Molecular Structure of Cyclic Deoxydiadenylic Acid at Atomic Resolution†

Christin A. Frederick,‡ Miquel Coll,‡ Gijs A. van der Marel,§ Jacques H. van Boom,§ and Andrew H.-J. Wang*,‡

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Gorlaeus Laboratories, Leiden State University, 2300RA Leiden, The Netherlands

Received March 29, 1988; Revised Manuscript Received May 26, 1988

ABSTRACT: The molecular structure of a small cyclic nucleotide, cyclic deoxydiadenylic acid, has been determined by single-crystal X-ray diffraction analysis and refined to an R factor of 7.8% at 1.0-Å resolution. The crystals are in the monoclinic space group $C2$ with unit cell dimensions of a = 24.511 (3) Å, b = 24.785 (3) Å, c = 13.743 (3) Å, and β = 94.02 (2)°. The structure was solved by the direct methods program SHELXS-86. There are 2 independent cyclic d(ApAp) molecules, 2 hydrated magnesium ions, and 26 water molecules in the asymmetric unit of the unit cell. The two cyclic d(ApAp) molecules have similar conformations within their 12-membered sugar–phosphate backbone ring, but they have quite different appearances due to the different glycosyl torsion angles that make one molecule more compact and the other extended and open. Three of the four deoxyribose rings are in the less common C3′-endo conformation. All four phosphate groups have their phosphodiester torsion angles α/ζ in the gauche(+)/gauche(+) conformation. One of the cyclic d(ApAp) molecules associates with another symmetry-related molecule to form a self-intercalated dimer that is a stable structure in solution, as observed in NMR studies. Many interesting intermolecular interactions, including base–base stacking, ribose–base stacking, base pairing, base–phosphate hydrogen bonding, and metal ion–phosphate interactions, are found in the crystal lattice. This structure may be relevant for understanding the conformational potentiality of an endogenous biological regulator of cellulose synthesis, cyclic (GpGp).

Many low molecular weight nucleoside derivatives play important roles in numerous biological functions. For example, cyclic (3′,5′) AMP functions as an essential factor in the regulation of complex hormonal activities by acting as a second messenger and transmitting information, through a series of biochemical reactions, from the cell surface receptors to the nuclear DNA. Another example is the group of riboguanosine nucleotide derivatives ppGpp and pppGpp (magic spots), which are important in signaling a response to a starving metabolic state in bacteria (Cashel & Gallant, 1969). These ribonucleoside derivatives are synthesized in vivo from precursor molecules by specific cellular enzymes when they are activated by the triggering inducer. cAMP is synthesized by adenylate cyclase from the precursor ATP, while ppGpp and pppGpp are synthesized by a nucleotide pyrophosphoryl transferase that transfers a pyrophosphate group from ATP to GDP or GTP, respectively (Haseltine & Block, 1973; Sy et al., 1973).

Given the great diversity of biochemical processes within a cell, one might expect that different small nucleotide biological factors are involved in many other functionally distinct metabolic routes. The observation that an intramolecular phosphodiester linkage can be introduced between the free 3′ and 5′ hydroxyl groups of a short oligonucleotide to form a closed circular molecule, as is true for the mononucleotides cGMP and cAMP, has led us to surmise that another class of effectors might involve derivatives of various cyclic oligonucleotides. Subsequently, a number of circular oligonucleotides (mostly di- and trinucleotides) were synthesized and tested for biological activity.

It was discovered that one of those compounds, cyclic (ribo) diguanylic acid, is a regulator of the biological synthesis of cellulose in the Gram-negative bacterium *Acetobacter xylinum* (Ross et al., 1987). The cyclic nucleotide is enzymatically synthesized from pppGpG by the enzyme diguanylate cyclase. This molecule exerts its function presumably by binding to membrane-bound cellulose synthase, thereby inducing a conformational change in the protein and subsequently activating the enzyme. In addition, other purine derivatives, including inosine and deoxyguanosine dinucleotides, also effectively activate this enzyme. It is, therefore, of interest to ask what possible conformations such a cyclic dinucleotide is capable of adopting, keeping in mind that a severe conformational constraint exists within such a molecule.

In this paper we describe the atomic resolution structure of a cyclic dinucleotide, cyclic deoxydiadenylic acid [denoted cyclic d(ApAp)], determined by X-ray diffraction analysis. Although the molecule exists in two distinct conformations in the crystal lattice, the 12-membered sugar–phosphate backbone rings of the two independent molecules are very similar. The two molecules adopt very different glycosyl torsion angles, however, leading to completely different orientations of the base planes. Many interesting intermolecular base-stacking and hydrogen-bonding interactions are also observed, including a novel method of self-intercalation between the two cyclic d(ApAp) molecules. Preliminary results described in this paper have been presented elsewhere (Frederick et al., 1987).

Materials and Methods

Synthesis of Cyclic Deoxydiadenylic Acid. Initially, the fully protected cyclic d(ApAp) molecule was prepared (de Vroom et al., 1988) by intramolecular condensation of the corresponding linear dimer having a free 5′-hydroxyl and 3′-*O*-(2-chlorophenyl phosphate) with [(2,4,6-triisopropylphenyl)sulfonyl]-3-nitro-1,2,4-triazole (TPSNT) in pyridine. The cyclic dimer thus obtained was purified by silica gel

† This work was supported by grants from NSF (DMB-8612268) and Burroughs-Wellcome Co. to A.H.J.W. C.A.F. was a fellow of the Leukemia Society, and M.C. is a fellow of the Fulbright Society.

* To whom correspondence should be addressed at the Department of Physiology and Biophysics, University of Illinois at Urbana–Champaign Urbana, IL 61801.

‡ Massachusetts Institute of Technology.

§ Leiden State University.

0006-2960/88/0427-8350$01.50/0

Table I: Fractional Atomic Coordinates[a] of Cyclic d(ApAp)

atom	x	y	z	atom	x	y	z
	Residue A1			C5	7131	2863	8669
P	3202	1214	6321	C6	7017	3415	8468
O1P	2821	0773	6543	N6	6533	3631	8610
O2P	3803	1103	6360	N7	6828	2461	9015
O5′	3139	1714	7042	C8	7142	2035	9011
C1′	3325	2855	8706	N9	7647	2127	8661
C3′	2954	2885	7061				
C2′	3234	3235	7832		Residue B2		
C4′	2632	2464	7659	P	8005	0067	10291
C5′	2594	1908	7225	O1P	8473	−0302	10575
O4′	2905	2460	8605	O2P	7821	0471	11011
O3′	2541	3178	6460	O5′	7477	−0259	9945
N1	5445	3032	8799	C4′	6996	−0866	8829
C2	5034	3415	8766	C3′	6562	−0450	8489
N3	4491	3333	8738	C5′	7521	−0682	9238
C4	4360	2797	8721	C2′	6016	−0696	8621
C5	4720	2372	8748	C1′	6122	−1230	9235
C6	5300	2508	8810	O3′	6631	−0316	7484
N6	5695	2131	8856	O4′	6713	−1129	9612
N7	4452	1883	8727	C5	5398	−1460	11456
C8	3939	2012	8742	C4	5651	−1621	10640
N9	3852	2570	8699	N3	5736	−2128	10296
				C2	5468	−2499	10917
	Residue A2			N1	5192	−2375	11767
P	2713	3482	5486	C6	5134	−1862	12138
O1P	2192	3729	5032	N6	5014	−1705	13094
O2P	3199	3845	5705	N7	5419	−0885	11527
O5′	2927	3014	4819	C8	5683	−0679	10813
O4′	3181	2312	3272	N9	5848	−1166	10205
C2′	3738	1689	4219				
C3′	3279	1879	4823		Magnesium Complexes		
C4′	2853	2132	4070	MG1	4365	0533	6724
C1′	3722	2091	3374	W1	5064	1004	6545
C5′	2541	2613	4442	W2	3774	−0050	6974
O3′	2990	1444	5273	W3	4939	−0082	7137
N1	5732	2733	3744	W4	4374	0786	8184
C2	5504	2245	3645	W5	4390	0283	5279
N3	4975	2097	3607	MG2	2245	0214	6144
C4	4657	2529	3645	W1	2549	−0215	7409
C5	4828	3070	3712	W2	1900	0722	5029
C6	5412	3164	3790	W3	2780	−0177	5273
N6	5619	3659	3889	W4	1696	0569	7085
N7	4393	3413	3689	W5	1671	−0371	5725
C8	3961	3106	3607				
N9	4101	2557	3583		Water Molecules		
				W1	5998	5430	13814
	Residue B1			W2	5890	0955	9238
P	6484	0275	7065	W3	4359	4517	3641
O1P	6509	0240	5985	W4	1226	3935	3806
O2P	5965	0468	7467	W5	0294	0165	0962
O5′	6961	0647	7530	W6	4353	4960	1844
C1′	8067	1741	8475	W7	8830	2513	6639
C2′	8173	1346	9352	W8	3304	10844	9230
C4′	7929	0865	7877	W9	3975	14337	8914
O4′	7884	1434	7655	W10	6500	14630	2907
C3′	7891	0823	8966	W11[b]	5000	04333	10000
C5′	7515	0553	7222	W12[b]	0000	0070	5000
O3′	8174	0361	9310	W13	0649	1267	2497
N1	7425	3713	8109	W14	8571	11753	4948
C2	7915	3471	7948	W15	3744	8585	13659
N3	8057	2967	8053	W16	4594	9133	14444
C4	7652	2674	8441				

