# Reports

## Heterogeneous and Epitaxial Nucleation of Protein Crystals on Mineral Surfaces

ALEXANDER MCPHERSON AND PAUL SHLICHTA

**Fifty different mineral samples were tested as potential heterogeneous or epitaxial nucleants for four commonly crystallized proteins. It was found, by conventional protein crystallization techniques, that for each protein there was a set of mineral substrates that promoted nucleation of crystals at lower critical levels of supersaturation than required for spontaneous growth. Numerous examples, involving all four proteins, were observed of modification of crystal habit and, in some cases, unit cell properties promoted by the presence of the mineral nucleants. In at least one case, the growth of lysozyme on the mineral apophyllite, it was shown by lattice analysis and x-ray diffraction that the nucleation and growth of the protein crystal on the mineral was likely to involve a direct lattice match.**

THE DETERMINATION OF THE STRUCTURE of macromolecules, particularly proteins and nucleic acids, by x-ray diffraction analysis is assuming an increasingly significant role in molecular biology. It currently provides the most powerful means for the visualization of molecules at the atomic level and, therefore, for the correlation of structural attributes with biochemical function. An essential component in the application of this technique is the growth of single, large crystals of macromolecules.

There are two fundamental phases, though they are in a sense continuous, that characterize crystallization: the nucleation step and the growth stage. Failure to obtain single crystals of adequate quality for analysis is generally a consequence of problems associated with the growth phase. Failure to obtain any crystals at all or failure to obtain single, supportable nuclei reflects difficulties in the nucleation step. The inability to promote the formation of periodically ordered arrays that by accretion become three dimensionally ordered aggregates and then crystals is frequently a major obstacle to the crystallization of specific proteins. In addition, failure to restrain the number of nuclei is the principal problem in growing large, single crystals rather than masses of microcrystals.

Ideally, one would like to ensure the formation of a limited number of nuclei, and at a level of supersaturation low enough to support continued, ordered growth. One would, furthermore, like to introduce a greater degree of reproducibility into the process than we currently experience.

An approach to this problem was suggested to us by a method occasionally used in the preparation of crystals of certain conventional inorganic molecules, the utilization of heterogeneous nucleants to promote epitaxial growth. In such procedures, a substance A, usually a mineral, is introduced into a supersaturated solution or melt of a second compound B. As supersaturation is increased, compound B nucleates on a specific face of mineral A and continues to grow as a crystal. The best recognized example of such epitaxy is the growth of GaAs crystals on substrates of crystalline silicon. An important point is that there need not necessarily be a perfect match of lattice dimensions for such epitaxy to occur. It is observed, for example, when the lattice dimensions of two faces on the pair of crystals are integral multiples of one another or even when the areas of integral numbers of unit cells are equal.

The various faces of natural and synthetic minerals display ordered and periodic arrays of chemical groups that in many cases are capable of interacting with the amino acid substituents of proteins. If such interactions occur in a regular manner, then there is the possibility that the surfaces of minerals, their crystal faces, might serve to organize protein molecules into complementary two-dimensional arrays that could subsequently serve as starting points for three-dimensional protein crystal growth. These would be cases of true epitaxial growth. Alternatively, the crystal faces of most minerals exhibit submicroscopic steps, imperfections, and dislocations that might also serve, in a mechanical or physical sense, to organize and order macromolecules at their surfaces. This type of phenomenon is referred to as heterogeneous nucleation. Either of the two types of nucleation we believed could contribute to the improved probability of obtaining protein crystals.

We conducted the following experiment. One hundred and thirteen 15-μl droplets of four protein solutions (total of 452 trials) were distributed into the depressions of nine-well glass depression plates contained in vapor diffusion boxes and equilibrated against appropriate reservoir solutions as described by McPherson (*1*). The four proteins used were canavalin (*2–4*) and concanavalin B (*3, 5*) from jack bean, beef liver catalase (*6*), and hen egg lysozyme (*7*). Into every trial sample, a small grain (50 to 500 μm) of one of 50 different minerals was introduced so that at least two samples of each of the four proteins were exposed to each mineral. In addition, one protein sample in every vapor diffusion box was left void of any mineral so that there were 52 control samples.

