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EVIDENCE in vitro FOR AN ENZYMATIC SYNTHESIS OF PHOSPHOCITRATE

Luigi Moro, Nicola Stagni, Elena Luxich, *John D. Sallis, and Benedetto de Bernard

Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Universita' degli Studi di Trieste, Via A. Valerio 32, 34136 Trieste, Italy

* Biochemistry Department, University of Tasmania, GPO Box 252C Hobart Tas 7001, Australia

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SUMMARY: A biological synthesis of phosphocitrate is described from precursors, citrate and adenosinetriphosphate reacting in the presence of rat liver homogenate. Identity of the newly formed product was examined by enzymatic digestion of reactions mixtures, HPLC chromatography and ^{1}H-NMR spectra. Authenticity of product was established by comparison to chemically synthesized phosphocitrate. Recognition of the existence of a biologically synthetic pathway adds credence to the known presence of phosphocitrate in mitochondria and a postulated role to control calcium phosphate deposition in that organelle.

In recent years, interest in compounds capable of exerting influence over biomineralization processes has revealed the exciting potential of phosphocitrate (PC). This phosphorylated carboxylic acid now is recognized to be a very powerful inhibitor of hydroxyapatite formation both in vitro and in vivo (1-4). Following reports of its synthesis in 1980 (2,5), studies have established that exogenous administration of PC offers potential to control events surrounding some of the calcific diseases states. The formation of "mixed" stones i.e. magnesium ammonium phosphate and magnesium hydrogen phosphate, in rats, for example, can be restricted by PC (6). Nephrocalcinosis and deposition of calcium phosphate granules in kidney mitochondria can be prevented by PC (3). Its influence under atherosclerotic conditions is also impressive. Monocyte adhesion to the aortic endothelium of rats maintained on high cholesterol diet is an early damaging event. In the presence of PC however, monocyte adhesion is virtually eliminated (7). In addition, the compound is known

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to restrict uptake of calcium and low density lipoprotein into smooth muscle cells (8), another important phase in the overall development of atherogenesis. Crystal-induced membranolysis such as can occur in certain forms of osteoarthritis is another potential target for PC with the compound providing a protective role against such damage (9).

PC does in fact occur naturally being found in soft tissue mitochondria (10). It is therefore not surprising that in rats at least, there is an absence of any form of cell toxicity when the compound is administered exogenously (7,11). Its true endogenous function however, has never been established. A considered role for the compound in mitochondria is to protect this subcellular organelle against the damaging effect of uncontrolled calcium phosphate precipitation (12). In preventing the transformation of amorphous calcium phosphate to crystalline hydroxyapatite, PC probably acts by binding and thereby interfering with the deposition and growth on nucleating sites of growing crystal surfaces (2,13). The high negative charge to size ratio combined with its stereochemistry is undoubtedly responsible.

The actual origin of the PC found in mitochondria has yet to be established. For synthesis to occur it seems likely that a kinase type reaction must be involved. In the current in vitro study, evidence is presented for the first time that a rat liver homogenate is capable of synthesizing PC in the presence of citrate and ATP. Identification of the product is described through HPLC and ^{1}H-NMR.

MATERIALS AND METHODS

Chemicals: Reagents obtained from commercial suppliers were all of analytical grade. PC standard was prepared by previously reported methods (2,5). Bovine semen acid phosphatase was a product from SIGMA, MO (USA).

Rat Liver Homogenate: A 10% rat liver homogenate was prepared essentially according to Schnaitman and Greenawalt (14), in 0.225 M mannitol, 0.075 M sucrose and 1 mM HEPES.

Assay Conditions: The typical microassay system had a volume of 100 μl containing 150 μg of proteins, 1 mM ATP and 20 mM sodium citrate in a 75 mM TEA buffer, pH 7.6. The incubation time was 60 min at 37° C and the reaction was stopped by adding 50 μl of 160 mM H_3PO_4. After centrifugation of the mixture at 6000 x g for 2 min, an aliquot (20 μl)

was subjected to HPLC. The amount of PC present was evaluated by peak area measurement.

Hydrolysis of phosphocitrate by acid phosphatase: The reaction product of the assay mixture (as profiled in Fig. 1b) was digested with bovine semen acid phosphatase as follows: (a) the volume of a typical assay was divided into two aliquots of 50 µl. One aliquot constituted the control; the second aliquot, after adjustement of pH to 4.5 with 100 mM acetate buffer, was digested by adding 2 µl of a solution of acid phosphatase (1 ng= 1 nU) in 50 mM acetate buffer pH 4.5. The incubation time was 66 h at 37 ° C. The reaction was stopped by adding 23 µl of a solution of 160 mM H_3PO_4. (b) Areas of peaks eluting at around 3.6 min (10 experiments) as profiled in Fig. 1b, were collected, lyophilized and dissolved in 100 µl of 100 mM acetate buffer pH 4.5. Digestion with acid phosphatase was then followed as described in (a).

