Why Do Protein Crystals Grown With the Aid of Porous Materials Show Better Diffraction Quality?

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Why do protein crystals grown with the aid of porous materials show better diffraction quality?

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Abstract: Well-diffracting protein crystals are indispensable for X-ray diffraction analysis, which is still the most powerful method for structure-function studies of biomolecules. A promising approach to growing such crystals is by using porous nucleation-inducing materials. However, while protein crystal nucleation in pores has been thoroughly considered, little attention has been paid to the subsequent growth of the crystals. Although the nucleation stage is decisive, it is the subsequent growth of the crystals outside the pore that determines their diffraction quality. The molecular-scale mechanism of growth of protein crystals in and outside pores is here considered theoretically. Due to the metastable conditions, the crystals that emerge from the pores grow slowly, which is a prerequisite for better diffraction. This expectation has been corroborated by experiments carried out with several types of porous material, such as Bioglass (“Naomi’s Nucleant”), Buckypaper, porous gold and porous silicon. Protein crystals grown with the aid of Bioglass and Buckypaper yielded significantly better diffraction quality compared with crystals grown conventionally. In all cases, visually superior crystals are usually obtained. We furthermore conclude that heterogeneous nucleation of a crystal outside the pore is an exceptional case. Rather, the protein crystals nucleating inside the pores continue growing outside them.
1. Introduction

X-ray diffraction studies account for 89% of structures deposited in the Protein Data Bank and, in most cases, continue to provide significantly higher resolution than other techniques, such as nuclear magnetic resonance and cryo-electron microscopy. However, well-diffracting protein crystals are notoriously difficult to produce. Porous materials help to mitigate this problem [1-11]; the synergetic effect of diffusion in the confined pore space and protein adsorption on pore walls can ensure a local supersaturation increase that is just sufficient for crystal nucleation to occur, whereas very high supersaturation is highly unlikely to be achieved [12]. Because the nucleation of crystals makes the difference between success and failure of the crystallization attempts, nucleation of protein crystals in pores was considered first, e.g. see [13]. That work has shown that to get 3D crystals it is vital to have 2D crystals nucleating first; protein layers of monomolecular thickness formed in pores can become a completely stable (i.e. destined to grow) crystal nucleus when the cohesive energy $\Delta G_v$ (that maintains the integrity of a crystalline cluster) and the destructive energy $\Delta G_s$ (tending to tear it up) become equal. However, relatively little attention was paid to the subsequent growth of the nucleated crystals. In [13] it was noted merely that “once nucleated, such crystallites continue their growth outside the pore orifice, forming 3D crystals.”

The aim of this paper is to consider the molecular-scale mechanism of growth of protein crystals in pores and outside them. It has been shown that the growth process is favored energetically in both cases – inside and outside the pore. Thus, it proceeds spontaneously, i.e., without the need for forming 3D nuclei, neither inside nor outside the pore. Most importantly however, this analysis explains why the use of porous materials as nucleants contributes to the growth of protein crystals of better diffraction quality.

2. Results

Growth of protein crystals in pores. There is a significant difference in the growth of crystals in the confined space of the pores and in the free space outside them. In the former case, only the volume of the crystals increases, whereas their exposed surfaces (which are subjected to the destructive action of the water molecules) remain constant - the pore size hardly changes on a molecular distance. This means that $\Delta G_s$ remains constant. In contrast,
both crystal volume and surface are constantly increasing during the growth of a crystal outside the pore.

After the formation of a stable 2D nucleus, its growth begins with the addition of molecules that are connected simultaneously to the wall of the capillary and to the lowest crystal layer (i.e., the stable 2D nucleus itself). The following molecules are then bound simultaneously to these and to the lowest crystal layer. This enables the continuous growth of the crystal in the pore and finally, a completed protein layer of monomolecular thickness is deposited upon the existing stable 2D crystal nucleus.

