Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 5, pp. 581-586, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00048-6

CHARACTERIZATION OF CYCLIC ADP-RIBOSE AND 2'-PHOSPHO-CYCLIC-ADP-RIBOSE BY ^{31}P NMR SPECTROSCOPY.

Klaus D. Schnackerz*, Chinh Q. Vu#, David Gani$, Rafael Alvarez-Gonzalez+, and Myron K. Jacobson#
Theodor-Boveri-Institut für Biowissenschaften, Physiologische Chemie I, Universität Würzburg, Am Hubland, D-97074 Würzburg, Germany, #Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, and Lucille P. Markley Cancer Center, University of Kentucky, Lexington, KY 40536-0082, $School of Chemistry, University of St. Andrews, St. Andrews, Fife, KY16 9ST, UK, and +Dept. of Microbiology and Immunology, University of North Texas Health Science Center, Fort Worth, TX 76107, U.S.A.

Abstract: The ^{31}P NMR spectra of the Ca^{2+} mobilizing nucleotides cyclic ADP-ribose and 2'-phospho-cyclic ADP-ribose have been studied. Both nucleotides show doublets of doublets in the range -9.5 to -11 ppm for the pyrophosphate group. The P-cADPR exhibits a singlet in the 0 to +4 ppm range for the 2'-phospho group. The pH dependence of the ^{31}P signals shows transitions with pK_a values of 8.37 and 8.95 for cADPR and P-cADPR, respectively.

Introduction

Cyclic ADP-ribose (cADPR), a metabolite of NAD, is a potent calcium releasing agent which is believed to be a second messenger involved in the regulation of calcium homeostasis.[1-3] The synthesis of cADPR from NAD involves the cleavage of the nicotinamide from the ribose and cyclization of the ribose to the N-1 of the adenine moiety[4,5] (Fig. 1). In mammalian cells, cADPR is synthesized and degraded by a multifunctional enzyme that contains cADPR synthase, cADPR hydrolase, and NAD glycohydrolase activities.[4] More recently, an analogous cyclic nucleotide synthesized from NADP, 2'-phospho-cyclic ADP-ribose (P-cADPR) (Fig. 1), has been shown to elicit Ca^{2+} release from rat brain microsomes.[6,7] The characteristics of Ca^{2+} release indicate that P-cADPR and cADPR elicit Ca^{2+} release by a similar mechanism, but by a mechanism distinct from inositol trisphosphate.[7] The presence of cADPR in animal

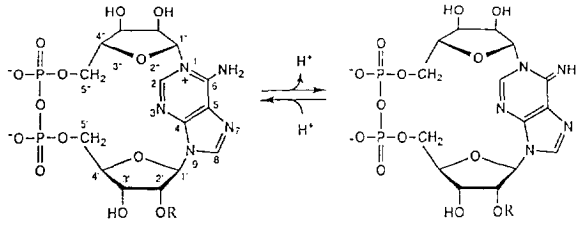

Figure 1. Possible structures for the acid and free base forms of cADPR and P-cADPR.
R = H, cADPR; R= PO_3^{2-}, P-cADPR

* *E-mail: schnacke@biozentrum.uni-wuerzburg.de.* Fax: (+49) 931-8884150

cells is well established[8] and the natural occurrence of P-cADPR has been recently detected.[9]

Characterizations of cADPR and P-cADPR by ^{1}H-NMR and UV spectroscopy have provided information concerning the configuration of these nucleotides.[7,10-12] While cADPR and P-cADPR are potent calcium mobilizing agents, ADPR and P-ADPR are totally inactive,[7] indicating that the cyclic nucleotides adopt a unique conformation(s) that results in the activation of membrane Ca^{2+} channels. We report here the ^{31}P NMR spectra of both cyclic nucleotides as a function of pH. Our results indicate that the ^{31}P signals of these nucleotides show a pH dependence which correlates to spectral changes in uv absorbance for the adenine chromophore. Such protonation/deprotonation events may be important in calcium channel activation.

Materials and Methods

Chemicals. cADPR and P-cADPR were synthesized and purified as described previously.[4,7] ^2H_2O was purchased from Sigma. All other chemicals were of highest purity commercially available.

