A Crystallographically Characterized Nine-Coordinate Calcium−Phosphocitrate Complex as Calcification Inhibitor in Vivo

Konstantinos D. Demadis,* John D. Sallis,†
Raphael G. Raptis,‡ and Peter Baran‡

Department of Chemistry, University of Crete
Heraklion, Crete, Greece 71409
Division of Biochemistry, University of Tasmania
Hobart, Tasmania, Australia 7001
Department of Chemistry, University of
Puerto Rico-Rio Piedras, San Juan, Puerto Rico 00931

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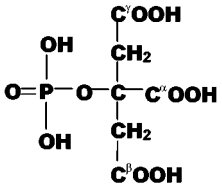
Calcium-containing crystal deposition diseases represent a group of clinically heterogeneous calcific diseases that are a significant source of morbidity.[1] Phosphorylated carboxylic acids are powerful inhibitors of biological crystallization as it relates to those diseases.[2] Phosphocitrate (PC), a naturally occurring compound (Chart 1),[3] is particularly potent. It has been demonstrated to inhibit the transformation of calcium phosphate to hydroxyapatite ($Ca_5(PO_4)_3(OH)$),[4] the deposition of calcium oxalates,[5] the crystallization of octacalcium phosphate ($Ca_8(HPO_4)_2(PO_4)_4{\cdot}5H_2O$),[6] and calcium pyrophosphate ($Ca_2P_2O_7{\cdot}2H_2O$),[7] and the formation of struvite ($Mg(NH_4)(PO_4){\cdot}6H_2O$) in vivo.[8] PC has also expressed inhibitory activity against the formation of scaling Ca salts, such as calcite ($CaCO_3$) and gypsum ($CaSO_4{\cdot}2H_2O$), related to industrial water treatment.[9] In addition, allied to these later actions, PC has been noted to prevent corrosion of carbon steel surfaces.[9] Overall then, the compound attracts keen interest from the viewpoint of its nontoxic nature[10] and potential to influence biomineralization in many diverse biological fields.

In this paper we describe the preparation and crystal and molecular structure of a polymeric mixed salt of PC, namely $[CaNa(PC)_2(H_2O)]_n$ (CaNaPC), and its improved calcification inhibition properties compared to its precursor, NaPC.[11]

CaNaPC forms by the reaction of NaPC and Ca^{2+} at pH ∼2 according to the balanced equation (protons on PC also shown):

$$CaCl_2{\cdot}2H_2O + 2Na_4(HPC){\cdot}3H_2O + 5HCl \rightarrow CaNa(H_3PC)(H_4PC)(H_2O) + 7NaCl + 4H_2O$$

Chart 1. Schematic Structure of Phosphocitric Acid (H_5PC)



The structure of CaNaPC[12] (Figure 1) can be described as polymeric in nature with $Ca(PC)_2(H_2O)$ "monomers" linked through Na^+ bridges.

Ca is coordinated by four phosphate, four carbonyl, and one water O-atoms defining an irregular polyhedron. Coordination number nine for Ca is rather rare.[13] In that regard, the unexpected presence of a coordinated H_2O is the result of the strain imposed by the PC ligand on the coordination geometry, making a wide site available to H_2O. Two biologically relevant examples of nine-coordinate Ca are β-calcium-pyrophosphate[14] and hydroxyapatite.[15] An intriguing feature in the structure of CaNaPC is the short distance of 2.477(1) Å between Ca and the ester *O* from C−*O*-PO_3H_2. For comparison, the Ca−O(pyrophosphate ester) distance in β-$Ca_2(P_2O_7)$ is 2.855 Å. Interestingly, this is consistent with the apparent resistance of the P−O−C moiety to hydrolysis in an acidic environment, suggesting that strong calcium coordination exerts a prominent protective effect on the overall molecule. The carbonyl oxygens are coordinated to the Ca center with Ca−O distances in the 2.446(2)−2.586(2) Å range, much shorter than those in Ca hydrogen citrate trihydrate (2.37−2.49 Å).[16] Similarly, the Ca−O(PO_2H) distance is 2.527(2) Å, much longer than Ca−O distances in related complexes (2.3−2.4 Å).[17] Ca−O distances become elongated as coordination number increases. Ca−O distances in CaNaPC are consistent with these observations. All −COOH groups are protonated due to the low pH of preparation. There are three dissociated protons *per two PC molecules*,[18] all coming from $-PO_3H_2$. Unexpectedly, the second proton from $-PO_3H$ is dissociated before that from α-COOH and is involved in a short hydrogen bond (2.453(3) Å)

* Corresponding author, University of Crete. E-mail: nitrido@onebox.com.
† University of Tasmania. E-mail: J.Sallis@utas.edu.au.
‡ University of Puerto Rico-Rio Piedras.

