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Simulation of Calcium Phosphate Prenucleation Clusters in Aqueous Solution: Association beyond Ion Pairing

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Abstract: Classical molecular dynamics simulations and free energy methods have been used to obtain a better understanding of the molecular processes occurring prior to the first nucleation event for calcium phosphate biominerals. The association constants for the formation of negatively charged complexes containing calcium and phosphate ions in aqueous solution have been computed, and these results suggest that the previously proposed calcium phosphate building unit, [Ca(HPO₄)₃]⁻, should only be present in small amounts under normal experimental conditions. However, the presence of an activation barrier for the removal of an HPO₄²⁻ ion from this complex indicates that this species could be kinetically trapped. Aggregation pathways involving CaHPO₄ ± [Ca(HPO₄)₂]⁻, and [Ca(HPO₄)₃]⁺ complexes have been explored with the finding that dimerization is favorable up to a Ca/HPO₄ ratio of 1:2.

1. INTRODUCTION

Calcium phosphate minerals are ubiquitous in nature and are found extensively in both geological settings and within living systems. The most studied crystalline phase of this mineral family is hydroxyapatite (HA). A carbonated form of HA is found in human bones where it is crucial as a structural element and as a mineral reservoir. It has also been extensively studied due to its wide range of properties, which leads to a wealth of potential applications including the development of new biomaterials or engineered bone tissue replacement, catalysis, liquid column chromatography, and heavy metal removal from soils. Nonetheless, the pathway by which this mineral nucleates from solution is still under debate, and understanding the molecular mechanism behind this process is essential to improve our ability to control the structure and tailor the final properties of this mineral for a desired application.

Many recent studies have tried to probe the structures arising from the association of solutes prior to the initial nucleation event (i.e., the formation of critical size nuclei). Recent interest was spurred by the observation of structures considerably larger than the ion pair, using analytical ultracentrifugation, before nucleation in the calcium carbonate mineral system. This observation was subsequently supported by cryo-TEM and further analysis using molecular dynamics simulations, which showed that clusters with linear, branched, and ring structures composed of close to a one-to-one mixture of Ca²⁺ and CO₃²⁻ ions can form. Whether these clusters simply follow a classical cluster distribution and therefore do not offer a significant change in the classical approach to nucleation remains a debate within the community. Recent work by Smeets et al. indicates that the clusters are unstable at high concentrations leading to the formation of a dense liquid phase, implying a two-step approach to the nucleation problem.

The importance of clusters larger than the ion pair has long been argued for in calcium phosphate, and they have been observed under various experimental conditions. Posner was the first to suggest both the existence of a calcium phosphate amorphous precursor and that formation of hydroxyapatite occurred through the aggregation of Ca₃(PO₄)₆ clusters, later called Posner’s clusters. These have been subsequently investigated both experimentally and via computational methods. The experiments of Onuma and Ito (using atomic force spectroscopy and dynamic light scattering to study the growth of hydroxyapatite) showed the presence of clusters with sizes between 0.7 and 1.0 nm in solution, which they proposed to be Ca₃(PO₄)₆ clusters. Additionally, they observed that hydroxyapatite grew in steps of 0.8–1.6 nm, suggesting that these clusters are the growth unit.

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et al. have used unbiased molecular dynamics to simulate the aggregation of calcium phosphate species toward the formation of Posner’s clusters. However, no quantitative thermodynamics was reported, and the simulations utilized a water model that is now known to freeze at ambient conditions. Kanzaki et al. used ab initio methods to study the thermodynamic stability of various cluster sizes and aggregates of \( \text{Ca}_x(\text{PO}_4)_y \), observing that a number of stable configurations existed on the potential energy surfaces of \([\text{Ca}_x(\text{PO}_4)_y]_n\). From the decrease of the energy as a function of the number of \( \text{Ca}_x(\text{PO}_4)_y \) units, they also proposed that the aggregation of these units could be an alternative to the formation of Posner’s clusters. However, the formation of neutral clusters, as suggested in that study, does not explain the recent observation that the amorphous precursor to the nucleation and growth of calcium phosphate minerals is negatively charged due to a calcium-deficient structure. This is almost certainly because the calculations of Kanzaki et al. considered the stability of clusters in vacuo, and the absence of solvent will have had profound consequences for the thermodynamics.

