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Exploiting fluorescence resonance energy transfer to probe structural changes in a macromolecule during adsorption and incorporation into a growing biomineral crystal

Lara A. Touryan^a, Gretchen Baneyx^a, and Viola Vogel^{a,b,*}

^aCenter for Nanotechnology, Department of Bioengineering, University of Washington, Seattle, WA 98195, USA ^bDepartment of Materials, ETH Zurich, CH-8093 Zürich, Switzerland

Abstract

The growth of natural biominerals is often tightly regulated by surface adsorption and subsequent incorporation of proteins into the crystal structure. Understanding how macromolecules intercalate into inorganic crystal lattices and how incorporation affects protein structure is crucial to learning how to engineer biomimetic materials with advanced properties, yet knowledge about the molecular-level interactions between organic guests and inorganic hosts remains sparse. Here we have used fluorescence resonance energy transfer (FRET) to probe conformational changes of a macromolecule as it adsorbs to, and becomes incorporated within, a biomineral crystal. Calcium oxalate monohydrate (COM) was used as a model due to its large size and kinetic stability under a wide range of pH values. Since the conformation of the extracellular matrix protein fibronectin (Fn) is highly sensitive to local ion concentrations, major conformational changes can be observed by FRET, as Fn senses and responds to varying local ionic conditions. When transferred from a physiological buffer to a supersaturated solution, Fn's crossed-over dimeric arms separate, indicating a weakening of the electrostatic interactions which otherwise stabilize the compact conformation of the protein. Fn returns to a more compact state when binding to the flat (−1 0 1) surface of the crystal, suggesting that Fn might sense a zone of ion depletion right at the interface of the growing crystal. As the crystal begins to grow around the absorbed protein, the dimeric Fn arms separate again, potentially driven by interactions with the newly formed charged step edges forming around it during the embedding process. FRET thus reveals for the first time how local changes in the electrostatic environment during the growth of a biomineral can cause major alterations in protein conformation. The insights derived using FRET and atomic force microscopy (AFM) could stimulate novel ways to tailor and tune the properties of organic–inorganic composites by exploiting dynamically changing electrostatic guest–host interactions.

Keywords

Fluorescence resonance energy transfer; (FRET); Protein adsorption; Protein; incorporation; Biominerals

1. Introduction

The conformation that proteins assume as they are incorporated into biominerals has a fundamental impact on crystal attributes, including their nucleation kinetics [1], morphology

[2], polymorphism [3,4] and materials properties [5]. Some proteins can become interred within biominerals without affecting the integrity of single inorganic crystals [3,6–8]. Understanding how such large molecules become incorporated into a crystal lattice is crucial to tailoring the properties of inorganic materials. Atomic force microscopy (AFM) was one of the first techniques that probed protein conformation and interactions with growing biomineral crystals. Single globular proteins bound to specific calcite planes [9] were imaged, to demonstrate protein attachment to the step edges of growing calcite [10,11]. Globular proteins were shown to attach to calcite edges, slowing and eventually stopping step growth, and atomic resolution measurements of the final lattice structure showed that a direct change from calcite to aragonite occurred in the presence of these soluble proteins [12].

More recently, thermodynamic and solid state NMR has been used to examine protein and biomineral surface interactions by probing interactions between the biomineral hydroxyapatite (HAP) and the saliva protein statherin, a protein known to inhibit HAP nucleation [13,14], and it was found that the interaction of statherin with HAP induces formation of secondary structure, while statherin in solution remains unstructured. While probing protein conformations by AFM or NMR remains limited to surface-bound states, probing peptides or protein conformations during or after the embedding process has mainly been done by demineralization, followed by scanning electron microscopy (SEM) [15,16], histo-chemical labeling [17], or TEM [18]. These studies have provided data about the roles of major functional groups of macromolecular matrices in directing biomineralization processes and enhancing mechanical properties, as well as providing techniques for mapping location and distribution of proteins within biominerals. Immuno-gold labeling, in combination with SEM, has also been used on fractured calcium carbonate to identify protein types and their distribution within the crystals [19]. However, fluorescence resonance energy transfer (FRET), which has been extensively applied in biomedical sciences and many other fields, has never been applied to the absorption and incorporation of macromolecules into a biomineral.

