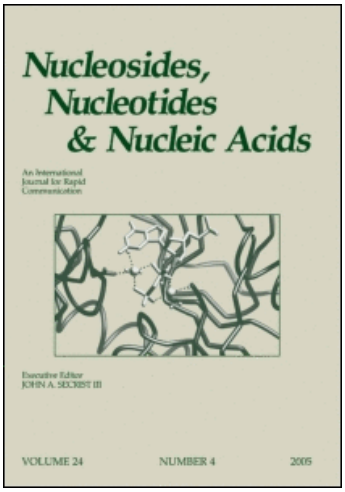

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Steven M. Graham[a]; Sarah C. Pope[b]
[a] Department of Chemistry, St. John's University, Jamaica, NY, U.S.A. [b] National Institute of Drug Abuse, Baltimore, MD, U.S.A.

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NMR SOLUTION STRUCTURE AND CONFORMATIONAL ANALYSIS OF THE CALCIUM RELEASE AGENT CYCLIC ADENOSINE 5′-DIPHOSPHATE RIBOSE (cADPR)

Steven M. Graham[1] and Sarah C. Pope[2,*]

[1]Department of Chemistry, St. John's University, 8000 Utopia Parkway, Jamaica, NY 11439
[2]Department of Chemistry, Oklahoma State University, Stillwater, OK 74078

ABSTRACT

A high-resolution NMR study of the solution structure of the calcium release agent cADPR has been performed. Pseudorotationals analysis reveals that in solution both sugar rings in cADPR adopt predominantly (∼75%) South conformations, with the A and N rings adopting approximately $^{2}T_{3}$ (C2′-endo(major)-C3′-exo(minor) and $^{4}_{3}T$ (C3′-exo-C4′-endo) conformations, respectively. The backbone torsion angles β and γ have also been determined. While the minor North conformers were not observed in the crystal structure of cADPR, the solution values of the major South conformers compare well to those found in crystal structure.

INTRODUCTION

Cyclic adenosine 5′-diphosphate ribose (cADPR) (1), along with nicotinate adenine dinucleotide phosphate (NAADP) (2) and inositol 1,4,5-trisphosphate (IP_3) (3) are known to cause calcium release from intracellular stores *in vivo*. Each of these molecules is formed as part of a signal cascade initiated by binding of an extracellular signal to a cell surface receptor. A feature shared by these small (molecular weight <750) organic molecules is they are all highly phosphorylated,

*Current address: National Institute of Drug Abuse, 5500 Nathan Shock Dr., Baltimore, MD 21224.

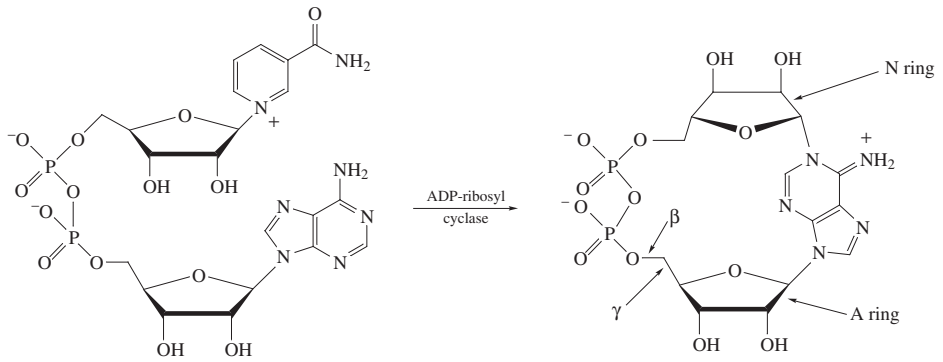

Figure 1.

suggesting that as a class low molecular weight polyphosphates may be important regulators of cellular function.

In biological systems, cADPR is formed from nicotinamide adenine dinucleotide (NAD) by the action of ADP ribosyl cyclase (Figure 1). The cyclase catalyzes a reaction whose end result is the loss of nicotinamide and formation of a new glycosyl bond between adenine and the ribose ring that formerly contained the nicotinamide moiety (the "N ring", Figure 1). In the initial studies (4,5) the exact nature of the new glycosyl bond was unclear, but UV spectroscopy (6) and x-ray crystallography (7) later established that the linkage was from the adenine-N_1 to the N ring $C_{1'}$ and that this bond had the β-configuration. The cyclase has a reasonably broad substrate specificity, and has been used to prepare cADPR analogs such as the corresponding cyclic triphosphates, along with analogs containing modified purine and/or ribose rings (8,9). As the number of cADPR analogs grew, we began to ask the question: is it possible to correlate the *solution structure* of these compounds with their *activity*? A logical starting point is then the determination of the solution structure of cADPR using NMR spectroscopy. In general, the literature NMR data for cADPR is of a quality insufficient for detailed structural analysis. One exception is an earlier report by Sekine (10), where it was found that lower temperature spectra (13°C versus room temperature) were of much higher quality. We had independently come to a similar conclusion. We then set out to improve the quality of the spectrum to the point that certain ambiguities in the earlier work regarding the ribose conformations could be resolved, and herein we report the results of our combined multi-pronged NMR and conformational analysis study.

