Polyhedral Instability of Glucose Isomerase Crystals

Mike Sleutel, Ronnie Willaert, Lode Wyns, and Dominique Maes

Cryst. Growth Des., Article ASAP

Downloaded from http://pubs.acs.org on December 19, 2008

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML
Polyhedral Instability of Glucose Isomerase Crystals

Mike Sleutel,* Ronnie Willaert, Lode Wyns, and Dominique Maes

Structural Biology Brussels, Flanders Institute for Biotechnology (VIB), Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

Received July 8, 2008; Revised Manuscript Received September 24, 2008

ABSTRACT: The problem of polyhedral (in)stability for the crystallization of macromolecules is discussed. We present both qualitative and quantitative data on the transition of face morphology in response to surface concentration gradients for the case of glucose isomerase. We show that in the absence of convective currents an isotropic protein depletion zone (PDZ) is installed. The mismatch between the spherical shape of the PDZ and the crystal habit leads to the Berg effect which gives rise to a localization of two-dimensional nucleation near the vertices and a retardation of step advancement at the center of the face. In response to this supersaturation inconstancy, a stabilizing microcompensation profile is setup that changes the local face kinetic constant $\beta$. Upon attaining a critical slope, $\beta$ reaches its maximum and the destabilizing mechanism, that is, surface protein concentration gradients, leads to the loss of polyhedral stability. Furthermore, we demonstrate that polyhedral instability is promoted even further by 0.1% agarose.

Introduction

The interplay between surface growth processes and the distribution of concentration around a growing crystal determines the shape and stability of the crystal habit. When the kinetics of protein attachment onto a growing crystal face removes protein more rapidly from the region adjacent to the crystal face than mass transport can replenish the supply of growth units, a significant concentration gradient is established. In the absence of relatively fast convective currents, an isotropic protein depletion zone (PDZ) is formed which creates local solutal conditions that can differ greatly from the bulk composition. The accompanied slow-down of the crystal growth process is considered to promote the growth of high quality crystals. 1 Also, a more stable mode of growth is attained. 2–4 By removing convection, spatial irregularities and temporal oscillations in solute transport that cause the formation of defective regions in the crystal are reduced. However, the effect of ablation of convection is system dependent and may either be advantageous or disadvantageous to the crystal quality. For instance, it has been shown that a reduction of the mass transport rate can enhance defect-causing step density and velocity fluctuations 5–7 or augment impurity uptake. 8–11 Such a mass transport regime that is governed by diffusion can be attained by working in a zero- or microgravity environment, 12–14 in gelled solutions, 15–17 or by lowering the ratio of the buoyancy and viscous forces of the system (capillary, 18,19 closely spaced glass plates, 20–23 high molecular weight PEGs 24).

There is one more case where elimination of convection will not be beneficial for protein crystallization and ultimately crystal quality, that is, the case of loss of polyhedral stability. 25,26 It is a kinetic phenomenon where, due to the specific interplay of surface kinetics and mass transfer, the crystal is no longer capable to retain its polyhedral shape and large depressions develop on the habit faces. Since the destabilizing mechanisms scale-up with crystal size and the limiting of nutrient supply, polyhedral stability is expected to occur in the same conditions required for the formation of a depletion zone. The geometry of this metastable halo encircling the crystal does not coincide with the polyhedral shape of the crystal. Consequently, edges and vertices of growing crystals are better supplied with nutrients than facet centers. This surface concentration inconstancy is referred to as the Berg effect 27 and can have a pronounced effect on surface kinetics and ultimately surface morphology. 28 Polyhedral instability is a long standing problem in the crystallization of small molecules due to their fast incorporation kinetics (for example, see refs 29–32), and it is important for applications where large protein crystals are required, for example, neutron diffraction. Initial reports of polyhedral instability of protein crystals were made by Nanev et al. 33,34 for ferritin, lysozyme, and trypsin. Further steps toward a deeper understanding were made by Vivares et al. who studied the concentration gradients in the vicinity of glucose isomerase crystals using confocal scanning fluorescence microscopy. 24 They achieved ablation of convection through the use of a high molecular weight PEG (10 kDa) and correlated the polyhedral instability of crystals to the presence of a depletion zone. However, the insights gained so far have been obtained from low resolution mesoscale data. A more detailed understanding of the problem requires microscopic data on both the stabilizing and destabilizing mechanisms.

