Citrate provides essential disorder for bone mineral

Erika Davies¹, Karin H Muller², Chris J. Pickard³, David G. Reid¹, Jeremy N Skepper² and Melinda J Duer¹

¹Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, UK ²Department of Physiology, Development and Neuroscience, Downing Street, Cambridge CB2 3DY, UK ³Department of Physics & Astronomy, University College London, Gower St, London, W1E 6BT, U.K

Submitted to Proceedings of the National Academy of Sciences of the United States of America

We provide evidence that citrate anions are included within the bone mineral crystal lattice and hypothesize that its presence acts to introduce disorder into the bone mineral atomic structure. In order to assess this hypothesis, we take as a model for a citrate-containing, hydrated calcium phosphate, a double salt, octacalcium phosphate citrate (OCP-cit). We use a combination of multinuclear solid-state NMR, powder X-ray diffraction and first-principles electronic structure calculations in order to propose a quantitative structure for this material, in which citrate anions reside in a hydrated layer, bridging between apatitic layers. In order to assess the relevance of such a structure in native bone mineral, we present for the first time 170 NMR data on bone and compare with 170 NMR data for octacalcium phosphate-citrate and other calcium phosphate minerals relevant to bone. The proposed structural model for bone mineral explains a number of known structural features of bone mineral: the thickness of the mineral platelets, the presence of strongly bound water molecules and the relatively high concentration of hydrogen phosphate compared to orthophosphate. We suggest that disorder in bone mineral is essential in order to limit the disordering of the surrounding organic matrix in bone that must occur upon ordered mineral crystal formation and that the incorporation of citrate into the crystal structure of bone mineral provides a simple mechanism to introduce disorder into the atomic structure.

Introduction

The presence of citrate in bone is an accepted fact. Its association with bone mineral was demonstrated in 2010 (1) by solid-state NMR, but while there are many hypotheses as to its role, there is little evidence to date to confirm or deny any of these (11 and see Supplementary Information – text, for other references). Here we propose a role for citrate as a means by which the degree of crystallinity or order in bone mineral is controlled. We present detailed characterization of a model compound, octacalcium phosphate citrate to understand how it could perform this role.

Bone is a complex organic-inorganic composite (2) with inorganic mineral nanoparticles held within a primarily collagen protein matrix. Bone mineral is widely believed to form initially from an amorphous calcium phosphate (ACP) phase within the organic matrix, which then re-organises into a crystalline lattice (3). Key is the fact that formation of an ordered mineral phase from ACP results in a significant decrease in entropy. In order that the second law of thermodynamics is not contravened, there must be a compensating increase in entropy in the surroundings in thermal contact with the bone mineral particles, namely the collagen matrix and surrounding water. However, increasing the entropy or disorder in the collagen matrix is an inherently random and uncontrolled process, undesirable in a system where order in the collagen matrix is required for both molecular and cellular recognition.

Thus the mineralising system needs to be able to control or at least limit the entropy change upon mineral formation so as to prevent potentially pathological disruption of the organic matrix. Significant reduction in the entropy change upon mineral formation can only come about by reducing the atomic order in the final mineral particles. In other words, the mineral particles themselves need the feature of being able to contain a degree of disorder. Solid-state NMR certainly suggests some structural disorder in bone mineral nanocrystals (4, 5), and powder X-ray diffraction studies often describe bone mineral as poorly crystalline (2, 6) so that disorder is present in bone mineral crystals is not in question. More interestingly, there are many biomechanical studies (7–10) that show that the degree of crystallinity of the mineral phase is of critical importance in determining the strength of the bone tissue (11). The answer is central to understanding bone strength and osteodegenerative diseases such as osteoporosis.

With this question in mind, we propose here a new model for bone mineral in which citrate ions reside within the mineral lattice; the ability of the citrate anion to coordinate to surrounding calcium ions via any or all of its one hydroxyl and three carboxylate groups allows it to adopt many possible orientations. The mineral crystals so that the citrate ion adopting different orientations or conformations merely requires the re-positioning of relatively mobile water molecules, and thus does not require substantial re-ordering of the crystal lattice. Therefore, it is likely that there are different citrate orientations/ conformations with relatively similar energies that can all be populated at biological temperatures – in other words, a naturally-arising disorder within the atomic structure of the crystal lattice. In order to provide additional support, we would like to propose here the feature of being able to contain a degree of disorder.

Significance

Bone contains ~2% wt citrate, however its role in bone remains a much-debated question. We provide evidence that citrate anions reside within the bone mineral crystal lattice and hypothesize that it acts to introduce disorder into the mineral atomic structure. The Second Law of Thermodynamics states that the entropy of the Universe can only increase. Thus, formation of an ordered mineral crystal lattice (decrease in entropy) must be accompanied by at least an equivalent increase in entropy (disorder) in the surroundings in thermal contact with the mineral crystals, namely the organic matrix.

We suggest disorder in bone mineral is essential in order to limit the entropy change upon formation of mineral crystals.
double salts characterized, albeit with varying water contents of OCP-citrate double salt on the basis of chemical analysis, with similar formulae for all other tricarboxylic acid double salts was first demonstrated in 1983 (11), with a the structural formula of C_{10}H_{2}O_{3}(PO_{4})_{3}(CIT)_{3}·16.4 H_{2}O, where CIT corresponds to the 3-anion, proposed for the OCP-citrate double salt on the basis of chemical analysis, with similar formulae for all other double salts characterized, albeit with varying water contents to the 3-anion, proposed for the OCP-citrate double salt on the basis of chemical analysis, with similar formulae for all other tricarboxylic acid double salts was first demonstrated in 1983 (11), with a the structural formula of C_{10}H_{2}O_{3}(PO_{4})_{3}(CIT)_{3}·16.4 H_{2}O, where CIT corresponds to the 3-anion, proposed for the OCP-citrate double salt on the basis of chemical analysis, with similar formulae for all other double salts characterized, albeit with varying water contents to the 3-anion, proposed for the OCP-citrate double salt on the basis of chemical analysis, with similar formulae for all other tricarboxylic acid double salts was first demonstrated in 1983 (11), with a the structural formula of C_{10}H_{2}O_{3}(PO_{4})_{3}(CIT)_{3}·16.4 H_{2}O, where CIT corresponds to the 3-anion, proposed for the OCP-citrate double salt on the basis of chemical analysis, with similar formulae for all other double salts characterized, albeit with varying water contents to the 3-anion, proposed for the OCP-citrate double salt on the basis of chemical analysis, with similar formulae for all other tricarboxylic acid double salts was first demonstrated in 1983 (11), with a the structural formula of C_{10}H_{2}O_{3}(PO_{4})_{3}(CIT)_{3}·16.4 H_{2}O, where CIT corresponds to the 3-anion, proposed for the OCP-citrate double salt on the basis of chemical analysis, with similar formulae for all other double salts characterized, albeit with varying water contents to the 3-anion, proposed for the OCP-citrate double salt on the basis of chemical analysis, with similar formulae for all other.
Results and Discussion

