The complex formed between the enzyme ribonuclease T₁ (EC 3.1.27.3) and its specific inhibitor 2'-guanylic acid (2'-GMP) has been refined to $R = 0.180$ using x-ray diffraction data to 1.9-Å resolution. The protein molecule displays a compact fold; a 4.5 turn a-helix packed over an antiparallel $\beta$-pleated sheet shields most of the hydrophobic interior of the protein against the solvent. The extended pleated sheet structure of ribonuclease T₁ is composed of three long and four short strands building up a two-stranded minor $\beta$-sheet near the amino terminus and a five-stranded major sheet in the interior of the protein molecule.

In the complex with ribonuclease T₁, the inhibitor 2'-guanylic acid adopts the syn-conformation and C2'-endo sugar pucker. Binding of the nucleotide is mainly achieved through amino acid residues 38-46 of the protein. The catalytically active amino acid residues of ribonuclease T₁ (His⁴⁰, Glu³⁸, Arg⁷⁷, and His⁹⁶) are located within the major $\beta$-sheet which, as evident from the analysis of atomic temperature factors, provides an environment of minimal local mobility. The geometry of the active site is consistent with a mechanism for phosphodiester hydrolysis where, in the transesterification step, His⁹⁶ and/or Glu³⁸ act as a general base toward the ribose 2'-hydroxyl group and His³⁸, as a general acid, donates a proton to the leaving 5'-hydroxyl group.

Ribonuclease T₁ (RNase¹ T₁, EC 3.1.27.3) from the fungus Aspergillus oryzae is the leading member of a family of microbial enzymes that cleave single-stranded RNA by a reaction mechanism involving formation of an intermediate 2'-3' cyclic phosphate followed by hydrolysis to yield a terminal 3'-phosphate. The RNase T₁ family of enzymes is defined by sequence and spatial structure homology (Hartley, 1980; Hill et al., 1983) and is distinct from the RNase A type mammalian ribonucleases (Blackburn and Moore, 1982). Being constituted of fungal as well as bacterial proteins, the RNase T₁ family spans the divide between the prokaryotic and eukaryotic kingdoms.

RNase T₁ has been the target of extensive biochemical and biophysical research (Egami et al., 1980; Takahashi and Moore, 1982). The stable and acidic protein contains 104 amino acid residues of known sequence (Takahashi, 1971, 1985). By chemical modification studies the likely involvement of the catalytic process of amino acid residues His⁹⁶, Glu³⁸, Arg⁷⁷, and His⁹⁶ has been demonstrated (Takahashi et al., 1967; Takahashi, 1970a, 1970b, 1976). Direct evidence for the location of histidine residues in the active site of RNase T₁ has also been obtained by NMR investigations (Fülling and Rüterjans, 1978; Arata et al., 1973; Inagaki et al., 1981) while the change of fluorescence of the protein upon nucleotide binding has been attributed to the location of tyrosine side chains near the binding site (Pongs, 1979; Campbell et al., 1976).

Ribonuclease T₁ cleaves phosphodiester bonds at the 3'-side of guanosine at least 10⁴ times more readily than other internucleotide linkages liberating products with terminal 3'-phosphate groups (Egami et al., 1980). Studies by ultraviolet difference spectroscopy (Walz, 1976; 1977b) and by NMR (Kyogoku et al., 1982; Inagaki et al., 1985) of the binding of various substrate analogues to RNase T₁ have identified 2'-guanylic acid (2'-GMP) as the inhibitor with highest affinity to the enzyme. At pH 5.5 the dissociation constant is 6.5 $\mu$M (Takahashi and Moore, 1982).

Heinemann and Saenger (1982) by x-ray crystallography have determined the three-dimensional structure of RNase T₁ complexed with the specific inhibitor 2'-GMP. They used an isoenzyme of RNase T₁, where Glu³⁸ of the originally sequenced protein (Takahashi, 1971, 1985) is substituted by a lysine.¹ A brief account of the refined structure of the Glu³⁸, RNase T₁·2'-GMP complex crystallized at a lower pH as well as a description of the mode of binding to Glu³⁸-RNase T₁ by the related substrate analogue 3'-GMP have been presented by Sugio et al. (1985a, 1985b). We have refined the structure of the RNase T₁·2'-GMP complex crystallized under standard conditions by the stereochemically restrained least squares method (Arni et al., 1987). Here we give a complete description of the three-dimensional structure of the enzyme, its hydration and inhibitor binding, and discuss implications for the RNase T₁-catalyzed specific RNA hydrolysis.

**EXPERIMENTAL PROCEDURES**

The Lys³⁵,RNase T₁·2'-GMP complex was crystallized by a variation of the published procedure (Heinemann et al., 1980), and x-ray diffraction data were collected to a nominal resolution of 1.9 Å. The crystallographic refinement used the reciprocal space stereochemically restrained least squares method (Hendrickson and Konnert, 1980; Hendrickson, 1985) combined with computer graphics-assisted manual model revisions (FRODO; Jones, 1978). The final model...

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Dedicated to Prof. Friedrich Cramer on the occasion of his 65th birthday.

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1 The abbreviations used are: RNase, ribonuclease; 2'-GMP, 2'-guanylic acid; 3'-GMP, 3'-guanylic acid; NOE, nuclear Overhauser effect; r.m.s., root mean square.

