Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 2279–2283

BIOORGANIC & MEDICINAL CHEMISTRY

α-Lactose Monohydrate Single Crystals as Hosts for Matrix Isolation of Guest Biopolymers

Harry C. Wang,[a] Miki Kurimoto,[b] Bart Kahr[b,*] and Jean Chmielewski[a,*]

[a]*Department of Chemistry, Purdue University, West Lafayette, IN 47907-1393, USA*
[b]*Department of Chemistry, Box 351700, University of Washington, Seattle, WA 98195-1700, USA*

Received 20 February 2001; accepted 22 March 2001

Abstract—Single crystals of α-lactose monohydrate show a remarkable tendency to include biopolymers, such as proteins, oligonucleotides and dextrans, within the growing lattice. Glycosylation increased the amount of protein contained within the crystals. The guest molecules were found only within the (010) growth sector of the hatchet shaped crystals, thereby binding preferentially to one of the seven developed crystal faces. The topographical features of the active surface are described.

Introduction

The growth of biogenic crystals is intimately controlled by proteins that often become trapped within the growing lattices.[1] In vitro studies have confirmed the effect of biopolymers on crystal morphology and internal texture. Although seemingly unrelated, biopolymer–crystal interactions have also been postulated to play a significant role in the matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS).[2] Indeed, we showed that crystalline MALDI-MS matrices incorporated proteins in specific growth sectors.[3,4]

Proteins and other biopolymers incorporated within the crystalline lattices of simple organic compounds are unusual examples of host/guest chemistry, and we sought to explore the scope and limitations of crystal face-specific molecular recognition processes. We initially investigated a wide range of biopolymers with the crystalline host phthalic acid. Phthalic acid (P) has a rich crystal chemistry and has been shown to orient and overgrow dyes in specific growth sectors.[5] Crystals of P also demonstrated a remarkable capacity to overgrow a variety of dye- or ^{14}C radio-labeled proteins, such as cytochrome *c* (cyt *c*), aprotinin and myoglobin (Fig. 1). In each case the {021} growth sectors[6] of P crystals were the most receptive to proteins, thus resulting in the hourglass or bow-tie pattern within the crystals (Fig. 1).[3]

Although P was a suitable medium for the single crystal matrix isolation of biopolymers, it was limited by its acidity and the need of organic co-solvents in crystallization. These drawbacks led us to evaluate a number of other potential host crystals, including α-lactose monohydrate (LM). We showed that LM crystals grown from solutions containing green fluorescent protein (GFP) were luminescent, indicating that the protein was still in its native conformation;[7] the emission was confined to the (010) growth sector (Fig. 2).[8] Denaturation in the crystal, as indicated by a loss of luminescence, was sharply retarded.[8]

In the current study, we sought to extend the use of LM for the single crystals matrix isolation of biopolymers

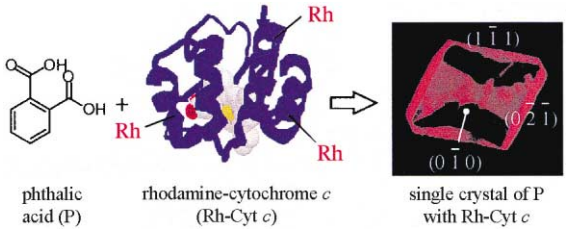

Figure 1. Matrix isolation of rhodamine-labeled cytochrome C within single crystals of phthalic acid (~2 mm).[3]

*Corresponding authors. Tel.: +1-765-949-0239; fax: +1-765-494-0135; chml@purdue.edu and tel.: +1-206-616-8195; fax; +1-206-685-8665; e-mail: kahr@chem.washington.edu

0968-0896/01/$ - see front matter
PII: S0968-0896(01)00129-8

and thereby shed light on the mechanism by which a growing crystal may orient and overgrow seemingly obstrusive guests.

Results and Discussion

Supersaturated solutions of LM were incubated with a variety of proteins at 30 °C. Crystals measuring 2–5 mm in length were harvested after 15–36 h.[9] LM crystals formed in the presence of Zn porphyrin cytochrome *c* (Zn-cyt *c*) or fluorescein labeled lysozyme (Fl-lysozyme), for example, displayed the typical hatchet morphology[10] with fluorescence associated with the (010) growth sector (Fig. 3), as was observed with GFP (Fig. 2).[8] Protein quantification in the mixed crystals was accomplished by acid hydrolysis and amino acid analysis of dissolved crystals. Zn-cyt *c* was found to be present within lactose crystals at a molar ratio of $1{:}6\times10^5$, as compared to $1{:}10^6$ for GFP[8] (Table 1).