Identification of Phosphocitrate:

(a) HPLC. Separation was accomplished with a Beckman system GOLD model instrument equipped with an UV-visible detector. Monitoring at due wavelengths (220 and 260 nm) permitted detection of all relevant compounds. Reverse-phase HPLC was performed using a 250 x 4.6 mm i.d. column of Ultrasphere ODS (C18, 5 µm), equilibrated for 120 h with a solution containing 10 mM KH_2PO_4 (adjusted with H_3PO_4 to pH 2) and 5 mM tetrabutylammonium sulphate. The elution solvent used was 10 mM KH_2PO_4 (adjusted with H_3PO_4 to pH 2) at a flow rate of 1 ml/min. The strong acid conditions enabled full resolution of assay mixtures as the molecules were entirely protonated.

(b) ^{1}H-NMR. Samples were lyophilized and dissolved in deuterated water. ^{1}H-spectra were obtained using a Bruker AM 300 WB spectrometer, with a 5 mm 1H-selective probehead. The chemical shift scale was referenced to the resonance of water at 4.8 ppm. Spectra were collected at room temperature using a 36 pulse with 8 s repetition rate. The solvent signal was suppressed by presaturation. For each sample 160 free induction decays were accumulated and Fourier transformed. A resolution enhancement was achieved by a Lorentz-Gauss exponential multiplication, using a line broadening of -0.75 and a Gaussian broadening of 0.1.

RESULTS

As revealed in Fig. 1a, PC and citrate show no absorbance at a wavelength of 260 nm but at 220 nm, the retention times for standard PC and citrate were 3.67± 0.12 (mean of 10 experiments) and 6.47±0.4, respectively. Figure 1b shows a typical chromatogram of the reaction mixture. At 3.65± 0.15 min a peak eluted which absorbed only at 220 nm. Addition of standard PC to the incubation mixture (Fig. 1a plus Fig. 1b) prompted an increase in peak height of material eluting at 3.60 min as shown by Fig. 1c.