² H. Kratzin, personal communication.
comprises all non-hydrogen atoms of the complex except for the
disordered side chain of Gln
. At this resolution, the electron density
map allows no discrimination of carbon versus nitrogen or of nitrogen
versus oxygen atoms. Therefore, the orientation with respect to a
180° rotation about the proximal single bond of the imidazole groups
of histidine and the amide groups of asparagine and glutamine was
chosen such as to maximize hydrogen bonding interactions. In order
to facilitate the assignment of hydrogen bonds, polar protein atoms
were hydrogenated if the position of the hydrogen atom could be
defined unambiguously after the completion of refinement.

The refinement converged at a crystallographic \( R \) factor of 18.0% for
6349 observed structure factors at the 3σ level between 6- and 1.9-
Å resolution (Arns et al., 1987). The r.m.s. deviation of bond lengths
from ideal values of 0.018 Å indicates reasonable stereochemistry of
the model. As suggested by the method of Luzzati (1952), the error
in most atomic coordinates should be less than 0.2 Å. The atomic
coordinates of the RNase T₁*·2'-GMP complex have been submitted
to the Brookhaven Protein Data Bank from which copies are avail-
able.

**RESULTS**

**Protein Conformation**—The r.m.s. distance between equivalent main chain atoms of the refined and the unrefined RNase T₁ models is 1.3 Å. Thus, no major changes in the overall architecture of the protein have been introduced with the refinement. The inclusion of solvent and the greater accuracy in the atomic coordinates, however, have made possible the assignment of a great number of hydrogen bonds,

**FIG. 1.** Stereographic representation of the RNase T₁·2'-GMP complex. The view is along the \(-z\) (top) and \(z\)-direction (bottom) of the unit cell. The bound inhibitor of RNase T₁·2'-GMP, the polypeptide backbone, and the amino acid side chains are drawn with decreasing line width.
salt bridges, and Van der Waals interactions within the protein molecule, between protein molecules in the crystal lattice, between protein and inhibitor, and between protein and solvent, that were not described previously (Heinemann and Saenger, 1982). All hydrogen bonds D-H...A mentioned hereafter obey criteria proposed by Baker and Hubbard (1984): distance donor (D) ... acceptor (A) less than or equal to 3.5 Å, distance, H...A less than or equal to 2.5 Å, angles D-H...A, C-O...D, and C-O...A greater than or equal to 90°.

Polypeptide Chain Folding—RNase T₁ is a very compact protein displaying a small cleft to accommodate the inhibitor molecule (Fig. 1). A well-defined hydrophobic core is for the most part enclosed between a long α-helix and a central β-sheet. Two disulfide bridges are formed, one of which, connecting amino acid residues 6 and 103, brings the polypeptide chain termini close together in space. A cartoon drawing of the α-helix and β-structure of RNase T₁ is presented in Fig. 2.

A remarkable feature of the protein architecture are four wide loops that are interspersed between the helix and the major β-sheet and between the strands of the sheet structure. These loops adopt a considerably irregular chain folding; they contain a number of reverse turns and 2 proline residues in the cis-conformation, Pro<sup>34</sup> and Pro<sup>55</sup>. Neglecting the two cis-peptide bonds, all peptide torsion angles in RNase T₁ are trans-planar, showing a mean deviation from planarity of 1.9° and a largest deviation of 9° at residue Thr<sup>34</sup>.

Secondary Structure—Secondary structural elements in RNase T₁ include an α-helix (α₁) covering amino acid residues Ser<sup>13</sup> to Asp<sup>99</sup> and seven strands in β-sheet conformation (β₁-β₇). According to our assignment, β-strands may include terminal residues that are not hydrogen-bonded to the other strand as long as the backbone torsion angles are located in the upper right quadrant of the standard Ramachandran plot (Ramachandran and Sasisekharan, 1968). There are two 5-turns, t₁ and t₄ with hydrogen bonds CO(i) ... HN(i+5) which both cover a β-turn, t₃ and t₄. A highly bent hydrogen bond closes the single γ-turn g₁ (Phe<sup>48</sup>-Asp<sup>49</sup>-Phe<sup>50</sup>). β-turns t₂, t₃, and t₄ are of the type I (Venkatachalam, 1968; Richardson, 1981); t₅ and t₆ are of the 3₁₀-helical type III. At position 2 of turns t₁ and t₅ (type II') and at position 3 of t₆ (type II') there are glycine residues as required sterically. Turn t₇ is at the N-terminal end of the helix; t₁ and t₇ are at its C terminus (Fig. 3). Following our assignment, 17 residues (16%) belong to the α-helix, 36 residues (35%) are part of the pleated sheet structure, and 43 residues (41%) are part of turns. Only 20 amino acid residues (19%) remain outside of regular secondary structure. The longest coil segment is Asp<sup>99</sup>-Ala<sup>70</sup>, located between strands β₁ and β₅.

