Journal of
Applied Crystallography

ISSN 0021-8898

Received 14 December 2007
Accepted 17 April 2008

Protein crystals can be incommensurately modulated

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For a normal periodic crystal, the X-ray diffraction pattern can be described by an orientation matrix and a set of three integers that indicate the reciprocal lattice points. Those integers determine the spacing along the reciprocal lattice directions. In aperiodic crystals, the diffraction pattern is modulated and the standard periodic main reflections are surrounded by satellite reflections. The successful indexing and refinement of the main unit cell and **q** vector using *TWINSOLVE*, developed by Svensson [(2003). Lund University, Sweden], are reported here for an incommensurately modulated, aperiodic crystal of a profilin:actin complex. The indexing showed that the modulation is along the **b** direction in the crystal, which corresponds to an 'actin ribbon' formed by the crystal lattice. Interestingly, the transition to the aperiodic state was shown to be reversible and the diffraction pattern returned to the periodic state during data collection. It is likely that the protein underwent a conformational change that affected the neighbouring profilin:actin molecules in such a way as to produce the observed modulation in the diffraction pattern. Future work will aim to trap the incommensurately modulated crystal state, for example using cryocooling or chemical crosslinking, thus allowing complete X-ray data to be collected.

1. Introduction

Crystallographers normally solve 'periodic' crystal structures (Fig. 1*a*). In an ideal periodic crystal, the contents of the asymmetric unit are perfectly replicated by the unit translations and the symmetry operators of the space group. One particularly fascinating deviation from ideality results in the appearance of distinct 'satellite' reflections around the 'main' Bragg reflections. These satellite reflections are often as sharp as main spots and result from a structural modulation contained in 'aperiodic' or 'modulated' crystals (Figs. 1*b* and 1*c*). These modulated structures contain a variation that perturbs the short-range translational symmetry and can be thought of as a form of systematic or smoothly varying disorder. The term aperiodic includes both positionally and occupationally modulated crystals and composite crystals (Dehling, 1927; Korekawa & Jagodzinski, 1967; Petříček & Dušek, 2004). Structural analysis of modulated crystals is based on the theoretical work of de Wolff, Janner and Janssen (de Wolff *et al.*, 1981; Janssen *et al.*, 1999). Today, modulated small-molecule crystals are frequently observed and can be solved (for examples see Daniels *et al.*, 2002; Gaillard *et al.*, 1998; Duncan *et al.*, 2002). Modulated crystals occur in molecular systems undergoing phase transformations, and the phenomenon is widespread and common in solid-state compounds and in structures of the elements (van Smaalen, 2007). For macromolecular crystals, satellite reflections from modulated crystals have only rarely been reported (Schutt *et al.*, 1989; Vila-Sanjurjo *et al.*, 2004; Gouaux & Lipscomb, 1989; Silva *et al.*, 1992; Warkentin *et al.*, 2005). When a modulated diffraction pattern is observed in protein crystallography, the sample is typically discarded in favor of a better behaving sample.

The diffraction pattern from a normal crystal (Fig. 2*a*) can be described in terms of an orientation matrix and a collection of indices. The indices have integer values (*hkl*) and describe the reflections observed on the diffraction image as shown in equation (1):

$$\mathbf{H} = h\mathbf{a}^* + k\mathbf{b}^* + l\mathbf{c}^*. \quad (1)$$

The modulation can be 'incommensurate' or 'commensurate' with the main lattice. For the commensurate case, the modulation is a special type of superlattice; the distortion is smoothly varying and can be described with an integer-multiple relationship to the main lattice (Fig. 1*b*) or supercell. Commensurate crystals can be indexed normally by three

integer indices and then solved as a supercell of the original unit cell. There have been several published cases of commensurately modulated protein crystals that have been solved using supercells (Schutt *et al.*, 1989; Vila-Sanjurjo *et al.*, 2004; Gouaux & Lipscomb, 1989; Warkentin *et al.*, 2005). For the incommensurate case, the relationship with the main lattice is non-integral (Fig. 1*c*). The position of a reflection from a modulated crystal is given by (Janssen *et al.*, 1999; Petříček & Dušek, 2004; van Smaalen, 2004)

$$\mathbf{H} = h\mathbf{a}^* + k\mathbf{b}^* + l\mathbf{c}^* + m_1\mathbf{q}_1 + m_2\mathbf{q}_2 + \cdots + m_d\mathbf{q}_d, \qquad (2)$$