Hence, in this paper we present for the first time quantitative data obtained from high resolution noninvasive in situ imaging of the microscopic surface processes during the loss of polyhedral stability of glucose isomerase crystals. We begin by addressing the most prominent destabilizing mechanism, that is, surface concentration gradients and their effects on surface kinetics. Next, we discuss the transition from a stable flat interface to a concave microcompensation profile. In the third section, we present quantitative data on the crystal’s compensation mechanisms and their ultimate failure beyond a critical point, followed by a last section where we ascertain the role and applicability of the hydrogel agarose as a putative microgravity mimic.

Materials and Methods

Crystallization of Glucose Isomerase. Glucose isomerase from Streptomyces rubiginosus was purchased from Hampton Research (California, USA). The protein solution was dialyzed against 10 mM Hepes buffer pH 7.0 and 1 mM MgCl₂. Protein concentrations were determined by UV absorbance at 280 nm. For the interferometry experiments, protein crystals were nucleated in situ inside the...
Mach–Zehnder interferometer (MZI) in a Hellma fluorescence cuvette (Jena, Germany) kept at a constant 20 °C using the macrobatch method. Mother liquor consisted of 40 mg/mL glucose isomerase, 100 mM Hepes pH 7.0, 200 mM MgCl₂, 5% PEG 1000 and 0.1% LM agarose. For the laser confocal microscopy experiments, crystals were grown at 25 °C in a Q516 quartz Hellma cuvette (0.5–11 mm solution thickness; Jena, Germany) from 30 mg/mL glucose isomerase, 100 mM Hepes pH 7.0, 200 mM MgCl₂, 5% PEG1000, and 0% or 0.1% agarose. For these conditions, the protein crystallizes exclusively in the orthorhombic form (I222, a = 94.01 Å, b = 99.37 Å, c = 103.01 Å). 

**Protein Depletion Zone Characterization.** Protein concentration gradients were measured using a computer controlled MZI-microscopy system using a quartz fluorescence cuvette as experimental cell with inner dimensions 10 mm × 4 mm × 45 mm (D × W × L) for the gelled solutions. For the nongelled experiments the solution thickness was either 0.5 or 1 mm. The experimental cell was placed in the interferometer in the object beam trajectory and kept at 20 ± 0.1 °C using peltier elements in combination with coolant water flows at the sides of the experimental cell. The Mach–Zehnder driven by a 680 nm laser diode was used to collect interferograms of the cell. Phases were obtained using the phase-shift method. Phase-shifts were induced by a piezo connected to the object beam mirror. Using the Haritharan algorithm phase interferograms could be reconstructed. Real phase values were obtained by subtracting the reference phase field (taken at the start of the experiment) from each phase map. Phase difference maps were transformed into concentration difference maps by using refractive index versus concentration calibration curves that we determined using an Abbe Refractometer model 60/ED. Such a conversion from refractive indices to protein concentrations does however require that the phase difference between two successive interferograms is smaller than one corresponding wavelength. The obtained raw data represents the integration of the protein concentration along the optical path, and as such are averaged values.

**The Accuracy of the Surface Concentration Determination Method Using Measured 2D Nucleation Rates and Step Velocities.** The surface concentration $C_{surf}$ is calculated from two-dimensional (2D) nucleation rates using the following expression

$$C_{surf} = \exp\left[\frac{B}{\ln J - \ln \frac{1}{T^2}}\right] C_e$$

with $A = \ln(\omega + T_Z), B = \pi \kappa_{HON}^2 sk^{-2}$ (1)

For an explanation of the used symbols, the reader is referred to the Results and Discussion section. The concentration at the edge is considered to be the bulk concentration, that is, $C_{surf} (x = 0) = C_{edge} = C_{bulk}$. The relative depletion (RD) in surface concentration is then defined as $RD(x) = 1 - C_{surf}(x)/C_{bulk}$. The error of $C_{surf}$ is the resultant of the propagation of a number of errors associated with both experimental parameters ($ln J, T, C, \kappa_{HON}$) and coefficients obtained through fitting ($ln(\omega + T_Z), \kappa_{HON}$). These uncertainties accumulate into an error of approximately 40% in the determination of $C_{surf}$. However, the calculation of the relative depletion RD is a little less error sensitive. RD is mostly affected by errors associated with $ln J, ln(\omega + T_Z)$, and $\kappa_{HON}$. For the determination of the nucleation rates as a function of imaging technique, the total number of nuclei appearing on 2700 µm² areas 40 µm apart were counted during a measuring period of 20 min. This translates into a maximum relative error of 1.5% and 12% in the determination of $ln J$ and $C_{surf}/C_{bulk}$, respectively. The errors in $ln(\omega + T_Z)$ (2.3%) and $\kappa_{HON}$ (5%) propagate into a combined relative error of 23% in RD. RD is much less sensitive to errors associated with the experimental parameters $T$ and $C_e$. For example, whereas an uncertainty of 0.5 K in the temperature leads to a relative error of 10% for $C_{surf}$, the quantity RD varies only by 0.3%. An even smaller dependency is observed for the associated error of 0.05 mg/mL in $C_e$. All these errors combined lead to an uncertainty of 36% for crystal 1 in the calculation of RD.