Let us consider the simplest crystal lattice, which is the so-called Kossel crystal (that is, a simple cubic symmetry crystal constituted by small cubes held together by equal forces) shown in Fig. 1. Evidently, the growth of the stable 2D crystal nucleus is an energetically driven process. The reason is that the cohesive crystal energy $\Delta G_v$ increases substantially due to the increased number of intermolecular bonds (importantly, bonding between the two protein layers of monomolecular thickness starts occurring), whereas the destructive energy $\Delta G_s$ remains the same. Denoting the number of molecules at the edges of the Kossel crystal by $n$ and $n_1$ (see Fig. 1), we see that the bonding between the first and second crystal layers is secured by $nn_1$ intermolecular bonds. Adding them to the $(n - 1)(n_1 - 1)$ bonds (which are the same as in the first layer of monomolecular thickness), the increase in bonding is:

$$\frac{[nn_1+(n-1)(n_1-1)]}{(n-1)(n_1-1)} = 1 + \frac{nn_1}{(n-1)(n_1-1)} > 2,$$

i.e., the growing crystal becomes more than twice as strong. Of course, this calculation is merely indicative, since it does not consider the interaction with the walls of the pore; the latter depends on the type of protein and the porous material, but it is weaker than the bonds between the molecules in the crystal; so, it does not change the course of the process.
Figure 1. Double-layered Kossel crystal growing in a pore having the shape of a rectangular prism \textit{(the so-called cuboid)}. The blue arrow shows the growth direction of the crystal.

Figure 2. Top view of a hexagonal pore orifice. Two closest packed layers of monomolecular thickness are formed: a second (ditrigonal) layer B (red balls) is deposited on the completely stable crystal nucleus, layer A (blue balls) with edge length $L = 3$).

It is worth emphasizing, however, that the increase in $\Delta G_v$ depends on the type of crystal lattice. The Kossel crystal in Fig. 1, which has a relatively low-density packing will be compared with closest packed crystal lattices (e.g., see Fig. 2). This comparison enables important conclusions about the energetically driven growth of protein crystals in pores.

For the comparison, hexagonal closest packed (HCP) crystals are considered. It is seen in Fig. 2 that the second layer of monomolecular thickness, deposited onto the completely
stable (destined to grow) crystal nucleus, is a ditrigonal layer, which is a little smaller than the first (hexagonal) layer and does not touch the pore wall. This means a smaller binding energy $\Delta G_{vd}$ between the molecules constituting it. However, as it will be shown below, this deficit is overcompensated because each molecule in the closest-packed surface layer (in this case the ditrigonal one) interacts with three molecules beneath it. In other words, the work (energy) needed for stripping off one overlaying layer is always three times the number of molecules in this layer. In HCP crystals, the third layer is also hexagonal. Importantly, the destructive energy $\Delta G_s$ tending to tear up the crystal, is always equal to the number of molecules in the uppermost crystal layer (those are alternatively ditrigonal and hexagonal layers) that are exposed to the destructive action of the water molecules.

The known (from crystallography) equations for the number of molecules and for the number of intermolecular bonds in closest packed (ditrigonal and hexagonal) monolayers enable numerical calculation of the relations between $\Delta G_v$ and $\Delta G_s$ for 1, 2, 3 etc. layered crystals. Denoting the number of molecules in the longer ditrigonal crystal edges in Fig. 2 by $L$ (which equals the number of molecules in the hexagonal layer beneath it), the number $Z$ of molecules in the ditrigonal monolayers is:

$$Z = 3(L - 1)^2$$

which gives $Z = 12, 27, 48, 75 \ldots$ for $L = 3, 4, 5, 6 \ldots$ respectively.

And denoting the number of molecules in the edges of the hexagonal layers by $\lambda$, we obtain the number $z$ of molecules in such layers:

$$z = 3\lambda(\lambda - 1) + 1$$

which gives $z = 19, 37, 61, 91 \ldots$ for $\lambda = 3, 4, 5, 6 \ldots$ respectively.

The formula for the number of bonds $\Delta G_{vd}$ in ditrigonal layers is:

$$\Delta G_{vd} = (3\lambda - 5)(3\lambda - 3)$$

(Recall that $\lambda$ is the number of molecules at the edge of a hexagonal layer, which coincides with the longer edge of the ditrigonal layer, for instance see Fig. 2.)

The crystallographic formula for the number of bonds $\Delta G_{vh}$ in the hexagonal crystal layer is:

$$\Delta G_{vh} = (3\lambda - 3)(3\lambda - 2)$$

Finally, the destructive energy $\Delta G_s$ is, respectively:

$$\Delta G_s^d = Z\psi_d$$
and:

\[ \Delta G_v^h = z\psi_d \]  

(6)

where \( \psi_d \) is the destructive energy per bond.