Helix Geometry—In the single long, peripheral α-helix from Ser<sup>13</sup> to Asp<sup>99</sup>, all the expected CO(i) ... HN(i+4) hydrogen bonds are present. Average backbone torsion angles are ϕ = −65(6)° and ψ = −40(10)° (numbers in parentheses are standard deviations). The large spread arises mainly from torsion
Ribonuclease T1: 2'-GMP Three-dimensional Structure

FIG. 4. Bending of the long α-helix of RNase T1. The α-helix is drawn in an orientation which emphasizes the bending.

FIG. 5. Irregular sheet structure between βα and βα. Carbon, nitrogen, and oxygen atoms are drawn with increasing radius. Main chain is drawn with solid bonds; side chains have open bonds. Thin solid lines represent hydrogen bonds.

angles $\varphi = -82.7^\circ$ and $\psi = -7.4^\circ$ of the C-terminal Asp^{29} which are slightly outside the normal helical range (Richardson, 1981); with this residue omitted, the mean values become $\varphi = -64(5)^\circ$ and $\psi = -42(6)^\circ$. The average geometry of the 13 α-helical hydrogen bonds of 3.1(2) Å (N...O distances), 2.1(2) Å (H...O distances), 158(7)° (N-H...O angles), and 152(7)° (C-O...N angles) does not deviate significantly from that usually found in globular proteins (Baker and Hubbard, 1984).

The deviation from ideal helical geometry at the C-terminal end of the helix allows the type I β-turn $t_3$ which is enclosed in the 5-turn $f$. At the N terminus of the helix, turn $t_5$ in the β5-helical conformation provides an additional hydrogen bond between Ser^{14}(O) and Val^{16}(NH). The presence of many polar residues in the N-terminal half of the helix further stabilizes the structure by side chain-backbone and side chain-side chain hydrogen bonds. Of the former category, hydrogen bonds from Lys^{29}(Ne) to Ala^{28}(O), from Thr^{30}(Oγ) to Ser^{14}(NH), and from Ser^{13}(NH) to Ser^{17}(Oγ) are noteworthy. The peptide nitrogen of Ser^{15} engages in a bifurcated (three-center) hydrogen bond with the carbonyl and side chain oxygen atoms of Ser^{12}. In addition, several water molecules make hydrogen bonds to peptide carboxyl oxygens.

Being located at the surface of the protein, the long α-helix faces the solvent with one side and the hydrophobic interior of the RNase T1 molecule with the opposite side. Based on a new hydrophobicity scale (Miller et al., 1987), the mean hydrophobic moment (Eisenberg et al., 1982) of the helix is 0.25 kcal mol$^{-1}$, while the mean hydrophobicity of the residues in the helix is −0.28 kcal mol$^{-1}$. The RNase T1 helix may thus be regarded as amphiphilic perpendicular to its axis; the side chain of Ser^{14} points to the hydrophilic side of the helix. Furthermore, the α-helix of RNase T1 is bent with a radius of curvature of about 60 Å (Fig. 4). The direction of bending is not correlated with the hydrophobic moment of the helix.

Sheet Geometry—Near the amino terminus of the RNase T1 molecule β-strands $\beta_1$ and $\beta_5$ form an antiparallel, small pleated sheet structure designated as the minor β-sheet. The major sheet is formed by the antiparallel arrangement of strands $\beta_3$ through $\beta_7$. Seven amino acid residues belong to the minor sheet, while the major sheet is composed of 29 residues. The three extended strands $\beta_6$, $\beta_7$, and $\beta_8$ harbor many hydrophobic residues and represent much of the hydrophobic core of the protein.

Excluding the peptide bond between residues Ala^{27} and Gly^{31}, the mean values for the backbone torsion angles are $\varphi = 110(24)^\circ$ and $\psi = 143(20)^\circ$. The rather wide spread of these values reflects the shallowness of the energy minimum in the torsion angle plot for the extended chain conformation (Ramachandran and Sasisekharan, 1968; Richardson, 1981). In the 21 peptide NH...O hydrogen bonds connecting different strands in the pleated sheets of RNase T1, the following mean values describe the hydrogen bonding geometry: 3.0(2) Å for N...O distances, 2.0(2) Å for H...O distances, 156(9)° for N-H...O angles, and 153(15)° for C-O...N angles. These values are within the normal range for hydrogen bonds in pleated sheets found by Baker and Hubbard (1984). The major and the minor pleated sheets of RNase T1 both display posi-
ative twist as normally observed in protein structures (Richardson, 1981).

The major pleated sheet contains an unusual bulge structure between strands $\beta_2$ and $\beta_6$, which is unlike the bulges described before (Richardson et al., 1978; Richardson, 1981). The register of hydrogen bonds between the anti-parallel strands is defined by the two hydrogen bonds linking residues Val$^{78}$ and Gly$^{54}$. If we consider the hydrogen bond from Asn$^{41}$(NH) to Asn$^{41}$(O) ($\beta$-turn 7) as part of the sheet, which seems legitimate due to the generally anti-parallel course of the two polypeptide segments, then residue Phe$^{68}$ of strand $\beta_6$ faces the tripeptide Gln$^{41}$-Leu$^{42}$-Ala$^{43}$ on $\beta_2$, i.e. 2 extra residues have been inserted in the latter strand. As evident from Fig. 5, this structure is stabilized by two additional backbone-backbone hydrogen bonds and two further hydrogen bonds from peptide nitrogen atoms to Asn$^{41}$(O$^\delta$). One of these, involving Gln$^{41}$(NH), is made possible by torsion angles $\varphi/\psi$ equal to 66°/39° of Asn$^{41}$ (in the left-handed helix region). While Ala$^{43}$ has adopted backbone torsion angles $-97^\circ/-38^\circ$ close to the $\alpha$-helical region of conformational space, all other amino acid residues involved in the bulge structure display torsion angles typical for pleated sheets.