**Grashof Number Calculation.** The dimensionless Grashof number ($Gr$) was used to approximate the ratio of the buoyancy to viscous forces in our system. $Gr$ was calculated using the following expression

$$Gr = C_{prot} \gamma \beta \delta^3 \nu^{-2}$$

with $\gamma = \frac{\partial \rho_{prot}}{\partial \rho_{sol}} - \frac{\partial \rho}{\partial \rho_{prot} \rho_{sol}}$ and $\nu = \eta / \rho_{sol}$

where $C_{prot}$ is the protein concentration, $\rho_{prot} - \rho_{sol}$ and $\rho_{prot} \rho_{sol}$ are the density of the protein, the solution and water, respectively, $\gamma$ is the protein solutal expansion coefficient, $g$ is the gravitational acceleration, $h$ is the characteristic length (solution thickness inside the experimental cell), and $\nu$ and $\eta$ are the kinematic and dynamic viscosity. The used values are summarized in Table 1.

**Results and Discussion**

**The Berg Effect Leads to Preferential 2D Nucleation at Facet Edges.** The impact of surface concentration gradients on step generation and advancement can be studied with a number of imaging techniques. However, since the typical length scale of the Berg effect is commensurable with crystal size, the classic surface imaging technique atomic force microscopy (AFM) is less ideal due to its limited surface scanning area. We therefore employ the less invasive optical method LCM-DIM which, given the fast scan rates (on average 5 s/image), large scanning area (mm range), and medium lateral resolution ($\pm 0.6 \mu m$), is ideal to probe microscopic kinetic surface phenomena. Crystals were grown in quartz cuvettes with a spacing of 0.5–1 mm. With this technique, we observe a clear impact of the Berg effect on the dominating step source mechanism (i.e., 2D nucleation for supersaturations $\sigma = ln(C/C_e) < 5.0$ at 25 °C) on the (011) face of orthorhombic glucose isomerase crystals. Figure 1a shows the manifestations of the Berg effect on 2D nucleation for supersaturations $\sigma = ln(C/C_e) < 5.0$ at 25 °C on the (011) face of orthorhombic glucose isomerase crystals. Figure 1b shows an $ln J$ versus distance from the edge plot obtained for two different crystals. A (kinked) linear decrease in nucleation rates is observed as a function of surface coordinate. We attribute the

<table>
<thead>
<tr>
<th>Table 1. Data for $Gr$ Calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{prot}$ (g/cm³)</td>
</tr>
<tr>
<td>$\rho_{prot}$ (g/cm³)</td>
</tr>
<tr>
<td>$\rho_{sol}$ (g/cm³)</td>
</tr>
<tr>
<td>$\rho_{sol}$ (g/cm³)</td>
</tr>
<tr>
<td>$\gamma$ (cm²/g)</td>
</tr>
<tr>
<td>$g$ (cm/m²)</td>
</tr>
<tr>
<td>$b$ (cm)</td>
</tr>
<tr>
<td>$\eta$ (g/cm s)</td>
</tr>
<tr>
<td>$\nu$ (cm²/s)</td>
</tr>
</tbody>
</table>

* Calculated for a 100 mM Hepes, 200 mM MgCl₂, 5% PEG1000, 5 mg/mL glucose isomerase solution. * Taken from ref. †. Taken from ref. †.
To summarize, the observed slowing-down of kinetics (depletion) can be found in the Materials and Methods section. Approximately 11% is measured at (250 nm (3-4 lattice parameters), well below the minimum interstep distances for Figure 1, that is, 2.8 μm. Assuming no significant temperature gradients along the surface coordinate and with \( \ln(\omega^* \Gamma Z) = 24.8 \pm 0.6 \) and \( C_c = 0.28 \text{ mg/mL} \) we obtain the relative surface depletion \( C_{surf}/C_{bulk} \) where \( C_{bulk} \) is the protein bulk concentration we equate with \( C_{edge} \). In reality, most likely \( C_{edge} \leq C_{bulk} \); however, we disregard this inequality since we focus here on the determination of relative surface concentration gradients rather than absolute gradients. This leads to the \( C_{surf}/C_{bulk} \) versus distance from edge plots in Figure 1c. A depletion of approximately 11 ± 4% is measured at ± 250 μm from the crystal edge. A discussion on the error associated with the calculated depletion can be found in the Materials and Methods section.