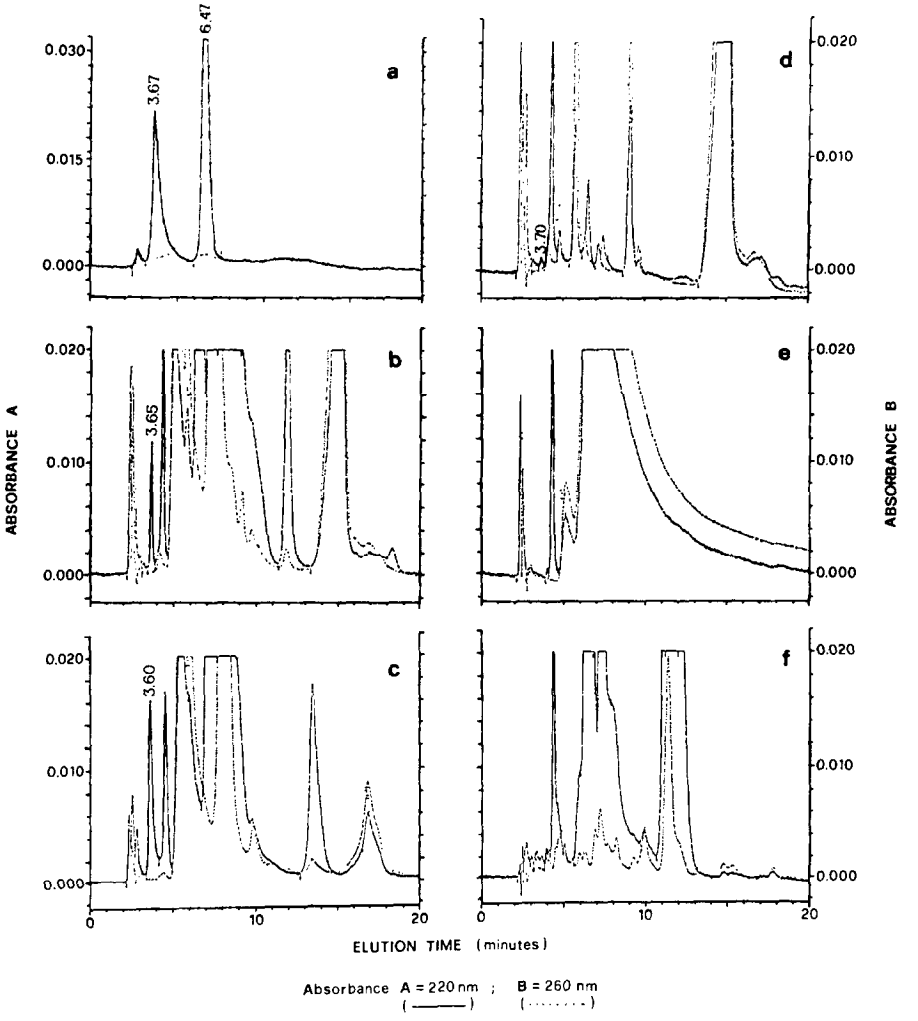

Fig 1: HPLC profiles of:
(a) standards of 2 mM PC (3.67 min) and of 5 mM citrate (6.47 min);
(b) enzymatic reaction mixture (see Methods);
(c) enzymatic reaction mixture with the addition of a standard 2 mM PC;
(d) enzymatic reaction mixture devoid of citrate;
(e) enzymatic reaction mixture devoid of rat liver homogenate;
(f) enzymatic reaction mixture, upon digestion with bovine semen acid phosphatase (see Methods).

Fig. 1d depicts the elution profile for a control mixture from which citrate was omitted. Only a very small peak was detectable at the elution time observed in the previous figures. An identical situation was observed with the control assay containing only homogenate (data not shown). No peak was detectable around 3.60 min in a further control experiment when homogenate was absent (Fig. 1e). The results demonstrate

that eluting material at a retention time of 3.65 min is formed only when citrate and the homogenate are present. The minor peak appearance in Fig. 1b can be considered result of the presence of endogenous compound.

Further evidence regarding the nature of the compound eluting at 3.65 min was gained from treating the assay incubation mixture with an acid

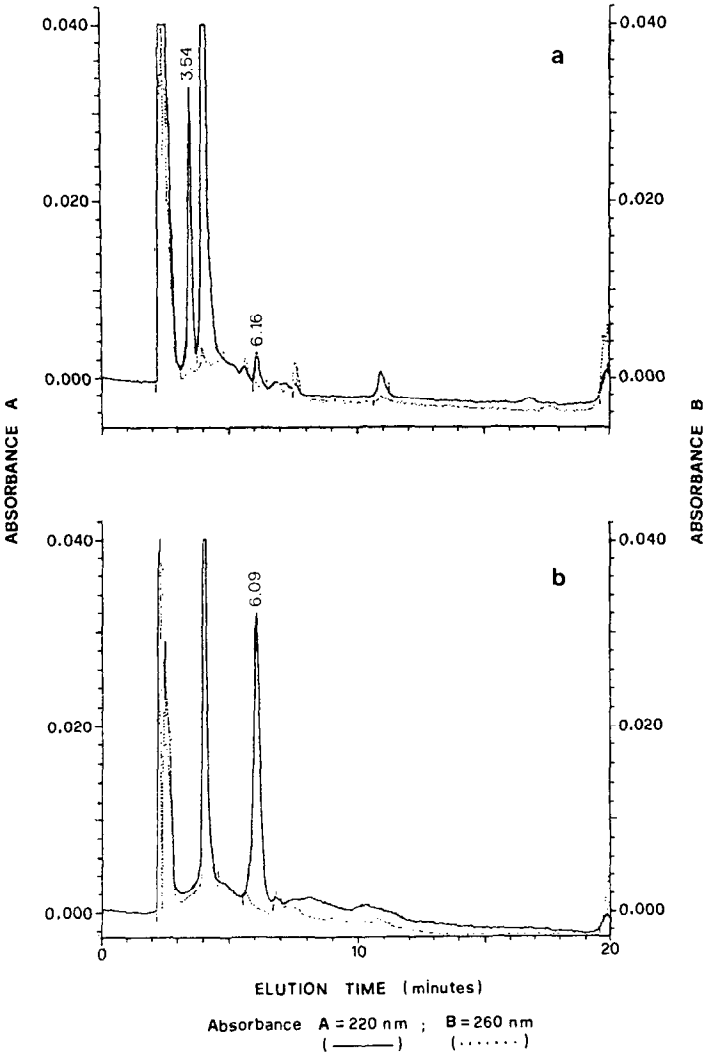

Fig. 2: HPLC profiles of:
(a) eluates around 3.6 min from 10 experiments, lyophilized and dissolved in 10 mM KH_2PO_4, adjusted to pH 2.0 with 160 mM H_3PO_4;
(b) the same eluates subjected to acid phosphatase hydrolysis (see Methods). Peak eluting at 6.09 min corresponds to citrate.

