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Analysis of isoprenoid phosphates and oligophosphates by capillary zone electrophoresis

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Abstract

Capillary zone electrophoresis (CZE) has been used for the first time to analyse water-soluble phosphorylated isoprenoids, key intermediates in the branched pathway of mevalonate metabolism. Following synthesis, isoprenoid phosphates and oligophosphates were isolated by flash chromatography and their purity was established using ^{31}P NMR spectrometry. In developing optimum conditions for CZE, several factors were considered: ionization properties of the solutes, stability of the solutes and maximum signal-to-noise ratio. At pH 8.5 in 0.3 *M* sodium borate buffer and monitoring UV absorbance at 200 nm, detection of farnesyl diphosphate was linear in the sub-picomol range; the limit of detection was ca. 12 fmol. For a given phosphorylated series (i.e., monophosphates, diphosphates, triphosphates), CZE cleanly resolved isoprenes of different chain length, and plots of relative mobility vs. M_r were curvlinear. Remarkably, neryl phosphate (C10, *α-cis*-isoprene), geranyl phosphate (C10, *α-trans-isoprene*) and citronellyl phosphate (C10, α-saturated isoprene) were resolved. In addition, isopentenyl monophosphate (C5,Δ^3) and dimethylallyl phosphate (C5,Δ^2) exhibited different electrophoretic mobilities. These studies pave the way for future work on determining levels of phosphorylated isoprenoids in various tissues under conditions of altered mevalonate production.

Keywords: Mevalonate metabolism; Isoprenoid phosphates; Oligophosphates; Phosphates

1. Introduction

Phosphorylated isoprenoid compounds are key intermediates in the branched pathway of mevalonate metabolism (Fig. 1). In animals, this pathway leads to several essential end products, including cholesterol, dolichol, ubiquinone and prenylated proteins (see Ref. [1] for a review). In most tissues, the bulk of the carbon flux is directed towards cholesterol. For example, in rat liver, the ratio of cholesterol to dolichol synthesis is on the order of 500:1 [2]. Several studies have indicated that the branched pathway is regulated at multiple steps [3–5]. It has been proposed that the purpose of such regulation is to maintain the levels of isoprenoid diphosphate intermediates (substrates for the synthesis of non-sterol products) under conditions in which the flux to cholesterol is greatly altered by dietary condi-

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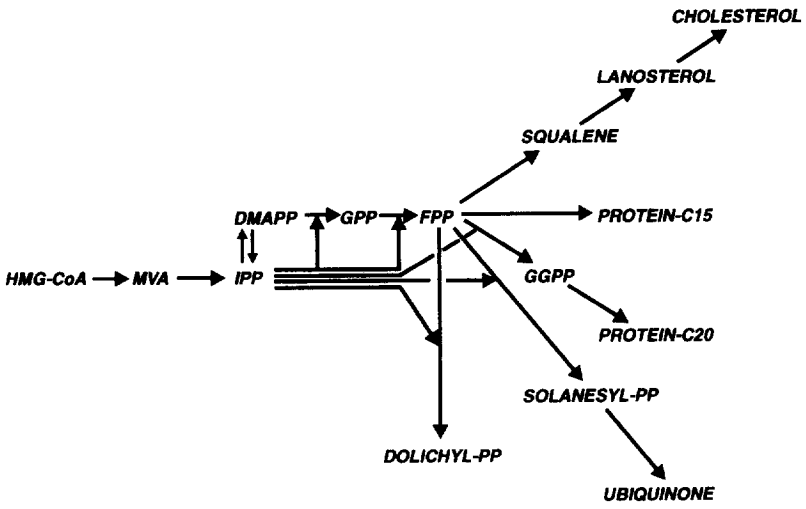

Fig. 1. The branched pathway of mevalonate metabolism. Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; Protein C-15, protein with farnesyl attached to C-terminal cysteine; Protein C-20, protein with a geranylgeranyl attached.

tions [6]. Indirect support for this proposal has been obtained from our studies on the regulation of isoprenoid metabolism in rat liver [7]. In order to test this hypothesis and to gain more information about the regulation of this important pathway, it is essential to develop assays to quantify the levels of the isoprenoid diphosphates. Previous assays have used either reversed-phase HPLC to separate the diphosphates [8,9] or silica HPLC to analyze the alcohols after dephosphorylation [10] or radioisotope dilution assays [4]. All of these techniques suffer from poor sensitivity (nanomole range); in addition, the separation of the isoprenoid compounds from endogenous contaminants by HPLC is often not sufficient for accurate integration of peaks (unpublished data).

