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SUBSTRATE CONFORMATION IN 5'-AMP-UTILIZING ENZYMES: 8,5'-CYCLOADENOSINE 5'-MONOPHOSPHATE

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SUMMARY

One of the two possible C_5' epimers of 8,5'-cycloadenosine 5'-monophosphate is a substrate for snake venom 5'-nucleotidase. A stereospecific, radiation chemical synthesis has produced one of the epimers in pure form whose configuration at C_5' is established in proton nuclear magnetic resonance studies. The conformation of adenosine 5'-monophosphate at the active site of 5'-nucleotidase is inferred. This conformation is not in agreement with a previous proposal.

INTRODUCTION

In 1972, Hampton, Harper and Sasaki (1) reported the synthesis of an epimeric mixture of 8,5'-cycloadenosine 5'-monophosphates (I a, b). They discovered that only one of the C_5' epimers was a substrate for the enzyme 5'-nucleotidase. In connection with radiation chemical studies (2-5) we had the occasion to prepare 8,5'-cycloAMP by a modification of Keck's procedure (6). The radiation chemical synthesis is stereospecific giving the C_5' epimer which is not hydrolyzed by 5'-nucleotidase. Hampton, et al (1) proposed that this epimer should be I a . We now present nuclear magnetic resonance evidence that, in fact, the non-hydrolyzable epimer is I b . These results have a bearing on the conformation of a flexible substrate such as adenosine 5'-monophosphate when bound to the active site of 5'-nucleotidase.

Abbreviations used: 8,5'-cycloAMP, 8,5'-cycloadenosine 5'-monophosphate; 5'-AMP, adenosine 5'-monophosphate; DDS, sodium 2,2-dimethyl-2-silapentane-sulfonate; tg, trans-gauche; gt, gauche-trans; NMR, nuclear magnetic resonance.

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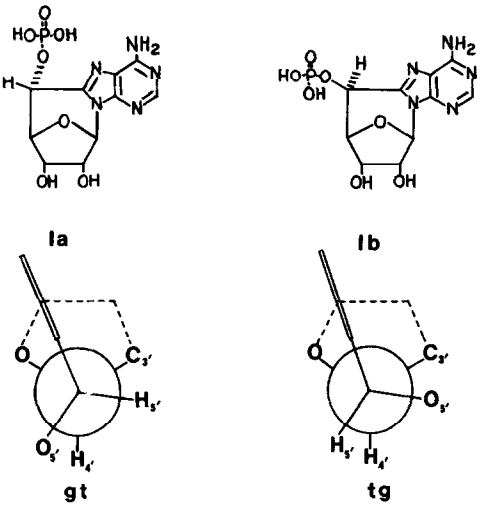

Figure 1: Structures of 8,5'-cycloAMP C_5, epimers and their respective Newman projections. Designations gauche-trans (gt) and trans-gauche (tg) for Ia and Ib, respectively, are adopted from terminology used in NMR and theoretical studies of nucleotides flexible about the $C_{4'}$-$C_{5'}$ bond.

MATERIALS AND METHODS

8,5'-CycloAMP was prepared as described previously (2). 5'-Nucleotidase (Crotalus adamanteus venom) and 5'-AMP were purchased from Sigma Chemical Company (St. Louis, Missouri). Nuclear magnetic resonance spectra were recorded on a Varian CFT-20 Spectrometer (79.54 MHz for proton studies). Chemical shifts were measured relative to DSS in a coaxial capillary tube. High pressure liquid chromatography was performed at ambient temperature on a 4.6 mm x 25 cm Partisil 10SAX column from Whatman, Inc. (Clifton, New Jersey) with 0.03 M K_2HPO_4 at pH 3.5 as eluant. The chromatography was used to monitor the 5'-nucleotidase hydrolyses with detection of nucleotides and nucleosides at 254 nm by means of a Chromatronix Model 230 flow spectrophotometer. The 5'-nucleotidase experiments were performed as described by Hampton, et al (1).