In order to develop a quantitative structure for OCP-citrate, we began from the OCP structure, since all previous work and our own shows that the OCP-citrate structure is strongly related to that of OCP. Multinuclear solid-state NMR spectroscopy was used to glean information about the environment of the phosphatic and citrate anions in the structure. We used this information to build a series of possible structural models which were then

Footline Author
then restriction of growth of the crystals in the [100] direction. Flat faces of those crystals corresponded to the (100) plane. That for OCP-succinate (18) where it was determined that the large reflections remain sharp indicates a preferred orientation for containing some element of atomic-level disorder. That some OCP, as also found in previous work (14), indicating a material of the reflection associated with the (100) reflection Figure 2; as found in previous work (14, 18), there is a significant shift in the scattering angle associated with the (100) reflection upon inclusion of citrate: 4.85° in OCP compared with 4.09° in OCP-citrate, indicating the expansion of the OCP unit cell along the a-axis to accommodate the citrate anions (d_{100} = 2.16 nm for OCP-citrate; d_{100} = 1.96 nm for OCP) in the (100) plane of the unit cell structure. Otherwise, the powder XRD pattern of OCP-citrate is similar to that of OCP, although the majority of the reflections are significantly broadened compared to those for OCP, as also found in previous work (14), indicating a material with a lower degree of atomic-level disorder. That some reflections remain sharp indicates a preferred orientation for the OCP-citrate crystals. Such a situation was found previously for OCP-succinate (18) where it was determined that the large flat face of those crystals corresponded to the (100) plane. That there is restricted growth of the crystals in the [100] direction would be consistent with disorder in the (100) plane, leading to imperfect stacking of unit cells along this direction. SAED on individual crystals (Fig S1) similarly indicates atomic position disorder within the crystals.

Thus, we begin our discussion here with an analysis of the NMR and powder XRD structural characterization of OCP-citrate that we used to derive possible structural models for geometry optimization.

**Characterization of OCP-citrate**

OCP-citrate was synthesized and its identity confirmed as described in the Supplementary Information - text, with the synthesis being performed six times to ensure a consistent product was obtained. Microanalysis of our OCP-citrate samples (see SI for details) gave a carbon content of 3.49 ± 0.16 wt%, Ca/P ratio of 2.01 ± 0.05 and a compositional formula of Ca_{10}(PO_4)(HPO_4)(HCIT)(11 ± 1)H_2O, all in agreement with the stoichiometric approximation to the structural formula (14, 22), in particular, the idea that there is one citrate anion per unit cell.

SEM and TEM (Fig S1) shows that OCP-citrate crystallizes into well-formed crystals of constant morphology, typical dimensions being 790×100 nm × 100×30 nm × ~20-50 nm. The powder XRD pattern for freshly-synthesized OCP-citrate is shown in Figure 2; as found in previous work (14, 18), there is a significant shift in the scattering angle associated with the (100) reflection upon inclusion of citrate: 4.85° in OCP compared with 4.09° in OCP-citrate, indicating the expansion of the OCP unit cell along the a-axis to accommodate the citrate anions (d_{100} = 2.16 nm for OCP-citrate; d_{100} = 1.96 nm for OCP) in the (100) plane of the unit cell structure. Otherwise, the powder XRD pattern of OCP-citrate is similar to that of OCP, although the majority of the reflections are significantly broadened compared to those for OCP, as also found in previous work (14), indicating a material with a lower degree of atomic-level disorder. That some reflections remain sharp indicates a preferred orientation for the OCP-citrate crystals. Such a situation was found previously for OCP-succinate (18) where it was determined that the large flat face of those crystals corresponded to the (100) plane. That there is restricted growth of the crystals in the [100] direction would be consistent with disorder in the (100) plane, leading to imperfect stacking of unit cells along this direction. SAED on individual crystals (Fig S1) similarly indicates atomic position disorder within the crystals.
Characterizing the atomic structural disorder is of course a key requirement in this work and requires a characterization technique that relies on local structure, rather than long range order as diffraction methods do. Solid-state NMR spectroscopy is an ideal technique from this point of view, and it is to this that we turned, both to characterize the nature of the atomic disorder in OCP-citrate and to glean information on the position of the citrate anion in the OCP-citrate structure. Solid-state NMR spectroscopy has previously been used to gain atomic-level insights into the structure of OCP-succinate (23), but there have been no reports of solid-state NMR spectra of OCP-citrate. The spectra presented in this section are therefore shown, where appropriate, with comparable spectra of OCP.

The $^1$H NMR spectrum of OCP-citrate (Fig S2), is dominated by a broad signal centred at ~5.5 ppm due to the water in the structure (24), and (presumably) overlies signals from citrate $^1$H as well. The water $^1$H signal in OCP is motionally narrowed, due to the mobility of the water molecules in that structure (24–26). The 5.5 ppm signal due to water in OCP-citrate is significantly broader than the water $^1$H signal of OCP (Fig S2), indicating that the water molecules in OCP-citrate are reorienting more slowly or through a smaller amplitude or both compared to OCP. This suggests that the inclusion of citrate within the structure is interfering with the water channel which lies along the crystallographic c-axis in the so-called hydrated layer of OCP (Fig 1). This is consistent with the citrate anion replacing one of the hydrogen phosphate ions which line the water channels (11, 14) with the citrate anion protruding into the water channel.