Using these equations, the calculations results are shown in Table 1. (Again, since the interaction with the walls of the pore is not considered, these results are indicative only. Evidently, the wider the pore the less important the positive effect of the capillary walls becomes.) The analysis is stopped at \( \lambda = 6 \) because, due to kinetic reasons, bringing together into the crystal nucleus more than 90 to 100 molecules via molecule-by-molecule assembly involves very large fluctuations - which in turn require very long waiting times.

**Table 1. Calculation results for HCP crystal**

| Thickness of crystal growing in pore | Number of molecules in the edge of the hexagonal layer (\( \lambda \)) | Crystal binding energy |
|-------------------------------------|-------------------------------------------------|-----------------------|
| \( \Delta G_v^h \) (in 2D nucleus layer) | 42 | 90 | 156 | 240 |
| \( \Delta G_v^h + \Delta G_d^d \) (in two layers)\(^a\) | 102 | 234 | 420 | 660 |
| \( 2\Delta G_v^h + \Delta G_d^d \) (in three layers)\(^b\) | 180 | 405 | 720 | 1125 |
| Ratios | | | | |
| \( \Delta G_v^h / \Delta G_s^h \) | 2.21 | 2.43 | 2.56 | 2.64 |
| \( (\Delta G_v^h + \Delta G_d^d) / \Delta G_d^d \) | 8.5 | 8.67 | 8.75 | 8.8 |
| \( (2\Delta G_v^h + \Delta G_d^d) / \Delta G_s^h \) | 9.47 | 10.9 | 11.80 | 12.36 |

\(^a\) The bonds between the two layers are added.

\(^b\) The bonds between the three layers are added.

**Table 1** shows a sharp increase in crystal stability (somewhat mitigated at larger crystal sizes; evidently, a very wide pore would approach the limit of a flat surface) with the thickening of the HCP crystal. The fourth crystal layer is again ditrigonal, i.e. one with reduced number of protein molecules exposed to the destructive action of the water molecules (and not touching the pore wall). Thus, the tendency is clear: the growth of protein crystals in pores - occurring by deposition of layers of monomolecular thickness - is energetically favored and thus, ceaseless. In other words, there is no need of forming a stable (post-critical) three-dimensional crystal nucleus for continuing the growth of the protein crystal in the pore. The comparison between HCP and Kossel crystals shows that with widening of the pores, the
growth of the protein crystals inside them is increasingly stimulated in the former case, while the energy stimulus decreases in the second case.

**Growth of the protein crystals outside the pores.** Undoubtedly, the point at which the protein crystal reaches the orifice of the pore is a turning point in the growth process. Perhaps the most important factor is the fact that the crystal grows outside the pore under reduced protein concentration, as compared to that inside the pore. Consequently, outside the pore, the growth of the crystal slows down. And the slower the crystal grows, the more perfect it is: During slow growth, the crystal swallows up fewer impurities, and thus has fewer defects - which means improved diffraction quality. Thus, an important advantage of porous materials can be that, by lowering the supersaturation under which the crystals nucleate and grow (compared to the one needed for the usual crystal nucleation), the porous nucleants can produce protein crystals of better diffraction quality. This theoretical suggestion has been confirmed experimentally.

Obviously, to grow in three dimensions outside the pore orifice, the protein crystal must also expand laterally. Indeed, the wider space outside the pore is already advantageous for growing 3D crystals - because they are fed from more directions (which somewhat mitigates the effect of the reduced protein concentration outside the pore). Provided the supersaturation is not too low, protein molecules can attach firmly enough (to two molecules in the crystal, as shown by the arrows in Fig. 3a). However, the open space outside the pore may by itself be insufficient; the shape of the pore orifice is also important. Sharply angular pore orifices (like in Fig. 3a) may not be very suitable in this respect. Fortunately, real pore orifices are generally devoid of sharp angles, but look more like, for example, the one schematically shown in Fig. 3b. Especially for HCP crystals, such pore orifices are more suitable for lateral growth of the crystal outside the pore, the reason being that the molecules on the right side of the pore orifice (in Fig. 3b) are connected more firmly – not only with two adjacent molecules, but also with the inclined surface of the pore orifice itself. To put it simply, slowly but surely, the protein crystal grows outside the pore orifice (and no 3D nucleation is needed).
Figure 3. Cross section of HCP crystal emerging from a pore. (a) The pore has the shape of a rectangular prism (like in Fig. 1); alternative layers: hexagonal A (blue balls) and ditrigonal B (red balls) are presented. For further growth outside the pore, protein molecules are attached (at places shown by the arrows) to the crystal. (b) The pore has one sloped orifice surface (shown on the right). Protein molecules adsorb on the inclined surface and facilitate the growth of the next crystal layer.