^{31}P NMR Spectroscopy. Fourier transformed ^{31}P NMR spectra were collected at 121.497 MHz on a Bruker AM300 SWB superconducting spectrometer using a 10-mm multinuclear probehead with broadband ^{1}H decoupling. The NMR tube spinning at 15 Hz contained the sample (2 mL) and ^2H_2O (0.2 mL) as field/frequency lock and was maintained at 20 ± 0.1 °C using a thermostated continuous air flow. Generally, a spectral width of 3000 Hz was acquired in 8K data points with a pulse angle of 60°. The exponential line broadening prior to Fourier transformation was 1 Hz. The samples were dissolved in 50 mM Hepes buffer and the pH was adjusted by addition of acid or base. pH values of the samples were determined before and after the NMR measurement. Positive chemical shifts in ppm are downfield changes with respect an external chemical shift reference standard of 85% phosphoric acid.

Results and Discussion

The $\{^1H\}^{31}P$ NMR spectrum of cADPR at pH 7.06 shows the expected doublet of doublets in the range -10 to -11 ppm for the pyrophosphate group (Fig. 2A). The similar location of the two doublets indicates that both phosphate groups are substituted. The singly substituted pyrophosphate groups of

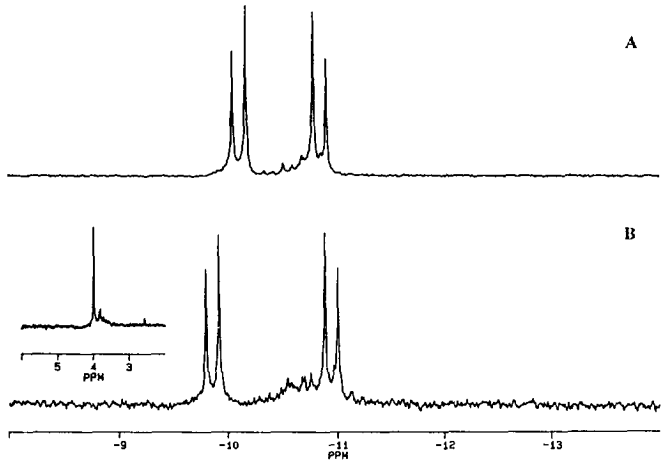

Figure 2: ^{31}P NMR spectra of cADPR (A) and P-cADPR (B) at pH 7.06 and 20°C. Conditions: 5,800 scans (A), 4,300 scans (B); ^{1}H broadband decoupling, line broadening prior to Fourier transformation 1 Hz. Inset in (B) shows the signal of the 2'-phospho group of P-cADPR.

thiamine diphosphate[13] and ADP[14] are separated by 4 and 4.6 ppm, respectively. The phosphorus-phosphorus spin coupling ($J_{\alpha\beta}$) in cADPR is constant at 13.6 Hz over a range of pH values (6.0-10.0), whereas the coupling constants of FAD and ADP are 20.9 and 22.0 Hz, respectively.[15] Note that in a control experiment the spin coupling constant for ADP-ribose was determined to be 21.9 Hz (data not shown). The smaller spin coupling constant for cADPR most probably results from the conformational restrictions imposed upon the pyrophosphate group by cyclization. The coupling constant for cADPR observed in this study closely matches the value of 14.5 Hz reported by Wada et al.[12] at unspecified pH. Fig. 3 shows the ^{31}P chemical shifts of cADPR for the pH range 6.8 to 10.0. One of the two phosphate moieties occurs at -10.2 ppm and shows very little change as a function of pH, while the second exhibits a strong pH dependence. The transition for this latter phosphate group shows a pK_a value of 8.30 and limiting δ values of -10.83, and -9.86 ppm at low and high pH values, respectively. Wada et al.[12] reported ^{31}P chemical shifts of -9.92 and -10.67 ppm in deuterium oxide for cADPR at unspecified pH and assigned the former to the adenosine unit and the latter to the ribose unit attached to N-1 using ^{1}H-^{31}P COSY experiments. We believe that these assignments for the ^{31}P NMR signals may not be correct and should be reversed. The ^{1}H-^{31}P and ^{1}H-^{1}H coupling constants for the 4' and 5' protons in each ribose furanosyl moiety are difficult to determine from the reported spectra and some are similar. Moreover, the symmetry of the cADPR about the pyrophosphate O atom, together with its cyclic structure, make comparative

assignments difficult. It has been shown that for noncyclic mono- and disubstituted adenosine diphosphates, the ^{31}P signal for the phosphate group closest to the adenosine unit, the α-phosphate, occurs at a higher field than that for the β-phosphate group.[13-15]

In contrast to cADPR, the ^{31}P NMR signals for each of the two phosphate groups of ADP-ribose occur as doublets at -10.47 and -10.64 ppm, respectively (data not shown) and the chemical shifts of these signals are pH-independent over the range 6.4 - 9.4. At pH values above pH 7.0, the distance between the doublets of cADPR diminishes until approximately pH 8.6, at which point a single signal remains. Above pH 8.6, the signals cross over and the distance between them increases.

