RNA ligase type 1 from bacteriophage T4 (Rnl1) is involved in countering a host defense mechanism by repairing 5'-PO₄ and 3'-OH groups in tRNA₃⁹⁰. Rnl1 is widely used as a reagent in molecular biology. Although many structures for DNA ligases are available, only fragments of RNA ligases such as Rnl2 are known. We report the first crystal structure of a complete RNA ligase, Rnl1, in complex with adenosine 5'-[α,β-methylene-diphosphonate] (AMPcPP). The N-terminal domain is related to the equivalent region of DNA ligases and Rnl2 and binds AMPcPP but with further interactions from the additional N-terminal 70 amino acids in Rnl1 (via Tyr37 and Arg54) and the C-terminal domain (Glu269 and Asp772). The active site contains two metal ions, consistent with the two-magnesium ion catalytic mechanism. The C-terminal domain represents a new all-α-helical fold and has a charge distribution and architecture for helix-nucleic acid groove interaction compatible with tRNA binding.

Bacteriophage T4 RNA ligase 1 (EC 6.5.1.3), the founding member of the RNA ligase family (1), is a very well studied representative of the nucleotide transferase superfamily, which includes RNA ligases, DNA ligases, and RNA capping enzymes. All members of this family hydrolyze a pyrophosphate bond of a ribonucleotide triphosphate and make a high energy phosphorimidate linking the nucleotide monophosphate with an essential lysine in the active site. This lysine is identified within a conserved motif XXD/NIG motif (motif I) and is responsible for the formation of the covalent bond to ATP, NAD, or GTP (2). DNA ligases and RNA capping enzymes share five amino acid sequence motifs: I, III, IIIa, V, and IV (3). Motifs III and IIIa are missing in the Rnl1 sequence (4) but are present in the recently discovered T4 RNA ligase 2 (Rnl2) (5, 6). Rnl2 shares greater sequence homology with DNA ligases and RNA capping enzymes compared with Rnl1. It may suggest that Rnl1 has a more specific role than Rnl2, although the function of Rnl2 still remains unknown.

The biological role of Rnl1 involves the countering of a host defense mechanism invoked following phage infection of the bacterial host. The bacterial RNA₃⁹⁰-specific anticondon nuclease (ACNase) is kept latent because of the association of its core protein, PrrC, with the endonuclease Ecorprl, which stabilizes PrrC and masks its activity (7). Upon infection, T4 bacteriophage expresses a T4 Stp peptide (8), which inhibits Ecorprl and activates the latent enzyme. Anticodon nuclease is involved in the 5’ cleavage of the wobble base of tRNA₃⁹⁰ (9). This modification of tRNA₃⁹⁰ acts as a defense mechanism by inhibiting phage protein synthesis and, as a consequence, stops the infection. Bacteriophage T4 has developed a counter-defense mechanism using Rnl1 and polynucleotide kinase (PnK) to repair the break in the tRNA anticodon loop. Thus Rnl1 plays an important in vivo role in the spread of the bacteriophage. It has been shown that if PnK and Rnl1 are not present, viral protein synthesis is blocked by depletion of tRNA₃⁹⁰ (10). A second biological role for Rnl1 has been reported, the promotion of tail fiber attachment (TFA)² to the phage baseplate (11). In the absence of Rnl1 the TFA reaction proceeds at a slow rate. Rnl1 can enhance the rate by up to 50-fold (12) but does not affect the final yield of attached tail fibers. Ligase and TFA activities may be mechanistically unrelated because the reaction requirements and the response to some inhibitors are different (11).