Evidently, the wider the pore orifice, the more energetically favored the growth of a monomolecular layer outside it (Fig. 3), the reason being the substantial increase of the binding energy: the binding energy between the two protein layers of monomolecular thickness is added to the binding energy between the molecules in the newly deposited layer of monomolecular thickness. It is worth recalling that the narrower the pore, i.e., the smaller the crystal nucleus, the higher the supersaturation required for its formation. Thus, too narrow pores are unsuitable for nucleation of protein crystals because the pore opening is reached, and the protein molecules enter the pore with the same probability with which they reach an equally large flat surface area (which means that narrower pore orifices are less accessible). However, the wider the pore the less important the positive effect of the capillary walls becomes (and a very wide pore would approach the limit of a flat surface). The conclusion is that there is an optimal range of pore orifice widths.

Experimental: diffraction quality of protein crystals grown by using porous materials. It is worth recalling that the usual approach to growing better crystals is by separating the nucleation and growth stages. Lowering the supersaturation at which the crystals grow (after their nucleation at a higher supersaturation value), yields visually better crystals. Using
porous nucleants, such an approach is superfluous because nucleants are added at conditions that are known from preliminary experiments to provide insufficient supersaturation to yield crystals. In other words, the supersaturation is already lowered before inserting the nucleants. Thus, practically, only protein concentrations in the metastable zone are applied for growing protein crystals with the aid of porous nucleants; labile, spontaneous nucleation conditions (i.e. supersaturations above the super-solubility curve, that are necessary for additional nucleation of 3D crystals outside the pore, as supposed by Page and Sear [14]) are never used.

Our data for actual diffraction of proteins on Bioglass and other solid nucleants are reported here. Another porous material has been successfully tested as a protein crystal nucleant: molecularly imprinted polymers (MIPs). It should however be noted that the pores in the MIPs are too small (of the size of one or, possibly, very few protein molecules), therefore, no growth inside such pores is possible. Thus, this part of the theory does not apply to the MIPs, which function via a different mechanism of specific molecular affinity. However, the MIPs do decrease the supersaturation that is needed for protein crystal nucleation. Thus, the protein crystals grow under lower supersaturation, and according to the theoretical expectation, they should be of better diffraction quality. This theoretical expectation has also been confirmed by our experiments with MIPs, where 6 out of 8 proteins tested under metastable versus higher supersaturation conditions (which were otherwise identical) gave better diffracting crystals [8, 10].

Diffraction data have been obtained for three target macromolecules, i.e. two proteins and one modified cyclodextrin, all of which required improved diffraction in order to determine their structure.

(i) Crystals of the C1 domain of the human cardiac myosin-binding protein-C (MyBP-C), obtained on Buckypaper made from an aggregate of single-walled carbon nanotubes and surfactant Triton X-100 (Fig. 4), diffracted to a maximum resolution of 1.6 Å (more typically in the 2.0-2.2 Å range), which is far superior to the best crystals obtained using standard techniques, which only diffracted to 3.0 Å [7]. The dominant pore size in that Buckypaper was 9 nm. The crystals grew at metastable conditions in the trials with porous material (10 mg/ml protein in 50 mM NaCl and 20 mM Tris ph 7.0, equilibrated by vapour diffusion against a reservoir solution of 18% polyethylene glycol (PEG) of mean MW 3350 and HEPES buffer, pH 7.3). The standardly obtained crystals were obtained from 20% PEG reservoir solutions [7]. Importantly, in all cases, the crystals in
the drops containing nucleant were single, i.e., not in clusters that may have appeared if repeated nucleation of novel 3D crystals outside the pore had occurred.

(ii) Crystals of InHr2 were obtained in the presence of Bioglass at metastable conditions as well as at ‘borderline metastable’ conditions, i.e. conditions that gave visible crystals overnight in the presence of Bioglass, but only after 6 days in the absence of porous material (10 mg/ml protein equilibrated against a reservoir solution of 11% PEG of mean MW 3350, 0.1 M imidazole at pH 7 and 75 mM MgCl2). The best of these crystals, obtained from the latter conditions, diffracted to 3.2 Å, as compared to ca. 5 Å for routinely obtained crystals at higher PEG concentrations.