Calcium oxalate monohydrate (COM) was used as a model biomineral system because it can be grown to comparatively large crystal sizes, has a relatively simple crystal structure and well-understood phase behavior, and is physiologically significant as the primary mineral component in human kidney stones [20]. Furthermore, its growth kinetics are insensitive over a wide range of pH values [21], which is important since a macromolecule carrying charges may alter local effective pH conditions [22–24].

Although a number of urinary proteins have already been shown to interact with COM and influence growth kinetics, morphology and cell attachment [25–27], how they bind and incorporate remains an open question. Stereospecificity between macromolecules and COM has been demonstrated for the urinary protein nephrolcalcin [25] and glycosaminoglycans [27], which are thought to preferentially bind to the (–101) face. It has also been demonstrated that proteins do incorporate into single crystals of COM both *in vivo* and *in vitro* [16], and that such intercrystalline proteins may play important roles in facilitating the breakdown of kidney stones, particularly after cell attachment and phagocytosis. Interestingly, COM crystals can upregulate the expression of Fn by renal epithelial cells, which may subsequently play a dual role in both preventing COM aggregation [28] and regulating the endocytosis of early COM crystals [29–31]. Fn is postulated to be a potent COM binding molecule [32], but little is known about its attachment to and incorporation into these crystals.

Fibronectin (Fn), a cell adhesion protein, was used as a model protein since it is known to undergo major changes of its quaternary structure in response to changes in ionic strength

and electrostatic surface interactions [33–44]. Focusing on Fn is of major physiological interest since it exists as a compact dimer in blood, interstitial fluids, and urine, and is assembled by cells into fibrillar networks that define extracellular matrix and connective tissues [45]. Fn furthermore plays a regulatory role in early phases of bone formation [46,47].

Molecular interaction studies with biomineral surfaces revealed that they are dominated by electrostatics rather than specific protein or lipid/lattice interactions [24,48]. Fn has a number of recognition sites that can bind to solid surfaces, other extracellular proteins and cells, including cell integrin binding via the Arg–Gly–Asp (RGD) loop and the proximal synergy site (for review see [49]). Exposure of these sites is regulated by conformational changes within the protein [50], and when adsorbed to surfaces is highly sensitive to the surface chemistry [42].

FRET, the non-radiative energy transfer from a donor to an acceptor [51], has frequently been applied as a “spectroscopic ruler” [52–54] to study layer thicknesses (for review see [55]), protein/ligand and protein/membrane interactions, as well as to examine protein conformational changes, with broad applications in biological research [56–58]. Here we apply FRET to investigate conformational changes as a macromolecule both adsorbs from a supersaturated solution to a well defined single crystal surface, and intercalates during crystallization. Resonant dipole coupling between a donor and acceptor can occur if their distance is less than 10 nm. Fn, however, is composed of more than 54 domains exhibiting an end-to-end distance of 120–140 nm, even when the secondary structure of all domains is intact [34,59]. To ensure that the FRET signature is sensitive to a broad range of conformational changes, Fn was labeled on average with 7 donors and 4 acceptors per molecule [41,44,60]. We thus sacrificed the ability to measure absolute distances, but gained sensitivity to probing major changes in the average donor-acceptor distances, from a tightly folded quaternary structure, to breaking the quaternary structure, to loosing secondary structure and upon denaturation [34,41,44,59]. To prevent intermolecular FRET, FRET-labeled Fn was always highly diluted with unlabeled Fn.