The single tryptophane residue Trp$^{68}$ of RNase T$_1$ is located within the major pleated sheet. The microenvironment of this residue, which has been the object of studies by various biophysical techniques (Hershberger et al., 1980; Eftink, 1983; Lakowicz et al., 1983; James et al., 1985; MacKerell et al., 1987), is depicted in Fig. 6. Although zero solvent accessibility is calculated for the Trp$^{68}$ residue (Kabsch and Sander, 1983), a water molecule is hydrogen bonded to atom Ne of the indole group. The backbone portion of Trp$^{68}$ is part of strand $\beta_6$, and it is linked to Val$^{78}$ of $\beta_2$ by two hydrogen bonds. This portion of the major sheet is rather rigid as judged by a mean crystallographic temperature factor of 7.2 $\AA^2$ for the backbone atoms of the tripeptide Gln$^{41}$-Trp$^{68}$-Pro$^{59}$. Despite the lack of a hydrogen bond linking the indole group to the protein matrix, the side chain of Trp$^{68}$ shows limited mobility as evident from a mean temperature factor of 6.0 $\AA^2$, which is significantly below the average B value of 12.9 $\AA^2$ for all side chain atoms of the protein molecule.

**Hydrogen Bonding**—For the analysis of hydrogen bonding and of hydration, the RNase T$_1$-2'-GMP complex is considered in a solvent sphere which contains all 154 water molecules within 5-Å distance from atoms of the complex. Of these, 91 are independent while the rest are generated by the unit cell symmetry. Our analysis places hydrogen-bonded atoms in three main categories: protein backbone, protein side chains, including the bound nucleotide, and water oxygens (Table 1).

Of the 309 hydrogen bonds of the structure, 213 involve a water molecule. The mean values for all hydrogen bonds of 2.95(0.27) Å (D...A), 2.08(0.21) Å (N-H...A), 151.9(14.5)$^\circ$ (N-H...A), 130.8(20.0)$^\circ$ (C-O...D), and 115.8(12.9)$^\circ$ (C-O...A) as well as average values and spread of these parameters in selected classes are in good agreement with data extracted from a larger sample of highly refined protein structures (Baker and Hubbard, 1984). No significant difference in hydrogen bonding geometry between individual classes of hydrogen bonds is observed, with the possible exception of protein side chain-side chain bonds that show an unusually small average donor-acceptor distance of 2.77 Å. The significance of this observation is obscured, however, by the small sample size of only 14 hydrogen bonds.

The degree of hydrogen bonding in the polypeptide backbone of RNase T$_1$ is very similar to the average of the survey by Baker and Hubbard (1984). All 164 hydrogen bonds in which the protein main chain participates are depicted in Fig. 7. Only four amino acid residues remain without backbone hydrogen bonding. This figure also gives a good impression of the stabilization of the overall folding of RNase T$_1$ by hydrogen bonds.

**Local Flexibility**—Since the mobility of amino acid residues in crystalline proteins is restricted by lattice contacts, in the thermal factor histogram (Fig. 8) those amino acid side chain or backbone portions that are involved in intermolecular hydrogen bonding (Arni et al., 1987) are marked. Most of them do not lie within the helix and sheet regions of the protein and adopt intermediate temperature factors, indicating that intermolecular contacts do not determine the segmental flexibility of the RNase T$_1$ molecule in the crystal. A
possible exception from this rule is around His$_{52}$, where mobility may be restricted by a hydrogen bond from the amino terminus of a symmetry-related molecule to the peptide carbonyl of this residue.

The temperature factor profile indicates high flexibility for the loop regions of the molecule while minima are within or near the strands of the major $\beta$-sheet. Without exception the backbone portions of the amino acid residues His$_{4'}$, Glu$_{5'}$, Arg$_{7'}$, and His$_{52}$, known to be important for the catalytic activity of RNase T$_1$, are at sites of minimal flexibility. Only for His$_{52}$ it may be questioned whether this is an intrinsic property of the molecule or a consequence of contacts within the crystal lattice. In contrast, the polypeptide backbone of residues Asn$_{4''}$ through Glu$_{5''}$, the major part of the base binding site, shows slightly higher than average B values. For this stretch of amino acid residues, one would expect even higher flexibility in the absence of bound inhibitor. The effect may be small, however, since there is remarkably good agreement between the relative mobility of amino acid residues, derived from a molecular dynamics simulation of inhibitor-free RNase T$_1$ (MacKerell et al., 1987), and local flexibility indicated by the temperature factor profile of the RNase T$_1$-2'-GMP complex.