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(11) In this paper the abbreviation "PC" is used with no specific reference to proton content, unless otherwise noted. Synthesis and characterization of NaPC, as the tetrakis-deprotonated $Na_4(HPC){\cdot}3H_2O$, has been described (Williams, G.; Sallis, J. D. *Anal. Biochem.* **1980**, *102*, 365. Tew, W. P.; Mahle, C.; Benavides, J.; Howard, J. E.; Lehninger, A. L. *Biochemistry* **1980**, *19*, 1983). For the synthesis of CaNaPC, NaPC (3.24 g, 7.83 mmol) was dissolved in 200 mL of distilled water. $CaCl_2{\cdot}2H_2O$ (2.86 g, 19.64 mmol; this amount of Ca^{2+} gave the highest yields) was added gradually as a solid under stirring. The pH started decreasing and some cloudiness formed. Final pH was adjusted to ∼2 with dilute HCl. The solution was then taken to dryness with a Rotovap. Excess Ca^{2+} and Na^{2+} were removed by washing briefly with distilled water. Yield 2.5 g (70%). Product purity was verified by ICP of an aqueous solution of the material. FT-IR spectrum (KBr disks): $\nu_{C=O}$ 1717, 1636 cm^{-1}, ν_{O-H} 3573, 3496 cm^{-1}, $\nu_{P=O}$(asym) 1260, 1230 cm^{-1}, and $\nu_{P=O}$(sym) 1090, 1075 cm^{-1}. Our attempts to prepare crystalline CaPC salts at higher pH have thus far failed due to rapid salt precipitation.

(12) Single crystals of CaNaPC were grown by slow evaporation of a concentrated aqueous solution of the salt prepared as above. They are colorless, monoclinic (space group *C*2/*c*), with $a = 22.331(3)$ Å, $b = 7.966(1)$ Å, $c = 13.233(2)$ Å, $\beta = 107.877(2)°$, $V = 2240.2(5)$ Å^3, $Z = 8$, FW = 311.13, and $d_{calc} = 1.845$ g/cm^3. Intensity data were collected on a CCD SMART diffractometer with Mo Kα radiation. A total of 4751 reflections were measured (1612 unique), 1478 with $I > 2\sigma(I)$ used in the structure refinement by full-matrix least-squares techniques (173 parameters). Final *R* indices: $R_1 = 0.0277$, $wR_2 = 0.0753$, and GoF = 1.072 (for all reflections $R_1 = 0.0303$ and $wR_2 = 0.0770$).

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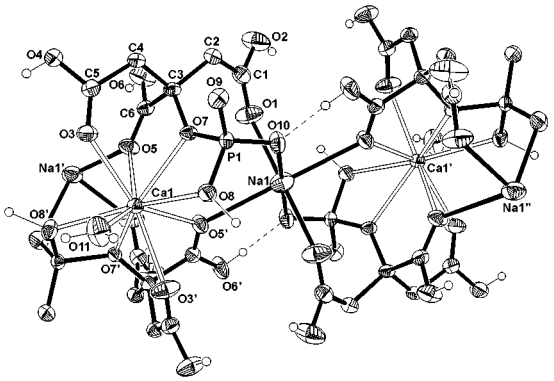

Figure 1. ORTEP diagram (50% probability ellipsoids) of CaNaPC. Important bond lengths and distances (Å): Ca···Ca 8.794(1), Ca···Na 4.3972(5), Ca(1)−O(11) 2.388(2), Ca(1)−O(3) 2.446(2), Ca(1)−O(7) 2.477(1), Ca(1)−O(8) 2.527(2), Ca(1)−O(5) 2.586(2). O-attached protons and two hydrogen bonds (dashed lines) are shown. See Supporting Information for further structural details.

