RESEARCH COMMUNICATION

Structure determination of a novel cyclic phosphocompound isolated from *Desulfovibrio desulfuricans*

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The structure of a novel diphosphodiester compound recently detected in *Desulfovibrio desulfuricans* cells [Santos, Fareleira, Pedregal, LeGall & Xavier (1991) Eur. J. Biochem. 201, 283–287] was fully elucidated using a combination of n.m.r. techniques in aqueous and in methanolic solutions. The novel metabolite was identified as 3-methyl-1,2,3,4-tetrahydroxybutane-1,3-cyclic bisphosphate, and the minimum energy conformation is presented. The two chiral centres have the relative configuration *RS*.

INTRODUCTION

Sulphate-reducing bacteria are strict anaerobes able to use a variety of low-molecular-mass organic compounds or hydrogen as electron donors for the reduction of oxidized sulphur compounds in an anaerobic respiration process [1,2]. Some strains can also utilize nitrate as an electron acceptor [2]. The bacteria belonging to the most extensively studied genus, *Desulfovibrio*, have been the source of a number of unique electron-transfer proteins [3].

Recently, an unusual phosphoric anhydride diester compound was detected in *Desulfovibrio desulfuricans* A.T.C.C. 27774 by ³¹P n.m.r. [4]. This novel metabolite was found to occur intracellularly in considerable amounts when cells of this strain were grown in media containing either sulphate or thiosulphate, but was absent when cells were cultivated with other electron acceptors such as nitrate. This phosphorus-containing compound was not present in seven other strains of *Desulfovibrio* which have been examined by ³¹P n.m.r. *in vivo*.

The extraction, purification and partial characterization by multinuclear n.m.r. of the novel compound have been reported [4]. The results obtained in this earlier study suggested a cyclic structure for the phosphoric anhydride diester compound with strong asymmetry of the two phosphorus nuclei with respect to the coupling to protons. The carbon skeleton of the molecule was found to be formed by one methyl group, two methylene groups, one methine group and one quaternary carbon.

In the present paper we report on the determination of the full structure of the novel phosphorus-containing metabolite by using one- and two-dimensional multinuclear n.m.r. spectroscopy. This approach led to the identification of the novel metabolite as 3-methyl-1,2,3,4-tetrahydroxybutane-1,3-cyclic bisphosphate. Analysis of the coupling constants in conjunction with molecular modelling shows that the two chiral centres have the relative configuration *RS*.

Part of this work was presented at the Fourteenth International Conference on Magnetic Resonance in Biological Systems held at the University of Warwick, Coventry, U.K., on 9–14 September 1990 [4a].

MATERIALS AND METHODS

Cell growth

Cells of *D. desulfuricans* A.T.C.C. 27774 were cultivated as described previously [4] on lactate/sulphate or lactate/thio-sulphate medium under an argon atmosphere.

Sample preparation and n.m.r. spectroscopy

The novel phosphorus-containing compound was purified by anion-exchange chromatography as previously reported [4].

For n.m.r. analysis, samples of the purified phosphocompound were freeze-dried from water and redissolved either in ²H₂O (99.9 atom % ²H) or in C²H₃O²H (99.8 atom % ²H). N.m.r. spectra were run either in a Bruker AMX-300, a Bruker AMX-500 or in a Varian VXR-500 spectrometer, using 10 mm broad-band probe heads for ¹³C and ³¹P observation and 5 mm selective proton probe heads for ¹H detection. ³¹P-n.m.r. spectra were obtained at 121.49 MHz or 202.45 MHz, with and without proton broad-band decoupling; 32000 data points were acquired covering a spectral width of 10 kHz, using a 45° flip angle and 1.2 s recycle time. Whenever necessary, resolution enhancement was achieved by Gaussian multiplication of the free-induction decays. Chemical shifts were referenced with respect to external 85 % H₃PO₄.

¹³C-n.m.r. spectra were obtained either at 75.47 MHz or 125.77 MHz and run with proton broad-band decoupling, using a 30° flip angle and a repetition time of 3.7 s. 132000 data points were collected over a 19 kHz spectral width. Chemical shifts were referenced to external methanol designated at 49.3 p.p.m. Two-dimensional spectra correlating the chemical shifts of the protonated ¹³C nuclei with the shifts of the attached protons were obtained as described in [5] at 125.7 MHz, with 4096 points acquired for each free-induction decay in the dimension of ¹³C shifts (*N-type* peak selection). The evolution period was incremented in 512 steps of 455 μs, corresponding to spectral widths of 13329 × 2198 Hz, and results are presented in the absolute-value mode.