Table 1. Quantities of Biopolymer in α-Lactose Monohydrate Crystals

Biopolymer	Guest:Host (mole)
Zn-Cyt C (12.5 kD)	$1{:}6\times10^5$
Fl-Lysozyme (14.7 kD)	$1{:}6\times10^5$
GFP[a]	$1{:}1\times10^6$
TR-Lectin (30 kD)	$1{:}3\times10^4$
Ac-DNA (4 kD)	$1{:}1\times10^5$
Fl-Dextran (10 kD)	$1{:}7\times10^4$
Fl-RNAse A	ND[b]
Fl-RNAse B (15.9 kD)	$1{:}9\times10^4$
Fl-NeutrAvidin	ND[b]
Fl-Avidin (16.5 kD)	$1{:}6\times10^4$

[a]Ref. 8.
[b]Protein not detected by fluorescence microscopy and amino acid analysis (see ref 14).

Since the crystalline host was a carbohydrate, we hypothesized that a carbohydrate binding protein, such as lectin, might be an especially effective dopant. Incubation of a lactose solution with a texas red-labeled lectin derived from *Arachis hypogaea* (TR-lectin) resulted in LM crystals with luminescent (010) growth sectors (Fig. 3). The molar uptake of TR-lectin was an order of magnitude greater than that of Zn-cyt *c* and GFP grown under similar conditions (Table 1). The lectin of *A. hypogaea* is specific for terminal β-galactose residues of glycoproteins,[11] and lactose (composed of β-galactose and α-glucose) has been found to bind to this lectin.[12] A similar binding interaction, therefore, may enhance the contacts between the lectin in solution and the face of the growing LM crystal. Indeed, Addadi and coworkers have shown that antibodies raised to crystals will bind preferentially to particular facets.[13]

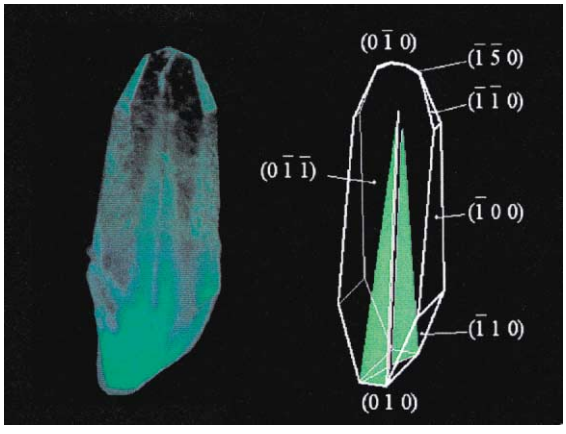

Figure 2. Crystals of lactose monohydrate (LM) as hosts for the guest green fluorescent protein (GFP): left: a mixed crystal of LM with GFP (2.0 (h) ×0.8 (w) ×0.5 (d) mm^3); right: a schematic representation of an LM crystal containing GFP with growth sectors indicated.[8]

Alternatively, we tested the possibility that guest protein glycosylation might enable the uptake of proteins that otherwise did not form mixed crystals with LM. To this end ribonuclease A (RNAse A) and NeutrAvidin (both fluorescein-labeled and not detected within LM crystals) were evaluated in their glycoprotein forms: ribonuclease B (RNAse B) and avidin, also labeled with fluorescein. RNAse B contains a single glycosylation site at Asn 34 that consists of an *N*-linked Man_{5-9} $GlcNAc_2$ carbohydrate, whereas avidin contains a heterogeneous carbohydrate structure also composed of Man and GlcNAc. LM crystals grown in the prescence of the two glycosylated proteins did indeed have luminescent (010) growth sectors (Fig. 3). A typical hatchet morphology was observed for Fl-avidin whereas LM crystals grown with Fl-RNAse B showed an increase in the relative size of the (010) face, the only example where significant habit modification was observed. Molar ratios of $1{:}9\times10^4$ and 6×10^4 were obtained for Fl-RNAse B and Fl-avidin, respectively (Table 1), whereas the unglycosylated proteins showed no detectable incorporation by fluorescence microscopy and amino acid analysis.[14] It is possible, therefore, that the carbohydrates on the protein surface serve to increase their binding to LM crystal facets enabling the protein to become overgrown; in other words the longer the protein is bound to the surface, the greater the chance of trapping it by the deposition of additional lactose molecules.[15] Conversely, the bound proteins could expose the surface carbohydrates to the solution, thereby increasing the rate of overgrowth. Either way, glycosylation increased the amount of the two proteins in the LM crystals.