Capillary zone electrophoresis (CZE) is a relatively new procedure which combines high sensitivity with excellent resolution. It has been employed to analyze a wide range of biological compounds, including peptides, oligonucleotides and various metabolites (see Ref. [11] for a review). Monitoring of UV absorbance is probably the most common method of detection. In this work, we explored the potential of CZE for the analysis of phosphorylated isoprenoid compounds. Since these compounds contain carbon–carbon double bonds, they can be easily monitored in the far-UV region. In addition, they exhibit high charge-to-mass ratios, ensuring rapid migration in free zone electrophoresis. We show that CZE can be used to (i) assay phosphorylated isoprenoids in the sub-picomol range and (ii) resolve these compounds based on isoprene length, number of phosphates and geometric conformation.

2. Experimental

Isopentenol (3-methyl-3-buten-1-ol), dimethylallyl alcohol (3-methyl-2-buten-1-ol), citronellol, geraniol and farnesol were obtained from Aldrich and geranylgeraniol from TCI Chemicals. The alcohols were converted into the mono-, di-, tri- and tetraphosphates and isolated by flash chromatography according to Keller and Thompson [10]. Solutions of isoprenoid phosphates were standardized by phosphate assay [12]. Molar absorptivities were determined using a Shimadzu 160 recording spectrophotometer.

Samples for electrophoresis were prepared from stock solutions of 1–10 m*M* isoprenoids in

methanol–10 m*M* NH_4OM (7:3) by transferring aliquots into samples tubes (400 μl, polypropylene) and removing the solvent in a Speed Vac apparatus. The dried samples were immediately dissolved in the sample buffer for analysis.

Capillary electrophoresis was performed on a Biofocus 3000 instrument (Bio-Rad Laboratories) using a 24 cm × 25 μm I.D. LPA-coated capillary. The running buffer and sample buffer were 0.3 and 0.03 *M* sodium borate, respectively, both of pH 8.5. The running voltage was constant at 10 kV with positive polarity applied at the detector side and the run temperature was maintained at 20 ± 0.1°C. Samples were injected for 4 s at 5 p.s.i. (1 p.s.i. = 6894.76 Pa). Detection was at 200 nm for most runs (see Figure legends).

^{31}P NMR spectra were obtained on an 8.5 T Bruker AMX-360 wide-bore FT-NMR spectrometer, operating at a ^{31}P frequency of 145.78 MHz, using a 5-mm standard broadband probe. Originally, isoprenoid phosphate samples were present at ca. 10 m*M* concentration in 100 m*M* ammonium hydrogencarbonate buffer (pH 8.4 ± 0.3), but 10% ^2H_2O was added to provide a lock signal. In addition, the geranylgeranyl monophosphate sample was diluted another 25% (v/v) with methanol to aid dissolution. Spectra were obtained at ambient temperature, with a variable number of transients co-added depending on analyte concentrations. An exponential line-broadening function of 5 Hz was applied to the data prior to Fourier transformation. Spectra were referenced using the ^{31}P chemical shift of external 85% H_3PO_4 at 0.00 ppm, with upfield shifts represented by negative values. ^{31}P chemical shifts for farnesyl diphosphate in ^2H_2O (pH 8.0) were previously reported as P-β 6.2, P-α 10.4 using an opposite sign convention [13].