To summarize, the observed slowing-down of kinetics (depletion) can be found in the Materials and Methods section. Approximately 11% is measured at (250 nm (3-4 lattice parameters), well below the minimum interstep distances for Figure 1, that is, 2.8 μm. Assuming no significant temperature gradients along the surface coordinate and with \( \ln(\omega^* \Gamma Z) = 24.8 \pm 0.6 \) and \( C_c = 0.28 \text{ mg/mL} \) we obtain the relative surface depletion \( C_{surf}/C_{bulk} \) where \( C_{bulk} \) is the protein bulk concentration we equate with \( C_{edge} \). In reality, most likely \( C_{edge} \leq C_{bulk} \); however, we disregard this inequality since we focus here on the determination of relative surface concentration gradients rather than absolute gradients. This leads to the \( C_{surf}/C_{bulk} \) versus distance from edge plots in Figure 1c. A depletion of approximately 11 ± 4% is measured at ± 250 μm from the crystal edge. A discussion on the error associated with the calculated depletion can be found in the Materials and Methods section.

**Transition from a Stable Flat Interface to the Appearance of a Central Depressed Area.** In the presence of relatively small surface concentration gradients (moderate Berg effect), shape stabilizing factors\(^{44} \) (e.g., anisotropy of face kinetic constant, surface tension and capillarity, nonuniform distribution of growth-retarding impurities, temperature gradients) succeed in preserving the polyhedral shape of the crystal. However, shape-destabilizing factors (e.g., nonuniformity of concentration distribution) scale-up with crystal size and can lead to morphological defects. In the following section we discuss the various (micro)structures of the surface in response to supersaturation inhomogeneity while retaining a flat morphological interface. Figure 2a shows one of the “flattest” and most stable modes of growth, that is, \( ad \ random \) homogeneous multilayer 2D nucleation (note that layer-by-layer growth would be flatter) with an average slope of zero. The random nature of this process (average island density is constant as a function of surface coordinate) indicates the absence of any significant concentration gradients on the surface. Any local perturbations in surface height show no temporal stability and fade away. When the crystal size increases or supply becomes hampered by reactor edge effects, concentration gradients arise that affect both the kinetics and localization of 2D nucleation. Figure 2b illustrates this phenomenon: step generation becomes limited to specific local areas of higher surface concentration leading to the formation of a 2D hillock. In response to the concentration gradients, the step density increases as a function of distance to the nucleation center until eventually the interstep distance becomes smaller than the critical length of a 2D nucleus or the supersaturation becomes smaller than the critical supersaturation for 2D nucleation. This effect enhances the “confinement” of 2D nucleation and hence step generation to a specific region. While Figure 2b shows straight step trains, Figure 2c shows loss of step straightness at a specific distance from the crystal’s edge. We interpret this step concavity as the result of a nonconstant step velocity along the average direction of the steps. Since we observe this effect for all the face’s edges at specific edge distances, we attribute the observed concavity to surface concentration gradients and rule out other step retarding possibilities such as impurities. However, we do observe the influence of impurities in the face’s center under a different form, namely, through step pinning (will be discussed below).

Next, impurity pinning and very high step densities giving rise
Figure 2. The violation of the morphological stability of a flat, crystallographic low index face (011) leads to the appearance of a central depressed area: (a) step generation through 2D nucleation occurs ad random across the entire surface; (b) surface concentration gradients lead to preferential 2D nucleation at crystal edges, leading to step trains crossing the vicinal surface; (c) step train velocity becomes nonconstant along the average direction of the step resulting in increased step concavity toward the center of the crystal face; (d) loss of stability of semi-equidistant step trains leads to the coalescing of steps and formation of macrosteps downhill of the nucleation center; (e) step pinning combined with increasing diminishment of step velocity in the face center lead to a singular critical point where quasi-zero step velocity is reached, creating a depressed valley downhill of the critical point, dashed white line indicates direction of step advancement. Dashed white boxes in zoom-out pictures indicate zoom-in area (a–c). White x’s denote preferential 2D nucleation centers (b–e).

to diffusion-field overlap in the face’s center can eventually lead to loss of stability of the step trains. This gives rise to the step bunching and subsequently formation of macrosteps in the center of the face (Figure 2d). Macrosteps can then lead to an additional slowing down of step advancement in the central areas of the crystal face. All these effects combined finally result in the appearance of a singular point/region on the surface where quasi-zero step velocity is reached. Upon reaching this point a cascade of events enhances the instability even more; that is, step density increases further and faster by the arrival of new steps to the near-zero-velocity region and step advancement slows down due to step-step interaction. Ultimately, this nonmoving macrostep (shock wave) leads to the formation of a central expanding depression (Figure 2e). This microscopic transformation is the onset of loss of polyhedral stability. We note that it is a kinetic phenomenon and that it can be remedied by decreasing the supersaturation or increasing material supply through (forced) convection.