Other biopolymers, such as oligonucleotides and dextrans, were evaluated as guests for LM crystals. Acridine-labeled Dickerson's dodecamer oligonucleotide[16] (Ac-DNA) and a fluorescein-labeled dextran (Fl-dextran, 10,000 MW) also produced LM crystals with luminescent (010) growth sectors (Table 1).

The binding of a guest biopolymer to a growing crystal surface is a complex molecular recognition process. In an attempt to elucidate the recognition mechanism(s), we investigated the fine structure of the luminescence pattern and the topography of the growth active surfaces. Views of LM/GFP through the broad (0-1-1) face revealed a curious non-luminescent stripe (Fig. 2) running along the *b* axis; this is also clearly seen in the photograph of Fl-avidin in Figure 3. Analyses of the

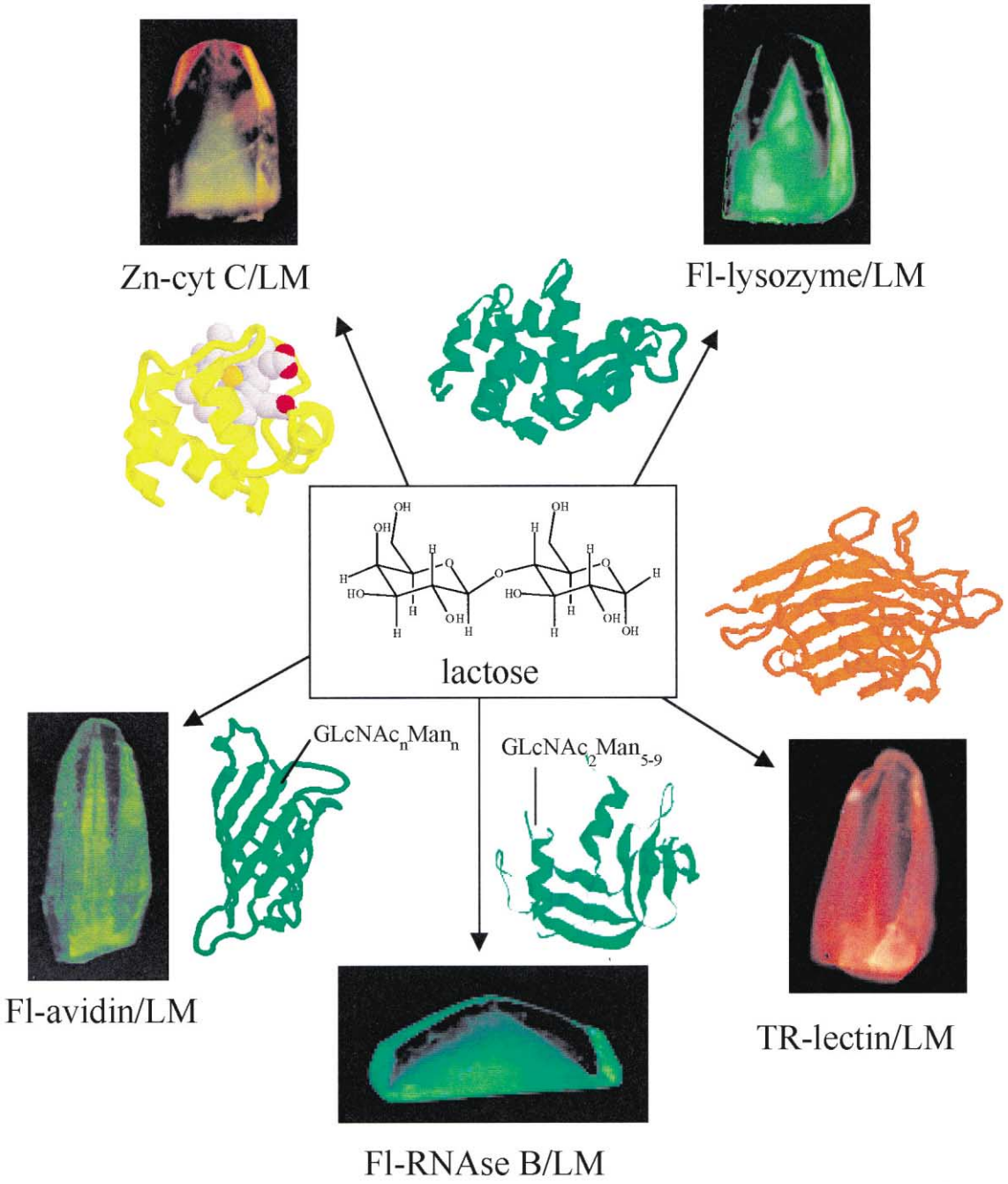

Figure 3. Crystals of α-lactose monohydrate containing corresponding proteins. In each case, the mixed crystal shows a characteristic fluorescence from the (010) growth sector.

growth active (010) surface showed that this feature was the result of a small spiral hill, or hillock arising from a screw dislocation emerging from the crystal surface.[17] Differential interference contrast (DIC) microscopy of a pure LM crystal revealed a single polygonized hillock that are partitioned the (010) surface into four vicinal slopes pair-wise related by the two-fold symmetry of the crystal.[18] Figure 4 compares an interference contrast image with atomic force micrographs made of the hillock center and of the steps on adjacent slopes.[19] Figure 5 compares another (010) DIC micrograph of LM/GFP with a fluorescence micrograph of the same surface indicating that only two of the four hillock slopes were fluorescent. This indicates that GFP recognized only the lateral slopes with greater step advancement velocity, an example of *intrasectoral zoning,* as opposed to the *intersectoral zoning* illustrated thus far.