¹H-n.m.r. spectra were recorded at 300.13 MHz or 500.13 MHz, using a 45° flip angle and 3.3 s recycle delay. Free-induction decays were acquired in 32000 data points covering a

Abbreviations used: NOESY, nuclear Overhauser enhancement spectroscopy; COSY, correlation spectroscopy.

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spectral width of 5 kHz. Spectra of the phosphoric anhydride diester compound in $^2\text{H}_2\text{O}$ were run with presaturation of the residual water signal. Chemical shifts were referenced with respect to sodium 3-trimethylsilyl[2,2,3,3- ^2H]propionate.

The long-range proton–proton correlation spectrum was obtained using a pulse sequence as described in [6]. $512(t_1) \times 2048(t_2)$ data points were collected over a spectral width of 5 kHz, with a recycle delay of 2.2 s and a delay for evolution of long-range couplings of 125 ms. The data were zero-filled in the f_1 dimension to 1024 data points and processed using magnitude calculation.

The NOESY (nuclear Overhauser enhancement spectroscopy) spectrum was recorded according to [7,8] in the phase-sensitivity mode using the 'TPPI' (Time Proportional Phase Increment) method. The spectrum was acquired over a spectral width of 3 kHz, collecting $512(t_1) \times 2048(t_2)$ data points, using a mixing time of 0.5 s and a recycle delay of 2.3 s, with presaturation of the water signal during relaxation delay and mixing time. The probehead temperature was kept at 298 K in all experiments.

Chemicals

Deuterated solvents ($^2\text{H}_2\text{O}$ and $\text{C}^2\text{H}_5\text{O}^2\text{H}$) were obtained from Sigma Chemical Co. Sodium 3-trimethylsilyl[2,2,3,3- ^2H]propionate was purchased from E. Merck (Darmstadt, Germany). All other chemicals were of reagent grade.

RESULTS AND DISCUSSION

The structure of the compound was determined initially by n.m.r. of the aqueous solution. Lines were generally broad except at very high dilution, possibly because of interaction with residual metal ions. ^1H -, ^{31}P - and ^{13}C -n.m.r. spectra of a nearly pure preparation of the compound are shown in Fig. 1.

The ^{31}P -n.m.r. spectra are characteristic of a diphosphodiester (J 23.2 Hz), one of the phosphorus nuclei having several couplings to protons (Figs. 1b and 1c).

The ^{13}C spectrum (Fig. 1a) indicates one CH_3 (16.98 p.p.m.), two CH_2 (66.32 and 67.58 p.p.m.), one CH (69.08 p.p.m.) and one quaternary carbon atom (84.51 p.p.m.). The quaternary carbon atom and one CH_2 have ^{31}P couplings (8.5 and 6.5 Hz respectively) characteristic of $^2J_{\text{PC}}$ [9]. All of the remaining carbons show couplings to ^{31}P in the range 2–6 Hz, which are compatible with $^3J_{\text{PC}}$. Crucially, the CH signal appears to be an unresolved triplet or doublet of doublets, indicating a cyclic structure.

The ^1H spectrum (Fig. 1d) shows a methyl group [chemical shift (δ) 1.44 p.p.m.], an AB system ($\delta_A = 3.79$; $\delta_B = 3.64$ p.p.m.) and a strongly coupled group of three protons (ABCX system) at ~ 4.2 p.p.m. The correlation between the carbon atoms and directly attached protons was elucidated by running a ^{13}C – ^1H heteronuclear shift correlation experiment (Fig. 2). NOESY spectra (not shown) revealed magnetization transfer from the methyl group to both protons of the AB system and to the CH region of the ABCX system. A long-range proton COSY (correlation spectroscopy) experiment clearly detected an unresolved coupling between the methyl and one proton of the AB system (Fig. 3). The ABCX system was first reduced to an ABC system by ^{31}P decoupling in a very diluted sample in order to simplify the analysis. Simulation of proton spectra obtained both at 300 MHz and 500 MHz (not shown) revealed a geminal coupling of -12.9 Hz and two vicinal couplings of 3.6 and 7.7 Hz. Using these values to analyse the ^{31}P coupled proton spectrum showed couplings of 10.7 and 16.7 Hz to the CH_2 group and essentially no coupling to the CH, confirming a P–O– CH_2 linkage.