A more complete accounting of our results with each of the 50 minerals used will be given elsewhere (*8*), but several observations are worth noting here, because they strongly suggest that both heterogeneous nucleation and epitaxial growth of protein crystals on mineral faces can occur.

For each of the four proteins examined, a set of mineral substrates was found to induce the nucleation and subsequent growth of crystals at substantially earlier times, and therefore at lower critical levels of supersaturation, than was observed for the corresponding controls. This set ranged in number from 18 in the case of catalase to as many as 30 of the 50 minerals for canavalin. The minerals that induced early crystallization varied according to the protein, although some were common to all. When nucleation occurred early, it invariably occurred on the surface of the mineral substrate rather than in the surrounding free solution. Some representative examples are shown in Fig. 1.

An alteration of the commonly observed crystal habit was noted for a variety of minerals for each of the four proteins included in the experiment; that is, the presence of a mineral nucleant occasionally promoted the growth of an anomalous protein crystal form. A sampling of these are shown in Fig. 2. In many cases, the alteration in morphology was very pronounced, which suggests that, for some proteins, the use of mineral nucleants might prove a valuable approach for favorably modifying crystal habits and unit cell properties.

Figure 2, A through E, for example, illustrates a series of results obtained for the crystallization of beef liver catalase on a

A. McPherson, Department of Biochemistry, University of California, Riverside, CA 92521, and Cryschem Inc., Riverside, CA 92507.
P. Shlichta, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, CA 91109, and Crystal Research Inc., San Pedro, CA 90731.

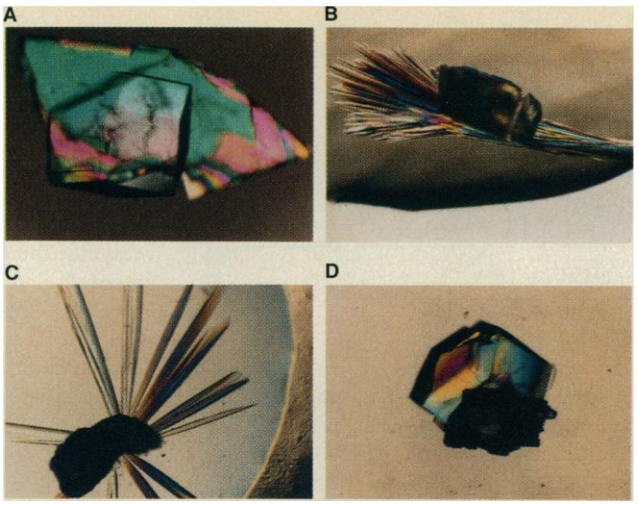


**Fig. 1.** Examples of protein crystals that nucleated and grew on mineral substrates. (**A** and **D**) Lysozyme on lepidolite and magnetite, respectively. (**B** and **C**) Concanavalin B on gypsum and aragonite, respectively.

