Single-Crystal Matrix Isolation of Biopolymers

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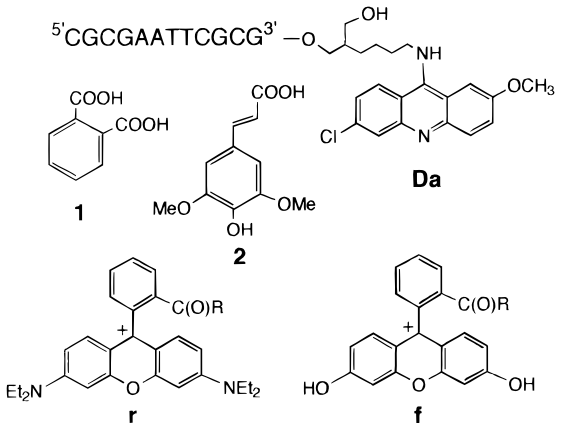
The absence of suitable single crystals of some biopolymers has encouraged structural chemists to partially orient such molecules with electric, magnetic, or flow fields,[2] by dissolution in liquid crystals or stretched gels,[3] and as monolayers.[4] Since it is often found that guests in crystals are oriented by the host lattice,[5] and that proteins are found within single crystals of biominerals,[6] it seemed possible that we might effectively isolate and orient biopolymers in suitable crystalline host matrices in the laboratory. Such mixed crystals might be used to analyze spectral anisotropies in biological molecules that could not otherwise be crystallized. Herein, we demonstrate the incorporation and orientation of proteins and an oligonucleotide in host aromatic acid crystals using three imaging techniques: fluorescence microscopy/spectroscopy, single-crystal desorption mass spectrometry, and autoradiography. This work is part of our continuing study of extra-ordinary solid solutions, mixed crystals with guests bearing no size, shape, nor constitutional similarity to the host molecules or ions.[7] Most relevant is our recent analysis of phthalic (**1**) and sinapic (**2**) acid crystals that have oriented and isolated dyes in particular sectors during growth from solution.[8]



Dye-labeled biopolymers were prepared to monitor their presence in host crystals **1** and **2**. The proteins cytochrome *c* (**C**), aprotinin, bovine serum albumin, lysozyme, and myoglobin were covalently labeled with the *N*-hydroxysuccinimide esters of the dyes rhodamine (**r**) or fluorescein (**f**) and then purified by gel filtration. Millimeter-sized crystals of **1** or **2** were grown at room temperature from solutions containing the labeled proteins.[9] A surprising number of the resulting mixed crystals showed patterns of elliptically polarized **r** or **f** emissions that were consistent with growth sectors (Figure 1a).[10] The most absorptive growth sectors for **1** and **2** were {021} and {10$\bar{3}$}, respectively.[11] The molar ratio of organic host acid to dye-labeled protein within the mixed crystals was approximately 10^4 as determined by nitrogen analysis and colorimetry. These results indicated to us that proteins can be routinely isolated in sub-millimolar concentrations within simple molecular crystals.[12]

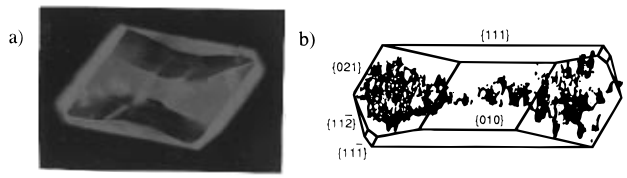


Figure 1. (a) Photomicrograph of **1Cr** under UV illumination and viewed along [010]. Horizontal dimension is 1 mm. (b). Autoradiogram of **1**[^{14}C]**C** upon which is superimposed the idealized habit of the crystal that produced it. Graininess is limited by film resolution.

Single crystals of **2**[13] containing proteins were analyzed by MALDI-MS.[14] The intensities of the protein signals in the mass spectra were highly dependent upon that region of the crystal irradiated with the focused nitrogen laser. Irradiation of **2Cr** (notation indicates sinapic acid crystals containing rhodamine-labeled cytochrome *c*) on the largest, uncolored {010} facets produced weak protein signal that increased 20-fold when beam spilled over the edges of the crystal associated with the colored {10$\bar{3}$} growth sectors.