[a] All coordinates are multiplied by 10^4. [b] Water molecules 11 and 12 are each assigned occupancies of 0.500.

chromatography and completely deblocked by oximate followed by aqueous ammonia treatment. The final product was then purified by DEAE-Sephadex G-25 anion-exchange chromatography and then converted to the ammonium salt. It was judged to be greater than 95% pure by HPLC analysis.

Crystallization and Structure Determination. The molecule was crystallized from a solution containing 10 mM cyclic d(ApAp), 30 mM sodium cacodylate (pH 6.0), 10 mM $MgCl_2$, and 25% 2-methyl-2,4-pentanediol (2-MPD) equilibrated against 60% 2-MPD by using the vapor diffusion technique. Long rectangular rod-shaped crystals appeared after several weeks. Crystal growth apparently required magnesium ions, since solutions containing only sodium as the counterion produced crystals of very poor quality. The crystals grown in the presence of Mg^{2+} were of extremely good quality and diffracted X-rays to better than 1.0-Å resolution. They were examined by X-ray precession photography and determined to be in the monoclinic space group $C2$ with unit cell dimensions of $a = 24.511$ (3) Å, $b = 24.785$ (3) Å, $c = 13.743$ (3) Å, and $\beta = 94.02$ (2)°. On the basis of the unit cell volume (8328.40 Å^3), the unit cell was judged to contain two independent molecules in the asymmetric unit.

A crystal with an approximate size of 0.15 × 0.15 × 0.60 mm was sealed in a thin-walled glass capillary and used for

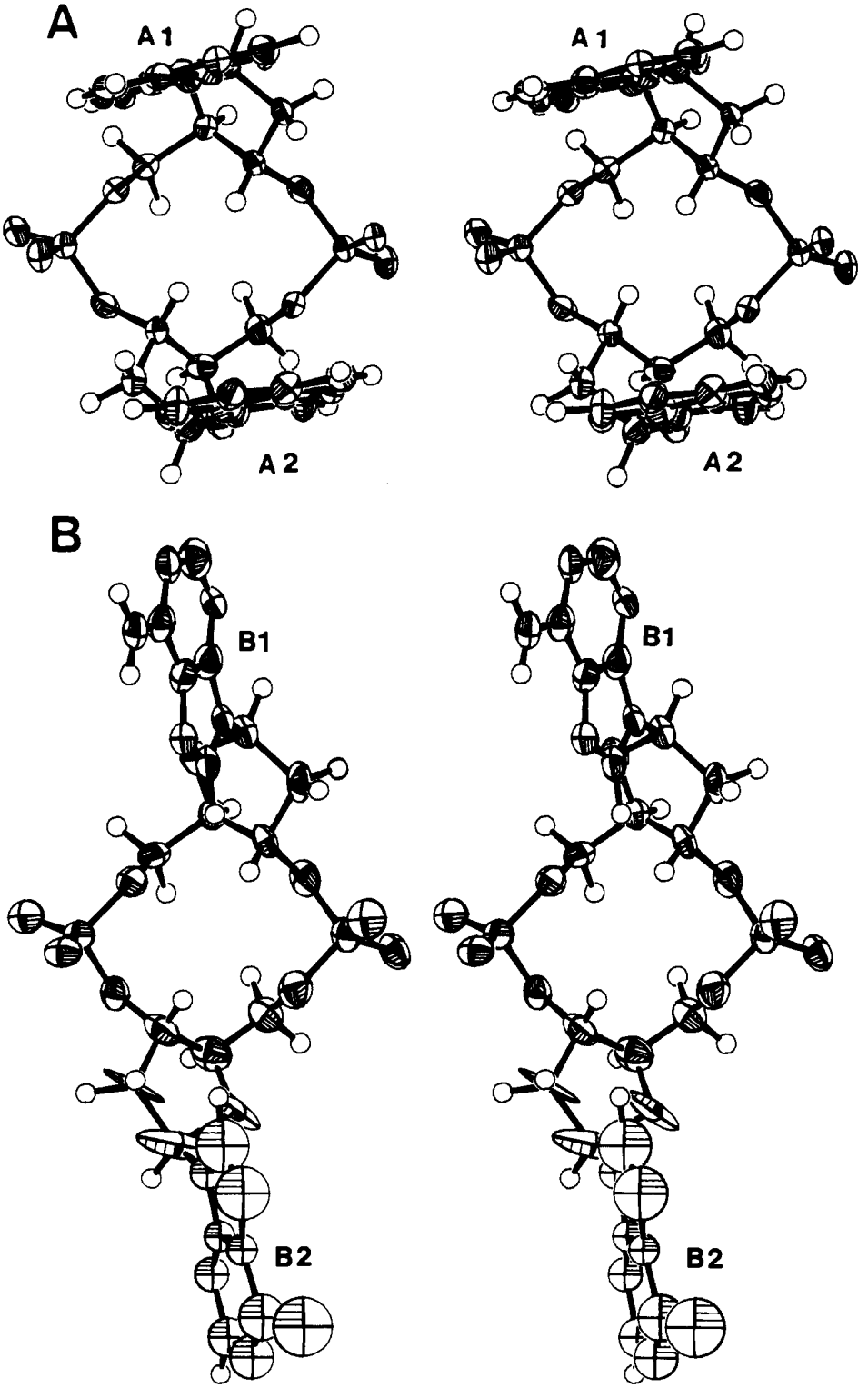

FIGURE 1: Stereo ORTEP representations of the two conformations of cyclic d(ApAp) drawn with their 12-membered backbone rings in the same orientation. All non-hydrogen atoms (except in base B2) are represented as thermal ellipsoids of 50% probability. (A) Molecule A, the closed conformer, has adenine bases A1 and A2 arranged parallel to each other with an average separation of 6.8 Å. (B) Molecule B, the open conformer, has adenine bases B1 and B2 extended away from the backbone ring. The atoms of base B2 are drawn as spheres with diameters proportional to their isotropic thermal factors.

data collection. Data were collected on a Nicolet P3 diffractometer using the ω-scan method to 1.0-Å resolution (2θ = 113° with Cu Kα radiation). Data were reduced with Lorentz and polarization corrections plus an empirical absorption correction (North et al., 1968). Of the 6205 reflections, 5360 unique reflections were considered to be observed at the 5.0σ(F) level above background, and they were used in the structure refinement.