3. Results and discussion

3.1. Purity of isopenoid phosphates and oligophosphates

The mono- and multiply-phosphorylated isoprenoid alcohols were subjected to TLC, HPLC and ^{31}P NMR spectrometry to assess purity. The TLC and HPLC results were characteristic of previous work from this laboratory [10]. Typical ^{31}P NMR profiles for the mono-, di- and triphosphate derivatives of farnesol and geranylgeraniol are shown in Fig. 2 and the chemical shift data for these compounds are given in Table 1. Assignments were made based on the work of Rao [14], where α is designated the phosphate closest to the isoprene group. Based on the combined results from these three different techniques, we deemed the compounds to be of

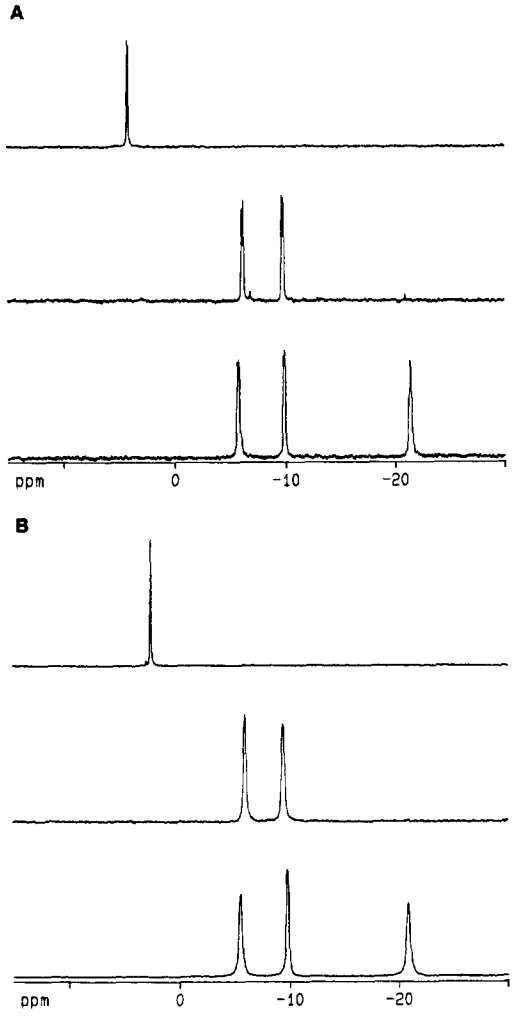

Fig. 2. ^{31}P NMR of (A) farnesyl mono-, di- and triphosphate and (B) geranylgeranyl mono-, di- and triphosphate. Solutions (ca. 10 m*M*) were analyzed as described under Experimental.

Table 1
^{31}P chemical shift data for isoprenoid phosphates and oligophosphates

Sample	^{31}P chemical shifts (ppm)	Sample	^{31}P chemical shift (ppm)
FMP	4.61	GGMP	2.57
FPP-α	−9.28	GGPP-α	−8.73
FPP-β	−5.26	GGPP-β	−4.68
FTP-α	−9.64	GGTP-α	−9.18
FTP-β	−20.73	GGTP-β	−19.21
FTP-γ	−5.00	GGTP-γ	−4.23

^{31}P NMR spectrometry was carried out as described under Experimental.

sufficient purity for analysis by capillary electrophoresis.

3.2. Optimization of conditions for CZE of isoprenoid phosphates and polyphosphates

Our first consideration in optimizing the conditions was pH, since the charge of the particular analyte plays an obvious role in electrophoretic mobility. Our choice of pH was dictated by the following considerations: (1) below pH 7.0, the allylic phosphates are unstable, spontaneously undergoing dephosphorylation and rearrangement [13]; (2) we preferred to analyze a fully ionized species, simplifying the electropherograms. Based on these criteria, we chose borate buffer (pH 8.5) as supplied by Bio-Rad. ^{31}P NMR indicated that all of the compounds tested, including farnesyl tetraphosphate, were fully ionized at this pH (data not shown). Several concentrations of running buffer and sample buffer were tried, but it was found that the conditions recommended by Bio-Rad (0.03 *M* borate sample buffer and 0.3 *M* borate running buffer) generated the most satisfactory electropherograms.