The Microscopic Compensation Profile Fails to Preserve Macroscopic Stability. For a moderate Berg effect the crystal remains able to retain its polyhedral faceted shape through the available compensation mechanisms. However, in the absence of convective currents, the concentration difference between edge and center \( C_{edge} - C_{center} \) scale-up with \( \frac{\beta_1}{l/\Omega} \) and eventually the crystal starts to change its habit, turning into a skeleton or dendrite, where \( \beta_1 \) is the face kinetic constant, \( l \) is the crystal size, and the \( \Omega \) is the diffusivity. Here, we discuss the most general (albeit limited) factor maintaining the regular polyhedral shape of a growing crystal,\(^{45}\) that is, the anisotropy of face growth rate \( V(n) \) as a function of orientation \( n \). Figure 3a shows the microcompensation profile in response to supersaturation inconstancy, that is, a gradual increase in step density toward the crystal center leading to a concave surface profile. The local slope \( p \) changes from 0.004° to 0.09° between the white markers along the dashed white arrow in Figure 3a, with \( p \) calculated as the inverse tangent of the ratio of the step height and the step distance. The reverse trend is measured for the tangential step velocity \( v_{step} \) (Figure 3b); that is, steps slow down farther from the edge. The crystal can offset this step retardation generated by the supersaturation inconstancy by increasing its local slope \( p \) (Figure 3c). The obtained surface curvature generates a higher kinetic coefficient in the central area compared to the face periphery and is able to counteract the destabilizing effect of supersaturation surface gradients. This is illustrated in Figure 3c, where \( v_{step} \times p \), which we use as a measure of the normal growth rate, remains constant over the first \( \pm 200 \mu m \) along the trajectory indicated by the dashed white arrow in Figure 3a. Note the smaller values of \( v_{step} \times p \) close to the edge. In this region, where 2D nucleation still occurs, the face growth rate cannot be approximated with \( v_{step} \times p \) and the normal growth rate is underestimated. Step density and velocity measurements become impossible closer to the center because here interstep distances become smaller than the lateral resolution of the LCM-DIM technique.

For regions closer to the center an “avalanche like loss of stability”\(^{26}\) sets in. The increase in \( \beta_1 \) \( l/\Omega \) as a result of the compensation profile leads to an additional rise in \( C_{edge} - C_{center} \) thereby increasing the demand for an increased surface curvature. Eventually, an abrupt decline is reached in the anisotropy of the kinetic constant (expected for larger \( p \)) and the kinetic coefficient \( b(p) \) attains its maximum \( \Theta b/\Theta p = 0 \). We calculate \( \Theta b/\Theta p \) using the following relationships:\(^{26}\)

\[
P_{center} - P_{edge} = \frac{1}{\Theta} \frac{\sigma_{edge} - \sigma_{center}}{\sigma_{edge}} \quad (3a)
\]

\[
\Theta = \frac{1}{b} (\Theta b/\Theta p) \quad \text{with} \quad b(p) = \beta(p)^{\sqrt{1 + p^2}} \quad (3b)
\]

where \( \Theta \) is the anisotropy of the face kinetic coefficient \( \beta(p) \). We derive the local slope \( p(x) \) from the surface profile (Figure 4a) and calculate \( \sigma(x) \) from the step velocity assuming no diffusion-field overlap using \( v_{step} = \Omega \beta_{step}(C - C_e) \) with \( \beta_{step} = 5 \times 10^{-4} \) cm/s. The results between 0–200 \( \mu m \) from the edge are summarized in Figure 4b. The decrease in surface supersaturation (~60%) is far more pronounced than in Figure 1c. Such significant depletion can arise when the crystal face is in close proximity to the reactor wall, rendering the face’s center poorly supplied and enlarging the Berg effect (will be discussed
below). A steady decrease in both \( \sigma_{\text{surf}} = \ln(C_{\text{surf}} / C_e) \) and \( \partial b / \partial p \) is observed, demonstrating the diminishing strength of the compensation mechanism at higher slopes. For low step densities, the kinetic coefficient increases strongly with increasing local slopes. At larger slopes, however, the kinetic coefficient of the face becomes practically independent of the orientation.