Numerous attempts, including sharpened Patterson functions and direct methods, were made to solve the structure but were unsuccessful. The structure containing 120 non-hydrogen atoms was finally solved by direct methods using a program by G. Sheldrick, SHELXS-86. The first E-map produced by the program clearly revealed one complete cyclic d(ApAp) molecule and fragments of the second molecule. The second E-map, calculated by incorporating those known atoms for

TANGENT phase recycling, produced a complete second DNA molecule. The structure was then refined by a full-matrix least-squares refinement program (SHELX) (Sheldrick, 1982). Two magnesium ions and 26 water molecules (10 of which are coordinated to the magnesium ions) were located from a series of difference Fourier maps. All non-hydrogen atoms were refined isotropically to an R factor $[\sum(F_o - F_c)/\sum F_o]$ of 14%. They were then converted in stages, with the exception of one adenine ring and some water molecules, to anisotropic temperature factors and refined in two blocks. After continued refinement of the whole contents of the unit cell, the R factor was reduced to 11.5%. At this stage, hydrogen atomic positions were generated by using known geometry for all DNA atoms, and they were incorporated in the refinement and allowed to ride with the heavy atoms to which they were attached. The final R factor is 7.9%, for 5360 reflections with unit weights, with no shift/esd value greater than 0.05.

During the course of refinement, it was discovered that one of the four adenine rings was disordered. In addition, several atoms of the corresponding deoxyribose moiety displayed distorted thermal ellipsoids (see Figure 1), probably reflecting the movement of this base. That adenine ring is situated in a relatively open area where two adenines related by a 2-fold symmetry axis are partially stacked over each other. No intermolecular hydrogen-bonding interactions were found for those two adenines except to isolated (nonbridging) water molecules. This disordered adenine ring was constrained geometrically during the early stages of refinement, with no adverse effect on the refinement of the rest of the molecules. Once the structure was fully refined several additional cycles were run by using anisotropic thermal parameters for this base as well. Although, as expected, these atoms demonstrated large thermal ellipsoids, there was little improvement on the overall R factor or refinement of the other bases. Consequently, results obtained from the isotropic refinement for this adenine base were used. The final atomic coordinates are listed in Table I, and the thermal parameters are deposited as supplementary materials (Table IS).

RESULTS

Molecular Structure. In the asymmetric unit of the crystal lattice, there are two independent cyclic d(ApAp) molecules, which are shown in Figure 1 with the four nucleotides designated A1, A2, B1, or B2. This figure shows the three-dimensional structure of the two independent molecules, looking from the direction perpendicular to the plane through each 12-membered backbone ring. The closure of the 12-membered sugar–phosphate backbone ring leads to a rigid structure with a small cavity near its center, which is filled by two pairs of hydrogen atoms, the HC3′ and HC5′ atoms, with close van der Waals contacts between them. The distances between the two opposite HC3′ atoms in molecules A and B are 2.27 and 3.05 Å, respectively, while those between the two HC5′ atoms are 2.51 and 2.52 Å. It can be clearly seen that both molecules possess molecular 2-fold symmetry and that they have very similar backbone conformations. The root mean square deviations between the two halves of the molecule (backbone atoms only) are 0.025 and 0.041 Å for molecules A and B, respectively. The rms deviation between the two molecules is only slightly larger, 0.174 Å. This is reflected in the similar values of the torsion angles along the circular backbone as listed in Table II.

The bond distances and angles within each molecule are provided in supplementary materials in Tables IIS and IIIS,

Table II: Backbone Torsion Angles for Cyclic d(ApAp)[a]

base	α	β	γ	δ	ϵ	ζ	χ
A1	67.4	−163.0	53.1	96.4	−157.9	61.2	−184.1
A2	65.9	−164.7	54.9	93.7	−158.6	64.2	−173.8
B1	66.4	−169.1	48.1	84.8	−153.8	77.9	−103.4
B2	63.3	−167.3	46.2	88.9	−148.5	72.7	−108.1
AveA	66.7	−163.9	54.0	95.1	−158.3	62.7	−179.0
AveB	64.9	−168.2	47.2	86.9	−151.2	75.3	−105.8
A-DNA[b]	−50	172	41	79	−146	−78	−154
B-DNA[b]	−46	−147	36	157	155	−96	−98
ApA[c]	61	−174	46	147 (5′) 77 (3′)	−141	61	42 (5′) −164 (3′)
UpA 1[d]	82	−157	55	86 (5′) 85 (3′)	−154	81	−168 (5′) −143 (3′)
A⁺pA⁺[e]	93	−172	56	81	−151	77	−172 (5′) −152 (3′)

[a] The standard angular notations are as follows: O3′—P$^{\alpha}$O5′$^{\beta}$C5′$^{\gamma}$C4′$^{\delta}$C3′$^{\epsilon}$O3′$^{\zeta}$P. [b] Saenger (19884) and references cited therein. [c] Einspahr et al. (1981). [d] Sussman et al. (1972). [e] Suck et al. (1976).

Table III: Sugar Torsion Angles for Cyclic d(ApAp)[a]

base	ν_0	ν_1	ν_2	ν_3	ν_4	P	τ_m
A1	13.8	−27.5	29.6	−22.3	5.1	−9	30
A2	6.9	−23.7	30.6	−26.9	12.5	5	31
B1	−7.6	−13.5	28.1	−33.7	26.4	32	34
B2	−37.9	15.3	10.5	−32.8	44.6	76	45

[a] The standard angular notations are as follows: O4′$^{\nu_0}$C1′$^{\nu_1}$C2′$^{\nu_2}$C3′$^{\nu_3}$C4′$^{\nu_4}$O4′. P and τ_m correspond to the pseudorotation angle and amplitude, respectively.

and except for those associated with the disordered base B2, in general they agree well with the published average values of unprotonated adenosine nucleotides (Bugg et al., 1971; Arnott et al., 1972). However, there are noticeable differences in some values. For example, two bond angles in the deoxyribose ring clearly exibit conformationally dependent variations. The O3′–C3′–C4′ angle in molecule A (av 105.4°) is significantly smaller than that of molecule B (av 108.9°). Similarly, the C3′–C4′–C5′ angle in molecule A (av 114.9°) is also smaller than that of molecule B (av 118.5°). These differences are probably associated with the distinct glycosyl torsion angle in the two molecules as will be described below.

Another interesting modification is found in the phosphate geometry, in which a concerted twisting appears to occur in all four phosphate groups. Specifically, the bond angles associated with the non-ester oxygens, O1P and O2P (the convention for numbering these oxygens is that O1P is on your right when looking in the O5′–P–O3′ direction with the phosphorus atom at the top of the arch), are nonequivalent due to the tight configuration of the 12-membered backbone ring. This is despite the fact that the bond lengths are quite normal with the following average values: O3–P (1.61 Å), O5–P (1.59 Å), P–O1P (1.50 Å), and P–O2P (1.50 Å). Here the O5′–P–O1P and O3′–P–O2P angles are expanded to values of 111.0° and 110.6°, respectively, definitely larger than the commonly observed value of 108° (Taylor & Kennard, 1982a). In addition, the other pairs (O5′–P–O2P and O3′–P–O1P) have somewhat smaller angles with values of 105.5° and 105.8°, respectively. Thus, there is a considerable difference between the two values (5°). All other angles appear to be normal (e.g., C3′–O3′–P, C5′–O5′–P, O3′–P–O5′, and O1P–P–O2P angles are 120.3°, 118.5°, 104.1°, and 119.0°, respectively), suggesting that there is a concerted and symmetrical twisting of the O1P and O2P atoms with respect to the