**Hydration**—In the refinement of the RNase T$_1$-2'-GMP complex, all solvent peaks had to be assigned as water oxygens since the resolution does not allow the unambiguous identification of ions which have to be present to neutralize the charge of basic and acidic amino acid side chains. Out of 154 water molecules that constitute the 5-Å solvent sphere of the protein (see above), 79 are in direct hydrogen bonding distance to polar protein or nucleotide atoms. 43 of these primary hydration shell waters are located at positions that would allow the formation of one hydrogen bond to the protein, two hydrogen bonds may be formed by 25 waters, three by nine, and four by two water molecules. Binding of a water molecule by multiple hydrogen bonds is likely to have a stabilizing effect on the protein structure.

---

**Table I**

<table>
<thead>
<tr>
<th>H-bond type</th>
<th>D...A (Å)</th>
<th>H...A (Å)</th>
<th>N-H...A (°)</th>
<th>C-O...D (°)</th>
<th>C-O...A (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>309 2.95 0.27</td>
<td>104 2.08 0.21</td>
<td>104 151.9 14.5</td>
<td>193 130.8 20.0</td>
<td>16 115.8 12.9</td>
</tr>
<tr>
<td>Water...all</td>
<td>213 2.94 0.30</td>
<td>29 2.04 0.24</td>
<td>29 153.0 15.6</td>
<td>98 124.4 18.5</td>
<td>10 119.8 15.2</td>
</tr>
<tr>
<td>Water...protein</td>
<td>128 2.96 0.28</td>
<td>29 2.04 0.24</td>
<td>29 153.0 15.6</td>
<td>98 124.4 18.5</td>
<td>10 119.8 15.2</td>
</tr>
<tr>
<td>Water...MC</td>
<td>82 2.98 0.26</td>
<td>23 2.06 0.20</td>
<td>23 157.7 12.6</td>
<td>58 128.3 16.7</td>
<td>10 119.8 15.2</td>
</tr>
<tr>
<td>Water...water</td>
<td>46 2.93 0.31</td>
<td>6 1.96 0.35</td>
<td>6 134.9 13.2</td>
<td>40 118.8 19.8</td>
<td>10 119.8 15.2</td>
</tr>
<tr>
<td>Intra-protein</td>
<td>96 2.97 0.21</td>
<td>75 2.10 0.20</td>
<td>75 151.4 14.2</td>
<td>95 137.4 19.4</td>
<td>16 115.8 12.9</td>
</tr>
<tr>
<td>MC...MC</td>
<td>51 3.00 0.17</td>
<td>51 2.09 0.20</td>
<td>51 153.2 13.1</td>
<td>51 145.3 18.4</td>
<td>16 115.8 12.9</td>
</tr>
<tr>
<td>MC...SC</td>
<td>51 3.01 0.23</td>
<td>21 2.13 0.20</td>
<td>21 147.3 17.1</td>
<td>30 127.0 17.8</td>
<td>8 116.6 15.1</td>
</tr>
<tr>
<td>SC...SC</td>
<td>14 2.77 0.24</td>
<td>3 2.08 0.30</td>
<td>3 150.0 17.0</td>
<td>14 130.7 18.5</td>
<td>8 116.6 15.1</td>
</tr>
<tr>
<td>MC...all</td>
<td>164 2.99 0.23</td>
<td>95 2.09 0.20</td>
<td>95 153.0 14.0</td>
<td>139 134.3 19.4</td>
<td>16 115.8 12.9</td>
</tr>
<tr>
<td>SC...all</td>
<td>91 2.93 0.28</td>
<td>30 2.09 0.24</td>
<td>30 145.1 16.0</td>
<td>84 123.7 18.5</td>
<td>16 115.8 12.9</td>
</tr>
<tr>
<td>MC CO...all</td>
<td>126 2.99 0.24</td>
<td>58 2.09 0.20</td>
<td>58 152.2 13.8</td>
<td>126 135.3 15.1</td>
<td>8 116.6 15.1</td>
</tr>
<tr>
<td>MC NH...all</td>
<td>89 2.99 0.18</td>
<td>88 2.09 0.20</td>
<td>88 153.7 13.7</td>
<td>64 141.0 20.2</td>
<td>16 115.8 12.9</td>
</tr>
</tbody>
</table>

* MC, main chain; SC, side chain.
Fig. 9. 5-Å hydration sphere around RNase T\textsubscript{i} *2'-GMP complex. Bold lines mark possible hydrogen bonds to water molecules (open spheres). The orientation of the RNase T\textsubscript{i} molecule is as in Fig. 1. Note the sparse hydration of a narrow section of the RNase T\textsubscript{i} surface spanning almost all around the molecule. The inhibitor 2'-GMP binds to this sparsely hydrated region.

Fig. 9 provides stereo views of the 5-Å solvent sphere around the RNase T\textsubscript{i} *2'-GMP complex. The inhibitor binding site is part of an apparently underhydrated surface portion which extends almost completely around the protein molecule. For the most part, the lack of water molecules binding to this region cannot be explained by lattice contacts occluding hydration sites. Instead, it seems that amino acid side chains in this surface area are on average less hydrophilic than the remaining exposed residues, suggesting a possible role of this surface region as binding locus for the basis of the RNA single strand.