where the reciprocal lattice of the main reflections is denoted by **a***, **b*** and **c***, d is the number of satellite directions [for Fig. 2(*b*), $d = 2$], and the satellite index, m, is a small integer describing the diffraction order of the satellite reflection [*e.g.* in Fig. 2(*b*), where $d = 1$ then $m = \pm 3$ with $m = 0$ for the main reflection, and where $d = 2$ then $m = \pm 1$]. In this way, a 'superspace' is defined for a $(3 + d)$-dimensional reciprocal lattice. Fig. 2(*b*) shows the case of a five-dimensional crystal with two modulation waves (*i.e.* $d = 1, 2$ and the satellites are observed in off-axis directions). In the description below, the simpler and far more common case of a four-dimensional crystal with a single modulation wave is considered:

$$\mathbf{H} = h\mathbf{a}^* + k\mathbf{b}^* + l\mathbf{c}^* + m\mathbf{q}. \qquad (3)$$

The satellite locations are defined using a '**q** vector' (Fig. 2*b*) to describe their direction and distance relative to the main reflections:

$$\mathbf{q} = q_1\mathbf{a}^* + q_2\mathbf{b}^* + q_3\mathbf{c}^*. \qquad (4)$$

The type of modulation within the crystal, commensurate *versus* incommensurate, can be distinguished by the spacing of the satellite reflection from the main reflection. This diagnostic information is held in the **q** vector. For commensurate crystals, all components of the **q** vector are rational, and for incommensurate crystals, at least one component is irrational (Figs. 1*b* and 1*c*). The direction of the modulation is given by the direction of the satellite reflections. Two examples of incommensurately modulated, small-molecule diffraction patterns are shown in Fig. 3. Fig. 3(*a*) is an axial photograph of the '*c*' axis of modulated thiourea, highlighting the single modulation vector along **c***; this structure has been solved (Gao & Coppens, 1989). Thiourea crystals can transition to a commensurately modulated phase under variations in temperature. Fig. 3(*b*) shows cubic Lazurite with its obvious satellite peaks in the three (equivalent) directions; this structure has also been solved (Bolotina *et al.*, 2003).

How are these incommensurate modulations in the diffraction pattern manifested in the unit cell? Incommensurately modulated diffraction is widespread in small-molecule crystallography, occurring in solid-state compounds and structures of elements, but is more often reported in phase-transition regions for compounds. In the majority of incom-

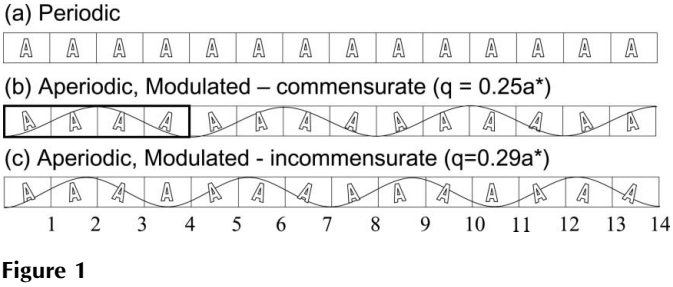

Figure 1
Nomenclature for modulated crystals: (*a*) A normal periodic crystal lattice; the A represents the asymmetric unit for the periodic case. (*b*) A commensurately modulated aperiodic crystal; the structure of A is smoothly varying over four unit cells and can be described by a superlattice (bold box) composed of an integral number of unit cells larger than the original unit cell [in this case four, with $\mathbf{q} = (1/4)\mathbf{a}^* = 0.25\mathbf{a}^*$]. (*c*) An incommensurately modulated aperiodic crystal; A is smoothly varying but cannot be simply described by a superlattice. The A structure will never repeat on a unit-cell boundary. Here $\mathbf{q} = 0.29\mathbf{a}^*$ and is an irrational number.