Investigation of the nucleation and growth of HA within living systems, specifically in murine and zebra fish, has shown that crystallization occurs through the initial precipitation of an amorphous structure (which is also a common mechanism for the crystallization of calcium carbonate). Intriguingly, recent analysis in vitro showed that, in contrast to previous observations where the calcium to phosphate ratio (Ca/P) was observed to be between 1.1 and 1.5, this structure carries a negative charge arising from a calcium deficiency that leads to a Ca/P ratio of 0.67. A similar structure appears to be important in vivo as well. Analysis of osteoblast vesicles lining the growth front of murine calvarial bone using electron dispersive spectroscopy showed a Ca/P ratio of 0.75. A recent in vitro investigation of biomimetic calcium phosphate precipitation by Habraken et al., proposed, by monitoring the pH and composition of the solution, that the inorganic polymeric chains observed before nucleation using cryo-TEM are composed of monomeric units of calcium trihydrogen phosphate complexes. They suggested that these complexes interact through a double hydrogen bond within the polymeric chains; however, this could not be verified. Subsequent densification of the cluster would then occur by the addition of a further calcium ion leading to an amorphous structure composed of \([\text{Ca}_2(\text{HPO}_4)_4]^{2-}\) fractals.

Previous analysis of the formation of clusters for the calcium phosphate system using molecular dynamics simulations and ab initio methods has shown a wealth of potential clusters in solution. Zahn looked at the thermodynamics of association of calcium and hydrogen phosphate using molecular mechanics and ab initio calculations and proposed that the stable initial complexes were \([\text{Ca}_x\text{HPO}_4]^{2+}\) units; however, other potential pathways were not examined. Mancardi, Terranova, and De Leeuw recently analyzed the formation of the negative calcium phosphate complex proposed by Habraken et al. by means of ab initio simulations. While they found a minimum in the free energy curve, the simulations used a small unit cell, and the free energy was sampled only up to 7 Å. Since the Bjerrum length for this reaction is 14 Å, it is therefore impossible to extract the standard free energy change. Furthermore, the length of time sampled was relatively short due to the computationally demanding nature of such simulations, and therefore any slow degrees of freedom (e.g., exchange of bound water at calcium) could not be fully explored. In other studies, Almora-Barrios et al. and Ma et al. looked at the interaction between calcium and phosphate ions in highly concentrated solutions in the presence of collagen using molecular dynamics simulations. They both observed the formation of calcium phosphate clusters. De Leeuw et al. analyzed solutions with only PO₄³⁻ anions present which are unlikely to exist in solution at a pH of 7.4 (the approximate pH of living systems). In contrast, Ma et al. did investigate solutions containing HPO₄²⁻ and a number of clusters carrying a charge were reported. However, Ma et al. obtained their force field for the HPO₄²⁻ molecule directly from the CHARMM database, and the ability of this parameter set to model the bulk structures of any calcium phosphate minerals has not yet been demonstrated, which may mean that the interactions are not fully reliable. This year, concurrently with the present work, a study by Yang et al. on calcium phosphate prenucleation clusters was published which represents the most rigorous investigation thus far as it included extensive sampling of the free energy landscape via bias enhanced sampling. Like the work of Ma et al., it used the CHARMM force field and attempted to validate the parametrization by comparing the ion pairing free energy to experiment and to the earlier values from the Demichelis et al. force field. Yang et al. reported an ion pairing free energy of -27.2 kJ/mol (compared to the experimental value of -15 kJ/mol) and the Demichelis value of -17.9 kJ/mol) indicating that the CHARMM force field overestimates the stability of Ca²⁺ and HPO₄²⁻ binding. As a consequence, they describe a mechanism by which the addition of further phosphate ions leads to a monotonic increase in stability despite the increasing overall negative charge of the cluster.

In the present study, we use molecular dynamics simulations based on a force field parametrized specifically for thermodynamic properties to probe the formation, structure, stability, and potential mechanisms of aggregation that can lead to the formation of the initial species containing two calcium ions in aqueous solution. Free energy profiles for both the formation of the monomeric complexes and their aggregation will be presented, allowing us to calculate their relative concentrations in solution. The results agree in part with previous experimental analysis and show that the complex suggested experimentally as part of the aggregation mechanism, \([\text{Ca}(\text{HPO}_4)_2]^{12-}\), has an exergonic formation. Even though our results suggest that it will not be the dominant species present in the solution, it could become kinetically trapped. However, \([\text{Ca}_2(\text{HPO}_4)_4]^{18-}\), the dimer of \([\text{Ca}(\text{HPO}_4)_2]^{12-}\), is endergonic, making it unlikely that dimerization is the method by which growth occurs. Additional potential species and aggregates were also found, giving new insights into the molecular mechanisms behind the initial precipitation of amorphous calcium phosphate minerals.