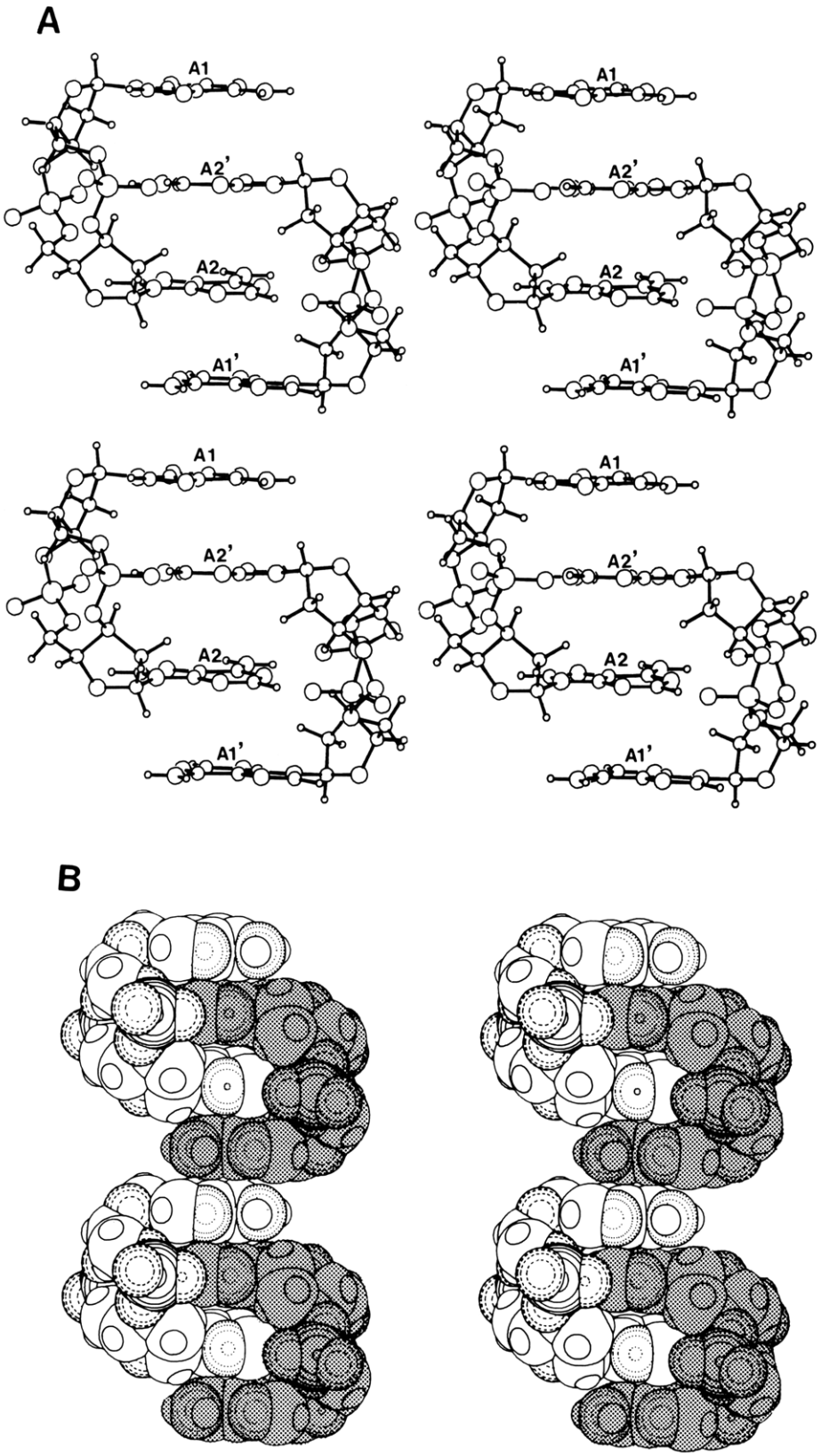

FIGURE 2: Stereo diagrams of end-over-end stacked self-intercalated cyclic dimers of molecule A. (A) ORTEP drawing showing the orientation of the adenine bases with respect to the opposing 12-membered rings. The intercalated molecules labeled with A1′ and A2′ are related to the main dimers (A1 and A2) by the symmetry operation $-X + 1, Y, -Z + 1$. The two pairs as related by a translation along the c axis. (B) Space-filling representation of the same dimers. The van der Waals radii emphasize the tight fit of the intercalated molecules. Oxygen atoms are drawn as dashed spheres, nitrogens as dotted spheres, phosphorus atoms with concentric circles, and carbons and hydrogens as open spheres. The symmetry-related molecules of each pair are shaded.

phosphorus atom. This distorsion is likely to be an intrinsic property, arising as a consequence of the 12-membered ring system, since all four phosphate groups exhibit the same changes even though they are in quite different environments.

Inspection of the backbone torsion angles (Table II) reveals that they all fall within the allowed regions found by theoretical studies for polynucleotides (Kim et al., 1973). This suggests that the closure of the 12-membered ring does not introduce

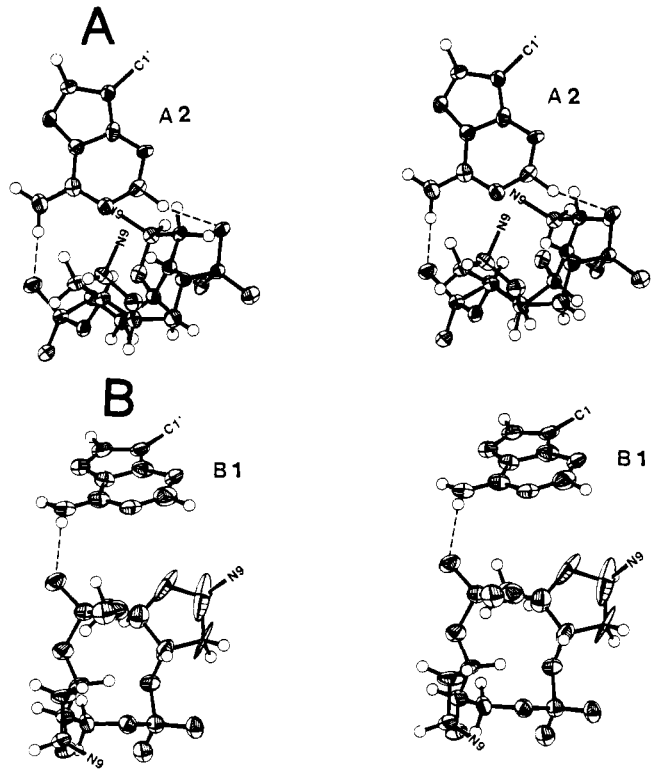

FIGURE 3: Stereo ORTEP representations of two base to backbone ring interactions. (A) Base A2 of a closed conformer hydrogen bonding to O2P of a symmetry-related ($-X + 1$, Y, $-Z + 1$) A2 backbone. This is a detailed view of the interaction shown in Figure 2. (B) Base B1 of an open conformer and its interaction with symmetry related ($-X + 1.5$, $Y - 0.5$, $-Z + 2$) O1P. In both panels hydrogen bonds between the two molecules are drawn with dashed lines.

any significantly unfavorable stress into the molecule. In fact, most torsion angles are not very different from those of the A-DNA double helix except those associated with the phosphodiester linkages α and ζ. In a right-handed double-helical polynucleotide, those two angles (α/ζ) are in general in the gauche(−)/gauche(−) region so that the complementary bases can form base pairs. In left-handed Z-DNA double helices, the repeating dinucleotide unit uses a combination of gauche(+)/gauche(−) and (near)trans/gauche(+) for the CpG and GpC steps, respectively (Wang et al., 1979, 1980). In both cyclic d(ApAp) molecules, the α/ζ conformation is gauche(+)/gauche(+), which is one of the seven allowed conformations based on the calculations combining ab initio and classical potential energy methods (Kim et al., 1973). This conformation, which has been found to occur in three other oligonucleotides (UpA, A^+pApA, and ApA) (Sussman et al., 1972; Suck et al., 1976; Einspahr et al., 1981), is prohibited in the construction of continuous helical polynucleotide chains. In fact, the backbone torsion angles of the cyclic d(ApAp) are remarkably similar to those of the three oligonucleotides described above, as can be seen in Table II. By examination of these four molecules, it can be seen that the two ends (3′ and 5′) of the three linear nucleotides are in close proximity with their O3′–O5′ distances in the range of 3.4–4.0 Å compared to those found in the cyclic molecules of 2.52 (±0.02) Å. This suggests that in the 5′ triphosphate precursor of the cyclic dinucleotides the terminal triphosphate group can easily position itself near the 3′-hydroxyl group such that a phosphodiester bond can be generated by the dinucleotide cyclase.