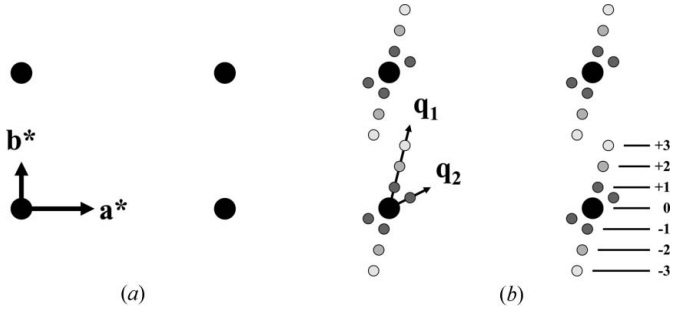

Figure 2
(*a*) A normal periodic diffraction pattern. (*b*) A five-dimensional modulated diffraction pattern. This pattern is described with two **q** vectors. The first **q** vector ($\mathbf{q}_1$) has six satellites described by m values ranging from +3 to −3. The second **q** vector ($\mathbf{q}_2$) has two satellites described by m values ranging from +1 to −1.

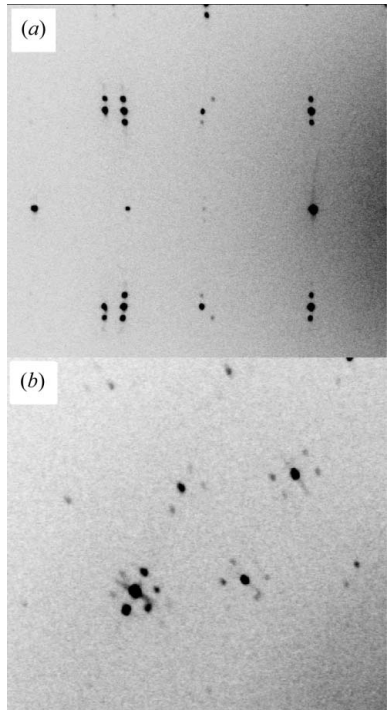

Figure 3
Examples of modulated diffraction patterns from small molecules: (*a*) thiourea (one-dimensional modulation); (*b*) lazurite (three-dimensional modulation).

mensurately modulated crystals that have been solved, the modulation occurs as the lattice is reorganized during a phase transition (Zuñiga *et al.*, 1989). There have been many types of modulation functions identified; in simple cases, the packing undergoes a sinusoidal oscillation that is not aligned to an integer value of the unit cell. Structure solution of these incommensurately modulated structures can be 'bootstrapped' by taking advantage of the fact that the main reflections contain the structural information for the 'average' structure. Thus, when only the main reflections are used to solve the structure, the displacement parameters (B values) for the atoms involved in the modulation are much higher than those for the surrounding atoms. This increase in B can be used as an indicator of where the modulated oscillations are occurring when trying to incorporate the modulated data contained in the satellite reflections. Since the incommensurately modulated component in small molecules is due to the minor movement of one atom over a very small range, it was believed that this phenomenon would not be observed in protein crystals because the data-set resolution would obscure these very small changes. Our work with a profilin:actin complex has resulted in a crystal whose diffraction pattern clearly indicates an incommensurate modulation.

1.1. Profilin:actin complex

Knowledge of the structure of actin in its various conformational states is essential for understanding the dynamics underlying the shape changes and migration of eukaryotic cells, as well as the actin-based motility of many bacteria (Halpain, 2003; Matus, 2000; Carlier *et al.*, 2003; Snapper & Rosen, 1999; Pollard *et al.*, 2000; Orlova *et al.*, 1994, 2001; Mendelson & Morris, 1994). A key regulator of actin polymerization is profilin, an abundant protein that associates with actin in a 1:1 complex that serves as a precursor for actin filament formation (Markey *et al.*, 1978; Carlsson *et al.*, 1977). Several crystal structures of actin in the monomeric state (Otterbein *et al.*, 2001; Rould *et al.*, 2006) and in complexes with actin-binding proteins, such as profilin (Chik *et al.*, 1996; Schutt *et al.*, 1993), have been solved.