GFP added to those steps whose risers are populated by the faces of the sugar rings as opposed to the edges (Fig. 6). Note, however, that because of the two-fold symmetry of the crystal along *b* axis, the hydrophobic edges and hydrophilic faces of the glucose moiety are presented to the interfacial solution with equal probability. The molecular basis of this step selectivity is unclear at this time.

Conclusion

During the course of our research, we have discovered a number of crystalline surfaces that have a remarkable ability to orient and overgrow guest molecules bearing neither size, shape, nor constitutional similarity to the host crystal molecules.[20] The (010) face of LM belongs to this class. Here we have shown that it binds and overgrows proteins, DNA, and dextrans in micromolar concentrations within the simple disaccharide crystal. Interfacial protein–carbohydrate and carbohydrate–carbohydrate interactions were manipulated so as to influence the extent to which the guests had become overgrown. The continued generalization of single crystal matrix isolation of biopolymers will require yet a more detailed understanding of the characteristics that define a highly receptive crystal face.

Experimental

Crystal growth

In a typical procedure, 500 μL of a deionized lactose[21] solution (41.5 g/100 mL H_2O) was added to 100 μL of an approximately 1–5 mg/mL solution of the desired biopolymer. Zn-cyt *c* was prepared according to the procedure of Fisher.[22] Fluorescein derivatives were prepared as previously described.[3] TR-lectin was purchased from Molecular Probes. Solutions were incubated at 30 °C for 15–36 h until crystals of a suitable size were obtained. The crystals were harvested and washed with hexanes. Typical crystals, 2–5 mm in length, were observed and photographed with a fluorescence microscope.

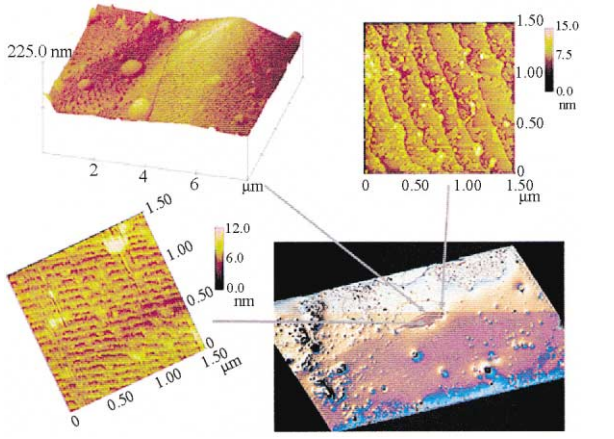

Figure 4. DIC micrograph of a pure LM crystal (010) face (~1 cm across, bottom right) and atomic force microscopy (AFM) images of vicinal sectors where GFP would (top right) and would not (bottom left) be recognized. An AFM image of the hillock core is at top left.

