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The only inositol tetrakisphosphate detectable in avian erythrocytes is the isomer lacking phosphate at position 3: a NMR study

Georg W. Mayr and Wolfgang Dietrich†

Institut für Physiologische Chemie, Abteilung für Biochemie Supramolekularer Systeme and †*Institut für Organische Chemie, NMR-Abteilung, Ruhr Universität Bochum, FRG*

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Avian red blood cells contain a millimolar amount of inositol polyphosphate which plays a role as an allosteric effector of hemoglobin. We confirmed the structure of this substance by NMR techniques as purely *myo*-inositol 1,3,4,5,6-pentakisphosphate. Based on present knowledge this effector is synthesized from inositol trisphosphate by successive phosphorylation. In a search for biosynthetic and degradative intermediates of inositol pentakisphosphate we found only one inositol tetrakisphosphate isomer. Its structure was unambiguously assigned by proton, ^{13}C- and ^{31}P-NMR to *myo*-inositol 1,4,5,6-tetrakisphosphate. As in mammalian cells the major inositol tetrakisphosphate isomer is *myo*-inositol 1,3,4,5-tetrakisphosphate, there seem to be differences between avian red blood cells and mammalian cells in the routes of inositol polyphosphate formation and/or degradation.

NMR; *myo*-Inositol 1,4,5,6-tetrakisphosphate; *myo*-Inositol 1,3,4,5,6-pentakisphosphate; (Avian red blood cell)

1. INTRODUCTION

Besides the discovery of phosphoinositides as essential components of cell membranes and thus of inositol as a constituent of cellular structures [1], soluble highly phosphorylated inositols, preferentially the hexaphosphorylated compound phytic acid, were identified early in soil [2] and plants, where they are preferentially accumulated in seeds possibly playing some storage function [3]. The first evidence that these highly phosphorylated inositols might also play functional roles in animal cells came from the early discovery by Rapoport of high concentrations of inositol polyphosphate in avian red blood cells [4] whose structure was later proposed to be Ins(1,3,4,5,6)P_5 [5]. Irvine and coworkers have shown for the first time that also in mammalian cells Ins(1,4,5)P_3, an agonist induced phosphoinositide breakdown product [6], can be further phosphorylated to inositol tetraphosphate and also to inositol pentaphosphate [7,8]. Whereas the structure of the tetraphosphate was shown chemically [7] and by a recent NMR study [9] to be Ins(1,3,4,5)P_4, the structure of the inositol pentaphosphate(s) generated remains unknown. We have performed a detailed NMR investigation of

Correspondence address: G.W. Mayr, Institut für Physiologische Chemie, Abteilung für Biochemie Supramolekularer Systeme, Postfach 10 21 48, Ruhr Universität, D-4630 Bochum 1, FRG

This paper is dedicated to Professor S.M. Rapoport, Humboldt-Universität zu Berlin, who was the first to point out the existence of inositol polyphosphates in vertebrate red blood cells in 1940

Abbreviations: Inositol polyphosphates are abbreviated in a form as exemplified: D-*myo*-inositol 1,4,5,6-tetrakisphosphate, Ins(1,4,5,6)P_4. Unless otherwise stated, in a phosphorylated inositol 'Ins' stands for *myo*-inositol in symmetric and D-*myo*-inositol in asymmetric isomers

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the structure of the inositol polyphosphates present in avian red blood cells, a source from which sufficient amounts of these substances for NMR investigations can be isolated [3]. To our surprise, the only *myo*-inositol tetraphosphate isomer found and structurally identified was Ins(1,4,5,6)P_4. This finding may either indicate different pathways in avian red blood cells and mammalian cells for a further phosphorylation of Ins(1,4,5)P_3 or a route of dephosphorylation of Ins(1,3,4,5,6)P_5 starting exclusively at the C(3)-phosphate.