Actin filaments are major contributors to the architecture of eukaryotic cells. They are also involved in muscle contraction and in many cellular activities, such as motility, cytokinesis and receptor-mediated endocytosis. Actin filaments are dynamic and their polymerization is central to all forms of cellular motility, including migration of eukaryotic cells (Hoglund *et al.*, 1980) and actin-based motility of bacteria (Gouin *et al.*, 1999). There are hundreds of actin-binding proteins that regulate the polymerization, organization and function of actin filaments (dos Remedios *et al.*, 2003). Polymerization of actin pushes forward the leading edge of a moving eukaryotic cell and several protein complexes are involved in this process (Borisy & Svitkina, 2000; Marx, 2003; Pollard & Borisy, 2003). Similar mechanisms are involved in bacterial actin-based motility (Kocks, 1994; Pantaloni *et al.*, 2001). All models for cellular motility show profilin binding to monomeric actin, exchanging the nucleotide and delivering actin to the growing filament (Cedergren-Zeppezauer *et al.*, 1994; Perelroizen *et al.*, 1996; Schluter *et al.*, 1997). Experiments using crosslinked profilin:actin in cells have demonstrated that the dissociation of profilin from actin is critical for actin-based motility (Nyman *et al.*, 2002; Hajkova *et al.*, 2000; Grenklo *et al.*, 2003). When profilin:actin is exposed to a slightly acidic pH, profilin dissociates from actin allowing stabilized actin filaments to form (Oda *et al.*, 2001; Carlsson, 1979). A fascinating and unusual X-ray diffraction pattern with off-lattice satellite reflections, indicative of a modulated structure, can be achieved by transfer of profilin:actin crystals to slightly acidic pH (Chik, 1996; Lovelace *et al.*, 2004; Schutt *et al.*, 1989). The strength and sharpness of these reflections are unprecedented in protein crystallography. It has been speculated that these modulated crystals contain actin filaments or an intermediate state of actin filament formation.

2. Experimental

2.1. Sample purification

Bovine profilin:β-actin from calf thymus was purified following protocols established by the Lindberg lab (Schuler *et al.*, 2005; Rozycki *et al.*, 1991) that are briefly summarized here. Thymus was purchased from Pel-Freez Biologicals. Thymus homogenate was centrifuged and passed over poly-L-proline sepharose, for which profilin has specific affinity. Profilin and associated actin were eluted from the column with dimethyl sulfoxide. The profilin:β-actin was then purified from profilin:γ-actin and free profilin by chromatography with a hydroxyapatite C column (Clarkson) using a phosphate/glycine gradient. Profilin:β-actin was subsequently concentrated and stored as an ammonium sulfate precipitate at 277 K for crystallization and structure analysis.

2.2. Crystallization

Profilin:β-actin was crystallized using batch crystallization and microseeding (Carlsson *et al.*, 1976). The protocol to crystallize the so-called 'open state' of profilin:actin is based on previous work (Chik *et al.*, 1996; Chik, 1996). The purified precipitated protein was resolubilized at 10–15 mg ml^{-1} in a 5 mM phosphate (pH 7.6) buffer containing 0.5 mM adenosine-5′-triphosphate (ATP), 0.2 mM $CaCl_2$ and 1.0 mM dithiothreitol (DTT), and clarified by centrifugation. The resolubilized protein was then dialyzed against 1.3 M phosphate (pH 7.3) containing 0.5 mM ATP, 0.2 mM $CaCl_2$ and 1.0 mM DTT. After 8 h, a microcrystalline precipitate of actin paracrystals formed and was removed by ultracentrifugation. The supernatant was filtered with 0.22 µm Millex-GV filter units from Millipore, and 5–30 µl hanging drops were suspended above the dialysis solution for crystallization. Microseeds were grown in unfiltered drops and were used to seed the filtered hanging drops. Crystals grew in 24–36 h, to an average size of 0.5 × 0.35 × 0.15 mm. Crystallization was performed in a cold room at 277 K (Fig. 4*a*).

2.3. Formation of modulated crystalline state

The profilin:actin crystals were transferred to a stabilizing solution, composed of 1.8 *M* phosphate (pH 7.3) buffer containing 0.5 m*M* ATP and 1.0 m*M* DTT at 277 K, and then slowly warmed to room temperature. To achieve a modulated state, the crystals were transferred into a buffer solution containing 1.8 *M* phosphate (pH 6), 50 µ*M* ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 4 m*M* $MgCl_2$, 0.5 m*M* ATP and 1.0 m*M* DTT.

2.4. Data collection

A plate-like crystal (500 × 500 × 200 µm) was mounted for data collection using a MiTeGen MicroMount and a MicroRT kit (for mounting at room temperature). The X-ray diffraction data were collected at 298 K with a Rigaku FR-E Cu *Kα* rotating-anode generator operating at 45 kV and 45 mA; the beam was focused with Osmic MicroMax optics and images were collected with an R-AXIS IV++ detector. The images were taken at a distance of 250 mm with a 1 min exposure and an oscillation of 0.5°.