The environment and structure of the citrate anion in OCP- citrate are both readily probed via cross-polarization (CP) NMR spectra of the material (Figure 3). The carboxylate carbons give three signals, two well resolved at 179.5 and 182 ppm (labelled COO(1)) and COO(2) respectively, see Fig 3 and a shoulder at ~183 ppm (labelled COO(3)). The quarternary carbon (Cq) gives a signal whose maximum intensity is at 75 ppm, with a broader tail of shoulders to high frequency, whilst the methylene carbons give two signals, a sharper one centred at 43 ppm (labelled CH(2)(1)) and a broader one centred at 46 ppm (CH(2)(2)). Analysis of the integrated intensities in the Bloch decay $^{13}$C NMR spectrum gives the ratio of COO : Cq : CH for the citrate anion as ~3 : 1 : 2, as expected given the chemical formula of citrate (C(6)H(5)O(7)(COO(1))C(2)O). Deconvolution of the three carboxylate signals shows that they have close to equal intensity within the limits of error so they can be assigned to the three carboxylate groups of a single citrate environment. The signals are however broader than one would expect for a highly ordered environment (the $^{13}$C spectrum of crystalline calcium citrate is shown in Fig 3 as an example of linewidths expected for highly ordered environments); thus it seems likely there is some disorder or heterogeneity in these three carboxylate environments.

The two methylene signals, CH(2)(1) and CH(2)(2), also have equal intensities and so can be assigned to the two methylene carbons of a single carboxylate environment as for the carboxylate signals. Clearly then, the two methylene groups of the single citrate environment in the structure are inequivalent, not surprising, given that the OCP unit cell and the citrate anion have incompatible symmetries: the OCP unit cell structure lacks any symmetry except a centre of symmetry, and citrate is not centrosymmetric. More importantly, the methylene group that gives rise to the broad $^{13}$C signal (again, comparing with the linewidths for crystalline calcium citrate in Fig 3) must be disordered in some way as it gives rise to a distribution of $^{13}$C chemical shifts. So at least one source of disorder in the OCP-citrate material is associated with one of the citrate methylene groups.

The most likely source of the disorder affecting a methylene group in citrate is conformational. Multiple conformations for one of the CH$_2$COO(H) branches of the citrate anion, i.e. multiple torsional angles about the C–COO group, would give rise to a different $^{13}$C chemical shift for the methylene carbon for each conformation, a consequence of the so-called y-gauche effect. Multiple conformations of one of the CH$_2$COO(H) branches would also be expected to give a chemical shift distribution for the quarternary carbon via the same effect, which is indeed observed: the tail of shouldes to the high frequency side of the main resonance (Fig 3). Likewise, one would expect some $^{13}$C chemical shift distribution for the terminal COO(H) in the disordered CH$_2$COO(H) branch; there is a broad tail of intensity on the low frequency side of the lowest frequency carboxylate signal (COO(1), 179.5 ppm) and so we tentatively assign this resonance to the terminal carbon of the disordered CH$_2$COO(H) branch of the citrate anion.

The conformational disorder of the CH$_2$(2)COO(H) branch may be static or dynamic. We investigated the possibility of dynamic disorder by cooling a sample of OCP-citrate to 200 K and re-recording $^{13}$C CP MAS spectra. Cooling the sample to 200 K does not change the relative intensities of the CH$_2$(1) and CH$_2$(2) signals, which remain 1:1, but it significantly increases the linewidth of the CH$_2$(2) branch of the $^{13}$C CP spectrum (Fig 4), with the broad CH$_2$(2) signal (46 ppm) beginning to resolve into three or more components. This observation is consistent with the citrate CH$_2$(2)COO(H) branch undergoing reorientational motion at room temperature and that motion being slowed by lowering the temperature so that signals from individual conformers are beginning to be resolved at the lower temperature. The resolution of the sharper CH$_2$(1) methylene signal also increases at 200 K, with a shoulder resolving on its low frequency side, suggesting that this methylene group too has more than one possible conformation or orientation.

Lowering the temperature to 200 K[1] also markedly increases the cross-polarisation efficiency the broad, CH$_2$(2) (46 ppm) methylene signal (Figure 4), the high frequency tail of the quarternary carbon signal (75 ppm) and the low frequency tail of the COO(1) carboxylate signal (179.5 ppm) relative to the other signals in their respective spectral regions. This is consistent with there being room temperature molecular motions affecting the CH$_2$(2) methylene. COO(1) carboxylate and the quarternary carbon environments giving rise to the high frequency tail above 75 ppm, reducing cross polarization efficiency at room temperature by averaging the $^1$H$^{13}$C dipolar coupling which mediates it. Thus, it would appear that the disorder of the citrate CH$_2$(2)COO branch is dynamic at room temperature (and still to some extent at 200 K). That the CH$_2$(2)COO branch is able to reorient dynamically strongly suggests that it is not coordinated to calcium (or any other species), but is relatively free to rotate. Since the citrate anion is contained in the hydrated layer of the OCP structure (see Fig 1), the most likely orientation of the CH$_2$(2)COO branch which permits relatively facile reorientation is for it to be protruding into the water layer itself. This feature will be used when constructing possible models of the OCP-citrate unit cell structure for subsequent geometry optimization.

The nature of the phosphate sites in OCP-citrate and how their environment differs from those in the parent OCP compound are readily assessed by $^{31}$P NMR. The $^{31}$P NMR Bloch decay spectrum of OCP-citrate (Figure 5) shows significantly broader lines than the corresponding spectrum for the parent OCP compound (also shown in Fig 5 for comparison). Some.