Three of the four deoxyribose rings are in the C3′-endo conformation, which is less common for deoxyribose rings (see Table III). The deoxyribose ring associated with the disordered nucleotide, B2, is of the O4′-endo conformation with a pseudorotation angle of 76°. However, the glycosyl torsion angles associates with each molecule adopt very different conformations. Molecule A has an anti conformation with an averaged χ value of −179.0°, which is close to that found in an A-DNA double helix. On the other hand, molecule B is close to the high anti range with an averaged χ value of −105.8°, which is similar to that found in B-DNA helices. This difference (73°) in the χ value produces a rather dramatic difference in the appearance of the two molecules. In molecule A, the two planar adenine bases "stack" over each other, with a C1′–C1′ separation of 6.8 Å. The two parallel bases point in the same direction away from the cyclic backbone. In molecule B, however, the two adenine bases point in opposite directions with their C8 atoms pointing their corresponding hydrogens toward the center of the circular backbone ring. These arrangements of the adenine bases have the net effect that molecule A looks quite compact, while molecule B is extended and open.

Intermolecular Interactions. There are a number of different interactions including base–base stacking, ribose–base stacking, base pairing, base–phosphate hydrogen bonding, and metal ion–phosphate interactions found in this crystal lattice. This extended network of interactions provides the stability of the lattice and explains the high-resolution diffracting nature of the crystals. In addition, they provide a series of high-resolution structural models for possible nucleic acid interactions other than the more generally seen base-pairing and base–solvent hydrogen bonds of double-helical DNA structures.

The two independent molecules utilize completely different sets of interactions to maintain their individual conformations. Molecule A (closed conformer) uses one of its two parallel adenine rings to intercalate into a 2-fold symmetry related molecule A in which the two adenines are separated by 6.8 Å (see Figure 2). This mutually self-intercalated dimer is stabilized by two hydrogen bonds (2.95 Å) from N6 of adenine A2 to O1P of a 2-fold related (A2′) residue. This dimer is

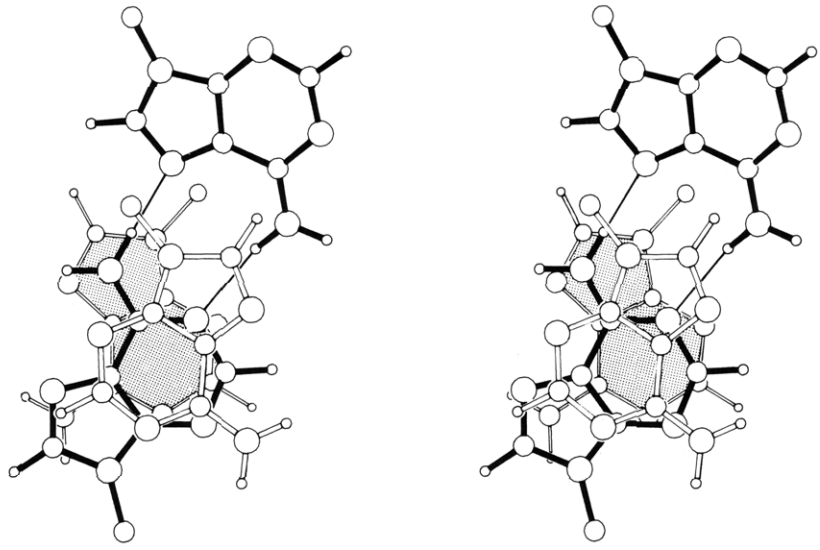

FIGURE 4: A-A base pair between one base from both the open and closed cyclic dimers. The bonds of the paired bases are drawn with solid lines, while the hydrogen bonds between base B1 and base A1 are drawn as dashed lines. Also included are the adenines from closed conformers stacked both above and below the plane. The lower base is shaded, and the upper is drawn with open bonds.

stacked end-over-end on another dimer of the next unit cell along the *c* axis, which produces an infinite column of stacked dimers. This self-intercalated dimer of two cyclic d(ApAp) molecules leaves two adenine rings (A2 and A2′) shielded from solvent with their N1 and C2 atoms facing toward the 12-membered sugar–phosphate ring of the opposite molecule. This orientation of the adenine ring leading to the base N6 to phosphate oxygen hydrogen bond as well as the potential interaction of the C2 atom with the symmetry-related backbone ring is clear in Figure 3A. The distance of the adenine C2 hydrogen atom to the O1P oxygen atom is 2.25 Å, which is shorter than the sum of the van der Waals radii of hydrogen and oxygen atoms. This might be considered a weak C-H–O hydrogen bond similar to C8-H–O bonds that have been observed in other nucleotide structures (Taylor & Kennard, 1982b). This may contribute to the stability of the dimeric interaction seen here. The very tight fit of these self-intercalated molecules is obvious in the van der Waals representation of Figure 2B. The intercalated base fits snugly between two other bases with the distal end shielded behind the backbone phosphate groups. Only the N3 positions are free for interaction with other molecules. The N1 atom of each A2 adenine base is in close contact with the two opposing deoxyribose C3′ hydrogen atoms (2.69 and 2.58 Å, respectively).

The clear N6–O1P hydrogen bonding resulting from an almost perpendicular orientation between the backbone ring and the base is detailed in Figure 3A. The N–O distance is 2.95 Å, and the N6–HN6–O2P angle is 167.7°, both well within accepted hydrogen-bond limits. Another interaction of this type, i.e., between an N6 amino group from the adenine base and phosphate oxygen from a different molecule, is also found in this crystal lattice as shown in Figure 3B. Here, one base (B1) of the open conformation dinucleotide is shown hydrogen bonding with a neighboring backbone ring of another symmetry-related open molecule. This interaction helps hold columns of dimers together in the overall crystal packing. Here, the orientation angle is different than was seen in Figure 3A, with the base being more nearly parallel to the plane of the 12-membered ring. This perhaps leads to a weak dipole interaction of the adenine ring and a ribose moiety underneath the adenine. In this case the N–O distance is 2.87 Å, again well within accepted limits; however, the N6–HN6–O1P angle is somewhat small (127.7°).

The base B1, shown in Figure 3B, is also hydrogen bonded to the other adenine ring (A1) of the closed conformer shown in Figures 2 and 3A. These two bases are paired through an unusual hydrogen-bonding scheme in which N1 and N6 of A1 form H-bonds with N6 and N7 of B1, respectively. (This is the same N6 of B1 that is H-bonded to the phosphate oxygen in Figure 3B.) The N1–N6 distance is 3.08 Å, and the corresponding angle around the HN6 is 154.0°; the N6–N7 distance is 2.89 Å with an angle at the N6 hydrogen atom of 176.8°. The two adenines are not coplanar, having a dihedral angle of 22°. Figure 4 shows these two hydrogen-bonded bases as well as the bases from the closed conformer that are stacked above and below the paired base A1. Although the relative orientations of these three bases are such that the functional groups are all pointing in different directions, all three 6-membered rings are almost perfectly stacked. They exhibit the characteristic amino–imidazole stacking interaction seen in the crystal structures of other adenosine nucleosides (Bugg et al., 1971; Einspahr et al., 1981). These tight interactions contribute to the high degree of stability of the structure.

Both bases from the closed conformer are involved in hydrogen-bonding interactions and van der Waals interactions with other molecules. In addition, one base from the open conformer (B1) is also involved in both types of interactions. The hydrogen bonding has been described in Figures 3 and 4, but there is also a close van der Waals interaction with ribose moieties from closed conformers both above and below the plane of the adenine ring. Figure 5 demonstrates in both ORTEP (Johnson, 1967) and space-filling diagrams how this base (B1) is sandwiched between these two ribose groups. The O4′ from the ring above is pointing directly down into the center of the imidazole ring of the adenine. Such dipole interactions of oxygen atoms with aromatic ring π electron clouds have been seen in a number of small nucleic acid structures (Bugg et al., 1971). In addition, this type of O4′–aromatic π interaction is the predominate feature of the CpG step in Z-DNA, which has very little base–base stacking interaction (Wang et al., 1979). Similarly, this is also believed to be an important force in stabilizing the interactions between minor groove binding compounds, i.e., netropsin, distamycin, and Hoechst 33258, and the DNA double helix, as described in detail elsewhere (Teng et al., 1988).