Here, we investigate changes in Fn conformation by FRET and AFM. The acceptor versus donor fluorescence intensity (I_A/I_D) was measured for Fn either adsorbed to mature crystals or incorporated during the growth of COM. This was compared to the I_A/I_D ratio probed for Fn under partial denaturing conditions [44,60]. These results suggest a model in which the protein's compact conformation is disrupted by supersaturated solution conditions, as well as short-range electrostatic interactions on the surface of a growing mineral crystal, ultimately revealing unexpectedly complex conformational changes during the process of incorporation.

2. Materials and methods

2.1. Protein labeling

Human plasma fibronectin (Chemion) was labeled by conjugation of fluorescent dyes to specific protein residues, as described previously [41,60] and shown in Fig. 1A. Briefly, Fn was specifically conjugated with the acceptor dye, Alexa 546 maleimide (Molecular Probes), on the free cysteine residues in modules FnIII₇ and FnIII₁₅, resulting in four possible sites per dimeric Fn protein (Fig. 1A). Amine residues of unknown location were randomly labeled with Oregon Green 488 succinimidyl ester. There are over 80 amine residues per Fn dimer. The results reported here are all from a single batch yielding an average of 6 donors to 3.8 acceptors per dimeric Fn protein. Consistent results were obtained using several other batches of labeled Fn.

2.2. Crystal growth

Calcium oxalate monohydrate ($\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$ or COM) crystals were grown at room temperature (22–25 °C) from supersaturated solutions prepared by mixing appropriate amounts of stock solutions consisting of salts ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$, Aldrich) dissolved in ultrapure water (18 M $\Omega \cdot \text{cm}$ resistivity). The CaCl_2 stock was added drop-wise to a 10 mmol $^{-1}$ ionic strength $\text{K}_2\text{C}_2\text{O}_4$ solution resulting in an equimolar mixture with $[\text{Ca}^{2+}] = [\text{C}_2\text{O}_4^{2-}] = 220 \mu\text{mol}^{-1}$. Spontaneous precipitation occurs at this molarity, which has relative supersaturation (σ) values of 1.44 as calculated by a speciation program similar to EQUIL [61]. Crystals were grown on cover slips that were placed in either 20 ml clean glass vials or in Petri dishes for at least 48 h. The majority of the COM crystals had a characteristic hexagonal prismatic shape [62], with sizes ranging from 10 $\mu\text{m} \times 5 \mu\text{m}$ to 60 $\mu\text{m} \times 20 \mu\text{m}$. Prior to crystal growth experiments, labeled Fn was mixed with various concentrations of unlabeled Fn to ensure that only intermolecular energy transfer occurred. A ratio of 95% unlabeled and 5% labeled Fn-D/A was well below the threshold at which we noticed intermolecular energy transfer, yet this ratio still gave sufficient signal intensity to be detected by the system.

In adsorption experiments, Fn in concentrations ranging from 0.02 to 20 $\mu\text{g}/\text{ml}$ was added directly to supersaturated solutions that included mature crystals, and Fn was allowed to adsorb for 1 to 4 h. In crystallization experiments, mixed crystals of protein and calcium oxalate were grown as described above except that Fn was pipetted into the calcium oxalate solution prior to crystal nucleation in concentrations ranging from 0.02 to 20 $\mu\text{g}/\text{ml}$ (with 95% unlabeled, 5% Fn-D/A). These adsorption and crystallization experiments were repeated in glass vials, in Petri dishes, and in situ, directly on the microscope.

2.3. Characterization of labeled fibronectin in calcium oxalate solution

A range of concentrations of labeled Fn in fresh calcium oxalate solution ($\sigma = 1.44$) was measured in 40 μl droplets on a coverslip using the imaging system described below. The data given here are for a concentration of 45 $\mu\text{g}/\text{ml}$ in calcium oxalate solution at a relative supersaturation of $\sigma = 1.44$.