2. MATERIALS AND METHODS

Inositol polyphosphates from avian red blood cells were isolated by an upscaled procedure essentially as described in [3]. 5 l of fresh turkey blood were immediately stirred into 5 l of ice-cold 1 M perchloric acid and kept on ice until further processed. The precipitate was removed by a 20 min centrifugation at 10000 × *g*. The pelleted material was reextracted by homogenizing with 2 l of 0.5 N perchloric acid and the combined supernatant was neutralized at 0°C by slowly adding solid $KHCO_3$. The precipitate was removed by filtration, and the filtrate was freeze dried. After resuspending the freeze dried matter in 400 ml of ice-cold water and removing the remaining insoluble $KClO_4$ like above, the pH value was adjusted to 5 by adding formic acid. The solution was degassed, diluted with water until the conductivity was 10 mS, and finally applied onto a column of Dowex 1X2 (100–200 mesh, chloride form, column dimensions 2.5 × 60 cm) at a flow rate of 400 ml/h. A linear gradient of 4 l from 0 to 1.2 M HCl was applied at a flow rate of 200 ml/h. The resulting separation is shown in fig.1.

Other inositol phosphates were isolated and their purity checked as in [10]. NMR measurements: For ^{1}H-NMR and ^{13}C-NMR spectra, 5–20 mg of the neutral ammonium salt of each compound was lyophilized 3–5-times from D_2O of 99.7% deuteration. Samples were finally dissolved in 0.7 ml of D_2O of 99.996% deuteration and the pH* value [11] was adjusted to either 6.0 or 9.0 by adding DCOOD or ND_3. Acetone (HPLC grade, 0.7 μl) was added as an internal standard. The reported deshielding of its methyl-^{1}H resonance by 2.04 ppm and of its methyl-^{13}C resonance by 29.8 ppm relative to external TMS was used to set the scaling of ^{1}H-NMR and ^{13}C-NMR spectra, respectively. For ^{31}P-NMR spectra, samples were dissolved in 3 ml of D_2O and measured in 10 mm tubes. Spectra were recorded on a Bruker AM 400 spectrometer equipped with an ASPECT 3000 computer, pulse programmer, and hard disk at ambient temperature. Standard pulse sequences were applied and all spectra were recorded at a field strength of 9.4 T. ^{1}H-NMR spectra were accumulated at 400.13 MHz, ^{13}C-NMR spectra at 100.62 MHz, ^{31}P-NMR spectra at 161.98 MHz. Chemical shifts are reported in ppm downfield of TMS for ^{1}H- and ^{13}C-NMR spectra and in ppm downfield of external phosphoric acid (85%) for ^{31}P-NMR spectra. Assignments of nuclei are proposed based on homonuclear and heteronuclear correlation and decoupling data and on the comparison with structural analogs varying in the number and positioning of phosphates.

3. RESULTS AND DISCUSSION

After separation by hydrochloric acid gradient elution from a Dowex anion exchanger (fig.1), about 63 μmol of inositol tetraphosphate, 960 μmol of inositol pentaphosphate and 4 μmol of inositol hexaphosphate were recovered from 5 l

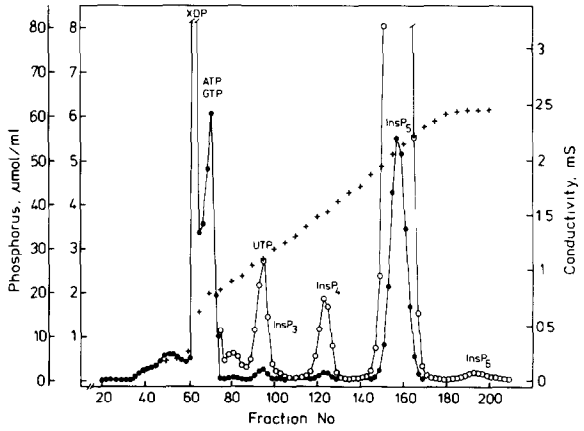

Fig.1. Separation of avian inositol polyphosphates by Dowex 1X2-chromatography. Conditions are as described in section 2. Fractions of 21 ml were collected. Total phosphorus (○, expanded inner left scale; ●, outer left scale) was determined as in [3], conductivity (+) was measured from 50 μl samples diluted to 10 ml. UV spectra between 220 and 320 nm (not shown) allowed the assignment of nucleotide-containing peaks.