2. METHODS

Classical molecular dynamics simulations were used to probe the formation of calcium and hydrogen phosphate complexes and their subsequent aggregation using a force field specifically parametrized with a thermodynamic focus. This force field reproduces the thermodynamics of the phosphate and calcium ions in solution and has been recently used to explore the water structure and dynamics at a surface of the calcium phosphate mineral brushite. A similar method was previously used to obtain a force field for the calcium carbonate system and was shown to capture the stability of the different polymorphs, as well as the solubility, more accurately than...
The water molecules were simulated using the flexible SPC/E force field. In order to be able to simulate the full speciation of phosphates, the force field developed by Demichelis et al. includes parameters for all the relevant anions. Here we concentrate on the hydrogen phosphate anion (HPO$_4^{2-}$) because experimentally it was observed to be the main species during the initial association with Ca$^{2+}$ ions. In the subsequent text, the different oxygen of the HPO$_4^{2-}$ ion and water will be defined as O$_p$ in the case of the nonprotonated oxygen, O$_{HP}$ for the protonated one, and O$_w$ for water.

Multiple-walker well-tempered metadynamics was used to explore the possible association mechanisms involving [CaHPO$_4$]$^{0}$, [Ca(HPO$_4$)$_2$]$^{2-}$, [Ca(HPO$_4$)$_3$]$^{3-}$, [Ca(HPO$_4$)$_4$]$^{4-}$, [Ca$_2$(HPO$_4$)$_3$]$^{5-}$, [Ca$_2$(HPO$_4$)$_4$]$^{6-}$, and [Ca$_2$(HPO$_4$)$_5$]$^{7-}$ complexes. (All the reactions sampled are summarized in Table S1.) Molecular dynamics was performed using LAMMPS with the PLUMED 2.4 plug-in in order to bias the collective variables and compute the pairwise free energies. All simulations were run using a 1 fs time step within the isothermal--isobaric ensemble with a Nosé--Hoover thermostat and barostat. Ions were placed in a cubic box of water molecules with a cell length of 49.843 Å that initially contained 4178 water molecules. Overlapping water molecules were removed leading to systems between with 4142 and 4174 waters, depending on the species inserted. Equilibration for 500 ps resulted in box sizes with cell lengths of 49.6–49.9 Å. All simulations were run at 300 K and 1 atm. Metadynamics simulation times were greater than 150 ns, with specific simulation times per association reported in Table S1.

One of the main challenges in mapping the free energy landscape for cluster formation is in determining the optimal set of collective variables (CVs) onto which to project the free energy. Unfortunately, the simulation cost rapidly increases with the number of CVs due to the dimensionality of the free energy surface, while the analysis also becomes complex. Hence, we have limited the number of simultaneously used collective variables to three. Thus, for the systems with a single Ca$^{2+}$ and up to three hydrogen phosphate ions, it was possible to define a CV between each anion and Ca$^{2+}$, e.g., an association of the form Ca$^{2+}$ + HPO$_4^{2-}$ + HPO$_4^{2-}$ + HPO$_4^{2-}$ → Ca(HPO$_4$)$_4^{4-}$, such that a cluster can form from initially separate ions within a single simulation and theoretically could fully explore multiple aggregation pathways. However, even here this requires the assumption that water exchange at Ca$^{2+}$ is fast on the simulation time scale and that the relative orientation and position of hydrogen phosphate ligands is similarly fully sampled. Once the situation is reached where there are two Ca ions and multiple ligands, then three CVs are generally insufficient to unambiguously map the pathways for association from single ions. In such cases, a stepwise association was performed, e.g., an association of the form Ca$^{2+}$ + HPO$_4^{2-}$ → Ca(HPO$_4$)$_2^{2-}$ + Ca(HPO$_4$)$_2^{2-}$ → Ca(HPO$_4$)$_4^{4-}$. There are now two simulations, but they each require fewer CVs. Here the assumption is made that any reacting species, such as ion pairs, remain bound during aggregation, and this is enforced through applying restraints. To verify that the use of different restraints for different reactions did not lead to a significant error, the stability of all complexes was determined via multiple pathways (see Table S2 for all summed paths). By comparing the overall thermodynamics for different routes between the same species, Hess’s Law was used to check the consistency of the free energy cycles. In some cases, the reduction of number of CVs through restraints was used to allow the inclusion of the Ca–O$_w$ coordination number(s) to account for the expectation that the slowest degree of freedom will be the exchange of water molecules around the cation.

