsilon2}$ Glu31†	2.6			3	1	−1	0
Asn9 $N_{\delta2}\cdots O_{\epsilon1}$ Glu31†	3.0	2.0	153	3	1	−1	0
Glu28 O···O_γ Ser72	2.8			3	1	0	0
Glu28 $O_{\epsilon1}\cdots$N Asn44	2.9	2.0	151	2	0	1	0
Thr32 N···O_γ Ser63	3.0	2.3	122	3	1	0	0
Thr32 $O_{\gamma1}\cdots O_\gamma$ Ser63	2.8			3	1	0	0
Ser35 $O_\gamma\cdots O_{\epsilon1}$ Glu46	3.5			4	0	0	0
Ser35 $O_\gamma\cdots$O Phe100	3.2			4	0	0	0
Asn43 $N_{\delta2}\cdots$O Asn83	3.0	2.8	93	2	0	1	−1

* Symmetry-equivalent atoms are on the right-hand side of each possible hydrogen bond. The symmetry operations mean: (2) $-x+\frac{1}{2}, -y, z+\frac{1}{2}$; (3) $-x, y+\frac{1}{2}, -z+\frac{1}{2}$; (4) $x+\frac{1}{2}, -y+\frac{1}{2}, -z$; and T_x, T_y, T_z, translations in x, y, z.

† This carboxylate group is probably protonated.

The catalytic and guanine recognition sites

In the catalytic site of free RNase T_1*Ca^{2+}, two water molecules are located. One, Wat121, has a low temperature factor (17.2 $Å^2$) and is tightly hydrogen bonded to all the active-site residues (except His40), Tyr38 O_η, Glu58 $O_{\epsilon1}$, Arg77 N_ϵ,$N_{\eta2}$, His92 $N_{\epsilon2}$; the other, Wat213, is less tightly bonded to Tyr38 O_η, His40 N_ϵ and Glu58 $O_{\epsilon1}$, and has a significantly higher temperature factor (43.3 $Å^2$). In the present RNase T_1*Zn^{2+} complex, there is only one water, Wat122, with a low temperature factor of 13.5 $Å^2$ in an identical position and hydrogen-bonding scheme as Wat121 in the Ca^{2+} complex; the position of Wat203 is unoccupied which we associated with the high temperature factor of Wat203 and differences in resolution, 1.5 Å in the Ca^{2+} complex and 1.8 Å in the present study. The amino acids at the catalytic site are further engaged in several intramolecular hydrogen-bonding contacts which are comparable to those in RNase T_1 complexed with Ca^{2+} (Martinez-Oyanedel, Choe, Heinemann & Saenger, 1991), with vanadate*Ca^{2+} (Kostrewa, Choe, Heinemann & Saenger, 1989), and with 2′-AMP*Ca^{2+} (Ding, Koellner, Grunert & Saenger, 1991).

The specific guanine recognition site in RNase T_1 is formed by amino acids 42–46 and Asp98. In the present RNase T_1*Zn^{2+} complex, the conformation of the amino acids in the guanine binding site is typical of an 'empty' site as observed in the guanosine-free enzyme (Martinez-Oyanedel, Choe, Heinemann & Saenger, 1991), in the vanadate complex (Kostrewa, Choe, Heinemann & Saenger, 1989) and in the 2′-AMP complex (Ding, Koellner, Grunert & Saenger, 1991). The base recognition site in the present structure accommodates three water molecules, Wat133, Wat148, Wat183. They occupy similar positions and are engaged in comparable hydrogen-bonding interactions as in free RNase T_1*Ca^{2+} (Wat140, Wat129, Wat194; Martinez-Oyanedel, Choe, Heinemann & Saenger, 1991), and in RNase T_1*vanadate*Ca^{2+} (Wat139, Wat179, Wat177; Kostrewa, Choe, Heinemann & Saenger, 1989). If compared with RNase T_1 complexes in which the base recognition site is filled with guanine, Wat130 and Wat148 simulate guanine N_7 and O_6 positions respectively. The hydrogen-bonding contacts of these and of eight additional water molecules in and around the guanine binding site are comparable to those in the free RNase T_1*Ca^{2+} complex.

Zinc coordination and binding site

Interestingly, the zinc ion is *not* located at the active binding site and coordinated to His40 and/or His 92 of the RNase T_1 molecule, as suggested by Itaya & Inoue (1982). This is probably due to the acidic crystallization conditions (pH 5.2), at which both histidine side chains with pK_a values around 7 are protonated (Takeuchi, Satoh & Harada, 1991). In consequence, they are not accessible for Zn^{2+} coordination.

Zn^{2+} is located at the same site as Ca^{2+} in those structures where RNase T_1 free or complexed with nucleotides was cocrystallized with Ca^{2+}. Like Ca^{2+}, Zn^{2+} is coordinated to the two carboxylate O atoms of Asp15 and to six water molecules in the form of a distorted square antiprismatic dodecahedron, see

Table 3. *M*···O *distances in the cation binding site* (Å) *and metal-ion temperature factors B* (Å^2)

RNase T_1 [a]		RNase T_1 [b]		RNase T_1*vanadate [c]		RNase T_1*2′-AMP [d]	
Ligand	Zn^{2+}···O	Ligand	Ca^{2+}···O	Ligand	Ca^{2+}···O	Ligand	Ca^{2+}···O
Asp15 $O_{\delta1}$	2.63	Asp15 $O_{\delta1}$	2.56	Asp15 $O_{\delta1}$	2.53	Asp15 $O_{\delta1}$	2.53
Asp15 $O_{\delta2}$	2.50	Asp15 $O_{\delta2}$	2.54	Asp15 $O_{\delta2}$	2.34	Asp15 $O_{\delta2}$	2.45
Wat109	2.37	Wat109	2.40	Wat112	2.42	Wat136	2.39
Wat114	2.46	Wat114	2.49	Wat118	2.50	Wat139	2.30
Wat115	2.46	Wat118	2.35	Wat119	2.48	Wat143	2.45
Wat121	2.43	Wat122	2.47	Wat120	2.37	Wat151	2.40
Wat123	2.50	Wat132	2.42	Wat121	2.39	Wat155	2.43
Wat154	2.67	Wat154	2.62	Wat161	2.62	Wat156	2.75
Mean	2.50	Mean	2.48	Mean	2.46	Mean	2.46
B	21.9	*B*	12.6	*B*	14.7	*B*	7.4

References: (*a*) present study; (*b*) Martinez-Oyanedel *et al.* (1991); (*c*) Kostrewa *et al.* (1989); (*d*) Ding *et al.* (1991).