of turkey blood. A small amount of inositol trisphosphate (3 μmol) was also obtained from the tailing edge of the peak of UTP (see fig.1) which is in agreement with older data [5]. The number of phosphates present per inositol was proved by a comparison of the elution positions with those of corresponding inositol polyphosphates from partly hydrolyzed phytic acid from wheat grain. The free acid forms obtained after freeze drying were neutralized with ammonia and stored frozen.

As shown in figs 2 to 4, the structure of inositol pentaphosphate as first proposed by Johnson and Tate [5] could be confirmed by our high resolution ^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR spectra. For a better comparison, the homologous series of isomers carrying three and four phosphates (see below) at corresponding positions are aligned below each other. The proton NMR (fig.2C) clearly indicates from the coupling pattern of C(2)H, the resonance from the only member of the *myo*-inositol ring carrying an axial OD-group neighboured by two *cis*-orientated equatorial OD-groups, that no phosphate is attached at C(2). As seen from the ^{1}H-NMR spectrum of the con-

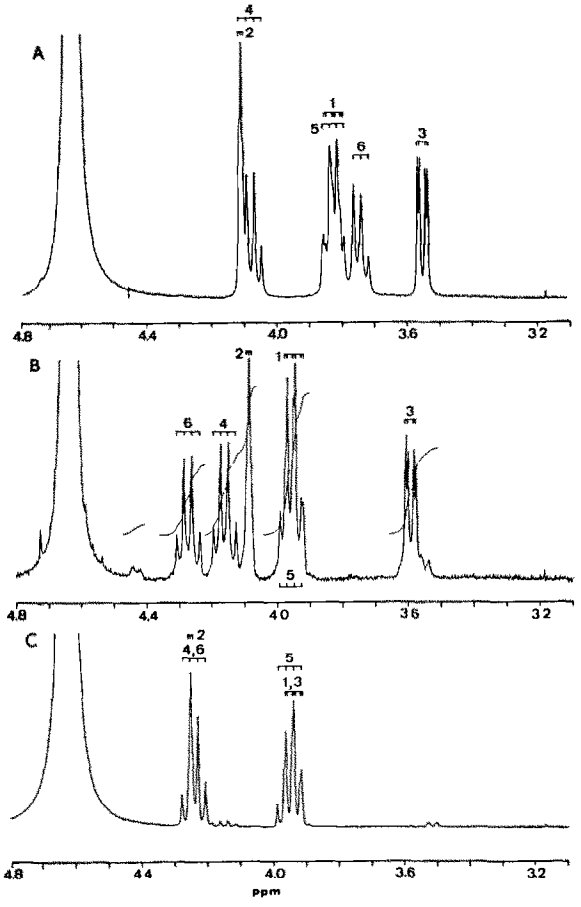

Fig.2. 400 MHz ^{1}H-NMR spectra of Ins(1,4,5)P_3 (A), and of Ins(1,4,5,6)P_4 (B), and Ins(1,3,4,5,6)P_5 (C) from avian red blood cells. Micromoles present and pH* values were 30 and 6.03 in (A); 10 and 6.03 in (B); and 58 and 6.06 in (C). Proton assignments and their multiplet patterns are depicted in the spectra. The broad resonance at about 4.65 ppm is from contaminating HDO.