These data demonstrate that crystals larger than a critical size will inevitably lose their polyhedral stability and a central starvation flaw will form. When this critical point is reached, the faceted form is no longer capable to remain similar to itself. In practice the depression is offset from the center of the face and step generation is limited to only a single vertex as opposed to multiple vertices. We believe this to be the result of a nonzero angle between the average crystal face orientation and the reactor wall.

The low reflectivity and high roughness of the depletion defects excludes techniques such as AFM and Michelson interferometry to visualize starvation flaws. With LCM-DIM, however, direct imaging of these regions is possible. A typical example of a starvation flaw is shown in Figure 5. Individual steps are not discernible, indicating high step densities. The cone-like structures are areas of strong step pinning, that is, step retardation by surface adsorbed impurities. These pinning cones are oriented radial with respect to the depression center indicating that growth occurs toward the depression center. The density of these pinning cones is also far higher than for the flat periphery of the singular face, suggesting a higher surface concentration of growth retarding impurities in the defect. This might be due to longer terrace exposure times as a consequence of slower step advancement. For the specific crystal in Figure 3, we can estimate the terrace exposure time \( \tau \) as a function of distance to the edge by calculating the ratio of interstep distance \( \lambda \) and the step velocity \( V_{\text{step}} \). As can be clearly seen in Figure 3c, \( \tau \) decreases strongly as a function of edge distance. We cannot evaluate \( \tau \) for areas closer to the starvation flaw or the starvation flaw itself because of too high step densities. This would suggest that a mechanism of enhanced impurity incorporation due to longer terrace exposure closer to the face center does not apply. A different mechanism of impurity uptake might be responsible for the increased step pinning at the center.

Because of the rising inclination of the crystal surface toward the center, the face may decompose into multiple facets with singular orientations other than the (011) direction. Indeed, two facets appear to be present in the starvation flaw shown in Figure 5. These new faces may incorporate impurities at different rates than the (011) face at the periphery. Such face-dependent incorporation of impurities has already been observed for macromolecules (e.g., dimer incorporation into lysozyme crystals\(^{47}\)), and may be in effect here as well. Or, if the impurity is
Figure 5. LCM-DIM image of the central depressed area of a crystal exhibiting loss of polyhedral stability. Severe step pinning suggests high density of growth-retarding impurities. Pinning cones are directed (white arrows) at the center of the depression indicating radial growth (image area 450 × 450 μm); (inset) optical macroscopic image of the crystal.

rejected by the growing crystal, its concentration will be highest at the center of the faces. This would lead to an additional distortion of the facet plane.

Currently, we cannot yet identify the exact mechanism that enhances the impurity uptake in the starvation flaws. Additional research is required to elucidate this further.

**Agarose Enhances Polyhedral Instability.** In the previous sections, we discussed crystal growth in nongelled solutions between two glass plates. Here, we study the polyhedral stability of crystals in the presence of 0.1% agarose gel. Using Mach–Zehnder interferometry, we determined the phase difference map in the vicinity of the growing crystal. A near-spherical protein depletion zone (PDZ) was observed (Figure 6a). This would suggest that crystal growth is diffusion-limited. With \( D = 3.5 \times 10^{-7} \text{cm}^2/\text{s} \), \( \beta_{\text{face}} = 2 \times 10^{-4} \text{cm/s} \) or \( 2 \times 10^{-5} \text{cm/s} \) (kinetic roughening and 2D nucleation respectively), \( \beta_{\text{face}}/D > 1 \) for crystals larger than 200 μm, thereby demonstrating that diffusion is indeed the rate-limiting step. Vivares et al. concluded that for their conditions glucose isomerase crystallizes in a mixed regime. However, they used PEG 10 kDa as a precipitant and at higher concentrations. Hence, no direct comparison with the results in this work can be made. From the isotropic nature of the concentration gradients the presence of the Berg effect becomes readily apparent, that is, a clear mismatch between the polyhedral crystal shape and the spherical PDZ. We calculate the protein surface concentration at the edge and the center of the crystal face as a function of time using the refractive index increment \( \text{dn/dC} = 0.232 \text{mL/g} \) (determined using Abbe refractometer) (Figure 6b). A relative depletion of roughly 7.5% for \( C_{\text{center}} \) with respect to \( C_{\text{edge}} \) is measured. Note that this value represents an average value for the concentration gradient because the concentrations obtained using MZI are integrated values along the optical path.