connecting adjacent "ribbons". An oxygen from PO_4 bridges Ca^{2+} and Na^+. The latter, are six-coordinate, a feature commonly found in Na-carboxylate salts.[19] Other structural features of CaNaPC compare well with those of NaPC.[20]

There is a considerable amount of evidence on the efficiency of PC to restrict pathological mineralization in vivo, despite its rapid excretion.[21] A slower, more sustained release form of this compound could therefore offer greater therapeutic benefits. Solubility of Ca^{2+} salts is typically much lower than that of the analogous Na^+ salts. This prompted the present comparison study of its effects to inhibit hardening of an induced plaque in rats. Chemical induction of calcergy has been described previously by Doyle et al.,[22] and this model has been used to demonstrate biological potency of PC.[23] In the present work, male Hooded Wistar rats (200 g) were randomly divided into three groups of five for treatment: A = controls; B = sodium phosphocitrate (NaPC, administered as $Na_4(HPC)\cdot 3H_2O$); and C = calcium phosphocitrate (CaNaPC, administered as $\{CaNa(H_3PC)(H_4PC)(H_2O)\}_n$). At all times, rats had access to water and chow ad libitum.

Plaque formation was chemically induced on day 1 by subcutaneous injection of a 0.1% $KMnO_4$ solution (200 μL dose) in two positions on either side of the interscapular region. Respective salts of PC were dissolved in 0.1 M Tris-HCl (pH 7.2) and 6 h after plaque initiation, treatment was commenced whereby Group A were injected intraperitoneally with 300 μL of buffer alone, Group B received 300 μL of NaPC solution (9.7 mg as H_5PC) in buffer, and Group C 300 μL of CaNaPC solution (9.6 mg as H_5PC). Thereafter, therapy with the aforementioned treatments was given on alternate days. Calcification of plaques was allowed to proceed over 10 days at which time the experiment was terminated and the plaque material present was excised and weighed. It had previously been established that there was a direct relationship between plaque weight and precipitation of hydroxyapatite.[23] Results are presented in Table 1.

Table 1. Inhibitory Action of NaPC and CaNaPC on Plaque Formation[a]

treatment groups	treatment dosage (as mg H_5PC)	plaque weight (mg)[b]	plaque weight reduction (%)
Group A	0	211 ± 9.244	0
Group B	9.7	147 ± 8.825	30
Group C	9.6	11 ± 4.444	95

[a] Data were processed to establish one way analysis of variance with significance determined as a pairwise comparison (Student−Newman−Keuls method). [b] Results are expressed as mean ± SEM for 10 plaques. Statistical significance was determined at the level of $P < 0.001$ for single groups and pairwise group comparisons.

NaPC is an effective plaque inhibitor but at higher and more frequently administered doses.[24] However, as shown in results from Group B, its effectiveness diminishes when a lower dose is used (9.7 mg as H_5PC), resulting in only 30% plaque reduction. Superior inhibition activity becomes evident by following the CaNaPC treatment (Group C), with an equal dose (9.6 mg as H_5PC) giving nearly quantitative (95%) plaque inhibition.

Possible explanations for the improved anti-calcification activity of CaNaPC compared to NaPC could lie with (a) the slower release of "active" PC, thus ensuring its bioavailability at all times by limiting the excreted amount, and (b) the more effective stereospecific interaction between CaNaPC and crystal face(s) of hydroxyapatite. This latter probability could be resolved through future molecular modeling, as the interaction of NaPC with other calcium crystallites has been reported.[25]

In summary, the results presented here reveal a unique neutral organic−inorganic hybrid system that can be synthesized under mild conditions. The polymeric structure of CaNaPC combines interesting features that include a nine-coordinate Ca center, a Ca−O(phosphate ester) linkage, and Ca−O═C bonding (from protonated carboxylate).[26] CaNaPC is a potent inhibitor of plaque formation in vivo, as demonstrated by calcification inhibition experiments on rats. Future studies in our laboratories will focus on efforts to delineate the inhibition mechanisms both in vitro and in vivo. Such studies are currently underway.

Acknowledgment. The assistance of Mr. N. F. G. Parry at the University of Tasmania with animal experiments is appreciated.

Supporting Information Available: Crystal data, bond lengths and angles, SEM images, and EDS spectra of the CaNaPC crystals (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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