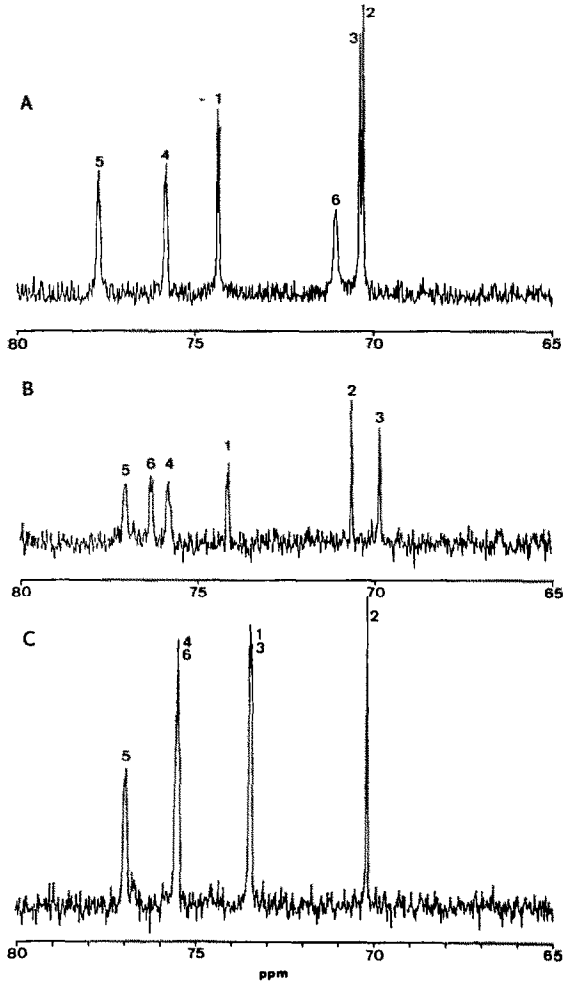

Fig.3. 100.6 MHz ^{13}C-NMR spectra of Ins(1,4,5)P_3 (A), and of Ins(1,4,5,6)P_4 (B), and Ins(1,3,4,5,6)P_5 (C) from avian red blood cells. Conditions were exactly as described in fig.2. Carbon assignments are given in the figures.

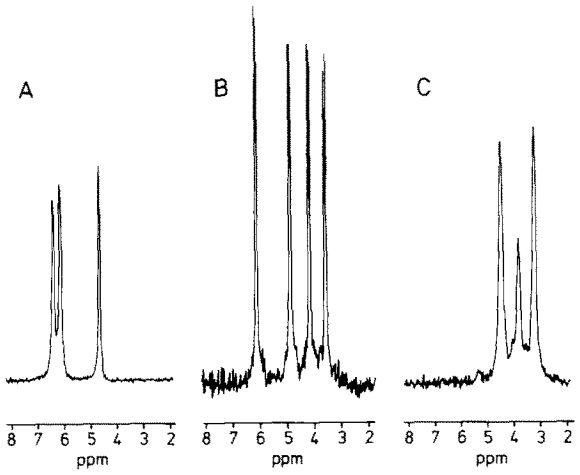

Fig.4. 162 MHz ^{31}P-NMR spectra of Ins(1,4,5)P_3 (A), and of *myo*-Ins(1,4,5,6)P_4 (B), and Ins(1,3,4,5,6)P_5 (C) from avian red blood cells. Micromoles present were as in fig.2, pH* values were 9.05 in (A), 9.01 in (B), and 9.1 in (C), respectively. No assignment of phosphorus nuclei was made. ppm values are downfield of external phosphoric acid.

taminating isomer in fig.2B (see below) a phosphate at position 2 leads to broad duplication (by about 10 Hz) of the narrow triplet (coupling constant about 3 Hz) characteristic for C(2)H. This unique resonance also proves the exclusive existence of the *myo*-form of inositol. The ^{13}C-spectrum of this compound (fig.3C) shows a splitting of three of the four observed resonances (best detectable by their broadening) which mainly results from a coupling with the geminal phosphorus nucleus. As only four resonances with approximate integrals of 1:2:2:1 were observed, the inositol must be symmetrically phosphorylated around the symmetry axis through C(2) and C(5). As both doubly integrating resonances were among the split ones, the phosphates can be assigned to C(1) and C(3), C(4) and C(6), and to C(5). The ^{31}P-NMR spectrum in fig.4C further confirms this structure by showing two doubly integrating ^{31}P-signals and one single resonance coming from C(5)OP.