RESULTS AND DISCUSSION

1D and VT Studies

The 1D ^{1}H NMR spectrum of cADPR in D_2O at several temperatures is shown in Figure 2. It was immediately apparent that the "room temperature" (27°C)

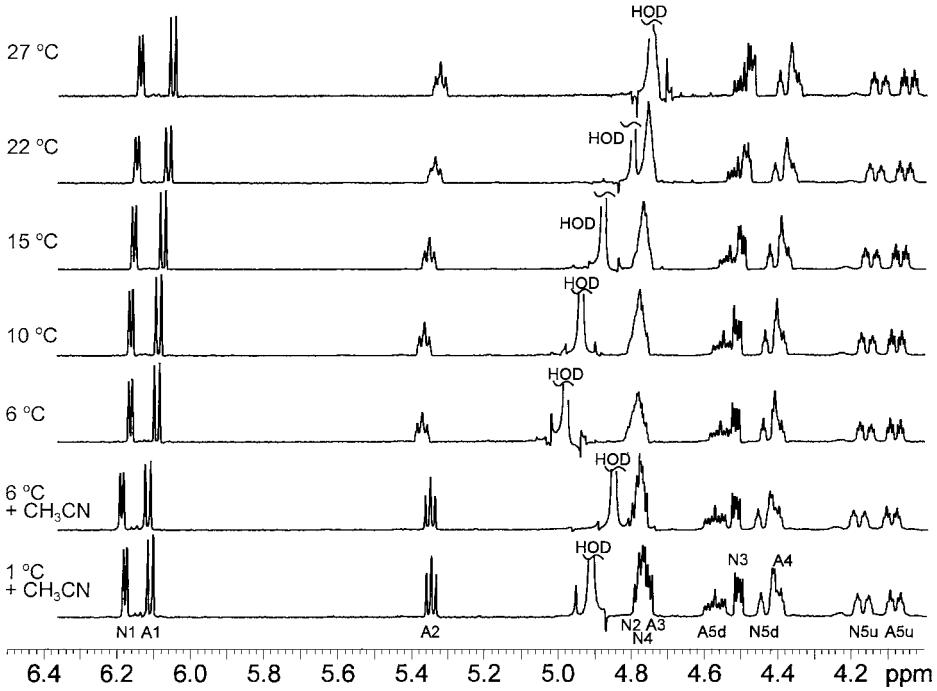

Figure 2. 400 MHz VT ^{1}H NMR spectra of the sugar ring portion of cADPR in D_2O and D_2O/CD_3CN. Assignments were made using COSY.

spectrum would be unsuitable for assignment of the sugar signals and subsequent conformational analysis. Most noticeable was the absence of three of the carbohydrate signals (14 non-exchangeable signals expected versus 11 observed by integration), but equally problematic was the lack of fine detail, needed to extract coupling constants, in the multiplet centered at approximately 4.35 ppm. As previously established (10), the missing signals were obscured by the residual HOD signal, and we undertook variable temperature (VT) experiments in an attempt to shift the position of the HOD signal. After gradually lowering the temperature to 6°C two significant improvements were noticed. First, the HOD signal shifted downfield by approximately 0.2 ppm, which led to the appearance of an ill-defined three-proton multiplet at 4.8 ppm. Second, the two-proton multiplet centered at ~4.5 ppm (27°C) became more dispersed, evolving into two separate, well-defined multiplets at 6°C.

In order to lower the temperature further, and to presumably continue the improvement of the appearance of the spectrum, an NMR solvent titration was performed. Addition of CD_3CN and continued lowering of the temperature produced further improvements, with the best results (bottom spectrum, Figure 2) obtained using an approximate 5:2 v/v mixture of D_2O/CD_3CN at 1°C. The improvement is significant in that now all signals are at least well enough resolved to proceed to the next step of the structure assignment process, namely assignment of the sugar ^{1}H signals using COSY.

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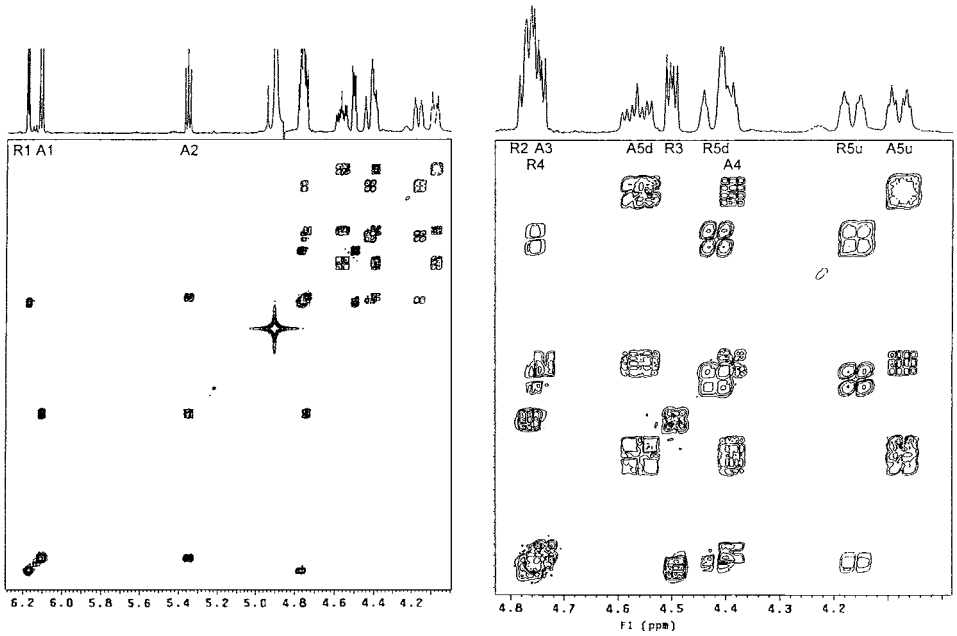

Figure 3. 400 MHz COSY spectra (1°C of the sugar ring portion of cADPR in 5:2 D_2O:CD_3CN.

COSY Assignments

The COSY spectrum of the $D_2O/CD_3CN/1°C$ sample of cADPR is shown in Figure 3. The assignment of sugar signals in ^1H NMR is greatly aided by the observation that the anomeric signals ($H_{1'}$) are usually the most downfield aliphatic signals and/or that the diastereotopic methylene signals ($H_{5'}$, $H_{5''}$ in riboses) are the most upfield. Given sufficient resolution, each ribose ring of cADPR (A or N, Figure 1) should show six cross peaks: $H_{1'}$–$H_{2'}$, $H_{2'}$–$H_{3'}$, $H_{3'}$–$H_{4'}$, $H_{4'}$–$H_{5'}$, $H_{4'}$–$H_{5''}$, and $H_{5'}$–$H_{5''}$ (11).