[1] Lowering the temperature below 200 K led to the formation of ice crystals on the sample its suggestive of water loss from the sample, and thus possible transformations of the crystal structure.
The question remains as to the assignment of the $^{31}$P signals at 1.1, 1.6 ppm in OCP-citrate. $^{1}H-^{31}$P 2D heteronuclear correlation (HETCOR) spectra allow assignment of the $^{31}$P signals by indicating which $^{1}H$ and $^{31}$P sites are close in space and $^{1}H-^{31}$P 2D HETCOR spectra for OCP-citrate (Fig S3) show that the overlapping signals 1.1, 1.6 ppm are correlated with both water and hydrogen phosphate $^{1}H$ signals at short mixing times (500\mu s), indicating that these signals are due (at least in part) to hydrogen phosphate sites. There may well be orthophosphate sites hydrogen bonded to water, i.e., P5 sites, giving signals in this same $^{31}$P spectral region, but these are impossible to distinguish from the hydrogen phosphate signals as their correlation signals will overlap with those of the expected hydrogen phosphate $^{31}$P - water $^{1}H$ correlation.

The apatitic orthophosphate sites (P1, P4 – see Fig 1) in OCP-citrate give the broad signal centred at 3.7 ppm (cf. 3.2 and 3.6 ppm in OCP for P1 and P4 respectively). The 2D $^{1}H-^{31}$P HETCOR spectrum of OCP-citrate shows a correlation between the apatitic orthophosphate sites and water $^{1}H$ even at short mixing times, indicating that there is significant dipolar coupling between water $^{1}H$ and the $^{31}$P nuclei associated with these sites. We note that given this, mobility of the water molecules dipolar coupled to the orthophosphate sites will affect the $^{1}H$ decoupling process for the $^{31}$P signals from these sites, rendering the $^{1}H$ decoupling less effective and will undoubtedly contribute to the linebroadening of their signals.

Also of interest in the 2D $^{1}H-^{31}$P HETCOR spectrum at long mixing times (10 ms, Fig S3) is that it shows an additional $^{1}H$ signal at 2.7 ppm, which we can assign to citrate methylene $^{1}H$ – this will be important when comparing NMR parameters calculated for trial OCP-citrate structures with experimental data later.

**Water content of OCP-citrate**

Previous studies of OCP-carboxylic acid double salts have found a wide variation in water content depending on the carboxylic acid involved (14, 16, 22). To some extent this is not surprising, as the water content will depend on the amount that the unit cell expands to incorporate the carboxylic acid anion. However, the water content is not entirely correlated with the degree of unit cell expansion and moreover, significantly different water contents were found for the OCP-citrate compound between samples in a previous study (14).

In our own work, we have observed mass loss during recording of NMR spectra and powder XRD, attributed to water loss and confirmed by $^{1}H$ NMR and FT-IR. Given that one of the citrate CH$_{2}$COO(H) branches is likely to be protruding into the water layer (as discussed with reference to the $^{13}$C NMR data), and therefore that the citrate conformation is likely to be at least to some extent interdependent on the water content, we undertook a more detailed study of the effect of changing the degree of hydration of the sample.

Fig S4 shows the powder XRD pattern from dehydrated OCP-citrate material in which the water loss of 6.6 % by weight corresponds to a loss of 7.6 water molecules per unit cell, the d00 reflection has clearly moved to higher 2$\theta$, indicating a shrinking of the unit cell along the a axis with removal of water. In addition, some of the reflections have broadened indicating further disordering. The further disordering is borne out by SEM (Fig S5), $^{31}$P NMR (Fig S6) and $^{1}H$ NMR (Fig S7) spectra. Figure 6 shows $^{13}$C CP and BD spectra of the dehydrated material. There is a clear shift in the intensity distribution towards higher frequency for the CH$_{2}$ and quaternary carbon signals, and significant linebroadening, consistent with conformational disorder now affecting both methylene groups. These data will be used when assessing the possible involvement of an OCP-citrate like structure in bone mineral.

**Constructing Structural Models of OCP-citrate**

Our next step was to use the structural information gleaned from the NMR data discussed above, along with that from powder XRD and FT-IR (14), to construct possible models for the unit cell structure of OCP-citrate that we then geometry optimized.

Specifically, the structural information used in constructing possible unit cell structures was that:

- There is only sufficient carbon content for one citrate per unit cell.
- Citrate replaces a hydrogen phosphate site and so citrate was mainly included in the trial structures as a singly protonated,
2-anion, denoted HCTI\(^{-}\), to achieve charge balance (though 3-citrate anions were included in some models – see Table S1).

- The citrate is most likely to replace a PS hydrogen phosphate anion (FT-IR data (14)), so one (or more) PS hydrogen phosphate anions was removed from the OCP structure.

- The hydrogen phosphate sites are all in the hydrated layer of the OCP structure (Fig. 1) and so the citrate anion was placed in the hydrated layer in all trial structures.

- Incorporation of citrate expands the unit cell along the \(a\) axis.

- The OCP-citrate chemical composition has been determined here to be close to \(\text{Ca}_4(\text{PO}_4)_3(\text{HPO}_4)_2\) (HCTI). (111 \(\pm\) 1) \(\text{H}_2\text{O}, although the water content is demonstrably variable.

- One \(\text{CH}_2\text{COO}(\text{H})\) branch of the citrate is conformationally disordered and is likely to be protruding, uncoordinated, into the water layer.

Ten possible models were geometry optimised using first principles electronic structure calculations as implemented in CASTEP (19, 29, 30), and the NMR parameters and powder XRD patterns associated with the resulting structures calculated; Table S1 in Supplementary Information details the eight geometry-optimized structures which give NMR parameters in some way comparable to experiment. The calculated \(^{13}\text{C}\) NMR parameters (Table S1 and Table 1 below) were then used to determine if a structural model had a realistic environment for the citrate anions, whilst bearing in mind that the calculated values of \(^{13}\text{C}\) shifts are those for 0 K, whereas experimental values are determined at room temperature. In addition, we took into account that the accuracy of the \(^{13}\text{C}\) chemical shifts in these calculations is \(~\pm 1.2\%\) of the chemical shift range of \(^{13}\text{C}\), i.e. \(\pm (2-4)\) ppm (31).

