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# Kinetic Stabilization of Biopolymers in Single-Crystal Hosts: Green Fluorescent Protein in $\alpha$ -Lactose Monohydrate

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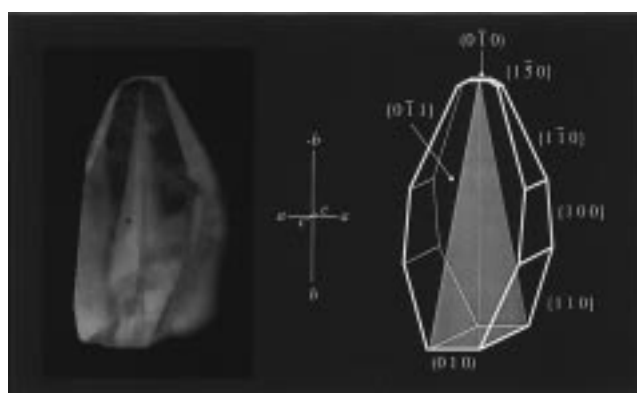
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We recently demonstrated that simple molecular crystals rich in hydrogen bonds adsorb, orient, and overgrow a surprising variety of biopolymers on specific faces.<sup>1</sup> Such protein- and oligonucleotide-containing mixed crystals inform biopolymer recognition mechanisms and are suited to studies of anisotropic molecular properties. Moreover, biopolymers trapped in single crystals might experience substantial kinetic stabilization. Here, we demonstrate that green fluorescent protein (GFP),<sup>2</sup> first isolated from the jellyfish *Aequorea victoria*, can be oriented and stabilized in its native conformation in single crystals of  $\alpha$ -lactose monohydrate, and subsequently released into solution in its native state by dissolution of the matrix.

GFP is fluorescent due to a *p*-hydroxybenzylideneimidazolinone chromophore that is formed in an autocatalytic posttranslational cyclization, dehydration, and oxidation of three residues, <sup>65</sup>Ser-Tyr-Gly<sup>67</sup>, buried within the cylindrical, 11-stranded  $\beta$ -barrel (~42 Å height and ~24 Å diameter, 238 amino acids, 27 kDa). Native GFP has chromophore-derived absorption maxima at 395 and 475 nm corresponding to the phenol and phenolate, respectively, both of which emit from the deprotonated excited state at 508 nm.<sup>3</sup>

GFP was chosen for this study because it is known to fluoresce only in its native conformation. Upon denaturation, the interior of the barrel is exposed, and the chromophore fluorescence is rapidly quenched.<sup>4</sup>  $\alpha$ -Lactose monohydrate (LM) was selected as a host because it is pH neutral and produces well-formed crystals ( $a = 7.982(2)$  Å,  $b = 21.562(3)$  Å,  $c = 4.824(1)$  Å,  $\beta = 109.57(3)^\circ$ , space group  $P2_1$ ,  $Z = 2$ ).<sup>5</sup> Crystals grow best by evaporation of water from a supersaturated solution only after deionizing the  $\alpha$ -lactose (Aldrich) by first passing the solution through both cation- and anion-exchange columns.<sup>6</sup> When 40  $\mu$ L of a 1 mg/mL GFP (Clontech) solution (10 mM tris-HCl, pH 8, 10 mM EDTA) was mixed with 0.5 mL of a 1.15 M aqueous



**Figure 1.** Photograph illustrating fluorescence from a LM/GFP mixed crystal (1.8 (h)  $\times$  0.8 (w)  $\times$  0.5 (d) mm<sup>3</sup>) with an idealized representation of habit. Sides of the crystal in the photograph are bright due to internal reflection.

lactose solution and allowed to stand for 3–4 days at room temperature in a 24-well plate, crystals were deposited. Crystals display a hatchet morphology having a broad base (010) further bounded by {100}, {110}, {110}, and {011}; small (010) and {150} faces are also occasionally present (Figure 1). LM crystals are polar and grow rapidly in the [010] direction, with virtually no growth in the  $[0\bar{1}0]$  direction. When illuminated with a long wavelength UV lamp, the crystals exhibited a bright green fluorescence (Figure 1), localized within a sharply defined pyramid corresponding to the (010) growth sector.<sup>7</sup> This indicates that the GFP is selectively recognized and overgrown by the (010) face in preference to the others.<sup>1</sup> More importantly, it is strong evidence that GFP is in its native conformation. The molar ratio<sup>8</sup> of GFP to lactose in one of these crystals is roughly 1:10<sup>6</sup>; each GFP molecule is as large as ~180 lactose molecules.