Our assignments, which are summarized in Table 1, are as follows: Of the downfield sugar signals, the ~6.18 ppm signal was assigned to $H_{N1'}$ (i.e., the $H_{1'}$ of the N ring) and the ~6.11 ppm signal to $H_{A1'}$ (5,10). The $H_{A1'}$ signal correlates to the $H_{A2'}$ signal at ~5.36 ppm; as $H_{A2'}$ is an apparent triplet in the 1D spectrum, $J_{A1',A2'} \sim J_{A2',A3'}$. Expansion of the COSY spectrum reveals that $H_{A2'}$ correlates with the upfield portion of the three-proton multiplet at 4.80–4.70 ppm, thus revealing $H_{A3'}$. The $H_{A3'}$ signal leads to $H_{A4'}$, which is the upfield portion of the two-proton multiplet at ~4.40 ppm. Finally, $H_{A4'}$ shows cross peaks to both the upfield (12) (H_{A5u}, ~4.08 ppm) and downfield (H_{A5d}, the seven peak pattern at ~4.57 ppm) signals of the A ring methylene group. The N ring analysis is complicated slightly by the near chemical shift coincidence of $H_{N2'}$ and $H_{N4'}$. Whereas Sekine (10) had found $H_{N2'}$ and $H_{N4'}$ to be chemical shift coincident (D_2O, 13°C), our $D_2O/CD_3CN/1°C$ sample of cADPR had $H_{N2'}$ and $H_{N4'}$ at least partially resolved ($\Delta\delta = 0.013$ ppm, or 5.2 Hz at 400 MHz). Careful analysis of the expanded COSY spectrum (Figure 3)

Table 1. Chemical Shift Assignments (δ, in ppm)[a]

	A Ring		N Ring	
	This work	Ref. 10	This work	Ref. 10
$H_{1'}$	6.11	6.08	6.18	6.14
$H_{2'}$	5.36	5.36	4.77	4.74
$H_{3'}$	4.75	4.76	4.50	4.49
$H_{4'}$	4.40	4.38	4.76	4.73
H_{5d}	4.57	4.53	4.43	4.41
H_{5u}	4.08	4.07	4.17	4.15
Other:				
	Ado H2	Ado H8		^{31}P
This work	9.08	8.45		-10.4 (d, $J = 14$ Hz) -11.7 (d, $J = 14$ Hz)
Ref. 10	8.97	8.38		-9.92 (d, $J = -14.5$ Hz) -10.67 (d, $J = -14.5$ Hz)

[a] See Figures 2 and 3.

reveals that $H_{N1'}$ correlates to the downfield portion of the three-proton multiplet at 4.80–4.70 ppm and that H_{N5u} and H_{N5d} correlate to the central portion of this multiplet.

Coupling Constants and Conformational Analysis

With a relatively well- resolved, assigned spectrum of cADPR in hand we were in position to extract the coupling constants needed for conformational analysis of the sugar rings and determination of the conformational equilibrium constants. The relationship between the observed coupling constants in sugar rings and the torsion angles required to produce these coupling constants has been elegantly described using the concept of pseudorotation (13,14). In this treatment, the conformation of a sugar is described in terms of a phase angle, P (a location on the pseudorotation wheel) and a sugar puckering amplitude, Φ_m. A two-state model is assumed (*vide infra*), from which it follows that a particular observed coupling constant is the weighted average of the respective coupling constants of the two rapidly equilibrating conformations. Analysis of a large number of nucleosides and nucleotides has shown that the majority of furanose rings adopt sugar puckers in the "North" (N, $P \sim 0^\circ$, C2′-exo-C3′-endo, or ${}^3_2\mathrm{T}$)[15] or "South" (S, $P \sim 180^\circ$, C2′-endo-C3′-exo, or ${}^2_3\mathrm{T}$) domains of the pseudorotation wheel (Figure 4). Statistically, the North domain centers about $P_N \sim 9^\circ$ and the South domain about $P_S \sim 162^\circ$ (13,14). The relationship between the observed coupling constants and the conformational equilibrium is described by Equation 1:

$$J_{a,b\ (obs)} = J_{a,b\ (N)}X_N + J_{a,b\ (S)}X_S \qquad (1)$$

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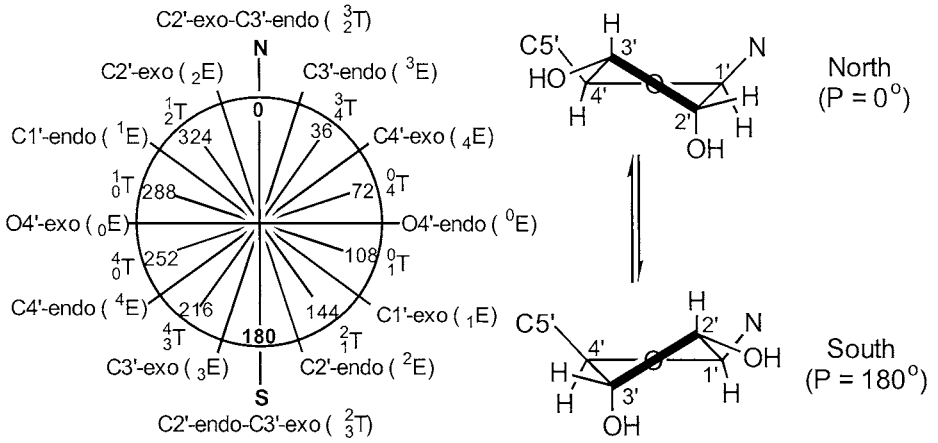