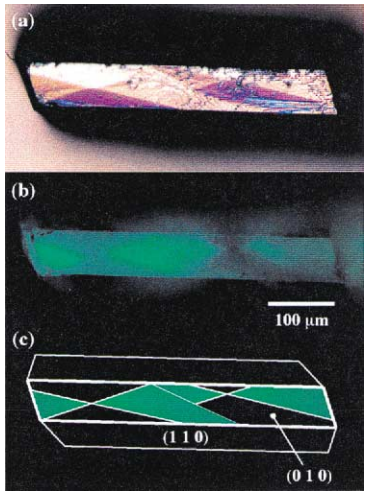

Figure 5. DIC micrograph of LM/GFP (010) face (a), corresponding fluorescence micrograph (b), and an idealized representation (c).

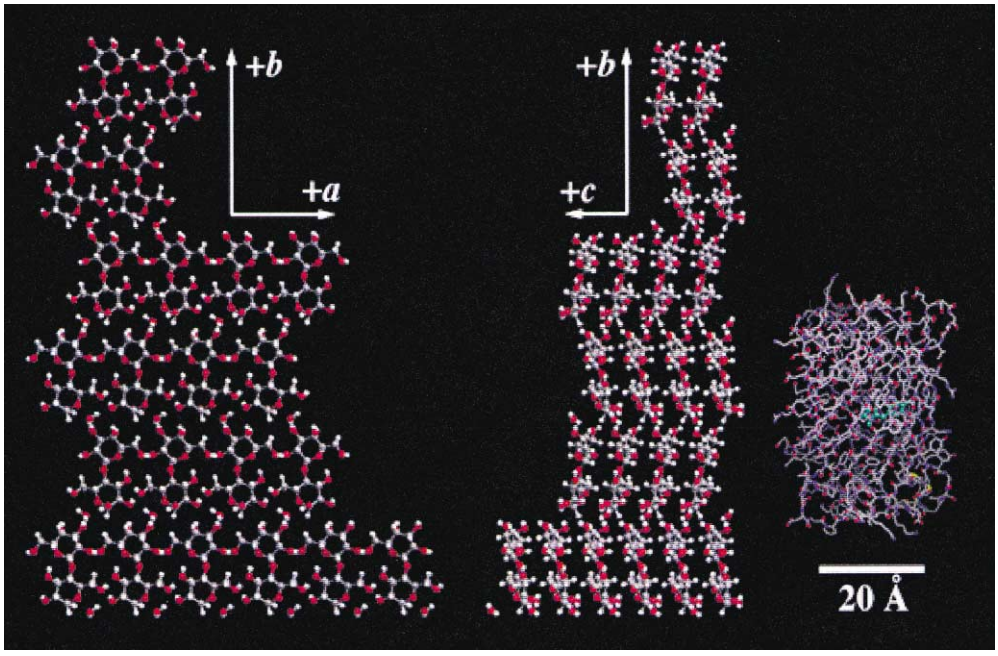

Figure 6. Views of the growth active steps in α-lactose monohydrate single crystals. GFP, drawn to scale at bottom right, recognizes the *b*/*c* steps.

Biopolymer quantification

Protein uptake was quantified by amino acid analysis following dissolution and acid hydrolysis of mixed crystals.[14] Norleucine was used as the internal standard. Dextran (10,000 MW, doubly labeled with fluorescein, Molecular Probes) and Ac-DNA were quantified by absorbance of dissolved crystals: dextran, λ_{494}, $\varepsilon = 68{,}000\,cm^{-1}\,M^{-1}$; DNA, λ_{260}, absorbance of 1 corresponds to 40 μg/mL of single-stranded DNA.[23]

Microscopy

Images of surface topography were made with a Leica DMLM reflected light microscope fitted with an interference contrast prism. TappingMode atomic force microgrpahs were made with a Digital Instrument Nanoscope IIIa, operated in air with a silicon probe.