The ^{31}P NMR spectrum of P-cADPR at pH 7.0 exhibits a singlet at 3.74 ppm and a doublet of doublets at -9.93, and -10.92 ppm (Fig. 2B). The singlet is assigned to the 2'-phosphomonoester group (Fig. 1). The pH dependence of the singlet shows a single transition with a pK_a of 5.89 and limiting δ values at -0.05 and 4.15 ppm for the low and high pH values, respectively (data not shown). The pK_a value is somewhat lower than those of normal phosphomonoesters.[16] The pH dependence of the doublets

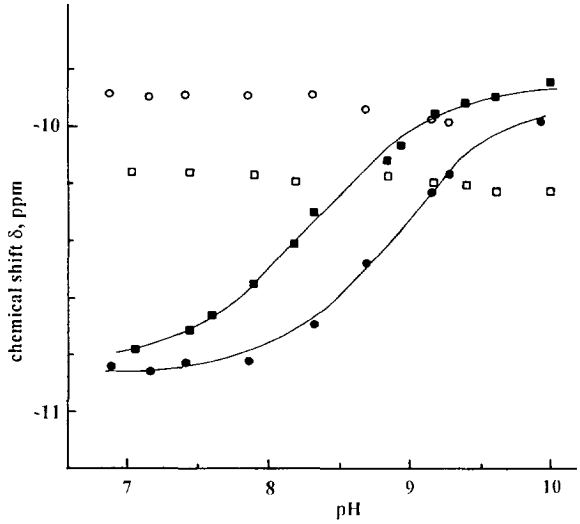

Figure 3. pH dependence of the ^{31}P chemical shift of cADPR and P-cADPR . The solid lines were derived by iterative computer analysis.[13] For cADPR (□, ■) a pK value of 8.30 and limiting δ values of -9.85 and -10.83 at low and high pH values, respectively and for P-cADPR (○, ●) a pK value of 8.96 and limiting δ values of -9.83 and -10.86 were calculated.

for P-cADPR in the pH range 6.8 - 10.0 shows that the phosphate moiety at -9.95 ppm is insensitive to pH changes while the other phosphate shows a single transition (Fig. 3). For this doublet, a pK_a value of 8.91 and limiting δ values of -10.87 and -9.88 ppm for the low and high pH values, respectively, were observed.

Previous work has shown that the third and fourth pK values (pK_{a3} and pK_{a4}) for inorganic pyrophosphate are 6.25 and 8.64, respectively.[13] Since the pK_{a1} and pK_{a2} values of a disubstituted pyrophosphate group are both below 2.0,[13] the observed pK_a value of 8.30 for cADPR must correspond to the protonation/deprotonation of the adenine moiety. In principle, deprotonation of the adenine ring system could cause a change in the chemical shift of either one or both of the ^{31}P signals. However, only one of the signals in both cADPR and P-cADPR is affected by the titration and, indeed, in each case it is for the same phosphate group. While anisotropic effects cannot be excluded, the magnitude of the change and the fact that completely analogous pH-dependent modifications occur in the UV spectra suggest the possibility of a direct interaction between the reporter phosphate group and the titrated chromophore. A possible structure for the acid forms would be the N-3 protonated vinylogous amidine of the adenine system which displays characteristic λ_{max} values at around 270 nm.[17,18] Any such N-3 protonated chromophore could form a hydrogen bond with oxygen atoms of the adenosine phosphate moiety and in so doing would provide a mechansim for transferring electronic information from the chromophore to the P-atom. Alternatively, when the protonation/deprotonation occurs distant from the phosphorus nuclei, conformational restrictions imposed upon the pyrophosphate group by cyclization could be sensed by the phosphorus nuclei since the ^{31}P chemical shift of phosphate esters correlate with small changes in O-P-O bond angles.[16,19]

Thus, we postulate that for cADPR and P-cADPR the signal which displays a pH-dependent chemical shift is that for the phosphate group that is nearest to the ribose attached at the N-9 position of the adenine moiety.