Figure 4.

where $J_{a,b\ (N)}$ and $J_{a,b\ (S)}$ are the particular coupling constants of the pure N and S conformers and X_N and X_S are the mole fractions of the N and S conformers. Given enough coupling constants, whose magnitudes are related to the furanose torsion angles, in principle one can calculate what equilibrium mixture of which two sugar puckers will lead to the observed coupling constants (16). The observed and simulated coupling constants for the A and N rings of cADPR ($D_2O/CD_3CN/1°C$) are summarized in Table 2. Briefly, the procedure was as follows: The $H_{N1'}$, $H_{N3'}$, and H_{N5u} signals were well resolved, and they provide enough information at least *estimate* all but one of the N ring coupling constants. Inspection of the $H_{N1'}$ doublet reveals $J_{N1',N2'} \sim 3.7$ Hz. $H_{N3'}$ is a doublet of doublets ($J = 4.9$, 2.9 Hz), giving the magnitude, but not the absolute assignments, of $J_{N2',N3'}$ and $J_{N3',N4'}$. H_{N5u} is an apparent doublet of broad triplets, with a large $^{2}J_{N5u,N5d}$ of ~12 Hz; the "triplet" portion of this signal is caused by the nearly similar values (2–3 Hz) of $J_{N4',N5u}$ and $^{3}J_{N5u-C5'-O5'-P}$. A similar treatment of the A ring coupling constants allowed estimation of all the coupling constants except $J_{A3',A4'}$. Next, spectral simulation was used to further refine the extracted coupling constants (17). The observed and simulated spectra are shown in Figure 5 (rms error = 0.2 Hz) and the chemical shift and coupling constant data are summarized in Table 1 and Table 2.

With the cADPR coupling constants now established, we turned our attention to the pseudorotational analysis—converting the coupling constants into descriptions of the ribose ring conformations. First, the decision was made to avoid using the simplifying approximation that the South-to-North ratio can be obtained from the ratio of $J_{1'2'}$ and $J_{3'4'}$ ($S/N = J_{1'2'}/J_{3'4'}$). Due to the novel, cyclic structure of cADPR, it seemed reasonable to expect that the A and N ribose rings might adopt geometries substantially different from the "typical" (18) values ($P_N \sim 9°$ and $P_S \sim 162°$) found in many nucleos(t)ides—and it is precisely these typical values from which the $S/N = J_{1'2'}/J_{3'4'}$ rule was derived (13,18). In other words, use of the $S/N = J_{1'2'}/J_{3'4'}$ rule *assumes* ribose conformations at or near values of

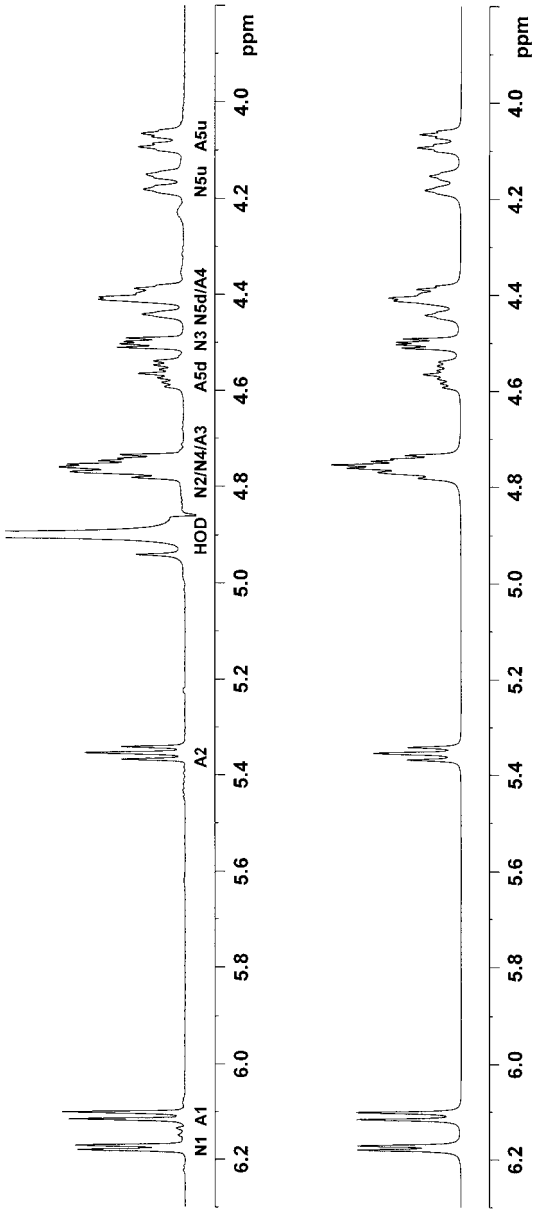

Figure 5. 400 MHz ^{1}H NMR spectrum of the sugar ring portion of cADPR for the 1° C D_2O/CD_3CN sample (upper) and the simulated spectrum (lower).