Fig. 3 shows electropherograms of farnesyl diphosphate (FPP) and isopentenyl diphosphate (IPP) at 5-nm intervals from 190 to 230 nm. Since FPP has three isoprene units, it has a substantially higher molar absorptivity than IPP. However, in both cases, the maximum signal-to-noise ratio was observed at 200 nm. Therefore, all subsequent electrophoretograms were carried out at 200 nm.

Fig. 4 shows that the response to FPP concentrations is linear over the range tested. Also shown is the electropherogram of 20 μM FPP (inset). Based on the signal-to-noise ratio, we calculate that the minimum level of detection for

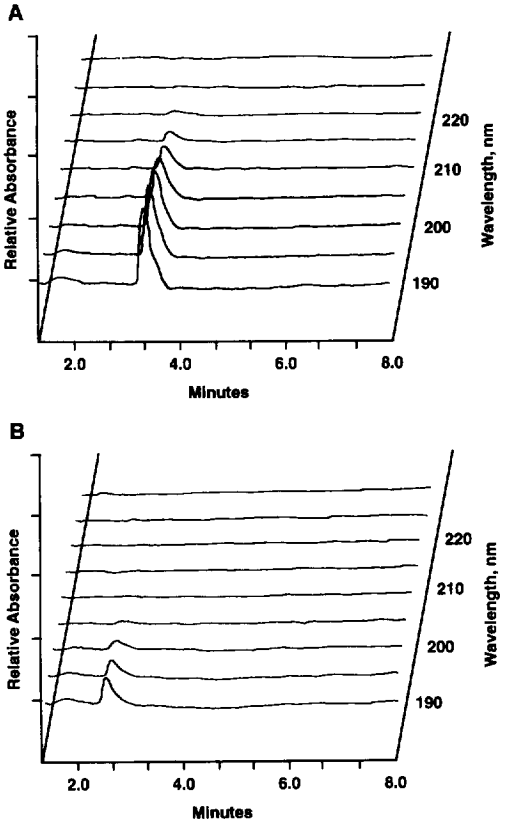

Fig. 3. On-line UV absorption scan of (A) FPP (1 mM) and (B) IPP (2.2 mM) during CZE. Conditions as described under Experimental. Scans were performed every 5 nm from 190 to 240 nm.

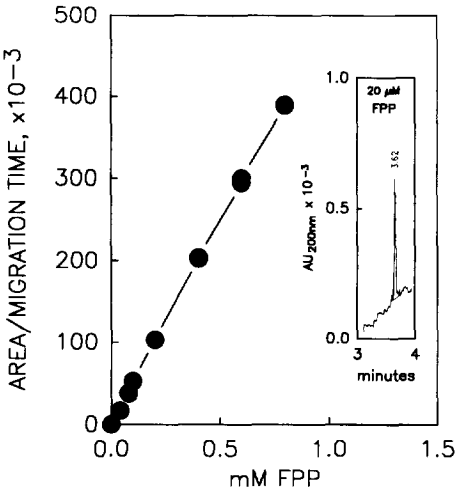

Fig. 4. Calibration graph for FPP. Concentrations from 0.02 to 1 m*M* for FPP were analyzed. The results of two separate runs for each concentration are plotted. Inset: CZE of 20 μM FPP.

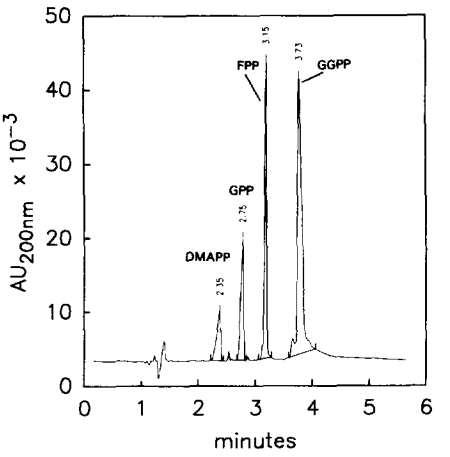

Fig. 5. Resolution of DMAPP, GPP, FPP and GGPP by CZE. The solution analyzed contained 1 m*M* concentration of each isoprenoid diphosphate.