The coordination number (CN) collective variables for both biasing and restraints were defined using the continuous Gaussian function:

$$\text{CN} = \frac{1}{r} \left( \frac{r - d_0^m}{r - d_0^m} \right)^n,$$

For the definition of the Ca–O$_w$ CN, $n = 4$, $m = 10$, and $r$ is the distance between Ca and O$_w$. When used to constrain clusters to remain together, $n = 6$, $m = 12$, and $r$ was taken to be the distance between Ca and P. The coordination number parameters for Ca (d$_{min} = 3.6$ Å for Ca–P, d$_{min} = 2.1$ Å for Ca–O$_w$) were chosen using the position of the first peak of the pair distribution function between Ca and HPO$_4$ and Ca + H$_2$O. In some cases, additionally, upper walls (UW) were used to limit cluster separation. These were defined using a half-harmonic restraint for $x_i^2 a_j^2$

$$\text{UW} = \sum k(x_i - a_i)^2$$

where $k_i$ is the force constant (for restraining a cluster to remain bound the upper wall $k_i = 2$ eV/Å$^2$, and for limiting the distance of separation between two species the upper wall $k_i = 10$ eV/Å$^2$), and the position of the upper wall ($a_i = 3.78$ Å) between calcium and HPO$_4$ was chosen based on the pair distribution function between Ca and HPO$_4$. The necessity of an ion pair upper wall was tested by running a simulation without this restraint, but the ion pairs were observed to break apart during the association process being mapped.

Once free energy curves are obtained as a function of separation, it is possible to obtain the free energy change for the formation of the different ionic complexes under standard conditions. Following the method detailed elsewhere and restated in the Supporting Information, the curves were offset so as to align with the analytic curve for the free energy in the long-range asymptotic limit, as shown in Figure 1. The maximum cluster separation was originally 16 Å for all associations, but for larger associations, where the curve was still not well-aligned to the long-range asymptotic limit, a larger separation of $a_i = 20$ Å was used (see Supporting Information for details of values for each specific association reaction). The maximum distance for the bound state depended on the specific association type and was chosen based on the furthest bound of a minimum which is discernible visually (generally ~11 Å). In this way, the free energy of the solvent-separated association was included in the calculation, thus preventing discrepancies between associations where there is a clear barrier between nonsolvent and solvent-separated associations (for example, all cases in Figure 1) and those where there was no significant barrier (for example, Figure S10). The specification of the upper bound for the association is important since it determines the volume of the complex relative to that for a 1 M concentration, thereby allowing the computed free energies to be corrected to the standard values.

Gaussians were laid every 1 ps along all CVs with a width of 0.1 Å and an initial height of $k_0 T$. Using the multiple-walker algorithm, 30 parallel simulations were run for each energy profile with a bias factor.
3. RESULTS AND DISCUSSION

3.1. Formation of \([\text{Ca(HPO}_4\text{)}_2]^{2-}\) Complexes. Given
that the experimental results suggest that negatively charged
\([\text{Ca(HPO}_4\text{)}_2]^{4-}\) complexes form prior to nucleation\(^{31}\) and that
calcium-deficient amorphous calcium phosphate (ACP)\(^{20,21}\)
forms subsequently, it is important to probe the mechanisms
by which these complexes can form and their relative stabilities
in aqueous solution. The free energy profiles (as a function of
the distance between the Ca and P atoms in the system) are
shown in Figure 1 for the formation of the following species:
\([\text{CaHPO}_4\text{]}^{0}\), \([\text{Ca(HPO}_4\text{)}_3]^{2-}\), and \([\text{Ca(HPO}_4\text{)}_4]^{4-}\). The ion
pair shows a split minimum at a distance of 3.3 Å, which
corresponds to two configurations with a small barrier
separating the states. The inner configuration relates to the
presence of a bidentate interaction (i.e., the HPO\(_4^{2-}\) ion is
interacting with the Ca\(^{2+}\) ion via two O\(_\text{P}\) atoms), while the
outer configuration is due to a monodentate interaction via a
single O\(_\text{P}\). The O\(_\text{H}^-\) bonded to a hydrogen, tends to remain
in solution and interacts with O\(_\text{W}\) through both O\(_\text{H}^-\text{-H}_2\text{O}\) bonding and H\(_\text{H}^-\text{-O}_\text{W}\) bonding. As HPO\(_4^{2-}\) ions are added
to the Ca\(^{2+}\) ion, the inner bidentate state shifts from being the
most stable configuration to a higher energy relative to the
monodentate one. The contact ion pair basin at 3.4 Å is
separated from the solvent-shared ion pair (SSIP) by a barrier
at 4.2 Å. This barrier is 15 kJ mol\(^{-1}\) for \([\text{CaHPO}_4\text{]}^{0}\), while for both
\([\text{Ca(HPO}_4\text{)}_3]^{2-}\) and \([\text{Ca(HPO}_4\text{)}_4]^{4-}\) it is slightly higher
at 17 kJ mol\(^{-1}\). All the curves also show one additional shallow
minimum at approximately 5.1 Å due to the formation of the
solvent-separated ion pair.