As such, real surface concentration gradients may be underestimated using this technique.

Figure 6. (a) Isotropic depletion zone encircling a glucose isomerase crystal growing in 0.1% agarose; (b) ratio of protein concentration at face’s center and edge \((C_{\text{center}}/C_{\text{edge}})\) as a function of time; (c) effect of the proximity of the reactor wall on the protein depletion zone; (d) amplification of the Berg effect due to the proximity of the wall: the relative surface depletion \((C_{\text{edge}} - C_{\text{center}})/C_{\text{edge}}\) is larger for the face closest to the wall (C-D) than for the opposite face (A-B).

Optical imaging in transmission mode showed that crystals growing in 0.1% agarose showed a severe loss of polyhedral stability (Figure 7). At 25 °C, all crystals reaching sizes >1 mm displayed deep (~100 μm) depressions in the faces making up the habit. An unambiguous exact determination of the critical stability length was not feasible since it is strongly correlated to the distance between the crystal face and the reactor walls. Figure 7a does show that it is possible for glucose isomerase to retain a polyhedral shape at crystal sizes of ~1500 μm when the crystal-reactor distance is ±175 μm with no agarose present. A strong difference in crystal habit at various temperatures (25 °C, 15 °C, 4 °C) is observed between gelled and nongelled solutions. At 25 °C, most crystal faces retained a polyhedral shape in the absence of agarose; however, macrosteps and mother liquor inclusions were frequently observed for larger crystals (>1 mm). Starvation flaws only appeared on the crystal faces closest to the reactor walls. For crystals grown in gel, these effects were enhanced greatly; that is, deeper and larger starvation pits not only restricted to a single face appeared. A strong temperature dependence was found as well, which, given the normal solubility dependence on temperature for glucose isomerase, can be attributed to a higher supersaturation at lower temperatures, thereby decreasing the critical length for polyhedral stability. For the agarose-free conditions, the crystal habit transforms gradually toward a dendrite (Figure 7a–c), whereas in the presence of agarose, the habit switches to a skeletal form and no dendritic side branches are formed (Figure 7d–f). Dendrite formation is expected to occur when the characteristic length of the skeletal side-branches exceeds a critical value, which is determined by the interplay of material supply and incorporation kinetics. From Figure 1c and Figure 6b we conclude that the surface concentration gradients are comparable in systems where buoyancy driven convection is either inhibited through the presence of a hydrogel (e.g., agarose) or partly suppressed (shallow depletion zones are still observed, see Supporting Information) through the specific reactor geometry characterized by a low Grashof number (here two closely spaced glass plates). This would suggest that agarose not only affects
protein crystallization at the level of mass transport, but potentially affects the molecular scale kinetics as well. LCM-DIM imaging of crystals growing in agarose lends credence to this hypothesis. Figure 8a shows loss of polyhedral stability of the (011) face growing in the presence of 0.1% agarose. A very high density of pinned steps can be discerned on the flat periphery of the central depression. Step pinning through surface adsorbed impurities is much more pronounced in 0.1% agarose compared to nongelled solutions (Figure 3a). We put forth three possible explanations: (i) there are additional impurities present in the agarose. For this we tested agarose from two suppliers (Hispanagar and Hampton Research) both of which gave similar results. (ii) The agarose polymers pierce the surface upon incorporation into the crystal bulk thereby pinning the steps. However, the density of pinned steps is smaller near the outer edges of the central depression. This would exclude the agarose polymers as pinning agents as they would be incorporated homogeneously in the crystal bulk. (iii) If the impurity (responsible for the step pinning in the ungelled solution) is repelled by the crystal, ablation of convection would generate a local environment that is strongly enriched in impurities. For this case, the gel will enlarge any impurity surface concentration gradients, leading to increased step pinning at the facet center.

The protein sample was analyzed by SDS-PAGE to ascertain the purity level (Figure 9a). Three bands of different molecular weight were observed. We identify the 87 kDa and the 42 kDa bands to be the glucose isomerase dimer and monomer, respectively. The intensity of these 2 bands was >99%, indicating a high level of purity. A third band corresponding to a molecular weight of 31 kDa could not be identified. High resolution imaging of pinned steps shows that the pinning agents are microscopic particles (µm size) adhered to the surface (Figure 9b,c). In Figure 9b we show an example where multiple steps remain pinned at the same time over a distance of 11 µm. These foreign particles might be composed of aggregates of the 31 kDa protein. However, as of yet, the exact identity of these pinning agents remains unclear.