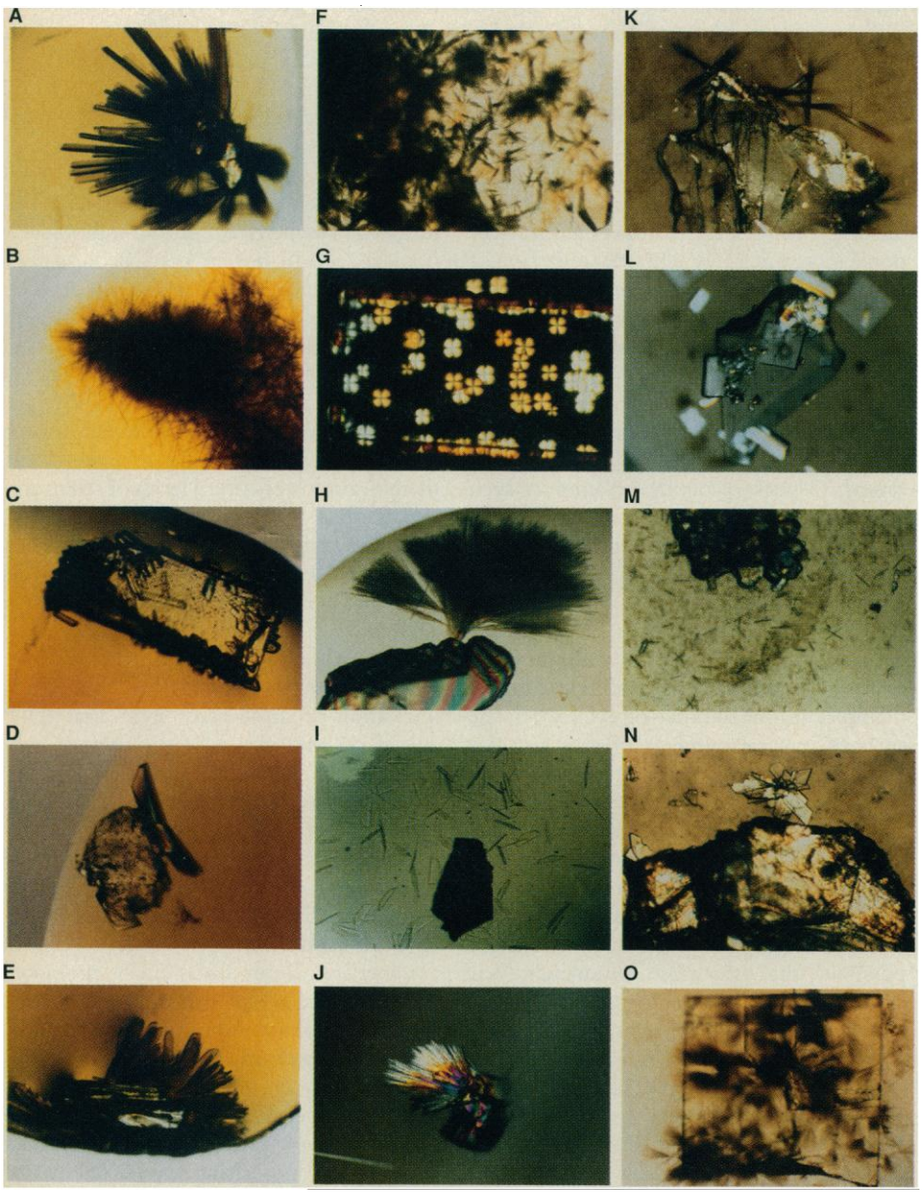


**Fig. 2.** Crystallization of catalase in the presence of (**A**) brucite, (**B**) cerussite, (**C**) MgO, (**D**) apatite, and (**E**) prehnite; lysozyme in the presence of (**F**) fluorite, (**G**) MgO, and (**H**) quartz; concanavalin B in the presence of (**I**) covellite and (**J**) sandbornite; canavalin crystallized in the presence of (**K**) apatite, (**L**) magnetite, (**M**) chalcopyrite, (**N**) calcite, and (**O**) MgO.

variety of mineral substrates. The habits range from fine trigonal needles in Fig. 2B to thick plates in Fig. 2D. Although the former is, at best, difficult for x-ray diffraction analysis, the latter is quite suitable, as in fact are the orthorhombic laths in Fig. 2, A and E.

In Fig. 2F a mass of fine needle crystals of lysozyme originated on the mineral substrate obscured in the upper left. In the lower right corner is seen a single tetragonal lysozyme crystal, the dominant habit. Unlike the flurry of needle crystals emanating from the mineral substrate, this tetragonal crystal nucleated spontaneously in the free mother liquor. Shown in Fig. 2, G and H, are additional examples of the nucleation of lysozyme crystals on mineral surfaces which resulted in habits dramatically different from the usual forms seen in Fig. 1, A and D.

More than five different crystal habits of canavalin were observed in addition to the common rhombohedral form (Fig. 2, K through O). Even concanavalin B which, in over 15 years of crystallization research, has never appeared in any crystal form save the hexagonal rods illustrated in Fig. 1, B and C, yielded two new morphologies (Fig. 2, I and J).

Although it was not possible, because of small size or unfavorable morphology, to analyze many of the modified crystal habits with x-ray diffraction, the unit cells of sever-

**Fig. 3.** Low-power microscope photograph (polarized light) of a crystal of lysozyme growing on a substrate crystal of the mineral apophyllite. The edges of the protein and mineral crystals appear to be parallel, which suggests possible alignment of the respective crystallographic axes.