The last base (B2) of the open conformer is stabilized in its position mainly by van der Waals interactions. It is partially

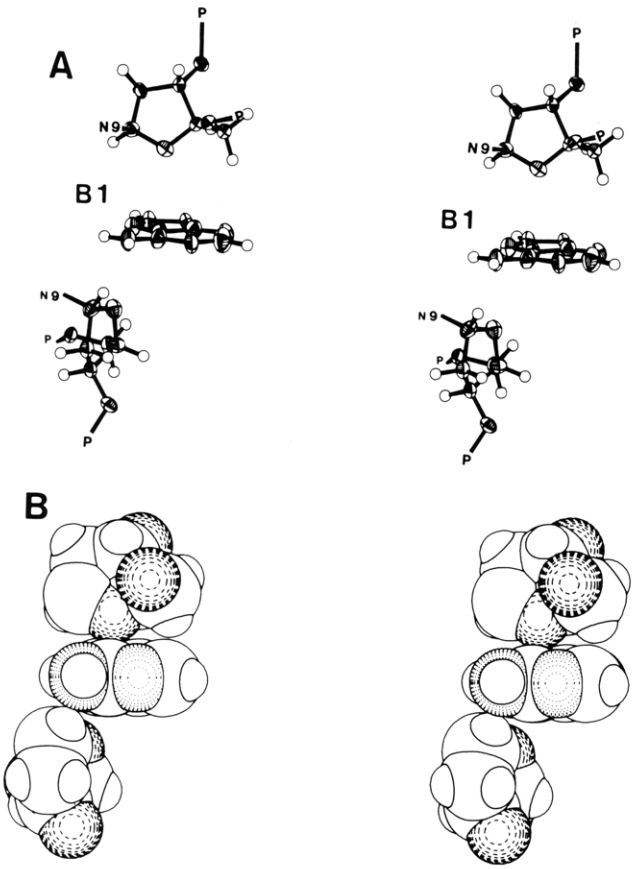

FIGURE 5: Adenine base B1 sandwiched between two ribose rings. (A) Stereo ORTEP representation of the atoms of base B1 from an open conformer with the ribose moieties from the two adjacent molecules above and below it. (B) Van der Waals diagram with oxygens as dashed spheres, nitrogen as dotted spheres, phosphates as concentric circles, and carbons and hydrogens as open spheres.

stacked on a symmetry related (B2′) base with its functional groups exposed to the solvent region. In contrast to the other three bases, all of the nitrogen atoms in B2 have hydrogen bonds only to solvent molecules. Therefore, all the intermolecular interactions involving B2 bases are solely van der Waals interactions, which may explain the more disordered structure associated with this base. This stacking is shown with two different representations in Figure 6. The space-filling diagram emphasizes the proximity of the N6 amino group of one base to a phosphate group of the symmetry-related molecule. This could help to account for the lack of planarity of this group with the rest of the base.

Magnesium Complex Interactions. There are two independent hydrated magnesium ion complexes associated with these adenine dimers that exactly neutralize the four negatively charged phosphate groups. Although each ion utilizes one phosphate oxygen as one of its coordination ligands, the relative positions of the two complexes with respect to the two dimer forms are very different. They are both illustrated in Figure 7. Complexes of the first type each interact with only one column of cyclic dimer molecules, while complexes of the second type are involved in holding adjacent columns together. In figure 7A the two parallel bases are from a closed conformer. Two of the water ligands are hydrogen bonding to N3 and N7 of these bases. As mentioned previously, one phosphate oxygen from this backbone ring provides the final magnesium ligand but, in addition, a third water ligand is hydrogen bonded to the other free oxygen of this same phosphate group. The two remaining associated water molecules are also each hydrogen bonded to one phosphate oxygen of two other symmetry-related open conformers. Finally, one of these last water molecules is also making a hydrogen bond with the N7 of a base (B2) from one of these open molecules. Thus, it is this magnesium pentahydrate and its symmetry-related complexes that in Figure 7 appear to be tucked next to the stacked bases of the closed dimers and behind the extended bases (B2 and B2′) of the open form. These complexes are linked in a continuous chain, through hydrogen bonds between symmetry-related ligands, that extends the length of the *c* axis of the crystal.

The other hydrated magnesium complex, shown in Figure 7B, forms hydrogen bonds through three of its water ligands to free oxygens of three different sugar–phosphate backbone rings. One other ligand is forming a hydrogen bond to a backbone O3′ of the same ring that supplies the phosphate oxygen for direct coordination to the magnesium. Finally, the last water ligand is hydrogen bonded to N1 of a base of an open conformer (B1). The symmetry-related copies of this complex appear at the edges of the columns of stacked dimers. They are coordinated to the other free oxygen of the same phosphate group to which the first complex is also coordinated. In addition, one of the other interactions between a coordinated water and a free phosphate oxygen is to a backbone ring of a neighboring closed conformer. By examination of all the hydrogen-bonding interactions for the two magnesium complexes as well as for the four adenine bases, it can be seen that, as expected for such a well-ordered structure, most possible interactions are satisfied, leaving only small regions of disordered solvent in the crystal lattice.

The detailed interactions surrounding these two hydrated magnesium ions allow us to visualize how a magnesium ion can be placed in a very tight space that is surrounded by several negatively charged phosphate groups and thereby neutralize the charge. Magnesium 1 is surrounded by three phosphate

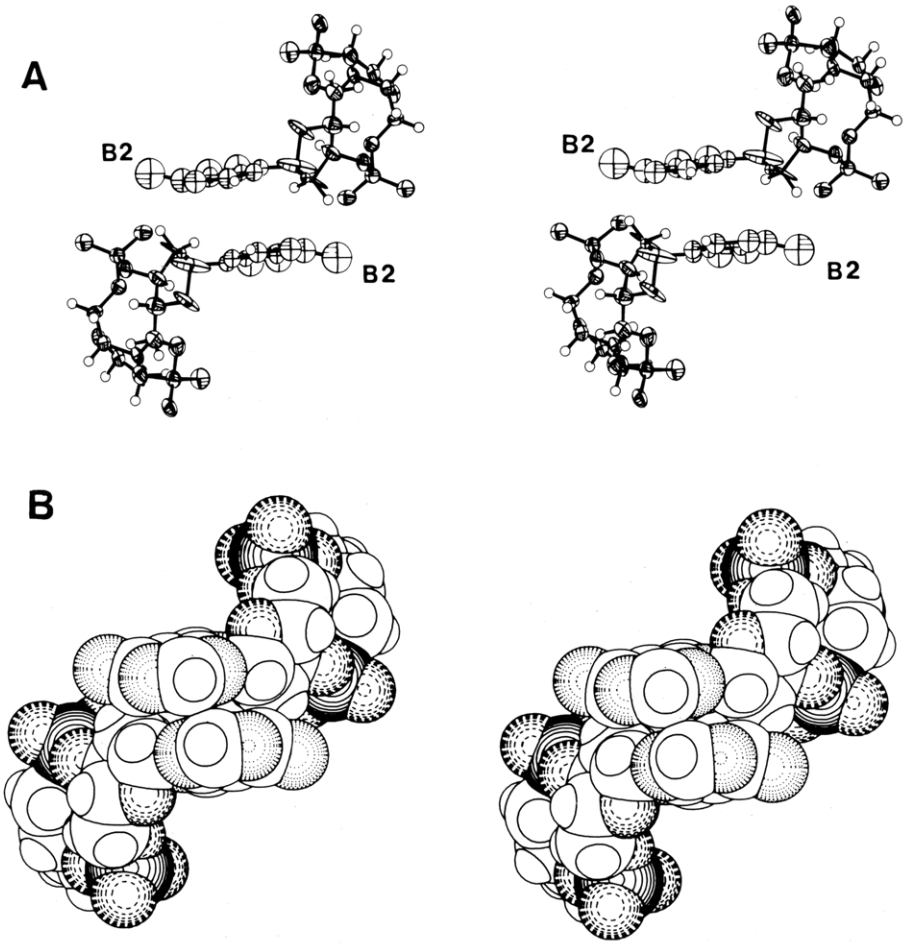

FIGURE 6: Stacking of two bases, B2, from open conformers. The two molecules are related by 2-fold symmetry ($-X + 1$, Y, $-Z + 2$). (A) Stereo ORTEP representation. (B) Space-filling van der Waals diagram.

groups (Figure 7A), while magnesium 2 is buried in a small hole with four phosphate groups around it (Figure 7B). This type of interaction is apparently important in many macromolecular nucleic acid structures. For example, in the yeast phenylalanine tRNA crystal structure, four magnesium hydrate ions are found in pockets created by nonhelical polynucleotide backbone segments, and they serve to stabilize the loop structure of the tRNA molecule (Jack et al., 1977; Holbrook et al., 1977; Quigley et al., 1978).