2.4. FRET imaging

Fluorescence imaging and spectroscopy were performed in this laboratory with an inverted epi-fluorescence microscope (Nikon TE 2000) and a 512 $\times$ 512 back-illuminated liquid nitrogen CCD camera (TEK512; Princeton Instruments, Trenton, NJ). Energy transfer was visualized with a FITC cube/filter combination that passes only the donor excitation and transmits both donor/acceptor emissions [41,60]. Images were taken in conjunction with emission spectra, which provided additional data and illustrated how efficiency depends on the protein conformation. Imaged light was converted to spectra using Metamorph software (Universal Imaging Corporation) and further analyzed using Igor Pro (Wavemetrics). A background was automatically subtracted from each spectrum.

2.5. FRET from Fn under denaturing conditions solution

As seen in Fig. 1B-D, FRET is sensitive to a wide range of conformational changes of Fn that occur upon denaturing the protein in increasing concentrations of Guanidinium Hydrochloride (GdnHCl). Since we did not correct our spectra for any crosstalk, the residual acceptor peak at 8 M GdnHCl is due to direct excitation of the acceptor. Fn began to extend from its compact conformation in buffer solution, to an intermediate range of conformations at 0–1.5 M GdnHCl which have previously been attributed to the disruption of ionic interactions within the protein, including those that stabilize the two dimers. At

concentrations above 1.5 M GdnHCl, Fn modules began to lose their sheet structure, and finally Fn became fully denatured at 8 M GdnHCl.

2.6. AFM

Scanning probe microscopy measurements were taken in air using at room temperature in non-contact mode with a Digital Mul-timode IIIa with an E scanner (Digital Instruments, Santa Barbara, USA).

3. Results

3.1. FRET from Fn in buffer, under denaturing conditions and in supersaturated calcium oxalate solution

To investigate how ionic conditions alter the conformation of Fn in solution, FRET was probed for Fn in PBS buffer and under denaturing conditions, as well as in supersaturated calcium oxalate solution. Fluorescence spectra of FRET-labeled fibronectin (Fn-D/A) in buffer normalized to the donor peak were measured at 0 M, 1 M, 2M, 4M and 8M GdnHCl (Fig. 1B,C). The residual acceptor peak at 8 M GdnHCL was due to direct excitation of the acceptor since the raw spectra as monitored on the microscope stage are reported uncorrected for residual cross-talk between the excitation and emission channel. As supported by circular dichroism spectra [35,41,44,63,64], Fig. 1D shows putative Fn conformations in solution [44], from the compact conformation in solution or the blood, to a partially extended structure that results when the intramolecular electrostatic interactions that stabilize the contact between the crossed-over dimer arms of Fn are broken by competing ions, to, finally, partially denatured states. When Fn was suspended in calcium oxalate solution with an ionic strength of 10 mM (NaCl), a slight decrease in I_A/I_D was observed as compared to Fn in PBS buffer (Fig. 1E). It has been found previously [34,41] that 1 M NaCl causes an expansion of Fn which is not associated with a loss of secondary structure. This suggests that the ionic strength of the supersaturated solution caused a slight disruption of electrostatic interactions between the two arms of the protein, concomitant with the reduction in FRET.

3.2. Fn adsorption to the flat planes of mature single crystals of calcium oxalate probed by FRET

Fn was found to adsorb directly to mature crystals of COM, and the FRET ratio was not a function of Fn concentration (Fig. 1F). A high ratio of I_A/I_D was measured on the (−101), (0 1 0) and apical faces of COM, with no significant difference in intensities between the different faces. The increase in I_A/I_D upon adsorption to the crystal implies that Fn initially adopts a conformation that is more compact than that in supersaturated calcium oxalate solution, approaching the I_A/I_D measured for the tightly folded conformation seen in PBS buffer. After the protein was left to adsorb to mature crystals over several hours (with no depletion of crystallizing solution) energy transfer was observed to decrease, implying that the dimeric arms of Fn become separated as it is embedded into a growing crystal. A representative fluorescence image and emission spectra are shown in Fig. 2A–C, along with a schematic sketch of the crystal and compact (adopted from [39]). The data suggest that while the globular Fn structure may be slightly disrupted in supersaturated calcium oxalate crystallizing solution, it assumes a tightly compact conformation as it adsorbs to the various faces of COM. The FRET data then give first indications that the protein might transition to a more extended conformation as it is incorporated over time into a growing crystal, a conclusion that is further corroborated when COM is nucleated in the presence of labeled Fn (see below).