Completion of the structure requires heteronuclei to be linked to the other CH_2 group and to the CH. Elemental analysis

revealed no trace of nitrogen or sulphur, and the ^{13}C chemical shifts are characteristic of hydroxy-group-bearing carbon atoms. The structure is therefore:

A Fischer representation of the molecule is included in Fig. 1.

The relative configuration of the quaternary and methine carbon atoms remained to be determined. Unfortunately, the similarity of the $^3J_{\text{PC}}$ couplings to the CH_3 , CH_2OH , and CHOH substituents of the quaternary carbon, which are 5.9, 4.7, and ≤ 3.5 Hz respectively, made it impossible to distinguish between isomers in aqueous solution. The compound was therefore studied in $\text{C}^2\text{H}_5\text{O}^2\text{H}$, in which these couplings become 8.3, ≤ 2.0 , and ≤ 2.0 Hz respectively. In addition, much sharper spectra

Fig. 1. One-dimensional n.m.r. spectra of an aqueous solution of the diphosphodiester compound isolated from *D. desulfuricans* (A.T.C.C. 27774)

(a) 75.47 MHz ^{13}C proton-decoupled spectrum. Directly measured carbon–phosphorus coupling constants (Hz) are indicated. (b) 121.49 MHz ^{31}P proton-coupled spectrum. (c) 121.49 MHz ^{31}P proton-decoupled spectrum. (d) 500 MHz proton spectrum. The intense peak at approx. 1.9 p.p.m. is due to residual acetate buffer. Minor peaks in the spectrum are due to hydrolysis products. A Fischer representation of the novel phosphoric anhydride diester compound is included. Labelling in the schematic representation of the compound is as in Table 1.

Fig. 2. Part of the ^1H - ^{13}C shift correlation n.m.r. spectrum of the phosphoric anhydride diester compound isolated from *D. desulfuricans* (A.T.C.C. 27774)

The peak with a chemical shift of approx. 1.9 p.p.m. in the ^1H dimension is due to residual acetate buffer.

Fig. 3. Section of the 500 MHz long-range COSY spectrum of an aqueous solution of 3-methyl-1,2,3,4-tetrahydroxybutane-1,3-cyclic bisphosphate revealing a clear coupling between the methyl group and one of the protons of the AB system

Table 1. Observed and calculated spin-spin coupling constants (J) of the phosphoric anhydride diester compound

Nuclei*	Observed J (Hz) in:		Calculated J (Hz)
	$^2\text{H}_2\text{O}$	$\text{C}^2\text{H}_3\text{O}^2\text{H}$	
P-P'	23.2	21.5	N.D.†
P-C	8.5	8.6	N.D.
P-CH ₃	5.9	8.3	8.1
P-CH ₂ OH	4.7	≤ 2.0	2.6
P-CHOH	≤ 3.5	≤ 2.0	1.5
P'-CHOH	≤ 3.5	≤ 2.0	2.1
P'-CH ₂	6.5	6.4	N.D.
P'-H _a	10.7	9.1	1.4
P'-H _b	16.7	20.7	22.9
H _a -H _b	-12.9	-11.9	N.D.
H _a -H _c	3.6	3.7	6.1
H _b -H _c	7.7	8.9	10.5

* Proton labelling as in Fig. 1.

† N.D., not determined.

Fig. 4. Minimum-energy conformation found for the *RS* isomer of 3-methyl-1,2,3,4-tetrahydroxybutane-1,3-cyclic bisphosphate

Oxygen atoms are represented by dotted circles and phosphorus atoms by striped circles.

were obtained, revealing a 1.5 Hz $^4J_{\text{PH}}$ coupling to the methyl group (result not shown).