In evaluating the geometry-optimized models of OCP-citrate, a number of trends became apparent.

- NMR calculations on seven of our models showed a significant separation of the two \(^{13}\text{C}\) NMR methane signals (~2 – 12 ppm difference in chemical shift, depending on the structural model) in agreement with \(^{13}\text{C}\) NMR experiments.

- In general, the CASTEP calculations indicated that the \(^{13}\text{C}\) COO\(^{-}\) signals at higher frequency are likely to correspond to Cq-COO\(^{-}\) or Cq-COO\(^{-}\) environments rather than -CH\(_2\)-COOH, consistent with reports of solution-state NMR of metal-citrate complexes (32, 33).

- In metal-citrate complexes, citrate can act as a tridentate ligand, coordinating to metal centres via the \(\alpha\)-hydroxyoxycarboxylate (i.e. Cq-OH and Cq-COO\(^{-}\)) and one of the terminal carboxyls (CtO\(^{-}\)). However, the structures that gave rise to \(^{13}\text{C}\) chemical shifts closest to experiment for the dominant Cq \(^{13}\text{C}\) signal (~75 ppm) are those in which Cq-OH is not coordinated to calcium; structures in which Cq-OH is coordinated to calcium give unrealistically high \(^{13}\text{C}\) chemical shifts for both Cq and Cq-COO\(^{-}\). This suggests that such a binding mode is not preferred in OCP-citrate and so these structures were not considered further.

- In order to predict the effect of hydration level on the \(^{13}\text{C}\) chemical shifts, the geometry of the two of the models (Models A and B in Supplementary Information, Table S1) were reoptimised after inclusion of additional water molecules, and the NMR parameters recalculated. With a single exception, the \(^{13}\text{C}\) chemical shift of all of the CH\(_2\) and Cq environments moved to lower frequency on addition of water (\(\pm (5\text{CH}_2) \sim (3-5)\) ppm, \(\pm (5\text{Cq}) \sim (1-2)\) ppm), and the \(^{13}\text{C}\) chemical shifts of the \(^{13}\text{C}\text{Qt COO}^{-}\) and \(^{13}\text{C}\text{Qt COOH}\) sites moved to higher frequency (\(\pm (5\text{Cq-COO}^{-}) \sim (5-2)\) ppm, \(\pm (5\text{COO}^{-}) \sim (1-3)\) ppm), consistent with the experimental \(^{13}\text{C}\) NMR spectrum of the dehydrated compared with the hydrated material.

- Structures in which one or more of the structural criteria outlined previously were ignored did not give pXRD and NMR data consistent with experiment.

- The lowest energy structure (structure A in the discussion that follows) which fits experimental \(^{13}\text{C}\) and \(^{31}\text{P}\) NMR and powder XRD data, and which is in accord with the expected composition is shown in Fig. 7 (a). The citrate anion in this structure has one CH\(_2\)-COOH\(^{-}\) branch "dangling" in the water channel of the OCP-citrate structure, not coordinated to calcium ions, but surrounded by water. This CH\(_2\)-COOH\(^{-}\) branch is primarily constrained only by the presence of the (mobile) water molecules and thus can be expected to have a distribution of rotational conformers at room temperature, giving disorder in the unit cell structure and a distribution of chemical shifts for the associated \(^{13}\text{C}\) methylene carbon signal and for the quaternary carbon signal, as observed experimentally. The terminal carboxylate group of this dangling branch is not coordinated to any calcium ions, and thus its chemical shift would not be expected to be very strongly affected by the orientation of the branch, which again, is as observed experimentally.

The calculated and experimental \(^{13}\text{C}\) chemical shifts for this structural model are compared in Table 1. Agreement between experimental and calculated chemical shifts is good, especially when one takes into account the fact that the \(^{13}\text{C}\) chemical shifts are calculated for a single 0 K structure whilst experimental measurement is made at room temperature where there is a distribution of citrate conformers. In particular, the lowest frequency carbonate \(^{13}\text{C}\) chemical shift calculated, 178.6 ppm, corresponds to the (protonated) carboxylate group of the dangling, uncoordinated CH\(_2\)-COOH\(^{-}\) branch of the citrate and the highest frequency methylene signal calculated, 45.6 ppm, the methylene group of that same branch in structure A, both features as assigned previously on the basis of experimental observations.

| Comparison of the experimental \(^{13}\text{C}\) chemical shifts for OCP-citrate and those calculated for structure A. For the experimental data, the observed ranges of chemical shifts are given for the broader signals. All values are given in ppm. The calculated chemical shifts for COOH(1) and CH\(_2\)(2) correspond to the uncoordinated CH\(_2\)-COOH\(^{-}\) branch of the citrate anion in structure A. |
| COOH(1) | COO(2) | COO(3) | C\(_3\) | CH\(_2\)(1) | CH\(_2\)(2) |
| Experiment | 182 | 183 | 75 - 77 | 42 - 44 | 44 - 49 |
| Calculated | 187.8 | 181.7 | 184.7 | 79.8 | 37.8 | 45.6 |

Comparison of the powder XRD pattern for the A structural model, assuming a (100) preferred orientation as initially predicted, is shown in Figure 8 and agrees well with the experimental pattern except that the (100) reflection in the calculated pattern appears at higher 2\(\theta\) than in the experimental pattern, i.e. the \(a\) unit cell parameter is smaller in the A structural model than the experimental structure. This discrepancy is to be expected as model A is a structure calculated at 0 K, whereas the experimental XRD measurement is at room temperature and we would expect some unit cell expansion between 0 K and room temperature. There are some other discrepancies, but these are small, especially in view of the uncoordinated CH\(_2\)-COOH\(^{-}\) branch of the citrate anion being conformationally disordered at room temperature, and each of those conformations is likely to be associated with a different spatial distribution of water molecules – in other words, the room temperature structure is in fact a dynamic combination of multiple structures.