3.3. AFM of Fn adsorbed to single calcium oxalate crystals

Mature COM crystals that were exposed for 1 h to a supersaturated solution containing Fn (in concentrations ranging from 2 to 5 $\mu\text{g/ml}$) were rinsed, dried and placed in the AFM under ambient conditions. AFM non-contact mode measurements confirmed Fn frequently adsorbed as compact, globular structures, 20–30 nm in width, resting on the wide step terraces found on the $(-1\ 01)$ faces of mature COM (Fig. 2D). Naturally AFM could not be used to image protein already interred within the single crystal. However, AFM images were also taken of mature COM crystals that first adsorbed Fn and then were allowed to rest in supersaturated solution for several hours. These measurements exhibited regions where Fn was in an extended conformation between step edges (Fig. 2E and G), but remained in globular, compact form where it rested on the wide step terraces (Fig. 2F).

3.4. Calcium oxalate crystals grown in the presence of FRET-labeled Fn

As investigated previously, Fn incorporates into growing COM over the full range of protein concentrations measured here [65]. At higher concentrations (generally $\geq 1\ \mu\text{g/ml}$) protein incorporation exhibited intersectoral zoning, appearing as a fluorescent “hourglass” shaped region within the crystal (Fig. 2H) and denoting incorporation along the fast growing apical planes. At lower protein concentrations, Fn still incorporated, but appeared more diffuse and the hourglass pattern was less clear. Overgrowth of protein by COM was confirmed by partial dissolution of fluorescent “hourglass” patterned crystals, followed by additional examination with the fluorescence microscope. I_A/I_D was measured from several points on at least 10 crystals per sample, and the from these mixed crystals were consistent over the range of protein concentrations (Fig. 1E). This insensitivity of the I_A/I_D ratio to the packing density of FRET-labeled FN and further confirms intramolecular FRET where the probe molecules were sufficiently diluted by unlabeled Fn. The I_A/I_D ratio of Fn incorporated into COM crystals was significantly lower than that from the protein in PBS buffer and in calcium oxalate solution (Fig. 2I). These results suggest that Fn is gradually converted back to a more extended conformation during the embedding process (Fig. 2J). The degree of Fn extension seen in COM crystals is similar to that measured in 1–2M GdnHCl in solution. Previous work has shown that GdnHCl at these concentrations causes Fn to open up in solution [41,44].

4. Discussion

Major conformational changes can be observed by FRET as Fn senses and responds to alterations in the local ionic conditions during biomineralization. When transferred from physiological buffer to supersaturated solution conditions, Fn loses its compact globular conformation and its dimeric arms partially separate (Fig. 1E). Unexpectedly, Fn returns to a more compact state after binding to the flat (-101) , $(0\ 1\ 0)$ or $(0\ 1\ 0)$ COM crystal surfaces under super-saturated conditions, as confirmed by two independent techniques, FRET (Fig. 2A–C) and AFM (Fig. 2D–G). Finally, FRET indicates that Fn's dimeric arms open up again during the embedding process as the COM crystal grows around it (Fig. 2H–J). Under physiological buffer conditions, electrostatic interactions stabilize the dimeric arms of Fn in a compact quaternary structure [39,60], while high salt concentrations are known to disrupt these interactions [39]. Breaking those intramolecular Fn arm–arm interactions facilitates major movement of those segments which will reduce or even eliminate the intramolecular energy transfer between them. The fact that Fn is observed by FRET to undergo major conformational changes indicates that Fn responds to major alterations of the local chemistry and electrostatic conditions as function of time, as well as its position within the crystal. Our data indicate that supersaturated Ca oxalate solutions may affect Fn conformation similar to 1–2M GdnHCl in physiological buffer (Fig. 3A and B). Based on

FRET and supported by AFM experiments, we suggest the following mechanisms regarding the surface binding and embedding process.