2.5. Data processing

2.5.1. Main reflections. The main reflections were indexed and processed with *CrystalClear* (Rigaku Americas, 2000). The main reflections were indexed as *P*222 with a unit cell of $a = 38.1$, $b = 72.3$, $c = 184.9$ Å. This unit cell is similar to that of the 'open state', indexed as $P2_12_12_1$ with cell dimensions of $a = 38.1$, $b = 72.2$, $c = 185.7$ Å for the periodic form of the crystal (Chik *et al.*, 1996).

2.5.2. Satellite reflections. The modulated data were processed with *TwinSolve* (Svensson, 2003). *TwinSolve* has the ability to index a modulated pattern with up to three **q** vectors. The unit cell given by *TwinSolve* from the main reflections was $a = 38.1$, $b = 71.9$, $c = 184.5$ Å, which is similar to the results from *CrystalClear*. The satellite diffraction data were then described by the addition of one **q** vector, which refines to approximately $\mathbf{q}_1 = 0.00\mathbf{a}^* + 0.29\mathbf{b}^* + 0.00\mathbf{c}^*$. This indicates that the modulation is only along the **b*** direction. Only first-order satellite reflections were observed.

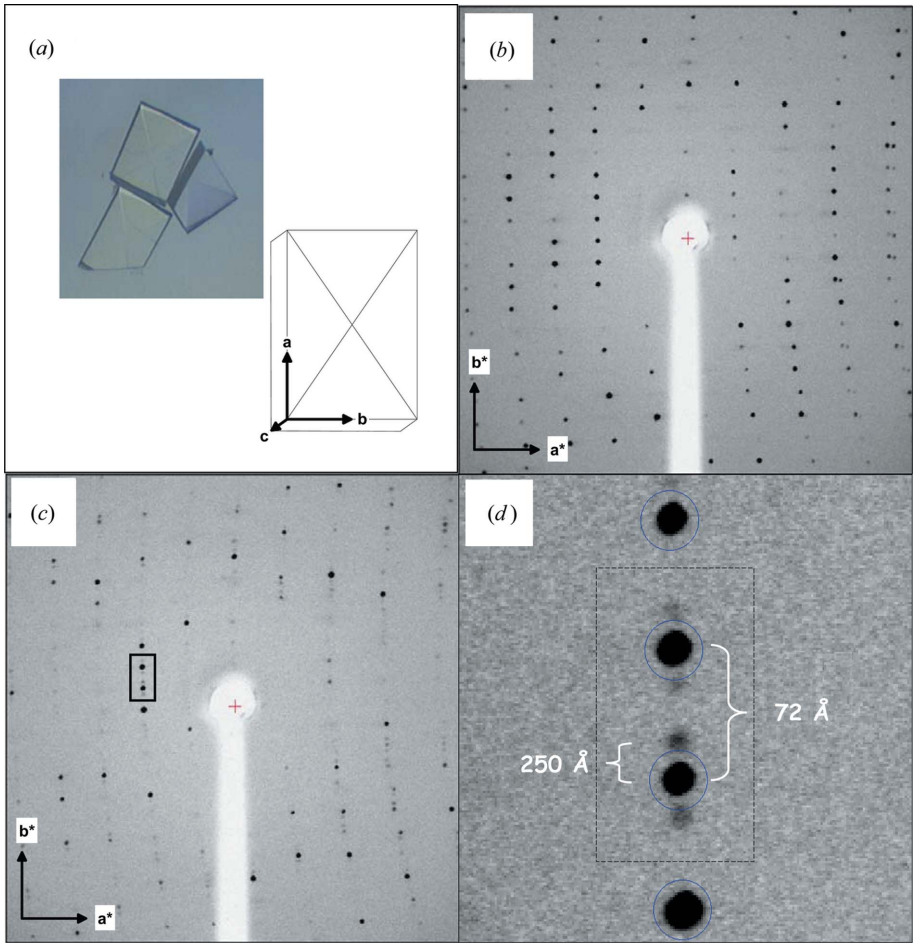

Figure 4
Profilin:actin crystals and diffraction patterns: (*a*) A typical profilin:actin crystal with a diagonal cross along the 001 plane. (*b*) A normal periodic diffraction pattern. (*c*) A periodic modulated diffraction pattern with satellite reflections in the **b*** direction. (*d*) A close-up of the modulated diffraction with approximated unit-cell dimensions.