A small fraction of another species of inositol pentaphosphate was also detected. By the appearance of a strongly upfield shifted duplicated doublet as is observed in fig.2A and B for C(3)H (below) and would be also observed for a non-phosphorylated C(1)H, one can conclude that this isomer must be devoid of phosphate at C(1) or C(3). A slight upfield shift of a quartet-like doublet of a triplet (average coupling constant about 10 Hz) characteristic for C(4)H or C(6)H is in agreement with a removal of phosphate from C(3) or C(1). The only possible structures compatible with these findings are D-*myo*-Ins(1,2,4,5,6)P_5 or its L-enantiomer which is identical to D-*myo*-Ins(2,3,4,5,6)P_5. Most probably this contaminant has been generated from the above isomer under the strongly acidic isolation conditions by acid migration of a phosphate to the C(2)OD-group [12]. The corresponding C(2)H resonance apparently was downfield shifted into the broad HDO resonance.

We next determined the structure of the inositol tetraphosphate isolated from the turkey blood. In contrast to the broad and heterogeneous peak of inositol tetraphosphate obtained from partly hydrolyzed phytic acid (not shown), this material elutes in a narrow homogeneous peak (cf. fig.1) which indicates only one or very similar inositol tetraphosphate isomers.

The ^{1}H-NMR spectrum shown in fig.2B reveals again a phosphate-free C-atom 2. A comparison with the spectrum of Ins(1,3,4,5,6)P_5 indicates that either C(1) or C(3) are unphosphorylated. Namely, an upfield shifted duplicated doublet (see above) is present now. A removal of one phosphate from the above inositol pentakisphosphate isomer at one of these two positions should render the structure asymmetric, and this is consistent with the presence of six magnetically distinct resonances in the ^{1}H- and ^{13}C-NMR spectra shown. Again only the *myo*-form of inositol is present. And the only contaminant detectable can be assigned by the criteria discussed above to an isomer in which phosphate has migrated from position 1 or 3, respectively, to position 2, most plausibly again by acid migration. Now also the duplicated C(2)H-resonance is visible at about 4.43 ppm. NMR techniques cannot clarify the remaining uncertainty, namely whether D-*myo*-Ins(1,4,5,6)P_4, or its L-enantiomer, D-*myo*-Ins(3,4,5,6)P_4, or a mixture of both is present in the extracts. Making the reasonable assumption that the biosynthesis of inositol pentakisphosphate starts from D-*myo*-Ins(1,4,5)P_3 like in mammalian cells [7,8] the identified Ins(1,4,5,6)P_4 should be the D-

enantiomorph. It either represents solely the degradation product of Ins(1,3,4,5,6)P_5, implicating a very low concentration of the biosynthetic tetraphosphate intermediate, or the latter has the same structure. Without uncertainty, however, one can conclude that Ins(1,3,4,5)P_4, proposed to be the major or sole inositol tetrakisphosphate species generated in mammalian cells [7,8], obviously is absent in avian red blood cells. From the detection limit of NMR spectra and the volume of red blood cells extracted the latter isomer, if present at all, must have a concentration far below micromolar and thus far below that determined in mammalian tissues [13]. HPLC profiles of mammalian cell extracts on the other hand do not indicate the presence of inositol tetrakisphosphate isomers other than Ins(1,3,4,5)P_4 [13,14]. Therefore the presented data indicate that different inositol tetrakisphosphates are prevailing in mammalian tissues and avian red blood cells.

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