Nucleotide Conformation—In the crystalline complex with ribonuclease T\textsubscript{i}, 2'-GMP adopts a conformation that is dictated by the multiple contacts with the protein (Table II). It was noted early on (Heinemann and Saenger, 1982) that 2'-GMP binds to RNase T\textsubscript{i} in the syn conformation. This orientation about the glycosyl bond is stabilized by a hydrogen bond from the free 5'-hydroxyl group to N3 of the guanine base. The proximity of these two groups is due to the exocyclic torsion angle $\gamma$ being in the (+)-gauche range and the ribose adopting the C2'-endo pucker. The degree of nonplanarity of the sugar as measured by the pseudorotation magnitude $\gamma_M$ is within the range observed in nucleoside and nucleotide single crystal x-ray analyses (Altona and Sundaralingam, 1972). Similarly, the value found for the torsion angle $\gamma$ of 2'-GMP represents one of several frequently observed orientations about the C5'-C4' bond in DNA, RNA, and fragments thereof (Saenger, 1984). In contrast to pyrimidine nucleosides, purine
nucleosides do not show a strong preference for anti-orientation about the glycosyl bond. In purine nucleotides in the syn form, the sugar pucker may be C2'-endo or C3'-endo (Saenger, 1984; de Leeuw et al., 1980) while syn purines in Z-DNA are always associated with C3'-endo sugar pucker (Wang et al., 1979).

There is a wealth of data in support of the syn conformation being adopted by both 2'-GMP and 3'-GMP upon binding to RNase T1. 8-Bromoguanosine and its 2'- and 3'-phosphates bind to the enzyme with roughly the same affinity as the unsubstituted parent compounds (Takahashi, 1972; Yoshida and Kanae, 1983). Since substituents at C8 of purines shift the syn/anti equilibrium to syn (Saenger, 1984), this may be taken as evidence in favor of binding in the syn conformation. Oshima and Imahori (1971) have interpreted their difference in circular dichroism spectra in terms of a change in the 3'-GMP conformation from anti to syn upon binding to RNase T1. The most detailed information comes from NMR experiments: One-dimensional nuclear Overhauser effect (NOE) data suggest that 2'-GMP and 3'-GMP bind to RNase T1, in C3'-endo-syn conformation (Ingaki et al., 1985). Thus, there is agreement with our x-ray results on the glycosyl torsion angle range of 2'-GMP but not on the sugar pucker. Finally, a recent two-dimensional NOE investigation of the solution structure of the RNase T1•2'-GMP complex supports the presence of a syn-2'-GMP (Rüterjans et al., 1987).

**Specificity of Inhibitor Binding**—The mode of 2'-GMP binding to RNase T1, as observed in the crystal structure of the complex can explain the enzyme’s specificity for guanosine residues in the RNA cleavage reaction. Refinement of the RNase T1•2'-GMP complex has revealed a slightly different pattern of hydrogen bonds between protein and nucleotide than originally postulated (Heinemann and Saenger, 1982). In a first approximation, the register of the nucleotide binding stretch had to be shifted by one residue relative to the edge of the guanine base, and the side chain of Glu46 had to be rotated to hydrogen bond with guanine. The presently observed 2'-GMP binding bears resemblance with that present in the low pH Glu70-RNase T1•2'-GMP complex (Sugio et al., 1985a), but differs in several important details.

In the crystalline complex with RNase T1, the hydrogen bonding potential of the guanine base is completely saturated (Fig. 10). In addition to the intranucleotide hydrogen bond from O-5' to N-3 the following hydrogen bonds between 2'-GMP and the protein are formed: N-3 to Asn46 (O), N-2 to Glu46 (O2), N-1 to Glu46 (O1), O-6 from Tyr46 (N), O-6 from Asn46 (N), and N-7 from Asn46 (N). The latter hydrogen bond is rather long (3.3 Å) while all of the others show almost ideal geometry. The main contribution to the substrate specificity of RNase T1, comes from the stretch between amino acid residues Asn46 and Glu46, the only other contact with the base is provided by Asn46. The importance of substrate recognition of the polypeptide backbone of RNase T1, which partakes in four out of the six hydrogen bonds, has been stressed previously (Heinemann and Saenger, 1982, 1983).

The involvement of the different functions of the base for guanine recognition by RNase T1, in solution were summarized by Egami et al. (1980). The bulk of the data can be explained in terms of the 2'-GMP binding to RNase T1, as seen in the crystal structure: 1) All guanine functions interacting with the protein (N-1, N-2, O-6, and N-7) are essential for binding in solution. 2) The loss of one hydrogen bond donor or acceptor group weakens the binding to RNase T1, without completely rendering the base a nonsubstrate as long as no stericly interfering group is introduced. 3) The loss of two functions leads to a base which is no longer recognized by RNase T1. For example, both polyinosinic acid, lacking the N-2 function, and poly(7-deazaguaninic acid), lacking the N-7, are cleaved by the enzyme, albeit at reduced rates (Egami et al., 1980; Seela et al., 1982), while 7-deazainosinic acid, lacking both groups, i.e. three hydrogen bonds, is no substrate. Accordingly, 7-methylguanosine or 2-dimethylguanosine are not bound by RNase T1 because of steric hindrance by the added substituents. In addition to the hydrogen bonding, the guanine base interacts with the phenolic side chains of Tyr46 and Tyr48. The arrangement of tyrosine side chains and base observed in the crystalline complex cannot be described as “stacking” in the sense of base pair stacking in nucleic acid helices (Saenger, 1984), since the phenolic rings approach the base at angles of 12 and 34°, respectively, rather than being oriented in parallel at 3.4 Å distance. The proximity with the guanine ring, however, should perturb the electronic structure of the aromatic side chains. Moreover, as the side chain of Tyr46 covers the 2'-GMP binding site like a lid, a movement of its phenolic ring upon substrate or inhibitor binding appears likely (Heinemann and Saenger, 1982). The interaction of Tyr48 with the guanine ring in the RNase T1•2'-GMP