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Table 2. Observed and Simulated Coupling Constants (J, in Hz)

	A Ring			N Ring		
	Observed[a]	Simulated	Ref. 10	Observed	Simulated	Ref. 10
$J_{1'2'}$	5.6 ± 0.2 (3)[b]	5.9	5.6	3.7 (1)	3.6	3.7
$J_{2'3'}$	5.2 ± 0.2 (4)	5.0	5.1	4.9 ± 0.1 (2)	4.9	5.1
$J_{3'4'}$	2.8 ± 0.2 (4)	3.0	3.2	2.9 ± 0.1 (2)	2.8	2.7
$J_{4'5u'}$	2.8 ± 0.3 (5)	2.6	2.6	2-3[c]	2.9	2.2
$J_{4'5d'}$	7.2 ± 0.2 (6)	7.2	6.8	2-3[c]	2.6	2.2
$J_{5u'5d'}$	-10.9 ± 0.2 (6)	-11.0	-11.0	-12.0 ± 0.1 (2)	-11.9	-11.9
$J_{P'5u'}$	2.9 ± 0.2 (3)	3.0	3.4	2–3[c]	2.9	3.7
$J_{P'5d'}$	3.5 ± 0.2 (4)	3.5	3.9	2–3[c]	2.6	2.2

[a] Observed $\pm$ average deviation.
[b] Number of measurements.
[c] Value estimated from the half-height linewidth; confirmed by simulation.

$P_N \sim 9^\circ$ and $P_S \sim 162^\circ$, and only when this assumption is justified can the rule be used to estimate the N $\rightleftarrows$ S equilibrium reliably. Because of this limitation that we used Altona's method (13) to explore wide *ranges* of conformations in the North and South pseudorotational domains.

A summary of the procedure used is as follows: The Altona values (13) of $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$ calculated for conformationally pure nucleos(t)ide ribose rings were used to calculate the time-averaged coupling constants one would observe as P_N, P_S, and the N:S ratio were systematically varied. We then sought the best fit of these calculated coupling constants to the coupling constants extracted from the simulated cADPR spectrum. For a given ratio of N and S conformers a good fit was defined as one where **each** calculated J was within ± 0.3 Hz of the value determined by simulation. The results are summarized in Table 3. For each ribose ring of cADPR we explored combinations of $P_N = 342^\circ - 36^\circ$ and $P_S = 144^\circ - 234^\circ$, using puckering amplitudes of $\Phi_m = 35^\circ$ and 40° (16). One of the significant findings (*vide infra*) was that in solution both the A and N rings of cADPR each contained $\sim$25% of the North conformer – neither of which was observed in the crystal structure (7) – and that the N ribose ring P_S was shifted to an atypically high value of $\sim 216^\circ$.

For the A ring eight pairs of N/S conformers were found (Table 3, Entries 1–8). The fit of the calculated versus simulated coupling constants, expressed as the rms error, was quite good, ranging from 0.12–0.21 Hz. The coupling constants for the A ring ($\Phi_m = 35^\circ$) are consistent with an N:S ratio of 25:75 for P_N values of 351°–27° ($_2T^3$–$^3T_4$) and P_S values of 162°–180° (^2E–^2_3T). When the A ring Φ_m was increased to 40° all of the previous N:S populations were now excluded. Recovery of N:S populations for $\Phi_m = 40^\circ$ was possible if the ΔJ fit criterion was relaxed from ± 0.3 Hz to ± 0.4 Hz (not shown). Under this relaxed fit criterion, the N:S populations were essentially unchanged (70–75% S) although P_N and P_S occupied slightly wider ranges ($P_N = 342^\circ$–36° and $P_S = 162^\circ$–189°) and the average rms

Table 3. Comparison of Coupling Constants Calculated for Various N:S Populations of a Ribose Ring (13) to Those Extracted from Simulation of the $D_2O/CD_3CN/1^{\circ}C$ Spectrum of cADPR

	Entry	Φ_m	P_N, P_S	%S:%N	Calculated J's			rms[b]
					$J_{1'2'}$	$J_{2'3'}$	$J_{3'4'}$	
A Ring			**SIMULATED**		**5.90**	**5.00**	**3.00**	
	1	35°	351, 162	75:25	5.92	5.27	2.88	0.17
	2	35°	0, 162	75:25	5.92	5.25	3.00	0.15
	3	35°	9, 162	75:25	5.94	5.27	3.08	0.16
	4	35°	0, 171	75:25	5.75	5.12	2.79	0.16
	5	35°	9, 171	75:25	5.77	5.13	2.88	0.13
	6	35°	18, 171	75:25	5.81	5.18	2.94	0.12
	7	35°	27, 171	75:25	5.88	5.25	2.97	0.15
	8	35°	27, 180	75:25	5.63	5.21	2.85	0.21
	9	40°	None found[c]					
Averages, Entries	**1–8:**		**10,169**	**75:25**	**5.83**	**5.21**	**2.92**	**0.16**
From Ref. 10:	10	35°	9, 162	64:36	5.35	5.25	3.73	0.35
From Ref. 10:	11	40°	9, 162	64:36	5.62	4.57	3.75	0.44
N Ring		**SIMULATED**			**3.60**	**4.90**	**2.80**	
	12	35°	None found					
	13	40°	351, 216	70:30	3.72	5.14	3.05	0.21
	14	40°	0, 216	75:25	3.89	5.17	2.81	0.23
	15	40°	9, 216	75:25	3.89	5.19	2.89	0.24
Averages, Entries	**13–15:**		**0,216**	**73:27**	**3.83**	**5.16**	**2.91**	**0.23**
From Ref. 10:	16	35°	9, 162	59:41	4.90	5.23	4.23	1.13
From Ref. 10:	17	40°	9, 162	59:41	5.13	4.55	4.30	1.28

[a]Only N:S ratios that produced all calculated J's within $\pm$ 0.3 Hz of the values determined by simulation are shown.
[b]Rms error in Hz, calculated vs. simulated (this work) or calculated vs. observed (Ref. 10) coupling constants.
[c]No good fit could be found with this value of Φ_m; see text.