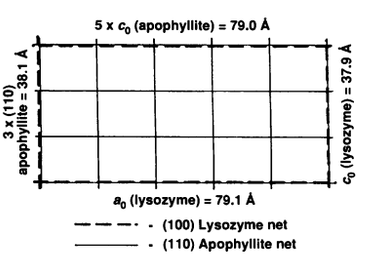


**Fig. 4.** Diagram showing the congruence of the lattices on the (110) face of the mineral apophyllite and the (100) plane of a tetragonal lysozyme crystal.

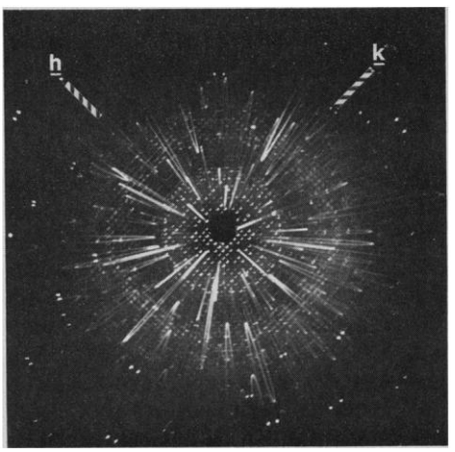


**Fig. 5.** A precession photograph ($\mu$ = 6°) of the *hk*0 zone of the reciprocal lattice of the lysozyme protein crystal and its apophyllite substrate seen in Fig. 3. A Buerger precession camera and nickel-filtered $CuK_\alpha$ x-rays generated by a rotating anode source were used. The x-ray beam was directed to strike both the protein and the mineral crystal so that both reciprocal lattices were recorded simultaneously. The *h* and *k* reciprocal lattice directions for the lysozyme crystal are indicated on the photograph. Evident from the photograph is the virtual alignment of the reciprocal lattice nets of the two crystals, consistent with the growth of the protein crystal by epitaxy.

al were determined. The rectangular crystals of canavalin induced by magnetite and seen in Fig. 2L were of space group $C222_1$ ($a$ = 136 Å, $b$ = 152 Å, $c$ = 131 Å) rather than the standard rhombohedral unit cell (*4*) of *R*3 symmetry ($a$ = 81.3 Å, $\gamma$ = 111°) seen in most other experimental trials.

The catalase crystals most frequently observed and consistently so (Fig. 2, A, C, D, E) in the presence of mineral nucleants are of orthorhombic symmetry $P2_12_12_1$ ($a$ = 89 Å, $b$ = 140 Å, $c$ = 231 Å) (*6*). The needle crystals seen growing on the mineral gypsum in Fig. 2B, however, were of the trigonal space group $P3_121$ ($a = b$ = 178 Å, $c$ = 241 Å), similar to those reported elsewhere (*9, 10*). Similarly, the standard crystal form of concanavalin B (*5*) is of hexagonal space group $P6_3$ ($a = b$ = 81 Å, $c$ = 101 Å), but in the presence of sandbornite (Fig. 2I) a new form of orthorhombic symmetry $P2_12_12_1$ was found ($a$ = 78 Å, $b$ = 62 Å, $c$ = 124 Å).

In general, the mineral substrates were quite irregular in form and faces were, in most cases, difficult to identify. Nonetheless, for most samples the growth of the protein crystals from substrates exhibited essentially random orientation and displayed no mutual alignment of crystal faces. This was not, however, true for all. In some samples, it did indeed appear that axial alignment was present between protein crystal and mineral crystal.

The most prominent example of such apparent alignment was the growth of lysozyme crystals on the mineral apophyllite (Fig. 3). The morphological edges of the mineral and protein crystals are virtually parallel. A calculation of the periodic spacings characterizing the major crystal faces of apophyllite and a comparison with the lattice spacings on the major faces of tetragonal lysozyme crystals (*11*) demonstrated that a 3 by 5 array of unit cells on the (110) face of apophyllite (*12*) superimposes on the (100) face of lysozyme with a discrepancy of 0.13 and 0.53%. This is illustrated in Fig. 4. Perhaps more significant, the areal match was within 0.40%. This is extraordinarily good even for the best cases of epitaxial growth of conventional inorganic crystals.