(iii) Crystals of the cyclodextrin derivative per(6-guanidino-6-deoxy)-γCD (gguan) that were obtained at metastable conditions in the presence of Bioglass (Fig. 5) diffracted to better than 1.08 Å, whereas all X-rayed crystals routinely grown in standard, conventionally optimized conditions, diffracted to 1.3 Å at best. This improvement in resolution enabled the determination of this unusual structure (manuscript in preparation).

To be completely convinced of the porous material's ability to induce the formation of crystalline nuclei, we always have used controls in each crystallization trial. While crystals were observed in the samples containing porous nucleants, the controls set up at exactly the same conditions but without addition of nucleant, were crystal-free. Undeniably, this is completely convincing evidence that the porous material acted as a nucleant.

Figure 4. Crystals of MyBP-C growing on Buckypaper at metastable conditions (Reprinted with permission from Asanithi et al. ACS Appl. Mat. Interf. 6, 1203-1210 (2009). Copyright 2009 American Chemical Society)
3. Discussion

Page and Sear [14] have found that, after the pore has been filled, the crystallization stops for a relatively long period of time. The authors attributed this pause to the existence of a second nucleation barrier, i.e., to the formation of an additional 3D crystal nucleus that is needed for further crystal growth. Page and Sear state that “the nucleation often proceeds via two steps: nucleation of pore filling, and nucleation out of the pore”. However, in our approach, secondary crystal nucleation is not needed for the growth of the crystal outside the pore. In fact, the protein crystal emerging from the pore orifice is a prolongation of the crystal grown in the pore (Fig. 3), and hence, this crystal can hardly be under-critically sized. Importantly, the surface of the crystal in the pore (which is a large part of the total crystal surface) is protected from the destructive action of the water molecules, while the crystal itself is strengthened due to the bonding to the pore walls. Thus, recalling that, according to nucleation theory, for the same crystal volume a lower surface energy corresponds to a lower supersaturation, we compare the crystal emerging from the pore with a heterogeneously formed 3D crystal nucleus of the same volume; it is evident that the formation of a 3D crystal nucleus requires higher supersaturation than the one that is sufficient for the protein crystal to grow outside the pore orifice. Hence, no additional 3D nucleation is required. The protein crystal emerging from the pore orifice (Fig. 3) simply grows outside it without impediment.

Figure 5. Crystals of gguan growing on a grain of Bioglass (shown by red arrow) at metastable conditions. Scale bar = 100 μm (First published in Khurshid et al. Nat. Prot. 9, 1621-1633 (2014)).
Besides, there is no guarantee that, if any, nucleation of a novel 3D crystal outside the pore (see [14]) would be limited to a single crystal nucleus only - repeated formation of nuclei cannot be excluded. Excess nucleation, leading to formation of sub-grains in the grown crystal, would also be possible. In such cases, the diffraction quality of the grown protein crystals would be deteriorated [15]; but this is not observed experimentally.

4. Conclusion

Growth of protein crystals in model pores of idealized shapes, rectangular and regular hexagonal cross-sections, has been considered here (the reason being that they are readily liable to be brought to quantitative accounts). Nevertheless, the *unimpeded* growth of the crystal in pores is proven in an irrefutable manner. The use of model (idealized) pore shapes is justified because, as already shown [13], the regularity of the pore shape is of no importance for nucleation of protein crystals in pores; non-regularly shaped pore cross-sections yield results that are very similar to those obtained with idealized pore shapes. Besides, only the binding energies between the protein molecules in the crystal lattices are compared. The reason is that the energy of adhesion of the protein molecules to the pore wall (which additionally stabilizes the crystal) increases by the same value for every deposited monomolecular layer. Thus, the addition of the energy of adhesion of the protein molecules to the pore wall would merely complicate the consideration but does not change the general tendency towards crystal growth (with increasing crystal size, the adhesion energy plays a lesser and lesser role).