The spectral data presented here may prove useful for future studies aimed at understanding the molecular basis of Ca^{2+} channel activation by cADPR and P-cADPR. The smaller phosphorus-phosphorus spin coupling constants of cADPR and P-cADPR compared to the noncyclized forms of these nucleotides and other dinucleotides indicate that cADPR and P-cADPR have a solution structure that has conformational restrictions for the pyrophosphate moiety. This conformation may be related to the ability of these nucleotides to interact with a Ca^{2+} channel protein to achieve activation. Additionally, the spectral changes observed upon protonation/deprotonation may be related to the mechanism of channel activation

as proton abstraction/addition to the protein-bound nucleotide may initiate a conformational change in the nucleotide that in turn translates into channel activation. The spectral differences between protonated and deprotonated forms should also be useful for the study of the interaction of these nucleotides with their binding proteins once these proteins have been identified.

Acknowledgment. This study was supported in part by NIH Grant CA43894 to MKJ and GM45451 to RAG and by a grant of the Deutsche Forschungsgemeinschaft to KDS. We thank Biotechnology and Biological Sciences Research Council for an International Scientific Interchange Scheme Grant to Würzburg for DG. We are indebted to Donna Coyle for excellent technical support and to Dr. Manfred Buehner for the computer analysis of the pH dependence of the chemical shifts.

References and Notes

1. Koshiyama, H.; Lee, H. C.; Tashajian, A. H. *J. Biol. Chem.* **1991**, 266, 16985-16988.
2. Takasawa, S.; Nata, K.; Yonekura, H.; Okamoto, H. *Science* **1993**, 259, 370-373.
3. Lee, H. C.; Galione, A.; Walseth, F. F. *Vitamin Hormon.* **1994**, 48, 199-257.
4. Kim, H.; Jacobson, E. L.; Jacobson, M. K. *Science* **1993**, 261, 1330-1333.
5. Lee, H. C.; Aarhus, R.; Levitt, D. *Nature Structure Biology* **1994**, 1, 143-144.
6. Zhang, F.-J.; Gu, Q.-M.; Jing, P.; Shih, C.J. *Bioorg. Med. Chem. Lett.* **1995**, 2267-2272.
7. Vu, C. Q.; Lu, P. J.; Chen, C.-S.; Jacobson, M. K. *J. Biol. Chem.* **1996**, 271, 4747-4754.
8. Walseth, T. F.; Aarhus, R.; Zeleznikar, R. J.; Lee, H. C. *Biochim. Biophys. Acta* **1991**, 1094, 113-120.
9. Vu, C. Q.; Coyle, D. C.; Jacobson, M. K. unpublished data.
10. Kim, H.; Jacobson, E. L.; Jacobson, M. K. *Biochem. Biophys. Res. Commun.* **1993**, 194, 1143-1147.
11. Gu, Q.-M.; Sih, C. J. *J Am.. Chem. Soc.* **1994**, 116, 7481-7486.
12. Wada, T.; Inageda, K.; Aritomo, K.; Tokita, K.; Nishina, H.; Takahashi, K.; Katada, T.; Sekine, M. *Nucleosides & Nucleotides* **1995**, 14, 1301-1314.
13. Lachmann, H.; Schnackerz, K. D. *Org. Magn. Reson.* **1984**, 22, 101-105.
14. Vogel, H. J.; Bridger, W. A. *Biochemistry* **1982**, 21, 394-401.
15. Kainosho, M.; Kyogoku, Y. *Biochemistry* **1972**, 11, 741-752.
16. Gorenstein, D. G. *J. Am. Chem. Soc.* **1975**, 97, 898-900.
17. Specker, H.; Gawrosch, H.; *Chem. Ber.* **1942**, 75, 1338-1338.
18. Brookes, P.; Lawley, P.D.; *J. Chem. Soc* **1960**, 539-545.
19. Schnackerz, K. D.; Wahler, G.; Vincent, M. G.; Jansonius, J. N. *Eur. J. Biochem.* **1989**,185, 525-531.

(*Received in Belgium* 20 *November* 1996; *accepted* 24 *January* 1997)