The present structural model, described above, accords with
X-ray near-edge structure results,\(^{18}\) which suggest that when a
HPO\(_4^{2-}\) ion forms a contact ion pair with a Ca\(^{2+}\) ion, two
water molecules are displaced from the coordination shell of
the metal cation (see Table 1 and Figure 2), although this does
not lead to a more centrosymmetric arrangement around the
cation since the hydrogen phosphate prefers to bind with two

![Figure 2. Representative geometries for configurations taken from the
local minima of the free energy curves of Figure 1 for the contact ion
pair state of (a) \([\text{CaHPO}_4\text{]}^0\); (b) \([\text{Ca(HPO}_4\text{)}_3]^{2-}\); and (c)
\([\text{Ca(HPO}_4\text{)}_4]^{4-}\). Ca is cyan, P is tan, O is red, and H is white.
Only those water molecules coordinated to calcium are shown for clarity.](image)

**Table 1. Thermodynamic and Structural Properties of Different Complexes of Ca\(^{2+}\) with Hydrogen Phosphate Anions in Water**

| Species            | \(n_{\text{W}}\) in coordination shell | \(n_{\text{W}}\) in hydration shell | \(\Delta G_{\text{assn}}\) (kJ/mol) | \(\Delta G_{\text{from lone}}\) (kJ/mol) |
|--------------------|--------------------------------------|-----------------------------------|----------------------------------|-------------------------------------|
| Ca\(^{2+}\)        | 7.22\(^{18}\)                        |                                   | -17.9                            | -17.9                               |
| \([\text{CaHPO}_4\text{]}^0\) | 5.06                                 | -2.16                             | -17.9                            | -17.9                               |
| \([\text{Ca(HPO}_4\text{)}_3]^{2-}\) | 4.05                                 | -1.01                             | -12.0                            | -29.9                               |
| \([\text{Ca(HPO}_4\text{)}_4]^{4-}\) | 3.05                                 | -1.00                             | +1.1                             | -28.7                               |

"The average number of water oxygens \((n_{\text{W}})\) in the hydration shell of the calcium cation, either as an isolated ion in water or within an ion pair or complex, is given in the second column. The change in average coordination number and free energy of one association \((\Delta G_{\text{assn}})\) are given relative to the species on the previous line of the table in the third and fourth columns, respectively. The free energy of the species formed from ions is reported in the fifth column.

The number of displaced water molecules per addition decreases,
mostly due to the shift of the preferred HPO\(_4^{2-}\) binding
configuration from a bidentate to a monodentate one.

As detailed in the Methods, it is possible to obtain the free
energy change for the formation of the different ionic complexes under standard conditions from Figure 1. For the ion
pair, the value obtained for the free energy of association is
exergonic, \(-17.9\) kJ mol\(^{-1}\), and within a few kJ mol\(^{-1}\) of the
experimental value,\(^{33}\) as previously reported and shown in
Table 1. Exergonic binding was also observed for the formation of the \([\text{Ca(HPO}_4\text{)}_2]^{2-}\) complex with a free energy change of
\(-12.0\) kJ mol\(^{-1}\). Despite the Coulomb repulsion between two
anions, the further addition of a HPO\(_4^{2-}\) ion to this complex,
leading to the \([\text{Ca(HPO}_4\text{)}_4]^{4-}\) complex described by Habraken
et al.,\(^{21}\) also showed an energy minimum at a Ca–P distance
of 3.6 Å but did result in a slightly endergonic addition (+1.1 kJ
mol\(^{-1}\)).