To ensure that the dye labels were not responsible for directing the inclusion of the proteins into the matrix,[15] we grew **1** in the presence of ^{14}C-radiolabeled cytochrome *c* ([^{14}C]**C**, Sigma) *without* the covalently bound dye. Indeed, autoradiograms of these crystals also showed an hourglass pattern associated with the {021} growth sectors (Figure 1b).[16]

Fluorescence anisotropy measurements indicated that the included proteins were oriented within the host crystals. For

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(9) In a typical crystallization 800−900 μL of a saturated solution of **1** in 2:1 CH_3CN/H_2O was mixed with 100−200 μL of approximately 100 μM protein or DNA solution. The solutions in Eppendorf vials were allowed to stand in air for several days, after which time crystals were removed from the walls of the vials.

(10) These include the following: **r**-labeled myoglobin in the {10$\bar{3}$} sectors of **2** and **r**- and **f**-labeled aprotinin in the {021} sectors of **1**. See Supporting Information.

(11) (a) Phthalic acid: Neuhaus, A. *Z. Kristallogr*. **1943**, *105*, 161−219. (b) Sinapic acid: Beavis, R. C.; Bridson, J. N. *J. Phys. D* **1993**, *26*, 442−447.
(12) Similar judgments have come from scientists studying MALDI-MS; see for example: Strupat, K.; Karas, M.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion Processes* **1991**, *111*, 89−102. King, R. C.; Owens, K. G. In *Proceedings of the 42nd ASMS Conference on Mass Spectrometery and Allied Topics*, Chicago, 1994; p 977. Dai, Y.; Whittal, R. M.; Li, L. *Anal. Chem*. **1996**, *68*, 2494−2500.

(13) Phthalic acid does not function as a MALDI host. Therefore, MS experiments could only be performed on crystals of **2**.

(14) Doktycz, S. J.; Savickas, P. J.; Krueger, D. A. *Rapid Commun. Mass Spectrom*. **1991**, *5*, 145−148.

(15) A stimulating paper (ref 11b) illustrated an hourglass coloration of **2** in the {10$\bar{3}$} growth sectors when crystals were grown in the presence myoglobin non-covalently bound to a triarylmethane dye. We have shown (ref 8) that aromatic acid crystals frequently orient cationic dyes even in the absence of a biological analyte, suggesting that proteins are only safely imaged in crystals with covalent dye labels or intrinsic chromophores.

(16) To our knowledge, imaging of growth sectors in crystals autoradiographically was first carried out in connection with the trace detection of Th, Pu, and Pb in simple sulfate crystals. See: Hahn, O. *Z. Kristallogr*. **1934**, *87*, 387−416.

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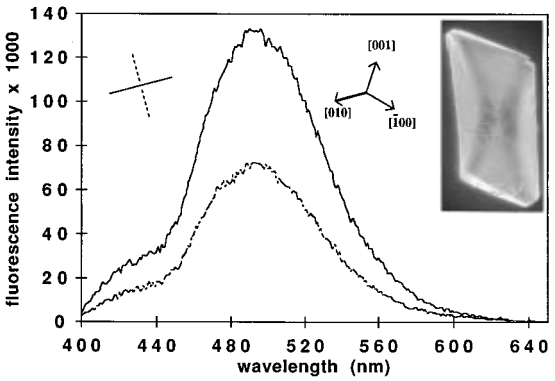

Figure 2. Luminescence of **1Cp** for exciting light polarized along the orthogonal directions shown in leftmost inset. Vertical crystal dimension in photomicrograph is 1 mm.

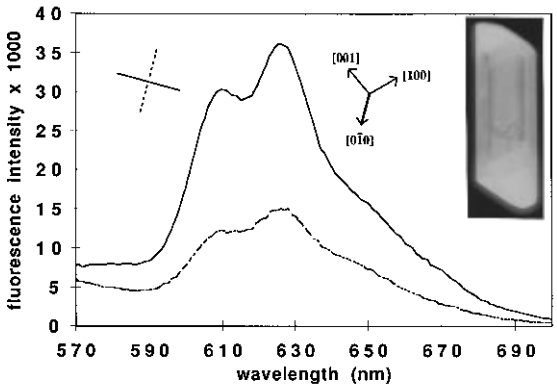

Figure 3. Luminescence of **1Da** for exciting light polarized along the orthogonal directions shown in leftmost inset. Vertical crystal dimension in photomicrograph is 1 mm.