Discussion

The structure of this cyclic dinucleotide provides information about a new type of small molecule metabolic effector as well as a possible model for certain nonhelical or single-stranded nucleic acid structures. While double-stranded base-paired nucleic acids must utilize certain preferential combinations of angular configurations in order to preserve their overall secondary structure, single-stranded regions are subject to much fewer restraints and therefore are much harder to predict. NMR studies have been used to describe small hairpin structures and to provide information about the dynamics of loop formation in solution (Haasnoot et al., 1986). Also, attempts have been made to infer the structure of oligomers of this type from crystallographic studies of nucleosides and short (1–3 base) nucleotide units. These structures have provided evidence for a broad range of configurations about the phosphodiester linkage, some of which could be used to generate turns as are actually seen in the present structure. Here, the DNA molecule turns back on itself within just a single base step. In addition, this structure demonstrates that closure of the sugar–phosphate backbone into a ring does not require serious distortion of the torsional angles elsewhere along the chain. However, the fact that these two independent molecules have almost identical 12-membered rings despite very different orientations of the adenine bases implies that the considerable steric constraints strongly favor this particular cyclic conformation.

One direct consequence of this constraint is that for all four bases in the cyclic dimers the α/ζ angular configurations are gauche(+)/gauche(+). On the basis of energy calculations with individual nucleotides, this is one of the allowed configurations at the phosphorus atom; however, it is not one that is possible in double-helical DNA. Such a combination of α/ζ angles would disrupt the direction of the helix axis and, therefore, would be perfect for turns in nucleic acid molecules with more complicated secondary structures. The fact that this angular combination is seen here in these circular molecules provides evidence that, when necessary, nucleic acid molecules will adopt whatever energetically allowed conformations they can. Such adaptations may be required for certain regulatory regions where constrained nucleic acid structures, i.e., turns, hairpins, bulges, or small loops, may be involved in the interaction of a particular segment of nucleic acid with other macromolecules. These circular nucleotides therefore represent an interesting class of molecules that may resemble altered DNA structures as mentioned above, which are proposed to occur during the initiation or regulation of transcription or other nucleic acid processing. Small circular RNA molecules have been implicated in a variety of cellular

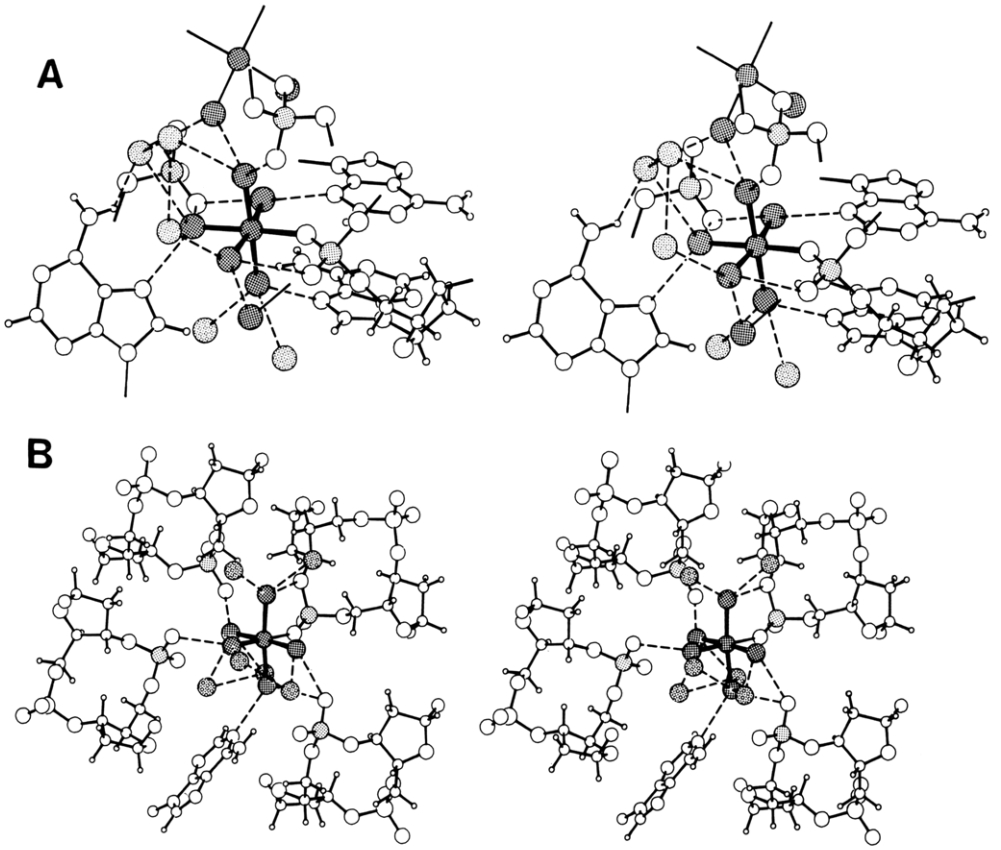

FIGURE 7: Hydrogen-bonding environments of two types of magnesium pentahydrate complexes found in the crystal lattice. (A) Magnesium 1 nestled against bases of the closed self-intercalated conformers and surrounded by three phosphate groups and one open conformer base. (B) Magnesium 2 coordinated between neighboring columns of dimers. In both panels the Mg ion and its water ligands are shown with dark shading, and the phosphorus atoms and additional water molecules are shown with lighter shading. The hydrogen-bonding interactions are drawn with dashed lines.

reactions (Cech & Bass, 1986), and it is also possible that these circular molecules may provide hints as to what structural features the intermediates of such processing reactions have in common. Along these lines, it will therefore be interesting to determine the structures of more cyclic molecules of larger sizes.

Several interesting intermolecular interactions result from the compact arrangement of molecules in this crystal lattice. The lattice is held together by hydrogen bonding between bases from adjacent molecules as well as ionic interactions between columns of dimers, mediated through bound Mg^{2+} ions in a manner that could also be used to help condense large cellular nucleic acid molecules. Nonconventional intermolecular adenine–adenine base pairs that utilize symmetrical N6–N1 hydrogen bonds were seen in the structure of the Ca^{2+}(ApA) anion (Einspahr et al., 1981). The more unusual adenine–adenine base pairing seen in this structure is similar to that found in the complex of adenosine 5′-monophosphate with a synthetic platinum-containing intercalator, chloro(pyridine)-platinum(II) (Wong & Lippard, 1977), and it represents another possible mode of interaction for longer stretches of single-stranded or otherwise nonhelical adenines. The stacking of adenine bases also provides another example of the previously noted tendency for the N6 amino groups to interact with an imidazole ring (Bugg et al., 1971; Einspahr et al., 1981), lending support to the idea that this hydrophobic interaction may be favored in larger structures as well. In the current structure this adenine stacking results from a novel method of self-intercalation between two cyclic dimers. This introduction of one adenine ring between two base planes may occur in single-stranded nucleic acid regions, as has been observed in tRNA structures (Rich et al., 1979).