Because RNA is more sensitive to degradation than DNA, it has been proposed that polynucleotidyltransferase ancestors catalyzed RNA repair/recombination and then may have evolved into other nucleotidyl ligases and capping enzymes by acquisition of different C-terminal domains. In the Rnl2 structure, there are no amino acid side-chain contacts with the adenosine ring, suggesting that changes occur in the active site to achieve GTP specificity in the case of RNA capping enzymes (13). Rnl1 catalyzes the formation of phosphodiester bonds between the 5’-phosphate and the 3’-hydroxyl termini of single-stranded nucleic acids (14, 15). Rnl1 catalysis involves three steps and requires ATP and divalent metals. In the first step, the α-phosphate moiety of ATP reacts with Lys⁹⁰ to form a covalent ligase-(lysyl-N)-AMP intermediate plus pyrophosphate. Formation of the (ε-amino)-linked adenosine monophosphorimidate is reversible. Secondly, AMP is transferred from the covalent intermediate to a 5’-phosphate RNA end. Finally, the RNA termini are sealed by the attack of the 3’-OH RNA via a phosphodiester bond and liberation of AMP, a process analogous to mRNA splicing (16, 17), although in the latter case two breaks in the RNA are required to excise the intron. The Rnl1 family members are narrowly distributed (4) and include a putative RNA ligase/polyadenylate kinase encoded by the baculovirus Autographa californica nucleopolyhedrovirus (ACNV), tRNA ligases of fungi, and RNA ligase from the bacteriophages RM378 (18) and TS2126 (19).

We report the crystal structure of the Rnl1-AMP analogue complex at 2.2 Å resolution, the first complete 3’-5’ RNA ligase structure solved; previously only structures of N-terminal fragments of Rnl2 (13) and an editing ligase, TbrEL1 (20), have been available. The structure high-

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The atomic coordinates and structure factors (code 2C5U) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: TFA, tail fiber attachment; AMPcPP, adenosine 5’-[α,β-methylene-diphosphonate]; MAD, multiple wavelength anomalous dispersion.
lights the interactions in the active site between AMPcPP, divalent metals, and the two different domains of the protein. Such data will be of value in the further characterization of the catalytic mechanism and in better understanding the role of the divalent metal cofactor in the enzyme reaction. Moreover, the C-terminal domain shows a new protein fold that is likely to play a role in tRNA binding.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of T4 RNA Ligase—The expression plasmid (pNHT4RNAligase) coding for an N-terminal histidine-tagged Rnl1 (MRGSH4GS-Rnl1) and the repressor plasmid (pDM1.1) were both transformed into Rosetta(DE3)pLysS for protein expression. Cultures were grown at 37 °C in Luria broth medium supplemented with 50 μg/ml carbenicillin, 34 μg/ml chloramphenicol, and 50 μg/ml kanamycin. When the A600 reached 0.7, the cells were induced with 1 mM isopropyl-1-thio-

TABLE 1
Statistics for crystallographic structure determination
Figures in parentheses are outer shell data. ESRF, European Synchrotron Radiation Facility; Se, selenomethionine; FOM, figure of merit; r.m.s., root mean square.

| Data collection details | Selenomethionine | Selenomethionine MAD |
|-------------------------|------------------|----------------------|
| Data set                | In-house         | ESRF BM14            |
| Detector                | MAR345           | MAR CCD 225          |
| Wavelength (Å)          | 1.5418           | 0.9791 (peak)        |
| Resolution range (Å)    | 30.0–2.2         | 0.9184 (remote)      |
|                        | (2.28–2.20)      | 0.9793 (inflection)  |
|                        | (2.07–2.0)       | 50.0–2.16            |
|                        |                  | 50.0–2.16            |
| Redundancy             | 8.2 (7.0)        | (2.24–2.16)          |
| Completeness (%)        | 98.6 (86.1)      | 3.6 (3.1)            |
| Average I/σ(I)          | 33.1 (7.51)      |                      |
| Rmerge*                | 0.059 (0.183)    |                      |
| Mean FOM                | 0.10 (0.335)     |                      |
| Mean FOM after RESOLVE | 0.065 (0.160)    |                      |
|                        | 0.065 (0.208)    |                      |

Phasing
Se sites found by SOLVE
Mean FOM
Mean FOM after RESOLVE

Refractive statistics
Resolution range (Å) 30.0–2.2
R-factor* (Rmerge/Rmerge) 0.198/0.258
r.m.s. bond length deviation (Å) 0.006
r.m.s. bond angle deviation (°) 1.24
Mean B-factor (Å²) 28.1/32.7/31.0/34.3

* Rmerge = ∑(I − ⟨I⟩)/∑I, b
  ‡ R-factor = ∑|Fo| − Fc|/∑Fc|, c
  † Main chain/side chain/ligands/water.