First, as Fn adsorbs to the $(-1\ 0\ 1)$ or $(0\ 1\ 0)$ face of COM crystals, it adopts a more compact structure. FRET and AFM have previously been used to probe conformational changes in Fn on hydrophilic and hydrophobic surfaces [40,41] and both types of measurements have indicated that Fn retains a compact form on hydrophobic surfaces but adopts a slightly more extended conformation on hydrophilic glass. Charge-charge interactions between Fn and negatively charged hydrophilic glass disrupt electrostatic interactions that stabilize the compact state, allowing Fn's arms to partially extend. Here, the binding of Fn to the flat $(-1\ 0\ 1)$ or $(0\ 1\ 0)$ faces of mature COM crystals caused a sharp increase in I_A/I_D , implying the protein assumes a more compact globular conformation (confirmed by AFM). Fn's unexpected return to a more compact conformation on the densely charged surfaces of COM may indicate that it senses a zone of ion depletion, which are typically generated if the local growth rate is transport limited (Fig. 3C).

Second, Fn extends as it is incorporated into a growing crystal. What might be the underpinning mechanism that again leads to the opening up of Fn after becoming incorporated into the crystal? The presence of a protein on a flat crystal will locally perturb the crystal growth kinetics. At the point of closest contact, the growth will be stopped while transport of calcium and oxalate ions proceeds with reduced rate in the proximity of the protein. Step edges will appear in the close proximity of Fn, and since the two building blocks of COM, calcium and oxalate ions, are both charged, the step edges present uncompensated charges as sketched in Fig. 3D. Because Fn carries a significant amount of positive and negative charges, these complementary Fn charges may be attracted to the step edges.

The electrostatic interaction of Fn with charges on these gradually emerging COM step edges could alter the conformation of Fn as the COM crystal starts growing around it. Since the surface chemistry can have a direct impact on Fn's conformation [40-42,66-71], the reduced FRET upon embedding suggests that environmental changes ultimately result in the disruption of intramolecular interactions that might be replaced by altering electrostatic guest-host interactions. Fn has a diameter of 20–30 nm in its compact globular form and after complete arm separation, and approximate length of 120–160 nm with a diameter of 2.5–3 nm as probed here by AFM (Fig. 2D-G) and reported in the literature [59,63,64,72,73]. AFM measurements of the (-101) face of COM have revealed wide (≈ 200 nm) stepped terraces with an average step height of 0.65 ± 0.23 nm, a value within the range of the elemental step height ($\sim 6\text{\AA}$) [62,74]. Therefore, when Fn binds to COM in its globular form, it can interact with up to six stacked planes, and multiple step edges, as the crystal continues to grow around it.

As found previously for fluorophores and other proteins [65], the presence of Fn does not alter the morphology of single COM crystals: COM commonly nucleates as elongated hexagonal plates with major faces characterized by the $(-1\ 0\ 1)$, $(0\ 1\ 0)$ and (110) planes and exposes a dense network of both positive (Ca^{2+}) and negative charges (Ox^{2-}) [75]. Nor does the presence of Fn during crystal nucleation alter the final COM morphology (unlike proteins such as nephrocalcin, which preferentially binds to $(-1\ 01)$ planes and inhibits growth in that direction [25]), indicating that the protein is not binding with charge/subunit specificity [48]. This is further supported by the data, which indicate that Fn binds to all faces of mature COM (Fig. 2A) and preferentially incorporates, along the (110) or $(1\ 2\ 0)$ apical faces during single crystal nucleation in the presence of the resulting in the observed fluorescent “hourglass” shape (Fig. 2H and J) that has been seen previously with several unbound dye molecules, as well as Protein G [65]. Proteins extracted from mineralized plant

tissue have also been found to preferentially interact with these apical (1 1 0) planes during COM nucleation, and it has been shown that these fast growing apical planes present crystal faces with balanced charge and (Ox^{2-}) anions that emerge at oblique angles without geometrically defined stereochemistry [76].