The method of intercalation seen in this structure can be compared to what has previously been observed for certain DNA–drug complexes. The structures of two bisintercalator antibiotics, triostin A and echinomycin, have been solved bound to short DNA oligomers (Wang et al., 1984; Ughetto et al., 1985; Quigley et al., 1986). These molecules are able to bind to DNA by bracketing two base pairs, due to their rigid octadepsipeptide backbone conformations that provide both the proper separation distance of aromatic quinoxaline groups and the flexible linkers (ester bond in quinoxaline antibiotics) connecting these groups to the backbone. The closed conformer of the cyclic dimer resembles a bisintercalator, but it differs in that the fixed distance geometry provided by the rigid 12-membered sugar–phosphate backbone ring is such that the molecule is well suited for nearest-neighbor intercalation, i.e., to bracket only one base pair. This type of nearest-neighbor bisintercalator has been synthesized with limited success (Atwell et al., 1985).

The present structure allows us to contemplate a new class of synthetic molecules that has the property of bisintercalation coupled with different affinities and specificities toward DNA. The tight fit of one adenine ring against another backbone ring allows speculation concerning the construction of other molecules that might interact with DNA helices by this method. Substitution of different functional groups, i.e., other purine derivatives, in this molecule may enhance binding. Alternatively, addition of other moieties may introduce new activities in close proximity to the DNA backbone. Further, it may be possible to engineer molecules with increased specificity for interaction in either the major or minor groove.

While this structure allows much speculation about models for nucleic acid secondary structure, several biological functions have been found for the ribo form of these cyclic dinucleotides. It has been demonstrated that cyclic r(UpUp) and r(ApUp) are effective inhibitors of *Escherichia coli* RNA polymerase during the initiation of transcription (Hsu & Dennis, 1982; Hsu et al., 1985) and, as mentioned above, that r(GpGp) is also involved in the regulation of the membrane-bound enzyme cellulose synthase. Although we have provided evidence for the existence of two conformers, we do not know which form an enzyme might recognize in solution. In a cyclic ribodinucleotide molecule, the two O2′ hydroxyl groups could be easily accommodated into the deoxyribose rings with the present conformation. The OH groups would present no steric hindrance at all, and they in effect should stabilize the C3′-endo sugar conformation.

Therefore, for example, it is not unreasonable to believe that the endogenous effector for cellulose synthesis, cyclic r(GpGp), has a very similar conformation to what we have observed here. It is possible that the closed conformer could interact with the protein by holding an aromatic amino acid side chain in a rigid conformation between its stacked guanine rings in much the same manner as an intercalated base. The biological activity that has been observed for other guanine derivatives (unpublished results) could result from a specific hydrogen-bonding interaction between the guanine residues and amino acid side chains of the synthase enzyme. In addition, it has been shown that *R* and *S* substituted monothioester derivatives of cyclic dinucleotides show very different abilities to activate the enzyme. Specifically, the *S* forms are still active while the *R* forms are not. In this context it is important to mention that the *R* form, which corresponds to substituting O1P, replaces the phosphate oxygen that would otherwise be available for binding to Ca^{2+} ions, an essential component for full activity. In our structure these are the oxygen atoms that are pointing away from the center of the backbone ring and are interacting with the magnesium ions to help stabilize the lattice.

Finally, the structure of this cyclic d(ApAp) molecule in solution has been determined independently from NMR data with subsequent energy minimization (Blommers et al., 1988). The structure seen under NMR conditions in high nucleotide concentrations appears to be the closed conformer that we refer to as molecule A. In general, the two methods have provided very consistent results in terms of distances and torsion angles. The only major differences is in the exact way in which the self-intercalated closed conformers come together in the two studies. In the crystal the mode of interaction is fixed, but in solution the dimers may come together with the adenine bases stacked at a number of orientations relative to each other. The existence of the form we have described can be probed by monitoring a potential NOE interaction between the deoxyribose C2′ hydrogen and the adenine C2 hydrogen from an intercalated base. In our structure they are just 2.5 Å apart, well within a detectable distance.

ACKNOWLEDGMENTS

We thank N. Usman for discussions and suggestions and A. Rich for generous support and encouragement.

SUPPLEMENTARY MATERIAL AVAILABLE

Refined thermal factors, either anisotropic or isotropic, for each atom and all bond distances and angles for the four adenine nucleotides (8 pages). Ordering information is given on any current masthead page.

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Solution Structure of the 3′-5′ Cyclic Dinucleotide d<pApA>. A Combined NMR, UV Melting, and Molecular Mechanics Study

M. J. J. Blommers,[‡] C. A. G. Haasnoot,[‡,§] J. A. L. I. Walters,[‡] G. A. van der Marel,[∥] J. H. van Boom,[∥] and C. W. Hilbers*,[‡]

Laboratory of Biophysical Chemistry, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands, and Gorlaeus Laboratories, State University Leiden, Einsteinweg 5, 2300 RD Leiden, The Netherlands

Received April 14, 1988

ABSTRACT: The 3′-5′ cyclic dinucleotide d<pApA> was studied by means of ^1H and ^{13}C NMR experiments, UV-melting experiments, and molecular mechanics calculations. The ^1H and ^{13}C NMR spectra were analyzed by means of 2-dimensional NMR experiments. *J*-Coupling analysis of the 1D and 2D ^1H and ^{13}C spectra was used to determine the conformation of the ring systems in the molecule. It appeared that at low temperature (283 K) the deoxyribose sugars adopt a N-type conformation. The geometry is best described by an intermediate between the ^3_2T and ^3E forms. In addition, we were able to derive all other torsion angles in the phosphate backbone ring system, i.e., α^+, β^t, γ^+, δ (=89°), ϵ^t, and ζ^+. When the molecule is subjected to an energy minimization procedure (using the program AMBER), the sugar ring system retains, practically speaking, the torsion angles found from the NMR experiments, while the torsion angles around the glycosidic bond adopt a value of 175° in the minimum energy conformation. UV-melting experiments indicate that two molecules can form a dimer in which the adenine bases are intercalated. The feasibility of this structure is indicated by molecular mechanics calculations. At higher temperatures the dimer is converted into separate monomers. In the monomer form the sugars exhibit S-pucker 20% of the time. Concomitantly with the conversion of the N- to the S-conformation, the torsion angles α and γ change.

Circular DNA and RNA molecules play an important role in molecular biology. Sometimes such molecules are formed as intermediates as is known, for instance, for cyclic RNA molecules (Cech & Bass, 1986; Tabak et al., 1987), and in other cases the circularity is the natural appearance of DNA and RNA, e.g., for plasmids, the DNA of some viruses and viroids (Lewin, 1983; Watson et al., 1987). The smallest conceivable cyclic oligo-DNA or -RNA molecules are the cyclic dinucleotides in which the nucleotide units are connected by 3′-5′ linkages as is illustrated in Figure 1. Recent studies have indicated that these small molecules may also play an important role in cellular reactions. Interesting examples are the compounds r<pUpU> and r<pApU>, which are effective inhibitors of the DNA-dependent RNA polymerase of *Escherichia coli* during the initiation phase of transcription (Hsu & Dennis, 1982; Hsu et al., 1985). A rather different activity is exhibited by r<pGpG>, for which it was reported that it acts as the natural activator of the enzyme cellulose synthase in *Acetobacter xylinum* (Ross et al., 1987). The analogous cyclic deoxyribonucleotide d<pGpG> was shown to be active as well during the cellulose synthesis (Benziman, private communication).

In the present paper the solution structure of d<pApA> is studied by means of high-resolution NMR spectroscopy, UV-melting experiments, and molecular mechanics calculations. The study of the deoxyribose was preferred over the ribose compound because the deoxyribose rings are more amenable to structural studies; adenine bases were chosen instead of guanine bases because the latter may easily form tetramers in solution, which would complicate the structural analysis. It was found that at low temperatures the deoxyribose rings of d<pApA> adopt a pure N-conformation, which is quite unusual for the deoxy type of sugar. In addition, at NMR concentrations, i.e., at about 5-20 mM concentrations, the d<pApA> molecules form dimers in which the adenine bases most likely are intercalated between one another. These results and the temperature-dependent behavior of this molecule are discussed. The presented study was set up and carried out independently from a crystal structure determi-

[‡] University of Nijmegen.
[§] Present address: Organon Int. B.V., Postbox 20, 5340 BH Oss, The Netherlands.
[∥] State University Leiden.

0006-2960/88/0427-8361$01.50/0