FPP is around 3 μM. Since only 4 nl are injected, this equates to a sensitivity of 12 fmol. Millimolar absorptivities at 200 and 210 nm for the biologically active isoprenoid diphosphates are given in Table 2.

3.3. Capillary electrophoresis of isoprenoid diphosphates

It was important to determine if CZE could resolve the biologically active isoprenoid diphosphates. Fig. 5 shows the electropherogram of a mixture of dimethyl diphosphate (DMAPP, 1 isoprene), geranyl diphosphate (GPP, 2 isoprenes), FPP and geranylgeranyl diphosphate (GGPP, 4 isoprenes). These compounds were clearly resolved. However, the peaks of DMAPP and GGPP showed considerable asymmetry, with DMAPP exhibiting a leading edge and GGPP a trailing edge. Interestingly, the DMAPP peak sharpened with decreasing concentration whereas the GGPP peak sharpened with increasing concentration. The basis for this phenomenon has not been determined, but a preliminary study failed to reveal any micelle formation of GGPP as analyzed by conductivity or ^{31}P NMR spectrometry.

Table 2
Millimolar absorptivities (l/mmol · cm) for isoprenoid diphosphates in 0.03 *M* sodium borate buffer (pH 8.5)

Compound	$\epsilon_{210\ nm}$	$\epsilon_{200\ nm}$
GGPP	16.6	32.0
FPP	14.0	23.0
GPP	7.44	12.5
DMAPP	1.27	3.50
IPP	1.28	4.13

Solutions (10–100 μM) of isoprenoid diphosphates were scanned in a Shimadzu 160 spectrophotometer.

3.4. Capillary electrophoresis of mono-, di- and triphosphorylated derivatives of isoprenoids

When farnesyl monophosphate (FMP) was coelectrophoresed with either FPP, farnesyl triphosphate (FTP) or farnesyl tetraphosphate, it was found that the di- and triphosphates were well resolved from FMP and each other, but that the tetraphosphate exhibited a migration time equal to that of the triphosphate (not shown). Fig. 6 shows the superimposed electropherograms in which FMP and FPP were run separately from FMP and FTP. It is clear from the migration times that the species do not migrate according to a simple charge/mass ratio. Apparently, as

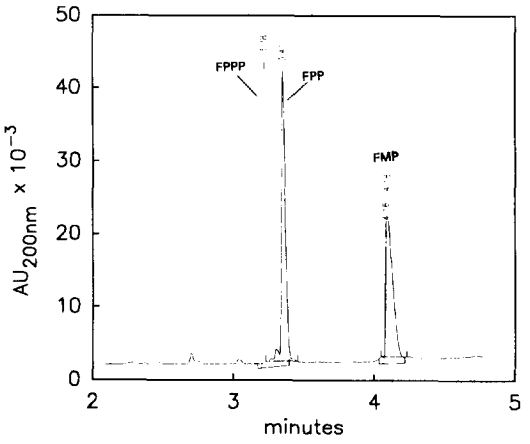

Fig. 6. CZE of FMP, FPP and FTP. Separate runs of FPP + FMP and FTP + FMP were superimposed. The solutions analyzed contained ca. 100 μM of each compound. Conditions as described under Experimental.

phosphate groups are added, bound water molecules and ions contribute a "drag effect", causing the tetraphosphate, for example, to migrate anomalously slower than would be predicted based on charge/mass ratio considerations alone.

Fig. 7 shows plots of the relative (to FMP) electrophoretic mobilities of the mono-, di- and triphosphate series of isoprenoids varying in chain length from one to four isoprenes. In all three cases, the plots are curvilinear upwards approaching the lower isoprenes. This finding is consistent with the molecules going from a rod shape to a more spherical shape with the lower isoprenes, approaching the frictional coefficient of a sphere.