The emission energy and excited-state lifetime of GFP in LM were measured while exciting at the long-wavelength absorption band ( $\lambda_{\text{ex}} = 470$  nm) to prevent photobleaching.<sup>9</sup> The data are comparable to those from GFP in other environments, although the lifetime is curiously shorter in condensed lactose phases. The relevant data are summarized in Table 1.

The emission anisotropy for LM/GFP as a function of polarization of the excitation in the (100) and (010) faces was marginal (10% differences in fluorescence). Nevertheless, the fluorescence maxima were consistent when the exciting light and the emitted light were polarized parallel to *b* for light incident on (100) and nearly parallel to *a* for light incident on (010).<sup>11</sup>

The GFP steady-state fluorescence intensity was measured as a function of time and temperature in three environments: saturated aqueous  $\alpha$ -lactose solution, lyophilized  $\alpha$ -lactose, and crystalline LM. As shown in Figure 2, both the solution and

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(8) Concentration of the GFP was calculated from the absorbance of a LM/GFP crystal of estimated volume using the known  $\epsilon_{395}$  ( $30 \times 10^3$  L M<sup>-1</sup> cm<sup>-1</sup>).<sup>2</sup>

(9) Fluorescence spectra were collected as reported previously using a SPEX FluoroMax-2 fluorimeter<sup>1</sup> coupled with fibers to an IMT-2 Olympus inverted microscope equipped with a Instec HS400 heating stage. Excitation and emission paths were parallel due to the front face collection geometry through the microscope objective. The polarization of the emission and excitation were analyzed with a fixed dichroic filter before and after the sample. Fluorescence lifetimes were recorded with a PTL LaserStrobe Timemaster fluorescence lifetime spectrometer.

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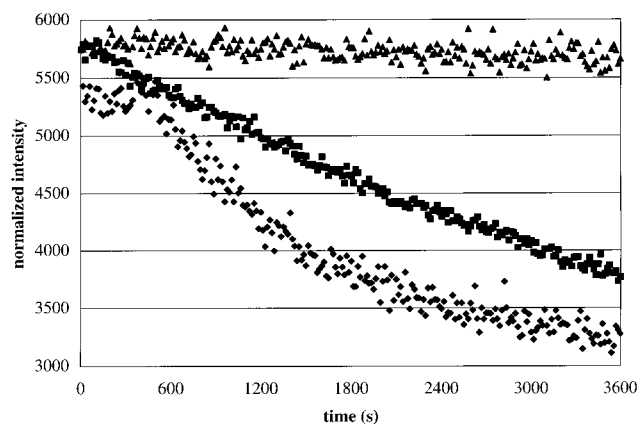
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**Figure 2.** Decay of steady-state fluorescence of GFP at 333 K in several environments: mixed crystal LM/GFP ( $\blacktriangle$ ), saturated lactose solution ( $\blacksquare$ ), lyophilized LM ( $\blacklozenge$ ).

**Table 1.** Energy and Lifetime of Fluorescence from GFP

	$\lambda_{\text{ex}}$ (nm)	$\lambda_{\text{em}}$ (nm)	$\tau(\sigma)$ (ns)	$\chi^2$
aqueous solution	470	507	2.86(6)	1.10
saturated aqueous LM solution	470	509	2.640(3)	1.07
single GFP crystal <sup>10</sup>	395	512	3.30(9)	
lyophilized lactose	470	503	2.29(6)	1.06
single crystal LM	470	515	2.2(1)	1.05

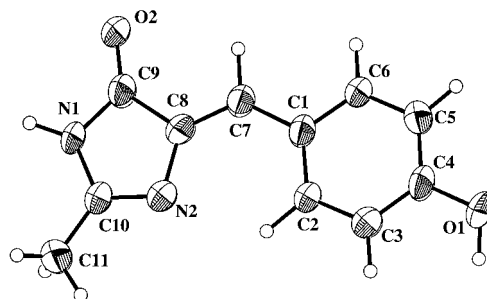
lyophilized preparations lost nearly half of the fluorescence at 333K within 1 h; surprisingly, the lyophilized sample was not more thermally stable than the solution over this time period. On the other hand, the crystal showed no change at 333 or even 343 K. It is reasonable to expect that encasing a protein in a crystal would retard the internal dynamics necessary for unfolding. The leveling off of the decay in the lyophilized sample may indicate that some of the GFP is genuinely incorporated in crystallites.