**Role of the Protein Depletion Zone in Polyhedral Instability.** Next, we tried to ascertain the role of the depletion zone in these polyhedral instability phenomena. Therefore, we calculated the dimensionless Grashof number in order to ascertain the relative role of convection and diffusion in material supply for the nongelled experiments. For a solution thickness of 0.5 or 1 mm, we obtained $Gr$ values of 1.4 and 11.1, respectively. This would suggest a mixed regime; that is, natural convection is not fully suppressed and it contributes significantly to the overall rate of mass transport. This was verified experimentally using Mach–Zehnder interferometry. Only faint, shallow depletion zones were observed around crystals growing inside the experimental cells (see Supporting Information). The maximum observed $C_{\text{bulk}} - C_{\text{surf}}$ was on the order of 1 mg/ml. Typical values for gelled solutions were larger by a factor of 20. As shown in the previous section, when convection is effectively suppressed using 0.1% agarose, clear wide and deep PDZs are formed. This indicates that convection is present in the ungelled solution and that it prevents formation of a deep PDZ. Nonetheless, Figures 1–5 clearly show that a significant Berg effect as well as a loss of polyhedral stability are possible in experimental cells where no clear macroscopic depletion zones are installed during growth. Vivares et al. concluded that loss of stability is correlated to the presence of a depletion zone. Here we propose a slightly different mechanism; that is, PDZ formation is not a prerequisite for stability loss but it enhances one of the destabilizing mechanisms, that is, surface concentration gradients. We also suggest that the distance between the crystal face and the reactor walls is a key parameter in stability.
control. To substantiate this claim, we measured the concentration profile surrounding a crystal in close proximity to the reactor edge. We added 0.1% agarose to ensure a diffusive environment (Figure 6c). The distance between the point C and the wall and D and the wall are 200 and 330 μm, respectively. In Figure 6d, the relative surface depletion \((C_{\text{edge}} - C_{\text{center}})/C_{\text{edge}}\) is plotted for the face closest to the wall (C-D) and the opposite face (A-B). For face A-B, \((C_{\text{edge}} - C_{\text{center}})/C_{\text{edge}}\) reaches a value of 8% (similar to the crystal in Figure 6a). For the face C-D oriented toward the reactor wall, \((C_{\text{edge}} - C_{\text{center}})/C_{\text{edge}}\) is more than doubled, that is, 17%. These data show that surface concentration gradients become larger due to edge effects. Although the effect is not as large as the 60% depletion observed for the crystal shown in Figure 3, here, however, the crystal was grown in a gelled solution. For a nongelled medium, gradients are expected to be larger. Convection will still be present at the edges thereby exposing them to a higher concentration and the center will experience a strong depletion due to a more limited material supply \((Gr = 0.2 \text{ for } h = 265 \mu m)\).

**Conclusion**

In this paper we have shown for the crystallization of glucose isomerase:

1. In the absence of convective currents an isotropic PDZ is installed. From an estimation of the relative weights of the kinetics of incorporation and the rate of mass transport we concluded that crystallization is diffusion-limited.

2. The influence of the Berg effect on the kinetics of the growth of glucose isomerase crystals was quantitatively determined.

3. In response to surface supersaturation inconstancies, a stabilizing microcompensation profile is setup that changes the local face kinetic constant \(\beta\). Upon attaining a critical slope, \(\beta\) reaches its maximum and the destabilizing mechanisms, that is, surface protein and possibly impurity concentration gradients lead to loss of polyhedral stability.

4. Polyhedral instability is promoted even further by the presence of 0.1% agarose. Hence, we conclude that for the diffusion-limited crystallization of glucose isomerase ablation of convection can have a detrimental effect on the polyhedral stability of large crystals.

The loss of stability is of kinetic nature and can be remedied by growing crystals in larger reactors (much larger than crystal size).

**Acknowledgment.** We are indebted to G. Nicolis and P. Vekilov for vital critical discussions. The authors wish to thank the reviewers for valuable comments and suggestions on the manuscript. This work was supported by the Flanders Interuniversity Institute for Biotechnology (VIB), the Research Council of the VUB and the Belgian Federal Science Policy Office (DWTC). We thank the European Space Agency for financing in the context of Propex project AO2004.

**Supporting Information Available:** MZI phase interferogram demonstrating the absence of a deep protein depletion zone for nongelled solution for glucose isomerase; glucose isomerase diffusivity as a function of PEG 1000, obtained using DLS. This material is available free of charge via the Internet at http://pubs.acs.org.

**References**

CG800728X