RESULTS

Figure 2 is a proton NMR spectrum of 0.1 M 8,5'-cycloAMP in D_2O at 48°. This temperature placed the HOD peak at a position where it would not interfere with nucleotide resonances. The spectrum is close to first order and, as expected, showed the absence of H_8 which is lost from 5'-AMP in the cyclization process. Assignment of the low field singlets to $H_{2'}$ and $H_{1'}$ is by analogy with 5'-AMP. The $H_{5'}$ resonance in 8,5'-cycloAMP is shifted well downfield from its position in 5'-AMP (7) but is readily identified as the doublet of doublets at 5.4 ppm

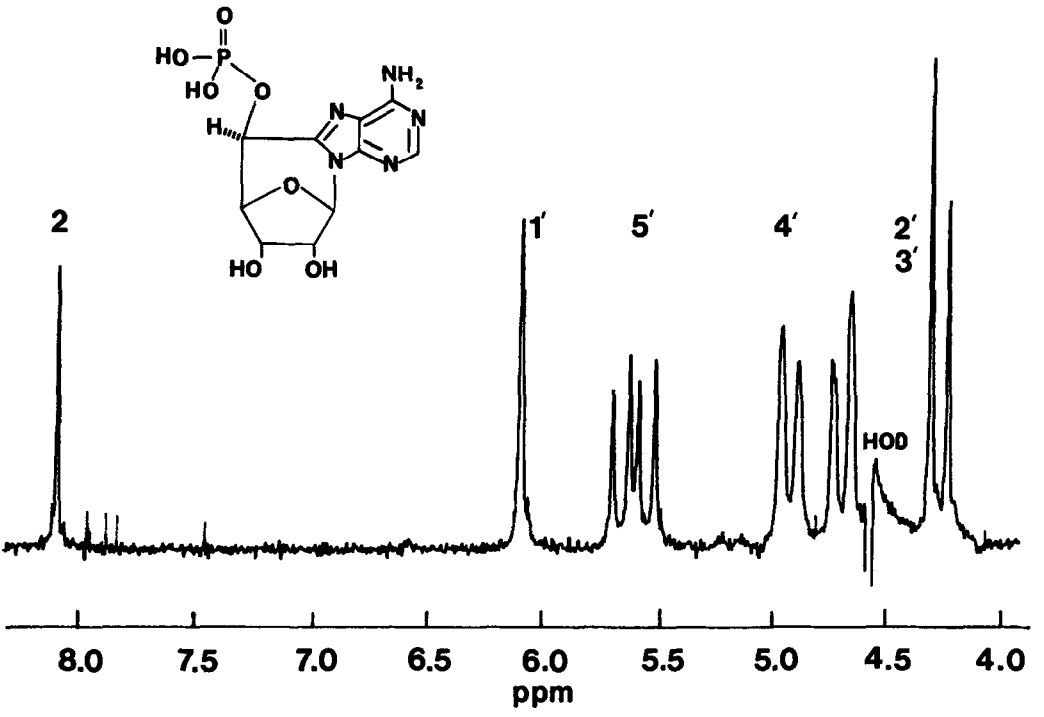

Figure 2: Varian CFT-20 (74.54 MHz) proton NMR spectrum of 8,5'-cycloAMP 0.1 M in D_2O at pD 5.7 and 48°. Chemical shifts (ppm) are relative to an external DDS reference. The sample was lyophilized in D_2O before the spectrum was run in order that OH and NH_2 groups with exchangeable protons were deuterated.

on the basis of a primary splitting of 8.9 Hz due to phosphate phosphorus coupling to $H_{5'}$ and a secondary splitting of 5.9 Hz due to coupling to $H_{4'}$ whose resonance appears as the doublet (5.9 Hz) at 4.9 ppm. Irradiation of $H_{4'}$ in a spin decoupling experiment caused the $H_{5'}$ quartet to collapse to a doublet ($J_{P,H_{5'}}$ 8.9 Hz) consistent with the assignments. The highest field resonances with a splitting of 6.1 Hz are due to $H_{2'}$ and $H_{3'}$ though it is uncertain which is which.

The stereospecificity of the radiation chemical synthesis was first revealed in a study of enzyme hydrolyses of 8,5'-cycloAMP. Although 8,5'-cycloAMP is completely hydrolyzed to the corresponding cyclonucleoside by alkaline phosphatase, no hydrolysis of the compound by 5'-nucleotidase could be detected even though 5'-AMP was readily hydrolyzed under the same conditions (figure 3).