The isomers were modelled using the program Macromodel version 3.0 (Dr. W. C. Still, Department of Chemistry, Columbia University, New York, NY, U.S.A.) with the MM2 force field [10], and in each case minimum-energy conformations were found with a pseudo-boat conformation having the hydroxy group in a pseudo-equatorial position. Both isomers give reasonable values for the $^3J_{\text{PH}}$ couplings to the CH₂ protons, calculated as $18.1\cos A^*\cos A - 4.8\cos A$, as described in [11], and for the vicinal coupling between these protons and the central CH using the expression described by Altona and colleagues [12]. The vicinal couplings calculated for the higher-energy conformations with the hydroxy group in a pseudo-axial position are both about 2 Hz and clearly do not match the observed values. The distinction between isomers therefore rests on the $^3J_{\text{PC}}$ couplings for which there is less published data.

We chose to consider the simplest possible Karplus equation, $K\cos A^*\cos A$, with the constant K fixed at 8.2 Hz by the sum of the couplings to the substituents of the quaternary carbon atom. Both isomers then give couplings of about 2 Hz between the central CHOH carbon and each of the phosphorus nuclei, in agreement with the measured values. The pseudo-axial carbon atom, a methyl group in the *RR* (or *SS*) isomer and a CH₂OH group in the *RS* (or *SR*) isomer, is then predicted to have a $^3J_{\text{PC}}$ coupling of about 3 Hz, and the pseudo-equatorial carbon atom to have a coupling of about 8 Hz. This clearly favours the *RS* isomer, which has the methyl group in the pseudo-equatorial position. This assignment of the relative configuration and of the most stable conformation is further supported by the 1.5 Hz $^4J_{\text{PH}}$ coupling to the methyl group. Couplings of 2.5–2.7 Hz have been observed between protons and phosphorus in the 'W' (trans anti-planar) configuration [13], which would then be reduced by methyl-group rotation, whereas no coupling would be expected in other configurations.

The observed and calculated coupling constants are summarized in Table 1, and the minimum-energy conformation found for the *RS* isomer is shown in Fig. 4.

The agreement between calculated and observed coupling constants is not expected to be very close, since specific electrostatic effects are not well represented and because they are undoubtedly averaged over a range of conformations in solution. The improved agreement obtained in methanol does not appear to result from a real change in the conformational-energy minimum, since the result was found to be essentially independent of dielectric constant, but it does suggest that a wider range of

conformations may be present in aqueous solutions, possibly as a result of interactions with metal ions.

The elucidation of the structure of the phosphocompound detected in *D. desulfuricans*, based on the n.m.r. data presented above, fully confirms our earlier predictions about the novelty of this metabolite and its cyclic structure [4]. This compound has been detected in only one of the several strains of *Desulfovibrio* searched; however, we found recently that its occurrence is not restricted to this group of bacteria. An identical compound was firmly identified in several strains of the aerobic bacteria *Acinetobacter* generously provided by Professor A. Zehnder, Wageningen Agricultural University, Wageningen, The Netherlands (H. Pereira, H. Santos and A. V. Xavier, unpublished work). Furthermore, unassigned resonances in the ^{31}P -n.m.r. spectra of *Propionibacterium acnes* [14] are possibly also due to the presence in these cells of an identical compound. These findings indicate that the novel metabolite may play a more general physiological role in bacterial metabolism than previously suspected.

All attempts to determine the physiological role of this interesting metabolite have been unsuccessful to date. The intracellular concentration in *D. desulfuricans* was found to be independent of the stage of growth and, therefore, there is no evidence for it serving as a phosphorus reserve in the cell. In experiments with non-growing cells, the concentration of the phosphocompound, as monitored by ^{31}P n.m.r., was insensitive to the presence of added substrate (lactate) and terminal electron acceptor (sulphate or nitrate).

Work is required to elucidate both the physiological role and the biosynthesis of this metabolite.

While this paper was in preparation a letter was published [15] reporting the occurrence of a phosphoric anhydride diester compound in *Brevibacterium ammoniagenes* and *Micrococcus luteus* displaying n.m.r. features coincident with the one-dimensional n.m.r. data shown in our earlier work [14]. The reported data are insufficient to enable the structure and stereochemistry of the compound to be determined unequivocally and are equivalent to our own data presented in 1990 at the Fourteenth

International Conference on Magnetic Resonance in Biological Systems [4a].

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