By summing consecutive associations, we can report the free
energy of association of the clusters compared to isolated ions
(see Table 1), which is a different way of showing the same
trend that the \([\text{Ca(HPO}_4\text{)}_2]^{2-}\) and \([\text{Ca(HPO}_4\text{)}_4]^{4-}\) complexes
are roughly energetically equal. These results differ from
the recent work of Yang et al.\(^{31}\) where it is suggested that addition
of HPO\(_4^{2-}\) ions leads to a systematic decrease in the free
energy compared to isolated clusters in solution. In addition
to the possible overestimation due to the CHARMM force
field, when we recalculate binding for their free energy curves
by alignment to the asymptotic limit, rather than using the force
integration method, we get values that are increasingly
discrepant as the total charge increases (see Table S3 and
Figure S21). In the case of \([\text{Ca(HPO}_4\text{)}_4]^{4-}\), Yang et al. report a
binding of \(-81\) kJ/mol for the cluster relative to the isolated
ions in solution; recalculated using alignment this value is
-57 kJ/mol. This value is still twice our reported value, but given
that the ion pair binding of Yang et al. is 1.8 times more
negative than the experimental value this result is consistent
given the systematic overbinding.

By using the changes in free energies detailed in Table 1 and
above, it is possible to calculate the concentration of the
different species in solution for a given starting concentration
and ionic strength. Using a concentration of 0.006 M Ca\(^{2+}\) ions
and 0.005 M HPO\(_4^{2-}\) ions, approximately that used by
Habraken et al.,\(^{21}\) and the same ionic strength (i.e., \(I = 0.02\)
M), the result suggests that in an equilibrated solution
approximately 74% of the Ca\(^{2+}\) ions would be unbound, 16%
would form ion pairs, and 11% would form a complex, namely,
\([\text{Ca(HPO}_4\text{)}_4]^{2-}\). The percentage of \([\text{Ca(HPO}_4\text{)}_4]^{4-}\) under
of S for the well-tempering. In the cases where a bias factor of S
returned an energy significantly larger than 15 kJ/mol, the simulations
for that CV were rerun with a bias factor of 15 to ensure that the
larger barrier was overcome by the simulations. Representative cluster
geometries were chosen by determining the lowest free energy
configuration via analysis of the free energy as a function of the CVs
(see Supporting Information).
these conditions would be insignificant. Similarly, under the conditions used by Xie et al., 0.00122 M Ca$^{2+}$ and 0.0073 M HPO$_4^{2-}$, 64% of the Ca$^{2+}$ ions would be free ions, 18% would form ion pairs, and 18% would form [Ca(HPO$_4$)$_2$]$^{2-}$, again with an insignificant concentration of [Ca(HPO$_4$)$_4$]$^{4-}$. However, depending on the solution mixing conditions, [Ca(HPO$_4$)$_3$]$^{4-}$ complexes could form and then be kinetically trapped for a short period, as the barrier to removal of an HPO$_4^{2-}$ ion from this complex is 17 kJ mol$^{-1}$.

The differences between our simulations and the experiments of Habraken et al. and of Xie et al. may arise from the height of the activation barrier that must be surmounted to escape the contact state. However, it is important to emphasize that our results indicate that negatively charged complexes in solution already represent stable structures, even without a counterion. When these complexes form, they could then become kinetically trapped due to the height of the activation barrier that must be surmounted to escape the contact state.

3.2. Aggregation of the Different Complexes in Solution. According to the computed equilibria for the [Ca(HPO$_4$)$_4$]$^{4-}$ complexes under representative experimental conditions, the main bound species present in solution are CaHPO$_4$ and [Ca(HPO$_4$)$_2$]$^{2-}$. As the concentration of phosphate increases, the more negative [Ca(HPO$_4$)$_3$]$^{4-}$ complex could also become important. To form larger stable complexes or aggregates, as seen experimentally, these species need to interact favorably in solution. The calcium phosphate complexes could aggregate directly in their current form, through the interaction with counterions or by the loss of HPO$_4^{2-}$. The free energy changes presented in Table 2 allow the comparison of the stability of the different complexes.