<table>
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<th>TABLE II</th>
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<td><strong>Torsion angles</strong></td>
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<tr>
<td>O-5' - C-5' - C-4' - C-3'</td>
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<tr>
<td>C-5' - C-4' - C-3' - O-2'</td>
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<tr>
<td>C-4' - C-3' - C-2' - O-2'</td>
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<tr>
<td>C-3' - C-2' - O-2' - P</td>
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<tr>
<td>O-4' - C-1' - N-9 - C-4</td>
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<td>P</td>
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**Pseudorotation parameters**
(Altona and Sundaralingam, 1972)

| τ0 | -21.5° |
| τ1 | 39.2° |
| τ2 | -48.3° |
| τ3 | 28.4° |
| τ4 | 5.0° |
| τm | 42.2° |
| P | 167.9° (C2'-endo) |
complex is also indicated by a change in RNase T$_1$ fluorescence (Pongs, 1970) and by photochemically induced dynamic nuclear polarization in combination with peptide analysis (Nagai et al., 1985). Thus, the binding mode of RNase T$_1$ in aqueous solution for the inhibitor 2'-GMP is likely to be identical with the one described here.

**Catalytic Site Geometry**—We define as the catalytic site of RNase T$_1$ that portion of the protein molecule which is in contact with ribose or phosphate moieties of the bound inhibitor, 2'-GMP (Fig. 11). The sugar-phosphate portion of the inhibitor protrudes into a shallow depression on the RNase T$_1$ surface where it interacts with several amino acid side chains, most of which are anchored in the major $\beta$-sheet. The catalytic site is fairly open to solvent and five water molecules are in hydrogen bonding distance to sugar or phosphate oxygen atoms.

His$^{46}$, Glu$^{88}$, Arg$^{77}$, and His$^{92}$, identified by solution methods to be indispensable for enzymatic activity of RNase T$_1$, are indeed located in the catalytic site. The imidazole ring of His$^{46}$ engages in a hydrogen bond and/or salt bridge with phosphate oxygen O-1P of 2'-GMP while the Glu$^{88}$ carboxyl group is in hydrogen bonding distance to both O-3P and Arg$^{77}$ (N). Although it is located in the vicinity of the ribose-phosphate moiety of 2'-GMP, the His$^{92}$ imidazole group cannot form hydrogen bonds to the inhibitor. Instead, a further hydrogen bond between the phenolic hydroxy function of Tyr$^{38}$ and O-1P may be present.

In order to describe properly the hydrogen bonding and charge balance in the catalytic site of RNase T$_1$, the protonation state of all functional groups must be known. This information is not available from x-ray crystallography. At the pH of crystallization (between 4.8 and 5.0; Heinemann et al., 1980) the imidazole groups of histidines and the guanidinium group of Arg$^{77}$ are likely to be protonated and positively charged. Since pH 5 is not far from the $pK_a$ value of free RNase T$_1$, the side chain of Glu$^{88}$ may be present in either possible state. The 2'-monophosphate probably carries one negative charge and one hydrogen atom (Saenger, 1984).

The $pK_a$ value of the Glu$^{88}$ residue in free RNase T$_1$ has been measured to be in the vicinity of 4.0 by spectrophotometric titration (Walz, 1977a) and by NMR (Inagaki et al., 1981). The latter study has predicted the interaction between the side chains of Glu$^{88}$ and Arg$^{77}$ actually observed in the crystal of the RNase T$_1$•2'-GMP complex to occur in the free enzyme as well. The close juxtaposition of the Glu$^{88}$ carboxylate group with the negatively charged phosphate monoester may shift its $pK_a$ to higher values. An increase to $pK_a = 7.8$ of a carboxylate group, presumably that of Glu$^{88}$, upon 2'-GMP binding in solution has been suggested to occur, based on a potentiometric titration of RNase T$_1$ and its complex with 2'-GMP (Iida and Ooi, 1969).

For these reasons we may assume that the side chain of Glu$^{88}$ is protonated and hydrogen bonded to the phosphate group. The latter is further involved in hydrogen bonds between O-1P and the Tyr$^{38}$ side chain and between O-3P and the water molecules bound to it. It is worth noting that no direct interaction of the protein with the ribose moiety of the bound nucleotide is seen.

**Possible Mechanism of Catalysis**—This crystallographic study of the RNase T$_1$•2'-GMP complex has revealed much detail about the specific protein-nucleotide interaction, as shown schematically in Fig. 12. Here, all protein residues and water molecules forming hydrogen bonds with the inhibitor (enclosed in rectangles) have been drawn in along with all groups hydrogen bonded to the primary binding groups (in ellipsoids). Wherever the assignment was unambiguous, the dashed arrows point from a hydrogen bond donor to an acceptor atom.