Acknowledgements

The authors are grateful to the National Institutes of Health (GM58102-01) for their support of this work. M.K. thanks the University of Washington Center for Nanotechnology for fellowship support.

References and Notes

1. (a) Weiner, S.; Addadi, L. *J. Mater. Chem.* **1997,** *7,* 689. (b) Addadi, L.; Weiner, S. *Angew. Chem., Int. Ed. Engl.* **1992,** *31,* 153.
2. Strupat, K.; Karas, M.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion Processes* **1991,** *111,* 89.
3. Chmielewski, J.; Lewis, J. J.; Lovell, S.; Zutshi, R.; Savickas, P.; Mitchell, C. A.; Subramony, J. A.; Kahr, B. *J. Am. Chem. Soc.* **1997,** *119,* 10565.
4. Kurimoto, M.; Bastin, L. D.; Fredrickson, D.; Gustafson, P. N.; Jang, S. –H.; Kaminsky, W.; Lovell, S.; Mitchell. C. A.; Chmielewski, J.; Kahr, B. *Morphology and Dynamics of Crystal Surfaces in Complex Molecular Systems, MRS Symposium Series,* in press (and references within).
5. Mitchell, C. A.; Lovell, S.; Thomas, K.; Savickas, P.; Kahr, B. *Angew. Chem., Int. Ed. Engl.* **1996,** *35,* 1021. See also ref. 20 and citations therein.
6. (a) Ermer, O. *Helv. Chim. Acta* **1981**, *64*, 1902. (b) Neuhaus, A. *Z. Kristallogr.* **1943**, *105*, 161.
7. Tsien, R. Y. *Annu. Rev. Biochem.* **1998,** *67,* 509.
8. Kurimoto, M.; Subramony, P.; Gurney, R. W.; Lovell, S.; Chmielewski, J.; Kahr, B. *J. Am. Chem. Soc.* **1999,** *121,* 6952.
9. Jelen, P.; Coulter, S. T. *J. Food Sci.* **1974,** *38,* 1182.
10. (a) Fries, D. C.; Rao, S. T.; Sundaralingam, M. *Acta Crystallogr., Sect. B* **1971,** *B27,* 994. (b) Beevers, C. A.; Hansen, H. N. *Acta Crystallogr., Sect. B* **1971,** *B27,* 1323.
11. Sharma, V.; Srinivas, V. R.; Adhikari, P.; Vijayan, M.; Surolia, A. *Glycobiology* **1998,** *8,* 1007.
12. Banerjee, R.; Das, K.; Raviashankar, R.; Suguna, K.; Surolia, A.; Vijayan, M. *J. Mol. Biol.* **1996,** *259,* 281.
13. Bromberg, R.; Kessler, N.; Addadi, L. *J. Cryst. Growth*, **1988**, *193*, 656–664; Kessler, N.; Perl-Treves, D.; Addadi, L.; Eisenstein, M. *Proteins*, **1999**, *34*, 383–394.
14. Amino acid analysis should be able to detect a molar ratio of at least $1{:}10^7$ depending on the quantity of lactose crystals interrogated.
15. McBride, J. M. *Angew. Chem., Int. Ed. Engl.* **1989**, *101*, 391.
16. Wing, R.; Drew, H.; Takano, T.; Broka, C.; Tanaka, S.; Itakura, K.; Dickerson, R. E. *Nature* **1980,** *287,* 755.
17. Van Enckevort, W. J. P. *Prog. Cryst. Growth and Charact.* **1984**, *9*, 1.
18. Pluta, M. *Specialized Methods: Advanced Light Microscopy;* Elsevier: New York, 1989; Vol 2, pp 146–196.
19. Dincer, T. D.; Parkinson, G. M.; Rohl, L.; Ogden, M. I. *J. Cryst. Growth* **1999,** *205,* 368.
20. Kahr, B.; Gurney, R. W. *Chem. Rev.* in press.
21. Visser, R. A. *Neth. Milk Dairy J.* **1980,** *34,* 255.
22. Fisher, W. R.; Taniuchi, H.; Anfinsen, C. B. *J. Biol. Chem.* **1973,** *248,* 3188.
23. Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning: A Laboratory Manual;* Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y., 1982; Vol. 3.