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error increased to 0.27 Hz. Analysis of the N ring revealed an opposite trend – no good fit with $\Phi_m = 35°$, whereas with $\Phi_m = 40°$ three pairs of N/S conformers were found (Table 3, Entries 13–15). The N:S ratios ranged from 30:70 to 25:75, with P_N spanning the range from 351°–9°($_2T^3/^3T_2$). Interestingly, the N ring P_S localized to 216°(^4_3T). The average rms error for theses three N:S pairs was 0.23 Hz. No populations of N:S conformers could be found for $\Phi_m = 35°$ until the ΔJ fit criterion was relaxed to ±0.5 Hz, and even this loosening of the fit revealed only a single N:S pair (N:S = 40:60, $P_N = 342°$, $P_S = 198°$) with a very large rms error of 1.12 Hz. In the published crystal structure of cADPR (7) the A ring was found to adopt a C2′-endo (major)-C3′-exo (minor) conformation ($P_S \sim 171°$, $^2T_3$); the N ring conformation was stated to be C3′-exo ($P_S \sim 198°$, $_3E$) with a C4′-O4′-C1′-C2′ torsion angle of +6°, which is probably better described as C3′-exo-C4′-endo ($P_S \sim 216°$, ^4_3T). Limiting our attention to the major solution conformers of the A and N rings ($P_S = 162$–$180°$ and 216°, respectively), we find the agreement between the crystal structure and our solution structure, at the current level of sophistication, to be quite good (19).

It is instructive to compare our pseudorotation parameters for the N ring of cADPR ($P_N = 351°$–$9°$, $P_S = 216°$, $\Phi_m = 40°$, N:S = 25:75) to those obtained by Sekine (10), who suggested that the N ring exists either as a 41:59 N:S mixture or that it exists in an undefined "flat" conformation. The Sekine proposal of a 41:59 N:S mixture for the N ring of cADPR is unlikely, for two reasons. First, the 41:59 N:S mixture was estimated using the $S/N = J_{1'2'}/J_{3'4'}$ rule (18). As discussed above, this rule is based on the assumption that P_N and P_S are at or near the statistical average values (9° and 162°, respectively). Using Sekine's N ring ribose coupling constants with $P_N = 9°$, $P_S = 162°$, and N:S = 41:69, we calculate ribose ring coupling constants with rms errors in excess of 1 Hz (Table 3, Entries 16, 17). Once P_N and P_S diverge from the average values $J_{1'2'}$ and $J_{3'4'}$ will change, and thus so will the S/N ratio. Second, our pseudorotational analysis revealed only one case where the N:S ratio was ~40:60 (*vide supra*), albeit under the relaxed fit criterion of $\Delta J = \pm 0.5$ Hz and with a poor rms fit of 1.12 Hz.

The alternative proposal that the N ring of cADPR is "flat" is also unlikely. It is well established that flattened ribose rings (small Φ_m) will show large values of $J_{2'3'}$ **and** small values of the sum $J_{1'2'} + J_{3'4'}$. Small Φ_m, large $J_{2'3'}$, and small $J_{1'2'} + J_{3'4'}$ are generally taken to mean smaller than 35°, larger than 5.2 Hz, and smaller than ~9 Hz, respectively (13,18). While the N ring of cADPR meets the last criterion ($J_{1'2'} + J_{3'4'} = 6.4$ Hz), the value of the N ring $J_{2'3}$ (4.9 Hz) is well within the range of normally puckered riboses for $P_N = 0 \pm 18°$ and $P_S = 180 \pm 18°$ of **any** N:S ratio. The observation that the sum $J_{1'2'} + J_{3'4'}$ is significantly different from ~9 Hz does not *by itself indicate an abnormally puckered ribose*; changes in $J_{2'3'}$ are also required. Indeed, deviations in the sum $J_{1'2'} + J_{3'4'}$ are usually attributed to shifts of P_N to values lower than ~9° and/or P_S to values higher than ~162°. This is exactly the trend suggested by our pseudorotational analysis.

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In fact, our data indicate that the cADPR N ring is slightly *more* puckered ($\Phi_m = 40^\circ$) than usual, not substantially *less*. Further evidence can be obtained from a more detailed analysis of $J_{2'3'}$, as this coupling constant is highly sensitive to changes in Φ_m. For a ribose of constant phase angle P, $J_{2'3'}$ always *increases* with decreasing Φ_m (increased flattening), except in the typically "disallowed" regions of $P \sim 90^\circ$ and 270°. For every 5° decrease in Φ_m, $J_{2'3'}$ increases by 0.55–0.72 Hz for $P_N = 0 \pm 36^\circ$ and $P_S = 180 \pm 36^\circ$(13). This analysis thus predicts that even a modest flattening of the N ring ribose to $\Phi_m = 30^\circ$ and a North population as high as 50% would yield a $J_{2'3'}$ of *at least* ~5.8 Hz for any combination of $P_N = 0 \pm 36^\circ$ and $P_S = 180 \pm 36^\circ$. As P_S tends towards the upper end of this range, the calculated $J_{2'3'}$ becomes even larger. Using our values from the N ring our pseudorotational analysis (average $P_N = 0^\circ$, $P_S = 216^\circ$, N:S = 25:75), we calculate an N ring $J_{2'3'}$ of ~6.3 Hz for $\Phi_m = 30^\circ$, which is substantially different than the observed value of 4.9 Hz.