RESULTS AND DISCUSSION

Overall Structure of Rnl1—The refined crystal structure at 2.2 Å resolution (see Table I for data collection, phasing, and refinement statistics) showed that the 374 amino acid Rnl1 was organized into two domain structure. The N-terminal region includes an α helix (a1) followed by an antiparallel β-sheet (β1–β6) (Fig. 1A). Although this sheet
is absent in the Rnl2 structure (apart from β4), it may play an important role in the Rnl1-catalyzed reaction because two amino acids of this region are involved in hydrogen bonding in the active site. The remainder of the N-terminal domain is structurally related to the N-terminal domain of the Rnl2 and consists of twisted antiparallel β-sheets flanked by α-helices. Organization of this core region is quite similar to the
other members of the Rnl2-like protein family. Although the β-strands containing the nucleotidyltransferase motifs are very well superimposed, the α-helices are positioned more divergently (Fig. 2A). Although Rnl1 shares the same nucleotidyltransferase domain architecture with capping enzymes and DNA ligases, the C-terminal domain structure is very different among members of this family. Capping enzymes or DNA ligase have an OB-fold domain that flanks the nucleotidyltransferase domain, this OB-fold is absent from Rnl1 and Rnl2. The secondary structure of the C-terminal Rnl1 domain consists entirely of α-helices (Fig. 1, A and C). There is a very long α-helix (α-9) of about 30 residues that interacts with a series of four α-helices that lie almost parallel to one another but at an angle of ~45° to α-9. DALI (26) searches did not find any structural fold in the Protein Data Bank similar to this C-terminal domain. BLASTP revealed that some proteins of unknown three-dimensional structure, such as the RnlA RNA ligase from bacteriophage RB69 or from bacteriophage 44RR2.8t, contain domains that share a high sequence identity (61 and 40%, respectively) with this C-terminal region of Rnl1 and thus will have the same protein fold. The TFA activity of Rnl1 may be related to the structural differences between it and other ligases. Although the second role of Rnl1 may have affected the evolution of the protein structure, it is difficult to draw any particular inferences about which structural features promote TFA activity.

**ATP Binding Site**—Analysis of the nucleotide binding site shows that both the N- and C-terminal domains are involved in forming interactions with ATP. The N-terminal domain makes hydrogen bond interactions via the side-chain hydroxyl group of Tyr37 to one phosphoryl oxygen from the γ-phosphate (Fig. 2B). The guanidinium group of Arg54 is linked by three hydrogen bonds to the AMPcPP, by two to the β-γ oxygen, and by one to the ribose 3′-OH. Arg24 and the Glu35 are conserved in all Rnl1-like proteins. Site-directed mutagenesis has indicated that Arg24 is essential for RNA adenylation (step 2) but not for enzyme adenylation or formation of the phosphodiester bond (4).