Finally, the exact cause of the frequent poor diffraction quality of protein crystals remained unclear till most recently. Koizumi *et al.* [15] have shown that it is not the dislocations, but the local strain due to sub-grains that degrades the diffraction quality of protein crystals. These authors demonstrate that the large stress exerted on the sub-grains of protein crystals could significantly lower their resistance to radiation damage, and that this strain can potentially be controlled by introducing grown-in dislocations in the crystal. Therefore, we may advance the hypothesis that the misfit between the crystal lattice and the supporting porous material can create grown-in dislocations, leading to a disappearance of local strain in the sub-grains – and thus, improve the diffractivity of the protein crystals. In fact, it has been observed [16] that some dislocations are grown from the seed crystals, chemically cross-linked by glutaraldehyde. Thus, it is logical to assume that the supporting
porous material can be far more efficient in creating grown-in dislocations, because the misfit between the crystal lattice and the supporting porous material is greater than in the former case.

References

1. Chayen, N. E., Saridakis, E., El-Bahar, R. & Nemirovsky, Y. Porous silicon: an effective nucleation-inducing material for protein crystallization. *J. Mol. Biol.* **312**, 591–595 (2001).

2. Rong, L., Komatsu, H., Yoshizaki, I., Kadowaki, A. & Yoda, S. Protein crystallization by using porous glass substrate. *J. Synchrotron Radiat.* **11**, 27–29 (2004).

3. Chayen, N. E., Saridakis, E. & Sear, R. P. Experiment and theory for heterogeneous nucleation of protein crystals in a porous medium. *Proc. Natl. Acad. Sci. USA* **103**, 597–601 (2006).

4. Sugahara, M., Asada, Y., Morikawa, Y., Kageyama, Y. & Kunishima, N. Nucleant-mediated protein crystallization with the application of microporous synthetic zeolites. *Acta Crystallogr. D* **64**, 686–695 (2008).

5. Di Profio, G., Curcio, E., Ferraro, S., Stabile, C. & Drioli, E. Effect of supersaturation control and heterogeneous nucleation on porous membrane surfaces in the crystallization of L-glutamic acid polymorphs. *Cryst. Growth Des.* **9**, 2179–2186 (2009).

6. Saridakis, E. & Chayen, N. E. Towards a ‘universal’ nucleant for protein crystallization. *Trends Biotechnol.* **27**, 99–106 (2009).

7. Asanithi, P. et al. Carbon-nanotube-based materials for protein crystallization. *Appl. Mater. Interfaces* **1**, 1203–1210 (2009).

8. Saridakis, E. et al. Protein crystallization facilitated by molecularly imprinted polymers. *Proc. Natl. Acad. Sci. USA* **108**, 11081–11086 (2011).

9. Kertis, F. et al. Heterogeneous nucleation of protein crystals using nanoporous gold nucleants. *J. Mater. Chem.* **22**, 21928–21934 (2012).

10. Saridakis, E. & Chayen, N. E. Imprinted polymers assisting protein crystallization. *Trends Biotechnol.* **31**, 515–520 (2013).
11. Khurshid, S., Saridakis, E., Govada, L. & Chayen, N. E. Porous nucleating agents for protein crystallization. *Nat. Protoc.* **9**, 1621–1633 (2014).

12. Nanev, C. N., Saridakis, E. & Chayen, N. E. Protein crystal nucleation in pores. *Sci. Rep.* **7**, 35821; [10.1038/srep35821](https://doi.org/10.1038/srep35821) (2017).

13. Nanev, C., Govada, L. & Chayen, N.E. Theoretical and experimental investigation of protein crystal nucleation in pores and crevices. *IUCrJ* **8**, 270-280 (2021).

14. Page, A. J. & Sear, R. P. Heterogeneous nucleation in and out of pores. *Phys. Rev. Lett.* **97**, 065701 (2006).

15. Koizumi, H. et al. Control of strain in subgrains of protein crystals by the introduction of grown-in dislocations. *Acta Crystallogr. D77*, 599-605 (2021).

16. Suzuki, R., Abe, M., Kojima K. & Tachibana, M. Identification of grown-in dislocations in protein crystals by digital X-ray topography. *J. Appl. Cryst.* **54**, 163-168 (2021).

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**Author contributions**

C.N.N. performed the theoretical investigation, L.G. and E.S. performed and analysed the experiments, C.N.N. and N.E.C. led the research, C.N.N., E.S. and N.E.C. wrote the paper. All authors reviewed the paper.

**Competing interests**

The author(s) declare no competing interests.