Interestingly, a second structure B (Fig 7(b)) with the same chemical composition as A and with only a slightly less favorable internal energy (by 29 kJ mol\(^{-1}\)) in which the citrate anion is flipped ~180° with respect to the \(b\) axis relative to structure A also
yields calculated $^{13}$C chemical shifts consistent with experiment. Thus, the OCP-citrate structure may be further disordered by virtue of the orientation of the entire citrate ion; this feature would certainly explain the two signals that the CH$_3$(1) $^{13}$C NMR signal begins to resolve into at 200 K, for instance.

A third structure C (Fig 7c), 83 kJ mol$^{-1}$ higher in energy than A, with the same atomic composition but with the chemical formula, Ca$_3$(PO$_4$)$_2$(HPO$_4$)$_2$(H$_2$PO$_4$)(CIT)·10H$_2$O, gives $^{13}$C NMR chemical shifts for the citrate anion that are consistent with those observed experimentally for partially dehydrated OCP-citrate. Structure C is a much more compressed structure; the hydrated layer is partially collapsed so that the citrate anion is wedged between the apatitic layers. This sort of structural collapse is a plausible model for a structure undergoing removal of water from the hydrated layer and is likely to be typical of the citrate environment found in the dehydrated material.

**Evidence for an octacalcium phosphate citrate-like phase in bone mineral**

The association of citrate with the mineral component of bone was previously demonstrated with NMR via a $^{13}$C($^{31}$P) REDOR experiment. This experiment identifies the carbon species in close spatial proximity to phosphorus, and as the very vast majority of phosphorus in bone is in the mineral component, the experiment effectively identifies the organic species in closest proximity to the mineral.

Figure 9 shows the $^{13}$C($^{31}$P) REDOR behaviour of adult horse limb bone. In each case, the pairs of spectra consist of a reference $^{13}$C spectrum (black) and a REDOR dephased spectrum (red) in which signals due to $^{13}$C sites close in space to $^{31}$P are dephased, i.e. reduced in intensity, enabling the identification of the organic moieties close to bone mineral. For the bone sample, there is, as observed previously by us and others (34–36), clear dephasing in the 180–183 ppm, 73–77 ppm and 44–48 ppm regions (Fig 9) of the spectrum, which corresponds closely to the signal frequencies for the citrate moiety in OCP-citrate, in particular those calculated for structure C. In which citrate anions are in compressed water layers, bridging between apatite layers.

In structure C, all the carbons in the citrate anion are between 0.36 and 0.46 nm from phosphorous atoms, which is consistent with the $^{13}$C($^{31}$P) REDOR dephasing behaviour observed for citrate in bone (2). It should be noted that bone subjected to REDOR experiments is inevitably partially dehydrated because of the effects of rf heating over the long period of time for which the NMR experiment runs and the nature of the sample preparation (37). Structure A predicts methylene $^{13}$C NMR signals at 38 and 46 ppm (at 0 K), structure B at 40 and 46 ppm and the REDOR spectra in Fig 9 also show dephasing in the 38–40 ppm region, as well as 44–48 ppm. Overall, the NMR data supports the existence of OCP-citrate like structures with varying degrees of hydration in bone mineral.

How could an OCP-citrate structure be incorporated into bone mineral? Bone mineral has a significant component with a structure related to hydroxyapatite of course (albeit with many substitutions). It is well known that hydroxyapatite and OCP can form an epitaxial interface with the minimum of interfacial energy (38, 39) between the (200) plane of hydroxyapatite and the (100) plane of OCP. Hydroxyapatite layers could be incorporated into the apatitic layers of OCP-citrate in a similar manner. The thickness of bone mineral platelets has been estimated to be $\sim 2.5$ nm (40); this measurement via NMR effectively estimates the thickness of mineral between mobile water layers. The thickness of the mineral layer in OCP-citrate between its mobile water layers is 1.2–1.4 nm. Thus adding one unit cell of hydroxyapatite to the (100) plane of OCP-citrate, i.e. intersecting the OCP-citrate structure with a unit cell of hydroxyapatite, which has a thickness of 0.91 nm along its $a$ dimension, would create a supercell with a mineral layer thickness similar to that in bone of $\sim 2.2$ nm.

If we take such a supercell as a model of at least a component of bone mineral, how much of such a structure might be present in bone mineral overall? The citrate content of bone is estimated at between 1–2 % by weight, whilst the mineral component is 60–70 % by weight. If we assume that all the citrate in bone is associated with the bone mineral, then the citrate comprises of order 1.5–3 % of the mineral by weight. Citrate in OCP-citrate constants 6.2 % of the weight of the hydroxyapatite-OCP-citrate super-unit cell formed by adding one unit cell of hydroxyapatite to the (100) plane of OCP-citrate; thus if we take the supercell structure as a model of bone mineral, 1/2 to 1/4 of the supercells would contain citrate. The absence of citrate in the remaining supercells could be modelled by replacing the OCP-citrate component with simply OCP, but with an extended unit cell along the $a$ direction for the OCP component relative to pure OCP. This structure could also incorporate water in the mineral in terms of significant observations. Several studies have shown that bone mineral has strongly bound water associated with it (4, 5, 41–43), with water $^1$H of order $\sim 2.3$–2.55 Å from mineral $^{31}$P (43). The water has previously been assumed to be bound to the surface of mineral platelets, but the HA-OCP-citrate supercell model introduces a further atomic disorder; in addition, the random presence of citrate in some supercells and not in others is a yet further source of disorder.

A stable structure for bone mineral naturally explains a number of significant observations. Several studies have shown that bone mineral is hydrated water associated with it (4, 5, 41–43).