In attempting to infer from this data a possible mechanism of hydrolysis, one has to observe two caveats. First, the complex has been crystallized at the optimal pH for inhibitor binding and not at the pH optimum for RNA cleavage (5.0 versus 7.5; Takahashi and Moore, 1982). At the higher pH the protonation state and hydrogen bonding properties of active site residues may be different from that in the crystalline complex. Second, substrate binding to RNase T$_1$ must be different from 2'-GMP binding since 2'-5' phosphodiester

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**Fig. 11.** The catalytic center of RNase T$_1$. Protein main chain is shown in full bonds and the nucleotide is shaded. Phosphorus, oxygen, nitrogen, and carbon atoms are drawn with decreasing radius. Solid narrow lines represent hydrogen bonds and water molecules are marked $W$.

**Fig. 12.** 2'-GMP binding cartoon. All protein residues and water molecules directly hydrogen bonded to the inhibitor are shown in rectangles; boxes; groups involved in secondary interactions are in ellipsoids. Thin arrows point from hydrogen bond donor atoms to acceptors. Where an unambiguous assignment was impossible, arrowheads have been omitted.
linkages are resistant to hydrolysis by the enzyme. In a crystallographic study of 3'-GMP bound to RNase T₁, guanine binding was found to be as in the 2'-GMP complex while the sugar-phosphate was unobservable due to disorder (Sugio et al., 1985b). One has to conclude that a 3'-phosphate is bound less tightly to the enzyme. However, the spatial arrangement of amino acid residues in the RNase T₁ active site provides insight in a possible mechanism of cleavage.

Using diastereomeric phosphorothioates, Eckstein et al. (1972) showed the stereochemistry of the transesterification step of RNase T₁ to follow an in-line mechanism. A concerted in-line transesterification requires general base catalysis toward the 2'-hydroxyl group and general acid catalysis toward the leaving 5'-OH (Usher, 1969). Thus, two functional groups of the enzyme having pKₐ values near the optimal pH of hydrolysis must be located on either side of the scissile phosphodiester group. A mechanism that meets these requirements has been proposed by Takahashi (1970a) where, in transesterification, the Glu⁵' carboxylate group activates the substrate 2'-OH which in turn nucleophilically attacks the phosphodiester group, and a histidine imidazole protonates the leaving 5'-OH. A cyclic 2',3'-O-phosphodiester is formed as an intermediate which, in a second reaction step, is hydrolyzed by a water molecule activated by the now deprotonated histidine imidazole ring while the carboxyl of Glu⁵' protonates the leaving 2'-hydroxyl group. The present and earlier crystallographic work (Heinemann and Saenger, 1982, 1983) support the described mechanism and propose His⁴₅² to be a residue directly involved in catalysis along with Glu⁵'. The other histidine in the active site, His⁴₆, appears to be too close to the Glu⁵' side chain to participate in acid base catalysis in concert with this residue. Furthermore, moving the phosphate group from the ribose 2'-position to 3' and leaving the nucleoside position and conformation unchanged would shift the phosphate closer to His⁴₅₂ and away from His⁴₆.

Recently, Nishikawa et al. (1986) have generated mutants of RNase T₁ that have Glu⁵' replaced with either an aspartic acid or a glutamine residue. Both mutants have retained their catalytic activity but the cleavage rates are drastically reduced. The authors suggest that Glu⁵' may not be directly engaged in catalysis and propose that the two histidines are involved, His⁴₅ and His⁴₆, in RNase T₁, as in bovine pancreatic ribonuclease (Wlodawer, 1985). Since the side chains of Glu⁵' and His⁴₅ are in fact rather close in the crystal structure, we cannot rule out the possibility that His⁴₅ and not Glu⁵' interacts with the 2'-hydroxyl group in catalysis. It must be pointed out, however, that there is ample evidence from biochemical and spectroscopic work in favor of a central importance of Glu⁵' for the enzymatic activity of RNase T₁ (Takahashi and Moore, 1982). A final, albeit indirect, argument supports this view from the observation that Glu⁵', as well as Arg⁷ and His⁴₅, are conserved in the sequences and tertiary structures of all ribonucleases belonging to the T₁ family, whereas His⁴₆ is only present in the fungal but not in the bacterial enzymes (Hill et al., 1983).

The role of Arg⁷ in RNase T₁ catalysis may be 2-fold. Through its interaction with Glu⁵' the guanidinium group may stabilize the position in the active center of this residue and influence its pKₐ value. More importantly, the positive charge of the side chain of Arg⁷ may lower the energy of the pentacovalent phosphate transition state through electrostatic interaction. The presence of a cationic group in the active site appears to be a common feature of nucleases; in bovine pancreatic ribonuclease there is a lysine residue (Wlodawer, 1985) and in deoxyribonuclease I there is a calcium ion near the phosphate group (Suck and Oefner, 1986).

While the refinement of the RNase T₁, 2'-GMP complex has added substantial new information regarding structure and mechanism of RNase T₁, we are far from fully understanding the enzyme's action. Important new data are likely to come from crystallographic investigations of mutant RNase T₁ molecules and complexes with different nucleotides. Work along these lines is in progress in this laboratory (Quaas et al., 1988).

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