The β-strands of the core region contain most of the residues involved in binding the ATP analogue. Lys99, Lys35 (motif I), Lys119 (motif II), Lys208 (motif V), and Lys242 (motif V) interact with the phosphate/phosphonate groups. Whereas Lys99 was found to be the site of adenylation in Rnl1 by fast atom bombardment mass spectrometric analysis (27), in our structure the α-phosphate is more than 3 Å from the Lys99 NZ (Fig. 2, B and C). This relative positioning of α-phosphate and Lys99 is presumably because of the presence of the methylene link in the ATP analogue but may also suggest that some conformational changes have to occur to allow formation of a covalent bond. A similar situation is observed in T7 DNA ligase, the Enterococcus faecalis DNA ligase, and in the Rnl2 structure, where the counterpart of lysine, catalytically equivalent to Lys99, is situated at a distance incompatible with covalent interaction with the phosphate. Moreover, Odell et al. (28) as well as Shuman and Schwer (3) have suggested from the Chlorella virus DNA ligase-adenylate intermediate structure that the nucleotide base conformation change, from syn to anti, accompanies the covalent attachment of the lysine. Indeed, it seems that a noncovalently bound ligand (ATP or NAD) in an RNA or DNA ligase has the nucleotide base in a syn conformation, whereas the conformation is anti if the covalent bond is made. This trend is supported by the Rnl1 structure where the AMPcPP adenine ring is in a syn conformation. It would, however, be difficult to infer whether the conformational change has to occur to permit the covalent bond to form or whether it is a consequence of covalent bond formation. Glu159 is conserved in the Rnl1 family members but is not found in other nucleotidyltransferase sequence motifs. Glu159 interacts with the ribose 2′-OH exactly as does Rnl2 Glu89 (Fig. 2C).

No side-chain contacts are made with the adenine ring, but rather main-chain interactions are present via two hydrogen bonds, i.e. one with the main-chain carbonyl of residue Thr98 and one with a water molecule stabilized by Leu179 and Asn180 (main-chain CO and side-chain CO, respectively) (Fig. 2B). Because the adenine ring only makes contacts with the protein main chain in Rnl2 as in Rnl1, it has been proposed that the nucleotidyltransferase ancestor was ATP-dependent and could have evolved into GTP- or NAD-dependent enzymes by acquiring side chains in the active site responsible for ligand specificity (13).

It has also been suggested, by comparison with the Chlorella virus motif IV Glu161, that Glu227 from motif IV coordinates a divalent cofactor ion (3, 4). In our structure, Glu227 interacts indirectly with Ca2+ via two water molecules. These are the only interactions between the protein and the metal ion, which is coordinated to six water molecules and the AMPcPP α-phosphonate. Interestingly, as the Glu227 carboxyl group is situated 2.6 Å from Lys99 NZ, this may indicate that the residue plays a role in the correct orientation of the lysine residue for forming the covalent adenylation intermediate. In the Rnl2 structure, the same interaction is observed between Glu204 and Lys35 (Fig. 2C).

There are no hydrogen bond contacts between AMPcPP and the C-terminal region, but there is an interaction between the carboxyl of Gly269 and the Mg2+ ion. The latter ion is also coordinated to side-chain hydroxyl of the conserved Asp72. Asp72 allows the correct placement of metal and thus the correct orientation of the phosphate.

**Divalent Cation Sites**—Using transient optical absorbance and fluorescence spectroscopy on T4 Rnl1 and DNA ligases, Cherepanov and de Vries (29) have proposed that nucleotidyltransferase catalysis proceeds via a two-metal ion mechanism. It has been demonstrated that metal ions such as Mg2+ are essential for the joining of nucleic acids by T4 DNA and T4 RNA ligases. These enzymes react via the same mechanism, although DNA ligase shows a higher rate of ATP binding. One reason for this difference is the decreased Kd for Mg2+. Studies on nick sealing by Rnl1 (and also by T4 DNA ligase) have shown that it cannot bind ATP-Mg2 directly, but rather that ATP-Mg2 binds first and subsequently a second Mg2+ ion. ATP-Mg2 is thus the true substrate in the adenylation reaction (29). The reverse reaction (ATP formation) is also possible using Mg3P2O7 as a substrate.