[2] The other signals which show (small) dephasing can all be assigned to lysine and arginine.
The crystalline lattice of bone mineral proposed here suggests a role to be associated with mineral crystal surfaces and the presence of mineral crystals, the strongly bound water, previously assumed to have been lost from ions during crystallization have substantial disorder upon crystalline mineral formation. Free water molecules reorient in thermal contact with the mineral. In a calcified tissue like bone, what is in contact with the mineral. In a biological system, disordering a matrix organised for cell and ligand recognition is an uncontrolled and therefore potentially dangerous process. In our proposed model of bone mineral, citrate anions bridge between apatitic mineral layers allowing the citrate to adopt a variety of conformations and orientations. In the model, the citrate anions are in hydrates layers of the mineral structure and different citrate orientations/ conformations are associated with different arrangements of water molecules to accommodate the citrate anions. Apatitic orthophosphate signals are expected in the 90 – 120 ppm range, whilst hydrogen phosphate, OH $^{17}$O will be much lower, around 60 – 90 ppm, and hydrated orthophosphate somewhere between these two regions. It is clear that neither hydroxypatite nor citrated bone mineral give spectra comparable to bone in this crucial region of the spectrum, crucial because it is the presence of acidic and hydrated phosphate groups in bone mineral that distinguish it from pure hydroxyapatite mineral. However, OCP-citrate provides a reasonable match to the bone $^{17}$O NMR spectrum.

**Conclusions**

The incorporation of citrate ions into the crystalline structure of bone mineral can account for many well known structural features of bone mineral, such as the very small thickness of mineral crystals, the strongly bound water, previously assumed to be associated with mineral crystal surfaces and the presence of significant quantities of hydrogen phosphate ions, in addition to the expected orthophosphate of pure hydroxyapatite.

The structural model of the citrate incorporation into the crystalline lattice of bone mineral proposed here suggests a role for citrate not previously encountered – that it acts to disorder the atomic structure of bone mineral. We further hypothesise that such a disordering role is of fundamental importance in biomineralization. The ordering of mineral ions into a crystalline lattice is always accompanied by a decrease in entropy for those mineral ions. The second law of thermodynamics says that the entropy of the Universe can only increase. Thus a decrease in entropy must be compensated by an increase in entropy elsewhere in the system, the system in this context being all that is in thermal contact with the mineral. In a calcified tissue like bone, what is in thermal contact with the mineral particles is the surrounding organic matrix and water. Thus it is these components that must suffer an increase in entropy, that is become more disordered, upon crystalline mineral formation. Free water molecules released from ions during crystallization have substantial disorder associated with them. However, water molecules released from ions during biomineralization within an organic matrix are not free. They are bound to mineral crystal surfaces and restricted to spaces with nanoscopic dimensions within the organic matrix where they further associate with protein molecules (including proteoglycans where the sugar components bind significant numbers of water molecules). Thus a large decrease in entropy of mineral ions because of formation of well-ordered mineral crystals is likely to necessitate at least some disordering of the surrounding organic matrix.

It has previously been proposed that citrate ions are coordinated to surfaces on the exterior of bone mineral particles (1) via all three of their carboxylate groups, as the available NMR data showed that all three carboxylate groups are in close proximity to phosphate. However, in this scenario, the citrate anion conformation would be significantly restricted, reducing the entropy of the organic matrix-mineral system in bone whose entropy has already been reduced by the ordering of mineral ions into crystalline platelets. This would mean a large compensating entropy change towards disorder would be needed in the surrounding organic matrix and water upon matrix calcification. But in a biological system, disordering a matrix organised for cell and ligand recognition is an uncontrolled and therefore potentially dangerous process. In our proposed model of bone mineral, citrate anions bridge between apatitic mineral layers allowing the citrate to adopt a variety of conformations and orientations. In the model, the citrate anions are in hydrates layers of the mineral structure and different citrate orientations/ conformations are associated with different arrangements of water molecules to accommodate the citrate anions. Apatitic orthophosphate signals are expected in the 90 – 120 ppm range, whilst hydrogen phosphate, OH $^{17}$O will be much lower, around 60 – 90 ppm, and hydrated orthophosphate somewhere between these two regions. It is clear that neither hydroxypatite nor citrated bone mineral give spectra comparable to bone in this crucial region of the spectrum, crucial because it is the presence of acidic and hydrated phosphate groups in bone mineral that distinguish it from pure hydroxyapatite mineral. However, OCP-citrate provides a reasonable match to the bone $^{17}$O NMR spectrum.

**Materials and Methods**

**Synthesis:** OCP-citrate was prepared using a previously published synthetic procedure (22) starting from α-tricalcium phosphate (Ca$_3$(PO$_4$)$_2$; α-TCP) prepared for us by the research group of Prof Serena Best, University of Cambridge using a standard procedure (47). All other reagents throughout the work were purchased from Sigma-Aldrich except where explicitly stated otherwise and used without further purification. Full details (including synthesis of OCP) are given in the Supplementary Information – text.

OCP-citrate (dehydrated): OCP-citrate (152 mg, 0.07 mmol) was stored under vacuum for 2 days (final mass 142 mg), and subsequently water molecules surrounding the citrate anions are reorienting approximately isotropically on a ~ms timescale and so also have a large number of different orientations accessible to them. Thus our proposed model for the structure of bone mineral would provide a much-needed source of disorder in the mineral phase, and so limit the compensating disorder that must occur in the organic matrix.

In this model, the presence of citrate is the key factor controlling the crystallinity of bone mineral, as its presence prevents the long range ordering that would be present in a pure hydroxyapatite structure, for instance, and its concentration controls the degree of order that can be obtained in the hydrated, citrated layers between the apatitic layers. This feature could become important in explaining the changes to bone mineral crystallinity in metabolic diseases (8) and understanding the mechanical properties of bone at a molecular level. Furthermore, the disordering imposed by the presence of citrate anions in the mineral structure naturally restricts growth of the mineral crystals in the [100] direction and so may explain the flat platelet morphology of bone mineral particles.
Sheep Bone: Distal sesamoid (heel) bone was taken from an adult sheep (male; age 4 years). All bone samples were cryopreserved before being packed into NMR rotors.