4.1. Implications of how the electrostatic sensitivity of macromolecules could be utilized to tailor properties of inorganic host crystals

When utilizing macromolecules to tailor the properties of single inorganic crystals, several different modes of guest–host interactions might be considered. Peptides and proteins that specifically recognize selective crystal faces might be exploited to induce oriented crystal growth, alter crystal shape or switch the crystal habit [10,77–79]. In contrast, proteins that primarily interact electrostatically [80] might be exploited to alter specific material properties without changing the crystal structure. As shown previously, Fn becomes embedded without perturbing the overall shape of COM crystals, nor does it cause the formation of microscopically visible defects [65]. Due to its condensation to a more compact structure when positioned at the crystal/solution interface, Fn might initially block a comparatively small surface area from further growth. During the subsequent incorporation process, separation of the arms ultimately allows single Fn molecules to interact with a large number of COM unit cells such that Fn is finally interlaced without altering the local COM structure. This could have a major impact on stabilizing the mechanical properties of biominerals, particularly when proteins are used that can elongate to more than 100nm in length just by eliminating quaternary structure.

In summary, this is the first time that FRET has been applied to probe protein–biomineral interactions. In fact, our data demonstrate that a FRET-labeled protein can be used as an optical sensor to probe local ionic conditions which could be of major interest for other (bio)mineralization studies. FRET thus sheds first light into how local changes in the ionic environment as crystals grow can cause major alterations in protein conformations.

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Fig. 1.

Conformational changes of fibronectin (Fn) probed by FRET. (A) Fn was specifically conjugated with the acceptor dye, Alexa 546 maleimide, on the free cysteine residues in modules FnIII7 and FnIII15, resulting in 4 possible sites per dimeric Fn protein. Amine residues were randomly labeled with the donor dye, Oregon Green 488 succinimidyl ester. (B) Fluorescence spectra of FRET-labeled fibronectin (Fn-D/A) in buffer normalized to the donor peak. Spectra were measured at 0M, 1 M, 2M, 4M and 8M GdnHCl. The residual acceptor peak at 8M GdnHCL is due to direct excitation of the acceptor since the raw spectra as monitored on the microscope stage are reported not corrected for residual cross-talk between the excitation and emission channel. (C) Reference curve probing the—FRET (I_A/I_D) ratio as a function of GdnHCL concentration for Fn in solution. Please note that the raw spectra are shown, not corrected for residual crosstalk between the excitation and emission channel. (D) Schematic sketch of putative Fn conformations in solution [44]. When $I_A/I_D > 1.6$, Fn-D/A is in a compact conformation. When $1.6 > I_A/I_D > 1.0$, Fn-D/A is partially extended due to the reduced electrostatic interactions that stabilize the contact