The differences between our simulations and the experiments of Habraken et al. and of Xie et al. may arise from the height of the activation barrier that must be surmounted to escape the contact state.
The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali- The other key di- energy curves shown in Figure 3 for (a) \([\text{Ca}^2+]\) simulations and potentiali-

Figure 4. Representative geometries for configurations taken from the minima in the region of calcium–calcium distances of ~4 Å for the free energy curves shown in Figure 3 for (a) \([\text{Ca}_2(\text{HPO}_4)_2]^{5–}\); (b) \([\text{Ca}_3(\text{HPO}_4)_2]^{5–}\); (c) \([\text{Ca}_3(\text{HPO}_4)_2]^{5–}\); (d) \([\text{Ca}_3(\text{HPO}_4)_2]^{5–}\); and (e) \([\text{Ca}_3(\text{HPO}_4)_2]^{5–}\). Ca is cyan, P is tan, O is red, and H is white. Only those water molecules coordinated to calcium are shown for clarity.

Figure 5. Free energy of formation from the component ions (\(\Delta G\)) for dimeric clusters \([\text{Ca}_2(\text{HPO}_4)_m]^{j+k–}\) shown in red, compared to the sum of the corresponding free energies for the single calcium species \([\text{Ca}(\text{HPO}_4)_l]^{2–}\) shown in blue (where \(j + k = m\)). The individual data points for the three methods for calculating \(\Delta G(\text{Ca}_2(\text{HPO}_4)_m)^{j+k–}\), as outlined in Table S2, are shown with red crosses, while the averages of the two methods giving the largest discrepancy are shown with black circles, where the error bar corresponds to the standard error.

4. NUCLEATION PATHWAYS

From the results discussed above, a number of conclusions can be drawn concerning the nucleation mechanism of calcium phosphate. However, it is important to emphasize that we are not claiming to have demonstrated a definite pathway to nucleation and crystallization since this lies beyond the scale that is readily accessible with current atomistic simulations. In this article, the aim was to critique, using molecular simulations, the conclusions drawn from experimental results reported for the nucleation and growth of calcium phosphate.
minerals by Habraken et al. and Xie et al. This gives a greater understanding of the mechanism involved and which molecular species could play an important role.\textsuperscript{20,21} The observation of a calcium-deficient amorphous precursor formed via the interaction of negatively charged complexes is surprising as it is not usual to think of natural processes evolving through electrically unbalanced reactions, and it is thus important to verify its existence.

The calcium phosphate complexes proposed by Habraken et al., \([\text{Ca(HPO}_4\text{)}_{\text{3}}]^{4-}\), are shown here to be (meta)stable. However, at experimental concentrations, the simulations suggest that 11% of the Ca\(^{2+}\) ions would form \([\text{Ca(HPO}_4\text{)}_{\text{2}}]^{2-}\), while 19% would form ion pairs and the rest would be free in solution. Even though equilibrium calculations suggest that \([\text{Ca(HPO}_4\text{)}_{\text{3}}]^{4-}\) complexes would form in negligible quantities (0.19%) in solution, they could become kinetically trapped for a short period as the calcium and phosphate solutions are mixed together. Additionally, the amount of each complex present in the solution will depend upon a number of factors that include the solution composition, the presence of organic molecules, and the physical constraints applied to the solution. In the experimental work suggesting the formation of \([\text{Ca(HPO}_4\text{)}_{\text{3}}]^{4-}\), a TRIS buffer was used and could have stabilized this complex. It is well-known that organic molecules can have an important impact on the nucleation and growth of minerals from solution.\textsuperscript{47-50}

This study shows that, for calcium phosphate, the formation of aggregates from the units that could be present in solution, i.e., \(\text{CaHPO}_4\), \([\text{Ca(HPO}_4\text{)}_{\text{2}}]^{2-}\), and \([\text{Ca(HPO}_4\text{)}_{\text{3}}]^{4-}\), is favorable for the smaller clusters. However, as the aggregation continues, the states become metastable with respect to the smaller clusters and could be important during nucleation. The work by Hu et al. used a modified version of classical nucleation theory (CNT).\textsuperscript{20,21} Hu et al. argued that if, in contrast to most presentations of CNT, we do not assume that the interfacial energy for clusters is the same as that for the bulk phase, but allow it to change as a function of the size of the forming nuclei, reaching a negligible value for the ion pair, then the transition free energy barrier will be scaled by

\[
\Delta G_i = B - \frac{\alpha^3}{(\sigma \pm C)^2}
\]