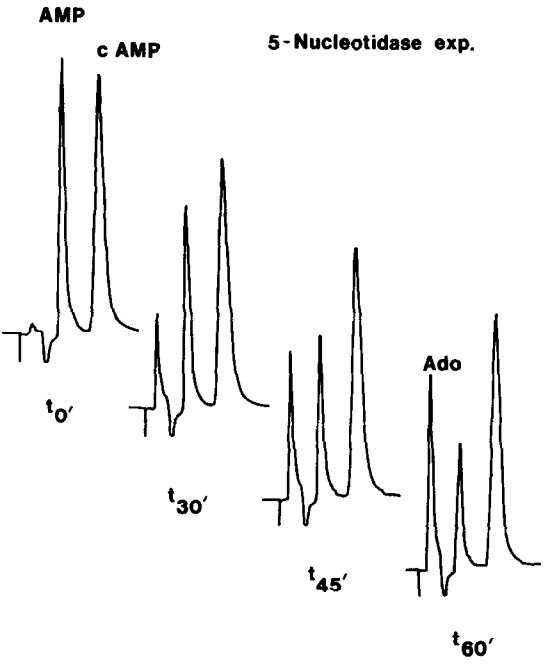

Figure 3: 5'-Nucleotidase (15 µg/ml in pH 8.0, 0.08 M Tris buffer, 37°) hydrolysis of a mixture of 5'-AMP (0.005 M) and 8,5'-cycloAMP (0.01 M) followed as a function of time (t') by high pressure liquid chromatography (Partisil 10SAX, 0.03 M K_2HPO_4, pH 3.5, ambient temperature).

DISCUSSION

One and the same C_5, epimer of 8,5'-cycloAMP has been shown to be a substrate for snake venom 5'-nucleotidase and pig muscle adenyl kinase. Additionally, in a coupled enzyme system, the 8,5'-cycloADP produced by adenyl kinase proved to be a substrate for rabbit muscle pyruvate kinase (1). The reactivity of the appropriate 8,5'-cycloAMP epimer was reported to be similar to that of 5'-AMP. The other epimer showed no detectable activity. These results imply that there is a limited degree of rotational freedom about the C_4, - C_5, bond in 5'-AMP when it is bound to 5'-nucleotidase and that the conformation of the active 8,5'-cycloAMP epimer fairly represents that conformation. An inspection of molecular models and Newman projections (figure 1) show that a clear distinction between the epimers can be made on the basis of expected H_4, - H_5, coupling constants. In epimer I a the

dihedral angle between $H_{4'}$ and $H_{5'}$ is close to 90^{o}. Accordingly, the NMR coupling constant, $J_{H_{4'},H_{5'}}$ should be close to zero (8) as it is between $H_{1'}$ and $H_{2'}$ in 8,5'-cycloAMP (figure 2) where the dihedral angle is similar to that between $H_{4'}$ and $H_{5'}$ in Ia. In contrast, the dihedral angle between $H_{4'}$ and $H_{5'}$ in Ib is close to 0^{o}. In this case, a substantial splitting of the respective proton resonances should be seen. In fact, a coupling constant of 5.9 Hz between $H_{4'}$ and $H_{5'}$ is observed and the epimer formed in the radiation chemical synthesis is Ib. Strong confirmation of this assignment comes from a recent radiation chemical synthesis of 8,5'-cyclo-2'-deoxyadenosine A stereospecific synthesis of the $C_{5'}$ epimer of 8,5'-cyclo-2'-deoxyadenosine corresponding to that of I a has been achieved, and, as expected, no coupling between $H_{4'}$ and $H_{5'}$ can be detected (9). The identity of Ib and the 8,5'-cycloAMP epimer prepared in the present work amends an earlier incorrect designation (2).

As epimer Ib is not hydrolyzed by 5'-nucleotidase, it follows that epimer Ia is the substrate for 5'-nucleotidase. A similar $C_{5'}$ configuration obtains in the rate-determining step for both adenyl kinase and pyruvate kinase. It has been established that Ib, the compound produced in the radiation chemical synthesis is not a substrate for porcine adenyl kinase (10). The rotamer conformation of 5'-AMP corresponding to the active epimer Ia is designated gauche-trans in NMR and theoretical studies and is not the most stable conformation of 5'-AMP in free solution (11). These observations may be of interest in attempts to establish patterns of substrate conformation on the surfaces of adenosine nucleotide-utilizing enzymes (e.g. 12, 13).

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