3. Results and discussion

We were attempting to transform open-state profilin:actin crystals into a modulated state that was first observed using a precession camera (Chik, 1996; Schutt *et al.*, 1989). For the modulated precession images there was no convenient way to handle the diffraction data and the data were shelved. With recent advances in solving modulated structures in the world of small molecules we thought it was a good time to look at this problem again. Profilin:actin crystals grow as small boxes with a diagonal cross along the biggest face (Fig. 4*a*). Several initial attempts were made at transforming the crystals into the modulated state, but unfortunately the diffraction patterns were normal [similar to Fig. 4(*b*)]. Eventually one crystal entered the modulated state, as indicated by the image in Fig. 4(*c*). It shows peaks that are flanked on either side by relatively weaker satellites (Fig. 4*d*). The satellites are about 15 times weaker than the main reflections on the basis of peak intensity.

About an hour later, the same region was re-imaged, and on this pass the modulated diffraction had disappeared (Fig. 4*b*) and only periodic

diffraction was recorded. This indicates that the modulated state was both metastable and reversible. As the modulation is linked to the pH of the mother liquor of the crystal, it is possible that the pH of the mother liquor was modified by the X-ray beam, causing the crystal to return to its original state. Another possibility is that the pH of the solution used to put the crystal into the modulated state was not quite low enough, and the crystal transformed into the modulated state temporarily and then returned to normal. The pH balancing to achieve the modulated state is critical; if the environment is too acidic the crystals will shatter into many pieces and if it is not acidic enough the crystals will remain in the normal state. Methods to stabilize modulated profilin:actin crystals are under development.

The fact that the modulation is along the **b*** direction is obvious from the diffraction pattern (Fig. 4*c*). The raw **q** vector value of 0.2897 (40), which was rounded to two decimal places, is indicative of incommensurate modulation or a situation where this pattern will never repeat itself no matter how many unit cells are stacked together. If the modulation had been commensurately modulated then a super unit cell could have been defined consisting of multiple unit cells, allowing the data to be processed in the normal way. The limited number of frames of data made it impossible to distinguish the space group other than as *P*222 and not possible to assign a superspace group. Currently there is no macromolecular crystallographic software that can determine the modulated protein structure from this diffraction pattern; however, we are working on software to fill this hole in the crystallographic toolbox.

4. Conclusions

Incommensurately modulated diffraction data were obtained from a protein crystal. The pattern was indexed with *TwinSolve*, which was designed to handle modulated small-molecule crystals. The indexing indicates that the modulation is along the **b** direction in the crystal, which corresponds to an 'actin ribbon' formed by the crystal lattice (Schutt *et al.*, 1993). The modulation was reversible. The best guess as to what may be occurring is that the protein is undergoing a conformational change that is affecting the neighbouring molecules in such a way as to produce the observed modulation in the diffraction pattern. The change back to the 'open state' seemed to be caused by the intense radiation emitted by the FR-E X-ray source. Work needs to be done on transforming the crystal more reliably into this modulated state so that more diffraction data can be collected. It would be even better if the state could be trapped, for example, with cryocooling or chemical crosslinking, allowing a much longer exposure and reducing the radiation-induced creation of free radicals, which may cause the protein to revert back to its original state. The amount of modulated data collected did not allow for assignment of a superspace group or any structure determination to be carried out. Even if there were enough data, the current suite of software for protein crystallography would not be able to handle the four-dimensional indexing (*hklm*). Nevertheless, these data demonstrate that protein crystals can be incommensurately modulated.

It is important to note that 'strange' diffraction patterns are not necessarily modulated. Modulated crystals are a relatively rare occurrence in protein crystallography. Any unusual diffraction pattern should be checked against more common issues such as twinning, parallel plates (more extreme twinning) and large mosaicity. Once these other possibilities have been eliminated then the notion that the pattern could be modulated should be tested using appropriate software. If the pattern is modulated, the easiest case to deal with would be the commensurate one, which can be solved using a supercell. If the reflections cannot be predicted with a supercell then most likely the pattern is incommensurately modulated and currently unsolvable with existing software.

We would like to thank Václav Petříček and Sander van Smaalen for useful suggestions and helpful discussions. This work was funded by NSF grant 0718661, Nebraska Research Initiative funding for this project and for the Nebraska Center for Structural Biology, and the Eppley Cancer Center support grant P30CA036727.

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