Adult Horse Bone: Horse limb cortical bone was taken from an adult horse used for general purpose exercise that was euthanized for humanitarian reasons unconnected with this work.

Prior to cryomilling, samples were stored at -80°C.

DFT Calculations

Working models of the OCP-CIT structure were created using the PyMOL Molecular Graphics System (Schrödinger, LLC). These models were then geometry optimised using CASTEP version 5.502 (conditions as per optimisation of OCP).

When determining 31P chemical shifts from the chemical shielding according to $\delta_{\text{ref}}$, with $\delta_{\text{ref}} = 275.3$ ppm. In the case of 13C chemical shifts, the calculated shielding were plotted against experimental shifts, and the reference shielding set to the intercept of the line of best fit, where the gradient of the line was set to -1. Finally, the 1H chemical shifts were calculated acquisition at 1H field strength 3.13 kHz, $\tau_{\text{H}}$, = 30.18 ppm, which was used effectively in a recent study by Webber et al. (2).

The calculations were performed using the Darwin supercomputer of the University of Cambridge High Performance Computing Service (http://www.hpc.cam.ac.uk), provided by Dell Inc. using Strategic Research Infrastructure Funding from the Higher Education Funding Council for England.

Physical characterization

FTIR-ATR spectra were acquired using a PerkinElmer Spectrum One FTIR spectrometer with Universal ATR sampling accessory over the range 650–4000 cm\(^{-1}\) (resolution 4 cm\(^{-1}\)).

PXD measurements were performed on a Philips X-Pert Pro powder diffractometer equipped with an X’celerator Ru(TMS) detector and using Ni-filtered CuKα radiation. Powder samples were mounted on an glast plates and data collection was performed over the range 20 = 3 to 80°.

To prepare samples for TEM, a small amount of material was suspended in pure ethanol, and a drop of the suspension was applied to a holey carbon film grid and allowed to dry. Bright-field TEM was performed in a FEI Tecnai G2 20 electron microscope run at 200–kV. Selected-area electron-diffraction images were acquired using the same microscope at 200–kV (spot size 7).

NMR Measurements

Solution-state 31P NMR spectra of the 10−O-enriched orthophosphate solution were acquired using a Bruker 400 MHz Avance III spectrometer, equipped with a QNP Cryoprobe, at a 1H field strength of 161.98 MHz and 1H frequency of 400.12 MHz. The standard Bruker-supplied “zg90gp” pulse program was used [2] in pulse length 8.63 μs, WALTZ-16 decoupling during signal acquisition at 1H field strength 3.13 kHz, $\tau_{\text{H}}$, = 30.18 ppm, which was used effectively in a recent study by Webber et al. (2).

All solid-state 1H, 13C and 31P NMR measurements were performed on a Bruker 400 MHz Avance spectrometer, equipped with a standard double resonance probe, at frequencies of 400.42 MHz (1H), 162.17 MHz (13C) and 106.05 MHz (13C). Samples were packed into 4 mm zirconia rotors and rotated at a magic angle spinning (MAS) rate of 12.5 kHz, except where otherwise stated.

Samples were characterised using standard Bloch decay (BD) and cross-polarization (CP) MAS techniques (1H 2π pulse length 2.50 μs, 13C π/2 pulse length 2.03 μs, 1H-13C CP 1H field strength 77 kHz, 13C-13C CP field strength 55 kHz, 1H-13C CP contact time 1–4 ms, 3 s recycle time for 1H CP and 300 s recycle time for 13C BD, 60 s recycle time for 1H BD (full relaxation was not required as no quantitative analysis of the 13C spectra was performed); SPINAL64 decoupling was used during signal acquisition at 1H field strength 100 kHz. The 1H CP spectrum of OCP-citrate was recorded at 200 K (MAS rate 12 kHz, contact time 2 ms) using the Bruker Avance spectrometer temperature controller unit.

Two versions of the 1H-13C heteronuclear correlation (HETCOR) experiment were performed: one with frequency-switched Lee-Goldburg (FSLG) decoupling during 1H (1H field strength 100 kHz) and another broadband TPPM decoupling or SPINAL64 decoupling during signal acquisition (1H field strength 87 kHz), and one without FSLG decoupling in it; to check the scaling of the frequency scale in the H-1 dimension, 6 points were collected in the 1H dimension (f1) and 512 in the 13C dimension (f2).

13C (1H) REDOR measurements were performed at a MAS rate of 12.5 kHz by applying a series of rotor-synchronised 1H π pulses (8.40 μs) separated by the rotor period (80 μs) after the initial 1H-13C CP step, with a 13C refocusing π pulse (9.90 μs) at the midpoint of the 1H π pulse train (the length of the pulse train determines the dephasing time).

All solid-state 13C NMR measurements were performed at the UK 850 MHz solid-state NMR facility in the University of Warwick included in the UK 850 MHz solid-state NMR Facility used in this research - via a special funding to the scientific community [1].

ED acknowledges funding by the Cambridge Commonwealth Trust (graduate studentship) and a SCI Scholarship. The authors wish to thank Prof. Grazzi, Dr. Peres for their assistance with the preparation of the manuscript. The UK 850 MHz solid-state NMR Facility used in this research was funded by EPSRC and BBSRC, as well as the University of Warwick including via a special funding through Birmingham Science City Advanced Materials Projects 1 and 2 supported by Advantage West Midlands (AWM) and the European Regional Development Fund (ERDF).

Acknowledgements: ED acknowledges funding by the Cambridge Commonwealth Trust (graduate studentship) and a SCI Scholarship. The authors wish to thank Prof. Grazzi, Dr. Peres for their assistance with the preparation of the manuscript. The UK 850 MHz solid-state NMR Facility used in this research was funded by EPSRC and BBSRC, as well as the University of Warwick including via a special funding through Birmingham Science City Advanced Materials Projects 1 and 2 supported by Advantage West Midlands (AWM) and the European Regional Development Fund (ERDF).
angle and torsional angle effects. Biochemical and biophysical research communications 65:1073–1080.