The crystal of lysozyme on the apophyllite substrate seen in Fig. 3 was mounted in a glass capillary by conventional methods (*13*) and photographed on a Buerger precession camera. With the x-ray beam directed to strike substrate and protein crystal, both the mineral and protein reciprocal lattices could be recorded simultaneously on the same film. One such photograph is shown in Fig. 5. Evident in this diffraction photograph is the virtual alignment of the reciprocal lattices of the substrate and protein crystals. This is convincing evidence for congruence of the crystalline lattices, and it suggests that epitaxial nucleation of protein on the mineral occurred.

The results presented here demonstrate not only that heterogeneous nucleation of proteins on mineral surfaces can occur at lower levels of critical supersaturation, but that epitaxial growth of protein on mineral crystals can occur. They further suggest that mineral surfaces have organizational properties with respect to biomolecules that might have significance in other regards. Mineral surfaces, for example, by their organizational capabilities, might have served as primitive catalysts of bioreactions or they could play an important role in the assembly of mineralized tissue. It remains to be seen whether protein-mineral epitaxial interfaces have unusual properties of value in processes other than the promotion of crystal nuclei. There is no reason why the promotion of nucleation of protein crystals by these mechanisms should necessarily be limited to mineral substrates. It seems reasonable that other ordered two-dimensional arrays, as obtained from synthetic materials, might serve as well.


REFERENCES

1. A. McPherson, *The Preparation and Analysis of Protein Crystals* (Wiley, New York, 1982).
2. J. B. Sumner, *J. Biol. Chem.* **37**, 137 (1919).
3. ——— and S. F. Howell, *ibid.* **113**, 607 (1936).
4. A. McPherson and R. Spencer, *Arch. Biochem. Biophys.* **169**, 650 (1975).
5. A. McPherson, J. Geller, A. Rich, *Biochem. Biophys. Res. Commun.* **57**, 494 (1974).
6. A. McPherson and A. Rich, *Arch. Biochem. Biophys.* **157**, 23 (1973).
7. G. Alderton and H. L. Fevold, *J. Biol. Chem.* **164**, 1 (1946).
8. A. McPherson and P. Shlichta, *J. Cryst. Growth*, in press.
9. M. G. Rossmann and L. W. Labaw, *J. Mol. Biol.* **29**, 315 (1967).
10. W. Longley, *ibid.* **30**, 323 (1967).
11. L. K. Steinrauf, *Acta Crystallogr.* **12**, 77 (1959).
12. J. D. H. Donnay and H. M. Oudik, *Crystal Data Determinations Tables* (Department of Commerce and JCPOS, Washington, DC, ed. 3, 1977), vol. 2.
13. M. V. King, *Acta Crystallogr.* **7**, 601 (1954).



20 August 1987; accepted 2 December 1987


# Enzymatic Oxidation of Cholesterol Aggregates in Supercritical Carbon Dioxide


T. W. RANDOLPH, D. S. CLARK, H. W. BLANCH, J. M. PRAUSNITZ



**Fundamental studies of enzyme-solvent interactions can be conducted with supercritical fluids because small changes in pressure or temperature may bring about great changes in the properties of a single solvent near its critical point. Cholesterol oxidase is active in supercritical carbon dioxide and supercritical carbon dioxide–cosolvent mixtures. Variations in solvent power caused by pressure changes or by the addition of dopants affected the rate of enzymatic oxidation of cholesterol by altering the structure of cholesterol aggregates.**


ZAKS AND KLIBANOV (*1*) HAVE shown that many enzymes can function catalytically in organic solvents that contain only small amounts of water. Enzymatic catalysis in nonaqueous media may provide information on enzyme-environment interactions (especially for lipophilic enzymes) and may also provide an escape from kinetic or equilibrium restraints imposed by the use of water as a solvent. For example, nonaqueous solvents offer higher solubilities for lipophilic compounds such as steroids, and they allow certain enzymatic


Department of Chemical Engineering, University of California, Berkeley, CA 94720.