The Rnl1 structure shows Ca2+ (temperature factor = 22.1 Å2) coordinated to six water molecules and also interacting with one phosphoryl oxygen of the AMPcPP α-phosphonate (metal ion site A). A Mg2+ (temperature factor = 11.5 Å2) interacting with one phosphoryl oxygen of the β-phosphate is also present (metal ion site B) (Figs. 1B and 2B). Rnl1 only crystallizes in the presence of Ca2+, which can be explained by the presence of two symmetry-related Ca2+ ions at the crystallographic dimer interface. The positive charge of the Ca2+ ions is presumed to allow the interaction between the negatively charged surfaces of two Rnl1 molecules (Fig. 3A). Each of these Ca2+ ions situated at the interface is coordinated to residue side chains belonging to each crystallographic monomer.

The presence of a Ca2+ ion is quite surprising in site A. It can be explained by the relatively much higher concentration of CaCl2 compared with MgCl2 (200-fold molar excess). However, a Mg2+ ion is also bound under these conditions at site B, so we can infer that the latter Mg2+ site is more specific and allows only certain types of coordination. Indeed, as mentioned previously, the site A Ca2+ does not directly interact with the ligase but via water molecules interacting with Glu1227, Glu139, Lys99, Glu100, and Tyr248 (Fig. 2B). In the Rnl2 structure, Glu99, Lys106, and Tyr246 superimpose with Rnl1 Glu1227. However, although the Rnl1 Ca2+ ion is replaced by water, three of the Ca2+-interacting waters are present in
FIGURE 2. Ligand binding to Rnl1. A, stereodiagram showing a comparison of Rnl1 with the Rnl2 N-terminal domain (13). The Rnl1 C-terminal domain is colored in yellow and the Rnl1 N-terminal domain in red and green (with green corresponding to the N-terminal part absent in Rnl2). Rnl2 is shown in blue. B, stereodiagram of AMPcPP recognition by Rnl1, highlighting important interactions. C, stereodiagram showing a comparison of Rnl1 (yellow) and Rnl2 (gray) with some essential active site amino acids indicated along with ligands (AMPcPP and AMP, respectively). The Rnl2 residue numbers are written in italics.
Rnl2. From this we conclude that the divalent metal site A is in fact the same in both Rnl1 and -2 and that Glu205 of Rnl2 also interacts via water molecules with Mg$^{2+}$. Metal site B Mg$^{2+}$ forms a closer interaction with the AMPcPP $\beta$-phosphoryl oxygen 1 (2.2 Å) than to the $\gamma$-phosphate oxygen which is 3.5 Å away. It also forms an interaction with three water molecules and two residues (Gly269 and Asp272), which belong to the Rnl1 C-terminal domain. Ho et al. (13) have inferred that the Rnl2 C-terminal domain is specifically required for the second step of the ligation (AMP transfer to the 5'-PO$_4$ RNA) and that the domain is an obstacle to adenosine diphosphate RNA binding or sealing. Because both enzymes share a similar catalytic mechanism and because the Rnl1 C-terminal domain is important in stabilizing metal site B, we suggest that site B Mg$^{2+}$ also plays a role in the ligation second step, most likely by interacting with the negatively charged RNA backbone.

RNA Binding Site—Despite significant efforts screening with a range of ligands including ATP, AMPcPP, or AMP, we were unable to crystallize an RNA-Rnl1 complex. Rnl1 seems to crystallize only with AMPcPP, which, on the basis of the proposed ping-pong mechanism, is incompatible with the presence of RNA in the active site. Analysis of charge distribution on the protein surface gives an indication of a possible site of RNA binding. The majority of the protein surface is negatively charged, apart from the C-terminal domain, which presents positive charges that could interact with the RNA backbone phosphates (Fig. 3A). Furthermore, we know that the RNA should be in close proximity to the ATP binding site to enable the AMP transfer from Lys99. Docking studies (Fig. 3B) suggest that RNA binds at the surface of the C-terminal domain, thereby positioning the anticodon loop toward the ATP binding site in the core region. Interestingly, the C-terminal architecture of the parallel $\alpha$-helices ($\alpha$-7 and $\alpha$-10–12) positioned along the $\alpha$-9 helix matches the tRNA groove architecture. As mentioned earlier, the Rnl1 C-terminal domain is unique among nucleotidyltransferase family members, suggesting that its topology evolved to bind specifically tRNA$^{\text{Lys}}$ in vivo.