The Sekine proposal of a flat N ring is based (1) their interpretation of the cADPR crystal structure, an interpretation which is almost certainly incorrect, and (2) their own ROESY data, for which alternate explanations are possible. In the cADPR crystal structure the N ring conformation was stated to be C3′-exo ($P_S \sim 198^\circ$, $_3$E), not flat, as Sekine says. An ideal $_3$E would have an endocyclic torsion angle C4′-O4′-C1′-C2′ (ν_o) of 0°; the observed ν_o torsion angle was $+6^\circ$, which shifts the conformation to $P_S \sim 216^\circ$ ($^4_3$T). A *single* endocyclic torsion angle near 0° is not evidence for a flat conformation (20). Further evidence for a flat N ring was the observation of weak ROESY cross peaks from the adenine ring H2 to $H_{N2'}$ and $H_{N3'}$, and the supposition that the A_{H2}–$H_{N3'}$ distance in a ^{2}E conformation and the A_{H2}–$H_{N2'}$ distance in a ^{3}E conformation is "too long" to observe an ROE. Implicit in this argument is the presence of only one conformer (^{2}E or ^{3}E) and that the N ring glycosidic torsion angle remains constant during N $\rightleftarrows$ S transitions. pseudorotational analysis clearly shows an N $\rightleftarrows$ S equilibrium, and the constrained nature of the cADPR macrocycle suggests that changes in ribose pucker will be transmitted to the glycosidic torsion angle. In our view, an N ring N:S ratio of 25:75 and a P_S of ~216 ($^4_3$T) can easily accommodate the ROESY cross peak intensities, and so neither the assertion that the N ring N:S ratio is ~40:60 (for *any* P_N and P_S) or that the N ring is unusually flat can be justified.

The observation of moderate populations of the North conformer in solution (and their absence in the crystal structure) raises an intriguing question: What is the biologically active conformation of cADPR? In the limiting case where one considers only the N $\rightleftarrows$ S equilibria of the A and N rings there are four possible structures, i.e., both rings could be S (A_SN_S), one could be S and the other N (A_SN_N or A_NN_S), or both could be N (A_NN_N). Using our 25:75 N:S ratios for each ring predicts an $A_SN_S : A_SN_N : A_NN_S : A_NN_N$ ratio of 56:19:19:6, any one of which could be the active conformer. Our solution data suggest that to a first approximation almost half (44%) of the molecules in solution *do not adopt the conformation found in the crystal structure*, demonstrating again that NMR solution data can be

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a valuable and necessary supplement to crystal structure data. This distribution is also based on the assumption that a conformational change in one ring does not *induce* a change in the other. We have not begun to address the relative energies of the various solution conformers, and additional studies with cADPR and analogs will be required before this idea can be pursued.

The coupling constant data has also been used to determine the backbone torsion angles β (P-O5′-C5′-C4′ via the ${}^3J_{\mathrm{P-H5'/5''}}$ coupling) (21) and γ (O5′-C5′-C4′-C3′ via the ${}^3J_{\mathrm{H4'-H5'/5''}}$ coupling) (22,23). For the A ring the coupling of the phosphorus to both H_{A5u} and H_{A5d} is quite similar (3.0 and 3.5 Hz, respectively), suggesting a symmetrical relationship. Reasonably assuming a staggered conformation about the β-bond leads to the conclusion that the phosphorus is *gauche* to both $H_{5'}$ and $H_{5''}$, and thus *trans* to C4′. Analogous behavior was observed for the N ring, and thus both the A and N ring β-bonds are likely to be almost exclusively ($>90\%$) *trans* (torsion angle $= \pm 180^\circ$). Sekine (10) came to a similar conclusion. The crystal structure β-bonds are in the same general range as our solution values, with an A ring β of -138° and an N ring β of $+160^\circ$. In the case of the N ring γ-bond (22,23) we observed near equivalence of $J_{\mathrm{N4',N5u}}$ and $J_{\mathrm{N4',N5d}}$ (2.9 and 2.6 Hz, respectively). Using the same logic as above gives thus places $H_{N4'}$ *gauche* to both H_{N5u} and H_{N5d}, i.e., the N ring C3′ and O5′ are *gauche* and the N ring γ-bond is ($>80\%$) γ^+ (torsion angle $= +60^\circ$). Using their coupling constant data, Sekine came to a similar conclusion, but their ROESY data led to the proposal that the N ring γ-bond has the γ^{t} conformation. Given that even small pertubations from the ideal γ^+ geometry could produce $H_{4'}$–$H_{5'}$ and $H_{4'}$-$H_{5''}$ ROESY cross peaks of differing intensity we see no compelling reason to assume an N ring γ^{t} conformation (24). Analysis of the A ring γ-bond is complicated because we have not yet made the stereospecific assignments of H_{A5u} and H_{A5d} to $H_{5'}$ and/or $H_{5''}$ (24,25). In the absence of the stereospecific assignments of H_{A5u} and H_{A5d} we prefer to interpret a "small" $J_{\mathrm{A4',A5u}}$ (2.6 Hz) and a "large" $J_{\mathrm{A4',A5d}}$ (7.2 Hz) coupling as evidence for approximately 35% γ^+ and either 65% γ^{t} ($\pm 180^\circ$) or 65% $\gamma^-(-60^\circ)$ (22–26). The solution values for the N and A ring γ-bonds (γ^+:$\gamma^{\mathrm{t}} \sim 80$:20 and $\sim$ 35:65, respectively, assuming negligible γ^-) differ from those found in the crystal structure (both γ^{t}, with the N ring ($\gamma = +174^\circ$ and the A ring $\gamma = -174^\circ$) (27). Besides crystal packing forces, another possible explanation is that the x-ray crystal structure was determined on the free acid of cADPR, whereas our solution structure is the potassium salt, and the ionization state of the phosphorus is likely to play a major role in determining at least some of the backbone torsion angles.

In conclusion, we have presented high-resolution NMR data supporting the following solution structure for cADPR: The A ring major conformation is approximately ${}^2\mathrm{T}_3$ ($P_{\mathrm{S}} = 171 \pm 9^\circ$), the N ring major conformation is approximately ${}^4_3\mathrm{T}$ ($P_{\mathrm{S}} = 216^\circ$), both ring β-bonds are *trans*, the N ring γ-bond is γ^+, and the A ring γ-bond is 35% γ^+ and either 65% γ^{t} or γ^-. Further work, especially modeling studies and NOE experiments to stereospecifically assign $H_{5'}$ and $H_{5''}$, will be reported in due course.