where \(C\) is a factor that depends on various parameters, including the shape of the cluster, the cluster radius, but most importantly the excess free energy of the cluster. Here \(\alpha\) is the interfacial free energy and \(\sigma\) depicts the favorable energy arising from the association of ions in solution. \(B\) is a constant that varies according to the shape and density of the nucleating solid. Whether there is a plus or minus sign in the denominator depends on whether the energy minimum occupied by the cluster is of higher or lower energy than the ions (or ion pair) in solution. In the latter case, a minus sign will apply, and the barrier to nucleation will be increased. In the former case, the barrier will be decreased, and the complexes are likely to lie on the nucleation pathway. As we found metastable states for the aggregation of complexes that could be present in solution, following the analysis of Hu et al., these could lie on the nucleation pathway. Habraken et al. extended the approach of Hu et al. to consider the case where the unit of growth for the critical nucleation cluster was a molecular cluster rather than individual ionic species. Evidence for such clusters was seen in their experiments. This implies a different nucleation pathway to that envisaged by simple classical nucleation theory. Our results in Figure 5 show that clusters with fewer than five \(\text{HPO}_4^{2-}\) ions are more stable than the units from which they are built. As argued above, this will tend to increase the nucleation barrier. However, it is still possible that the overall barrier is lower because the pathway is different. Unfortunately, it is beyond the ability of simulation to investigate this at present. The most we can say is simply repeating the dimerization process is unlikely to be the only mechanism for the precipitation of amorphous calcium phosphate. The dimer of \([\text{Ca(HPO}_4\text{)}_{\text{3}}]^{4-}\) is much higher in energy compared to its separate state, suggesting that \([\text{Ca(HPO}_4\text{)}_{\text{3}}]^{3-}\) is unlikely to be required for amorphous growth.

5. CONCLUSIONS

Our results, obtained from classical molecular dynamics simulations, provide further evidence that negatively charged complexes of calcium phosphate can indeed form and are stable in solution confirming previous experimental observations. However, calculation of the equilibrium constant for the different calcium phosphate complexes suggests that, in the experimental conditions used by Habraken et al.\textsuperscript{21} and other authors,\textsuperscript{20} the ion pair would be the dominant calcium phosphate species in solution. The barrier inhibiting the loss of an \(\text{HPO}_4^{2-}\) ion, 17 kJ mol\(^{-1}\), suggests that \([\text{Ca(HPO}_4\text{)}_{\text{3}}]^{4-}\) complexes formed could be kinetically trapped to some extent. In this scenario, the large negative charge could attract Ca\(^{2+}\) ions, which would then be able to bridge complexes to form larger charged aggregates, which are (meta)stable, as shown using metadynamics. These inorganic clusters are chemically similar to the amorphous calcium phosphate found experimentally in two separate studies. Additionally, the fact that vesicles in osteoblasts present at the growth front of bone showed a Ca/P ratio of 0.75 suggests the potential importance of this pathway for the in vivo mineralization of calcium phosphate.

The aggregation of \(\text{CaHPO}_4\) and \([\text{Ca(HPO}_4\text{)}_{\text{3}}]^{2-}\) leads to configurations that are more stable in energy than the separated state, suggesting that they could lie on the nucleation pathway. This would increase the barrier to nucleation as above. However, the idea that nucleating clusters could be made from units of molecular complexes, used by Habraken et al. to explain how amorphous calcium phosphate could form at their studied supersaturation, is still viable since the corresponding nucleation pathway could still have a lower barrier overall. Incorporating a dependence on cluster size of the interfacial energy was shown to decrease the nucleation barrier of amorphous calcium phosphate to a reasonable value.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.cgd.9b00889.

Supplementary methods containing an explanation on the determination of the lowest free energy structure; table containing every association reaction performed and its corresponding standard free energy, time simulated, biased CV(s), and walled CV(s); table containing the standard free energies for formation of each ion cluster with the corresponding uncertainty; recalculated free energies for formation of each ion cluster from the previous work of Yang et al. for...
comparison based on alignment of the free energy to the long-range asymptotic limit; figures containing the free energy as a function of distance and/or calcium-water coordination number and a minimum geometry for each association performed; free energy as a function of distance recreated from the previous work of Yang et al. for comparison; supplementary discussion on the alignment of free energy profiles (PDF)

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**Notes**

The authors declare no competing financial interest.

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