In the active site, a chloride ion (temperature factor $= 17.8$ Å$^2$) is positioned 3.1 Å from the ribose 3'-OH and at the same distance from a Ca$^{2+}$-coordinated water molecule (Fig. 2B). The negative charge of the chloride may mimic the 5'-phosphate of the incoming RNA as proposed for the sulfate ion in the Chlorella virus DNA ligase structure (28, 30). The chloride ion is situated above the AMP moiety on the active site face, which is accessible to RNA, and is sterically compatible with the position of a nucleic acid phosphate. Wang et al. (4) have reported the specific function of Arg54 and Lys119 in the RNA adenylation reaction. In our structure, Arg54 is 3.5 Å from the chloride ion and would be even closer to a phosphoryl oxygen if a phosphate occupied the same site. Thus, we suggest that Arg54 stabilizes and orients the RNA phosphate by hydrogen bonding in the second step of the reaction. However, as mentioned above, Arg54 is also involved in the first step of the reaction, stabilizing the ribose 3'-OH and the $\gamma$-phosphate, although it has been shown that its mutation to alanine partial activity is retained in the first steps, whereas activity was abolished in the second step of the reaction (4). This raises the question of why such a seemingly important residue is not conserved in other nucleotidyltransferases. By superimposing the structures of Rnl1 and Rnl2, we observed that the position of the guanidinium group of Arg54 is quite close to corresponding group of the Rnl2, Arg55 (Fig. 2C). Arg55 is conserved in Rnl2-like proteins and is essential for catalysis. Interestingly, the chloride ion is replaced in the Rnl2 structure by a water molecule 2.6 Å from Arg55. Thus we postulate that Arg54, which is conserved in Rnl1-like proteins and belongs to a domain not present in Rnl2, plays the same role as the conserved Arg55, i.e. that of orientating the RNA phosphate in the active site.

Lys119 has been shown to be as important for the second catalysis step as Arg54, because mutation of either residue interferes with adenylation of RNA (4). Although the Rnl1 structure does not directly indicate its role, we nevertheless suggest that Lys119, based...
on its placement at the entrance of the active site and the position of the chloride ion, may interact with RNA. Lys$^{119}$ may interact with a 3’-hydroxyl ribose group, for example, and thus could be involved in the 5’-P RNA recognition.

Although we have to keep in mind that AMPcPP might not bind as exactly as ATP because of its methylene group, and that Lys$^{39}$ has to be deprotonated to make a covalent bond, nevertheless the Rnl1 structure still provides information about nucleotide binding prior to the adenylation reaction. The structure also provides clues about the ligation second step. We suggest, as in the case of the Chlorella virus DNA ligase, that the chloride ion mimics the 5’-P-RNA. We also suggest that Arg$^{54}$, essential for the second step reaction, catalytically corresponds to the conserved Arg$^{55}$ of Rnl2. Because all five nucleotidyltransferase motifs are present in Rnl2, it has been proposed that Rnl2 is homologous to the nucleotidyltransferase family ancestor (5); thus we infer that acquisition of the additional N-terminal residues, and especially Arg$^{54}$, was an adaptation for Rnl1 to replace the missing Rnl2 Arg$^{55}$. Moreover, the structure confirms the presence of two divalent metal ions in the active site and shows the essential interacting residues. Both metals interact with the ATP analogue and water molecules, but the site B metal also interacts with two residues of the Rnl1 C-terminal domain. The latter domain is a completely new protein fold, which presents a structure and a surface charge profile compatible with tRNA binding. The work reported here has thus provided structural information for a complete RNA 3’-5’ RNA ligase.

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