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EXPERIMENTAL

Synthesis: The potassium salt of cADPR was synthesized and purified as described (28), substituting ADP ribosyl cyclase (Sigma) for pig brain acetone powder.

NMR: ^{1}H (400 MHz) and ^{31}P (121.5 MHz) NMR spectra were recorded on a Varian INOVA spectrometer. Pure D_2O (> 99.8%) samples were referenced to the residual HOD peak in a temperature-dependent fashion as described (29). Samples run in D_2O/CD_3CN were referenced to the previous non-CD_3CN containing sample. ^{31}P NMR spectra were referenced to external 85% H_3PO_4 in D_2O. NMR samples were lyophilized three times from D_2O prior to final dissolution in D_2O. 1D NMR spectra were acquired for 2.7 seconds (32 transients) with a 5 second pulse delay, using a spectral width of 6000 Hz, 32K data points, and zero-filled to 64K. No solvent suppression was used and the data was processed with a Gaussian (0.1) and line broadening (−1.0 Hz) weighting functions. The COSY experiment was performed in absolute value mode using 16 transients per increment, 512 increments, with an acquisition time of 1.11 seconds, a 2.0 second delay, a spectral width of 3600 Hz, and zero-filled to 8K × 8K points. After sine bell weighting (0.3 seconds) in both dimensions, the data was symmetrized. ^{1}H NMR (D_2O/CD_3CN, 1°C) δ 9.08 (s, 1H, adenine H2); 8.45 (s, 1H, adenine H8); 6.18 (d, J = 3.7 Hz, 1H, $H_{N1'}$); 6.11 (d, J = 5.9 Hz, 1H, $H_{A1'}$); 5.36 (d, J = 5.4 Hz, 1 H, $H_{A2'}$); 4.77–4.76 (m, 2H, $H_{N2'}$, $H_{N4'}$); 4.75 (dd, J = 5.0, 2.9 Hz, 1H, $H_{A3'}$); 4.57 (ddd, J = −10.9, 7.3, 3.5 Hz, 1H, H_{A5d}; 4.50 (dd, J = 4.9, 2.9 Hz, 1H, $H_{N3'}$); 4.43 (app dt, J = −11.9, ∼2 Hz, 1H, H_{N5d}); 4.40 (app dt, J = 7.0, 2.8 Hz, 1H, $H_{A4'}$); 4.17 (app dt, J = −12.1, ∼3 Hz, 1H, H_{N5u}); 4.08 (dt, J = −11.1 2.9 Hz, 1H, H_{A5u}). ^{31}P NMR (D_2O/CD_3CN) δ 10.4 (d, J = −14 Hz); 11.7 (d, J = −14 Hz).

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16. Two points should be stressed. First, in the full treatment described in the literature, ${}^{3}J_{\mathrm{HH}}$ is a function of five variables - P_{N}, Φ_{N}, P_{S}, Φ_{S}, and X_{N} (or X_{S}, since $X_{\mathrm{N}} + X_{\mathrm{S}} = 1$). Deoxy sugars can be completely determined, as there are five observable values related to the endocyclic torsion angles- $J_{1'2'}$, $J_{1'2''}$, $J_{2'3'}$, $J_{2''3'}$, and $J_{3'4'}$. Ribo sugars, on the other hand, have only three coupling constants related to the endocyclic torsion angles - $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$. Ribo sugars are therefore "underdetermined" and assumptions concerning two of the variable must be made. In practice, ${}^{3}J_{\mathrm{HH}}$ shows relatively little dependence on the puckering amplitude, Φ (the variation in Φ is typically 1 Hz or less over most of its allowed range). In our study we have constrained Φ to two values, 35° and 40°. The second point concerns the dynamic equilibrium between N and S conformers. Strictly speaking, **all** N conformers exist in **one** potential energy well (i.e., there is a *continuum* of N conformers), and all S conformers exist in another. There **is** an energy barrier to N/S interconversion, but the barrier for "interconversions" **between** various N conformers (and likewise, between S conformers) is small or non-existent. See also reference 18.
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20. All ideal envelope conformations have one endocyclic torsion angle of 0°.

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24. There is some question as to the degree of certainty of Sekine's (reference 10) $H_{5'}$ and $H_{5''}$ assignments. First, the procedure for the stereospecific assignments was not detailed. It appears to have been based single pairs of ROESY cross peak intensities (e.g., comparison of the $H_{4'}/H_{5'}$ to the $H_{4'}/H_{5''}$ cross peak) and in no cases was a confirming pair of cross peaks observed. Second, the $H_{5'}$ and $H_{5''}$ labels appear to be reversed, at least according the IUPAC/IUB Joint Commission on Biochemical Nomenclature guidelines (Ref. 11). In fact, the Sekine ROESY data, if interpreted using the IUPAC/IUB JCBN rules, suggests the γ^- , not the γ^t conformation for the R ring γ bond.
25. We have not invoked the rule of Shugar and Remin – that $H_{5''}$ is typically the upfield signal and $H_{5'}$ is the downfield signal – as the cyclic and presumably conformationally restricted nature of the sugar-phosphate backbone in cADPR may render this observation invalid. See Remin, M.; Shugar, D. *Biochem. Biophys. Res. Comm.* **1972**, *48*, 636–642.
26. We have confirmed our "sum rule" estimate of the γ populations (reference 23) using the more rigorous formulae described in reference 22.
27. In the crystal structure, the N ring and A ring γ torsion angles were given as $+54^\circ$ and $+66^\circ$, respectively, both of which would be γ^+. However, the *figure* in this paper appears to show both γ-bonds as *trans*. It is likely that the γ-bonds were defined using an older system where the γ torsion angle is defined by O5′-C5′-C4′- O4′, which would explain the 120° shift. The values in our discussion above have been corrected to conform with the IUPAC/JCBN guidelines.
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