As it is conceivable that GFP can unfold in LM crystals without fluorescence-quenching, we monitored the rise in fluorescence on dissolution of the mixed crystals. After placing several crystals in water, an immediate onset in the solution fluorescence was observed, followed by a steady increase as the crystals dissolved. To further test the possibility that denatured GFP *inside* the LM crystals might still be fluorescent we synthesized a derivative of the "naked chromophore" (**1**) using a modification of the procedure by Tsuji and co-workers.<sup>12,13</sup> Its X-ray crystal structure

(11) To interpret this anisotropy it is necessary to determine the electric transition dipole moment. INDO/S-CI calculations on the AM1-optimized geometry of **1** predicted that the transition dipole is oriented in the mean plane and is approximately parallel to the exocyclic double bond, disposed toward the phenolic group by 8°. Photoselection experiments of **1** in a 0.1 M KOH ethanol glass indicated that the angle between absorption (470 nm) and emission dipoles is 25(4)°.

(12) Compound **1** was isolated during the literature synthesis of its *t*-butyldimethylsilyl protected derivative.<sup>13</sup> Here, the removal of the protecting group was concomitant with purification of **1** on a silica gel column. Compound **1** was recrystallized from 1:1 mixture of ethanol and water: mp 232 °C; IR (Nujol) 1711, 1635, 1597, 1309, 1275, 1190, 1163, 1073, 1021, 975, 887, 838, 722; <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>-DMSO-*d*<sub>6</sub>)  $\delta$  9.38 (s, 1H), 7.96 (d, *J* = 8.9 Hz, 2H), 6.87 (s, 1H), 6.79 (d, *J* = 8.9 Hz, 2H), 2.26 (s, 3H); <sup>13</sup>C{<sup>1</sup>H}NMR (500 MHz, DMSO-*d*<sub>6</sub>)  $\delta$  171.4, 161.3, 159.1, 137.4, 133.7, 125.3, 124.1, 115.7, 16.1; EI (direct probe, 70 eV) 202, 201, 187, 133; high-resolution mass spectrum *m/z* 202.07447 (calcd for C<sub>11</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub> 202.07423).

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**Figure 3.** ORTEP representation (50% probability) of **1**.

revealed the *cis* stereochemistry at the exocyclic double bond (Figure 3).<sup>14</sup> Unfortunately, growing LM crystals did not incorporate detectable quantities of the synthesized chromophore, underscoring the fact that isomorphism is not the requisite condition for forming unusual mixed crystals, but rather the persistence of surface interactions.

Recently, proteins encapsulated in porous, silica-based sol-gels have been shown to retain their structure and activity.<sup>15</sup> In some cases, proteins were more stable,<sup>16</sup> but in others they were less so.<sup>17</sup> Crystals, like sol-gels, should also limit large conformational fluctuations of included proteins, thereby retarding denaturation, and consequently should be considered as containers for storing and analyzing biopolymers.<sup>18</sup> The kinetic stabilization of GFP in LM crystals shown here is one realization of this proposition. As a variety of GFP mutants are now available, we will in the future study the effect of primary and secondary structural perturbations on the process of mixed-crystal growth.

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**Supporting Information Available:** Crystallographic parameters, tables of atomic coordinates, bond lengths, valence angles, and anisotropic displacement parameters for **1** (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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(14) Crystal data: C<sub>11</sub>H<sub>10</sub>N<sub>2</sub>O<sub>2</sub>·3H<sub>2</sub>O, *M* = 256.26 g mol<sup>-1</sup>, crystal dimensions 0.20 × 0.20 × 0.08 mm, orange plate, triclinic, space group *P*1, *Z* = 2, *a* = 6.726(1) Å, *b* = 9.515(2) Å, *c* = 10.945(2) Å,  $\alpha$  = 114.647(7)°,  $\beta$  = 96.19(1)°,  $\gamma$  = 98.33(1)°, *V* = 618.8(2) Å<sup>3</sup>,  $\rho_{\text{calcd}}$  = 1.375 g cm<sup>-3</sup>,  $\mu(\text{Mo K}\alpha)$  = 0.109 mm<sup>-1</sup>,  $\lambda$  = 0.71070 Å, 14177 reflections collected, 1284 observed (*I* > 2.0 $\sigma$ (*I*)) 2° < 2 $\theta$  < 41°. Intensity data were recorded at -112 °C with a Nonius KappaCCD diffractometer. Heavy atoms were refined anisotropically, and hydrogen atoms were located during the Fourier synthesis, except for one water hydrogen and the hydroxyl hydrogen. *R*(*F*<sub>o</sub>) = 0.0637, *R*<sub>w</sub>(*F*<sub>o</sub><sup>2</sup>) = 0.1893, GOF = 1.008, 164 parameters. Largest difference peak and hole, 0.25 and -0.24 eÅ<sup>-3</sup>.

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