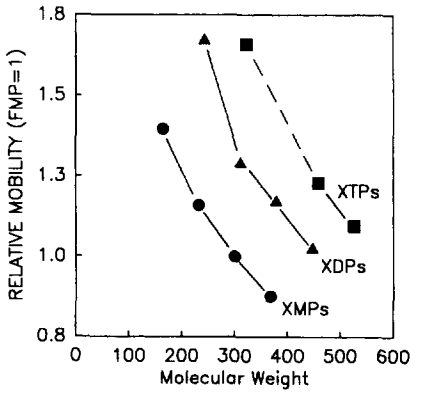

Fig. 7. Plots of electrophoretic mobility (relative to FMP) vs. molecular mass for the (●) mono-, (◆) di- and (■) triphosphate esters of dimethylallyl alcohol, geraniol, farnesol and geranylgeraniol (i.e., increasing molecular mass). Electrophoretic mobility of FMP, molecular mass = 300, set equal to 1 (actual electrophoretic mobility = $1.6 \cdot 10^{-4}$ $cm^2/V \cdot s$). FMP was used as internal standard in all runs. Geranyl triphosphate was not analyzed.

3.5. Capillary zone electrophoresis of isoprenoid phosphates with same charge/mass

To determine if CZE could separate isoprenoid phosphates of the same (or nearly the same) charge/mass ratio, we compared isopentenyl (C5,Δ^3) mono- and diphosphate with dimethylallyl (C5,Δ^2) mono- and diphosphate, respectively (nomenclature of isoprenoid compounds according Ref. [15]). Remarkably, these compounds exhibited slightly different electrophoretic mobilities relative to the FMP internal standard. Thus, IMP = 1.344 × FMP, DMAP = 1.397 × FMP and IPP = 1.595 × FMP, DMAPP = 1.675 × FMP (two runs for each sample, with variation ≤0.001). We also compared three C10 monophosphates: neryl phosphate (ω,Z), geranyl phosphate (ω,E), and citronellyl phosphate (ω,saturated). Fig. 8 shows a superimposition of three runs in which each of these compounds was analyzed using FMP as an internal standard. Surprisingly, all three compounds are resolved, indicating that the conformation of the compound affects the frictional coefficient and thus plays a substantial role in the electrophoretic migration of these molecules.

In conclusion, CZE has been shown to be a useful technique for the separation and quantification of phosphorylated isoprenoids in the femtomole range. Future studies will be aimed at using CZE to detect tissue levels of these important metabolites under conditions which greatly alter the flux through the branched pathway of mevalonate metabolism. Along these lines, it would be interesting to determine the levels of FPP and GGPP in the livers of rats treated with a new class of hypocholesterolemic drugs which block at the FPP→squalene reaction (i.e., squalene synthase inhibitors [16,17]).

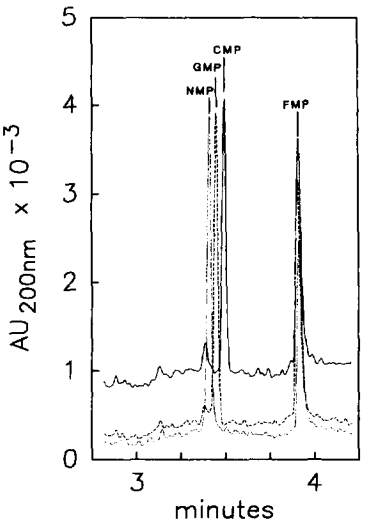

Fig. 8. Resolution of neryl monophosphate (NMP), geranyl monophosphate (GMP) and citronellyl monophosphate (CMP) by CZE. Separate solutions of NMP (ca. 100 μM) + FMP (8.0 μM), GMP (ca. 100 μM) + FMP and CMP (ca. 200 μM) + FMP were analyzed and the data superimposed.

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