1Cr observed normal to the (010) face (Figure 1a), λ_{em} was 586 nm (λ_{ex} 530) and the major axis of the elliptically polarized emission was parallel to the slow vibration direction.[17]

To establish that these facially selective adsorption phenomena were not restricted to proteins, we carried out the single-crystal matrix isolation of an oligonucleotide, Dickerson's dodecamer (**D**)[18] in **1**. **D** was bound to acridine (**a**) using a functionalized controlled pore glass and the standard phosphoramidite methodology.[19] Crystals of **1Da** showed a highly anisotropic blue fluorescence associated with the {021} growth sectors (Figure 2). Here, the major axis of the elliptically polarized emission in the (010) face was parallel to the *fast* vibration direction.

Since the orientations of the fluorescent labels were not fixed with respect to the biopolymers, it was desirable to study guest molecules with intrinsic luminophores. Therefore, Fe-free porphyrin cytochrome *c* (**Cp**) was prepared by reaction with HF.[20] Crystals of **1Cp**, grown at room temperature by water evaporation, gave rise to the luminescence spectrum shown in Figure 3. Emission from the Q-band, bright in the {021} and {010} sectors, has a tripartite structure which most resembles emission from monoprotonated free porphyrins.[21] Total ratios of the fluorescence of the Q-band polarized in orthogonal directions in the {010} and {111} faces were 2.7 and 1.6, respectively.

We examined the circular dichroism (CD) of **C** in saturated solutions of **1** prior to crystallization.[22] The CD spectrum associated with the Q-band of **C** is sensitive to denaturation with urea and heat but insensitive to saturating concentrations of **1**. One may conclude from these observations that **C** maintains its folded state prior to association with the {021} face of growing crystals of **1**.

Dyes in crystals have been found in a variety of aggregation states, and presumably, varied growth conditions would present both isolated and aggregated biopolymers in single crystals. Measurements of the lifetimes of the luminesence of **1Cr**, **1Cp**, and **1** containing both **Cp** and **Cr**[23] provided no definitive evidence for Förster energy transfer.[24]

The {021} faces of phthalic acid project carboxyl groups and aryl rings directly from the surface in alternating rows.[25] Such a surface structure does not lend itself to a straightforward characterization of the recognition mechanism as hydrophobic nor hydrophilic in nature. Moreover, emergent secondary surface structures, not characterized at this time, may be essential in directing absorption phenomena.

Our previous investigations of organic dyes in simple ionic crystals indicated the formation extra-ordinary solid solutions required a rare stereochemical match between guest and host surface.[7] We were therefore surprised at the frequency and ease with which we could find evidence for oriented biomolecules in **1** and **2**. On the other hand, it is well-known, and often problematic, that biopolymers tenaciously adhere to a variety of surfaces.[26] And, Addadi and co-workers have shown that proteins can attach to specific crystal faces, as surmised from the influence of insulin antibodies on the morphology of insulin crystals.[27] The burgeoning field of MALDI-MS provides further evidence that biopolymers are often contained within single crystals.[12] Now, our challenge is to address specific structural questions about oriented bioguests in simple single crystals, while devising experiments to define the recognition mechanisms.

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Supporting Information Available: Single-crystal MALDI mass spectra, CD spectra, fluorescence lifetime measurements, photographs of additional biopolymers in crystalline matrices, and image of active {021} surface of **1** (6 pages). See any current masthead page for ordering and Internet access instructions.

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(17) Fluorescence spectra were recorded with a Spex Fluoromax2 spectrometer using a 90° detection geometry. Optical fibers carried the light to and from the sample mounted on an IMT-2 Olympus inverted microscope. Excitation and emission paths were parallel due to the front face collection geometry through the microscope objective. The polarization of the emission was analyzed with a fixed dichroic filter before the light exited into the return fiber. The crystal was rotated on a stage after carefully centering the microscope objectives. In this way, polarization bias due to the instrument, small due to scrambling through the input fiber and assessed using standard crystals with known fluorescence anisotropies, is canceled out. Magnifications were chosen so that reflective, flat crystal surfaces were normal to the light path and occupied the entire field of view. For reviews on fluorescence polarization measurements, see: Badley, R. A. In *Modern Fluorescence Spectroscopy 2*; Wehry, E. L., Ed.; Plenum Press: New York, 1976; pp 91−168. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Plenum Press: New York, 1983; pp 111−150.

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(22) CD spectra for 30 μM solutions of **C** were recorded with a Jasco 600 CD spectrophotometer.

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