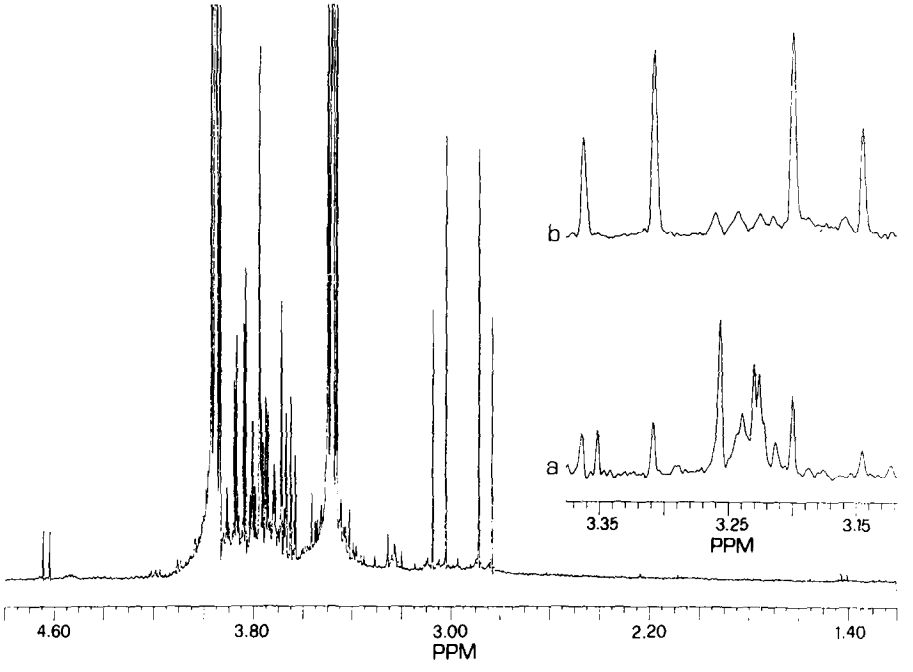

Fig. 3: ^{1}H-NMR spectra of the enzymatic reaction mixture adjusted to pH 2.0.
0.3 ml of the sample was lyophilized and dissolved in 0.5 ml of deuterated water. Spectra were collected as described in Methods section.
(a) expanded plot in the range of 3.12-3.37 ppm. The A_2B_2 doublet pattern of PC gave a J of 16.5 Hz;
(b) the same sample after addition of standard PC buffered at pH 2.0 (final concentration of the standard: 1 mM; the ordinate scales are different).

phosphatase from bovine semen. As shown in Fig. 1f, the peak corresponding to PC was completely abolished. The same result was obtained if the digestion with acid phosphatase was performed on the peak material eluting around 3.65 min followed by re-chromatography of the mixture. The peak at 3.54 min was shown to disappear whilst the peak at 6.16 min corresponding to citrate was noticeably increased (Fig. 2).

An examination by NMR provided further direct evidence of the presence of PC in the enzymatic reaction mixture. The peak positions were confirmed by adding an internal standard of PC to the sample (Fig. 3). The typical A_2B_2 pattern of PC (5), shifted downfield relatively to citrate, was recognizable and a quantitation was possible (data not shown).

DISCUSSION

The present studies demonstrating that PC can be produced by a biological synthesis add credence to previous documented report for the existence of PC in soft tissue mitochondria. Under suitable in vitro incubation conditions which include the presence of an energy source, we have been able to demonstrate for the first time that citrate can be phosphorylated by a rat liver homogenate to the corresponding 3-phosphocitrate molecule. Whilst it seems likely that a kinase type reaction is involved, details of the reaction mechanism must await the isolation and purification of the enzyme to gain understanding of the characteristics of the reaction.

Identification of the biologically synthesized product by HPLC and NMR systems leaves little doubt as to its authenticity. Retention time and peak profiles following chromatography of the reaction mixture on a reverse phase indicated that one of the products present displayed characteristics identical to the chromatogram produced with chemically synthesized PC. Enrichment of peaks assigned to PC and citrate was evident following chromatography with the respective compounds. Further, the anticipated absence of the relevant PC peak and the simultaneous appearance of citrate following phosphatase hydrolysis was added proof of identity. The ^{1}H-NMR patterns also proved confirmatory with the typical field strengths and doublet signals known to occur with PC being readily demonstrable.

The significance of the present findings is that eventually, they should lead to a greater understanding of the physiological role of PC. With a basic concept of how PC is biologically synthesized, it should now be possible to determine cellular conditions relating metabolite concentrations and ionic species which might promote an increased or decreased synthesis of the compound. Studies to determine the cellular distribution of the enzyme system also can now be contemplated. Thus, the role of PC as a natural inhibitor providing control over metastatic calcification and in particular, basic calcium phosphate precipitation in mitochondria may be more easily discernable.

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