between the crossed-over dimer arms of Fn. When $1.0 > I_A/I_D > 0.88$, Fn-D/A is extended. When $0.88 > I_A/I_D > 0.76$, Fn-D/A is partially unfolded. When $I_A/I_D < 0.76$, Fn-D/A is unfolded (denatured). Energy transfer between donors and acceptors bound to free cysteines are limited to within approximately double the Förster radius (~ 12 nm), denoted by gold circles around Fn III7 and Fn III15. Fn in solution assumes a compact conformation stabilized through ionic interactions between dimer arms [39]. To prevent energy transfer between adjacent Fn molecules, only 5% of the Fn used was labeled for FRET, and the remainder was unlabeled. (E) Intramolecular energy transfer (I_A/I_D) for Fn in solution and for Fn incorporated in COM crystals which were grown in the presence of the protein. I_A/I_D decreases for Fn suspended in supersaturated calcium oxalate solution, indicating that Fn has adopted a partially extended conformation ($1.6 > I_A/I_D > 1.0$). I_A/I_D further decreases for Fn incorporated into COM ($1.0 > I_A/I_D > 0.88$), suggesting a fully extended, but not unfolded, conformation. I_A/I_D is consistent for batches of 5% Fn-D/A+ 95% Fn-unlabeled added to supersaturated calcium oxalate crystallizing solutions with concentrations of 20, 10 and 2 $\mu\text{g/ml}$ prior to COM nucleation. Data are averages $\pm$ SD for at least 10 separate crystals. (F) Energy transfer (I_A/I_D) for 10 $\mu\text{g/ml}$ Fn (5% Fn-D/A+ 95% Fn-unlabeled) adsorbed to mature COM crystals as a function of time. For Fn adsorbed directly to crystals *in situ*, I_A/I_D increases over time until it stabilizes in the >1.6 range, which is similar to energy transfer seen in buffer and indicates a compact conformation. I_A/I_D for crystals remaining in supersaturated solution in separated crystallizing dishes over time eventually decreases indicating that Fn is extended ($1.0 > I_A/I_D > 0.88$) as it becomes incorporated within crystals. Data are averages $\pm$ SD for at least 10 separate crystals.

Fig. 2.

Imaging of fibronectin adsorbed to and incorporated within single COM crystals. (A) Fluorescent image of Fn adsorbed to mature COM. (B) Representative energy transfer (I_A/I_D) spectrum for 10 $\mu\text{g/ml}$ Fn (5% Fn-D/A + 95% Fn-unlabeled) adsorbed to mature COM crystals for 20 min (C) Schematic illustration of protein adsorption to all exposed faces of COM and the compact conformation that Fn adopts once it is adsorbed to the surface. (D) AFM image (250 nm $\times$ 250 nm) of the (-101) face of mature COM with Fn adsorbed for 1 h and adhering to terraces in a compact form. (E) AFM image (600 nm $\times$ 600 nm) of 0.04 $\mu\text{g/ml}$ unlabeled Fn adsorbed to COM and left in crystallizing solution for 3 h. (F) Zoomed image (120 nm $\times$ 120 nm) of Fn extended between two step edges. (G) Zoomed image (50 nm $\times$ 50 nm) of compact Fn resting on a wide terrace. (H) Fluorescent image of Fn incorporated into COM along fast growing (110) apical planes. (I) Representative energy transfer (I_A/I_D) spectrum for 20 $\mu\text{g/ml}$ Fn (5% Fn-D/A + 95% Fn-unlabeled) incorporated into COM during crystallization. (J) Schematic illustration of the “hourglass” pattern

indicative of incorporation along (110) and the extended conformation that Fn adopts as it is becomes incorporated into a growing crystal.

Fig. 3.

Schematic model illustrating how Fn might interact with a growing COM crystal. (A) Schematic sketch of putative Fn conformations in GdnHCl solution. (B) The ionic nature of the supersaturated crystallizing solution may disrupt the electrostatic bonds just enough to partially extend the protein. (C) The protein reverts to its most compact conformation once adsorbed to a charged, but electrostatically neutral COM face. The large protein blocks ions in solution from accessing the growing crystal surface, creating a zone of ion depletion and returning the protein to its compact, globular form. (D) As the crystal continues to grow, charged step edges may come in direct contact with the protein, transitioning from layer to layer growth to roughening caused by adding layers next to a large protein. The presence of the charged step edges poses a mechanical strain on the protein and results in the extension of Fn as growing planes surround it and it becomes embedded within the crystal.