Fig. 1. The water molecules are further hydrogen bonded on NH and O_γ of Ser12, to main-chain O atoms of Cys10, Ser63 of the same RNase T_1 molecule, and to Gly94 O of a symmetry-related molecule. This latter interaction appears to be of importance for crystal packing as it was observed in all the other Ca^{2+}-containing complexes of RNase T_1.

There is an interesting discrepancy between ionic radii and coordination number for Zn^{2+} and Ca^{2+} in the RNase T_1 crystal structures. According to the ionic radii, 0.74 Å for Zn^{2+} and 0.99 Å for Ca^{2+}, the average coordination number should be smaller for Zn^{2+} than for Ca^{2+}. This is actually observed in several protein crystal structures, with the smaller Zn^{2+} having *M*···O distances ~2.0 Å and coordination number 4 to 5 whereas the *M*···O distances for Ca^{2+} are around 2.3 Å, and the coordination number increases to around 8 (Bertini, Luchinat & Monnanni, 1985; Chakrabarti, 1990). In contrast, in the RNase T_1 complexes with Zn^{2+} and Ca^{2+}, the coordination sphere is virtually identical as are the *M*···O distances of around 2.5 Å (Table 3). We associate this finding with Zn^{2+} clathration rather than coordination, the basic idea being that Asp15 and the water molecules surrounding Zn^{2+} or Ca^{2+} are in a stable pre-formed cage-like configuration with an empty cavity into which the metal ion is inserted. With Zn^{2+}, the cage should collapse so that the average Zn^{2+}···O distance shortens by 0.5 Å to optimize the coordinative interactions. Since this is not observed, the clathrate effect dominates over metal coordination.

This view is supported by the high temperature factor of Zn^{2+}, 21.9 Å^2, which is much higher than that of Ca^{2+} in the corresponding RNase T_1 complexes, see Table 3. We explain the low temperature factors of Ca^{2+} by tight fitting into the clathrate cavity. Zn^{2+} is too small to fill it properly, and in consequence, it is disordered over the available space provided by the cavity, associated with an increase in temperature factor.

Otherwise, the complexation of Zn^{2+} by the Asp15 carboxylate follows known schemes and adopts the stable near-symmetrical bidentate mode (Christianson & Lipscomb, 1988). Zn^{2+} is located near the carboxylate plane and the distances to Asp15 $O_{\delta1}$, $O_{\delta2}$ differ slightly, 2.6 and 2.50 Å respectively, and the corresponding C—O_δ—Zn^{2+} angles are close to 90° (88 and 93° respectively), as found in other proteins (Chakrabarti, 1990) and in small biological molecules (Carrell, Carrell, Erlebacher & Glusker, 1988).

The observed differences in crystal morphology, needle-shaped for Ca^{2+} and stout prismatic for Zn^{2+} complexes, are not due to differences in molecular packing as the crystals are isomorphous. There are very slight differences in hydrogen bonding which might influence the crystal growth, or the precrystallization equilibria might be determined by addition of Ca^{2+} or Zn^{2+}. This opens up the possibility of doing crystal engineering with the addition of various cations or by producing an RNase T_1 mutant where Asp15 is substituted by another amino acid that cannot bind M^{2+}, and consequently will not support formation of the clathrate cage. Such studies have already been initiated by Pace, Heinemann, Hahn & Saenger (1991) and are in progress in our laboratory.

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Structure Determination and Refinement of Homotetrameric Hemoglobin from *Urechis caupo* at 2.5 Å Resolution

BY PRASANNA R. KOLATKAR,* STEPHEN R. ERNST AND MARVIN L. HACKERT†

Clayton Foundation Biochemical Institute, Department of Chemistry and Biochemistry, University of Texas, Austin, TX 78712, USA

CRAIG M. OGATA AND WAYNE A. HENDRICKSON

Howard Hughes Medical Institute and Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY 10032, USA

AND ETHAN A. MERRITT‡ AND R. PAUL PHIZACKERLEY

Stanford Synchrotron Radiation Laboratory, Stanford University, Stanford, CA 94309, USA

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Abstract

A 5 Å resolution multiple isomorphous replacement solution for hemoglobin isolated from *Urechis caupo* revealed a previously unobserved quaternary structure for tetrameric hemoglobin [Kolatkar, Meador, Stanfield & Hackert (1988). *J. Biol. Chem.* **263**(7), 3462–3465]. We report here the structure of *Urechis* hemoglobin in the cyanomet state refined to 2.5 Å resolution by simulated annealing yielding $R = 0.148$ for reflections $F > 3\sigma$ between 5.0 and 2.5 Å resolution. The starting model was fitted to a map originally derived from multiple-wavelength anomalous-dispersion phases to 3 Å resolution that was then subjected to cyclic twofold molecular

* Present address: Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA.
† Author for correspondence.
‡ Present address: Department of Biological Structure, University of Washington, Seattle, WA 98105, USA.

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