$\beta\gamma$ -Crystallination Endows a Novel Bacterial Glycoside Hydrolase 64 with Ca^{2+} -Dependent Activity Modulation

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ABSTRACT The prokaryotic $\beta\gamma$ -crystallins are a large group of uncharacterized domains with Ca^{2+} -binding motifs. We have observed that a vast number of these domains are found appended to other domains, in particular, the carbohydrate-active enzyme (CAZy) domains. To elucidate the functional significance of these prospective Ca^{2+} sensors in bacteria and this widespread domain association, we have studied one typical example from *Clostridium beijerinckii*, a bacterium known for its ability to produce acetone, butanol, and ethanol through fermentation of several carbohydrates. This novel glycoside hydrolase of family 64 (GH64), which we named glucanallin, is composed of a $\beta\gamma$ -crystallin domain, a GH64 domain, and a carbohydrate-binding module 56 (CBM56). The substrates of GH64, β -1,3-glucans, are the targets for industrial biofuel production due to their plentitude. We have examined the Ca^{2+} -binding properties of this protein, assayed its enzymatic activity, and analyzed the structural features of the β -1,3-glucanase domain through its high-resolution crystal structure. The reaction products resulting from the enzyme reaction of glucanallin reinforce the mixed nature of GH64 enzymes, in contrast to the prevailing notion of them being an exotype. Upon disabling Ca^{2+} binding and comparing different domain combinations, we demonstrate that the $\beta\gamma$ -crystallin domain in glucanallin acts as a Ca^{2+} sensor and enhances the glycolytic activity of glucanallin through Ca^{2+} binding. We also compare the structural peculiarities of this new member of the GH64 family to two previously studied members.

IMPORTANCE We have biochemically and structurally characterized a novel glucanase from the less studied GH64 family in a bacterium significant for fermentation of carbohydrates into biofuels. This enzyme displays a peculiar property of being distally modulated by Ca^{2+} via assistance from a neighboring $\beta\gamma$ -crystallin domain, likely through changes in the domain interface. In addition, this enzyme is found to be optimized for functioning in an acidic environment, which is in line with the possibility of its involvement in biofuel production. Multiple occurrences of a similar domain architecture suggest that such a " $\beta\gamma$ -crystallination"-mediated Ca^{2+} sensitivity may be widespread among bacterial proteins.

KEYWORDS $\beta\gamma$ -crystallins, Ca^{2+} binding, glycoside hydrolase, glucanase, crystal structure, calcium binding, calcium-induced activity

As in eukaryotes, Ca^{2+} regulates several important biological processes in prokaryotes (1), which necessitates the search for appropriate Ca^{2+} -responsive molecules. The $\beta\gamma$ -crystallin superfamily has recently emerged as a major group of Ca^{2+} -binding proteins among prokaryotes (2–7). In correspondence to its vast prevalence, this superfamily displays huge sequence diversity and architectural heterogeneity (8). Although their homologs in the vertebrate lens serve as structural components with little Ca^{2+} binding propensity (9), the functional significance of prokaryotic $\beta\gamma$ -

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Our analysis shows that a frequent partner of $\beta\gamma$ -crystallin is the laminaripentaose-producing β -1,3-glucanase (LPHase) domain. LPHase belongs to the GH64 family of glycoside hydrolases (as classified in the Carbohydrate-Active enZymes [CAZy] database) (11, 12). Of the multiple architectures that result from these domain combinations, we focused upon one shared at least by seven proteins from multiple organisms (the seven sequences have been aligned in Fig. 1). The representatives of this configuration are composed of three domains: an N-terminal noncatalytic carbohydrate-binding module 56 (CBM56; CAZy database) (12), a middle $\beta\gamma$ -crystallin domain, and the C-terminal LPHase region (Fig. 1). The multiple occurrences of this architecture in nature allude to an evolutionary preference and a probable functional collaboration

between the associated domains. We chose one representative of this architecture, a predicted multidomain glucanase from the Gram-positive bacterium *Clostridium beijerinckii*, to unravel the significance of this association between the two domains. We named this 589-amino-acid protein (accession no. [WP_012059029.1](#)), with a calculated molecular weight of 65.27 kDa, glucanallin. The first 26 amino acids of the protein form a signal sequence (predicted by SignalP 4.1) (13). Next to it, the N-terminal region was identified as CBM56 based on sequence homology. The residues 122 to 202 constitute a Ca^{2+} -binding β -crystallin domain, which has been structurally characterized earlier as clostrillin (5), and residues 223 to 588 were predicted to form the LPHase region.

C. beijerinckii bears industrial significance for its ability to produce acetone, butanol, and ethanol via a fermentation process (14). The major advantage in the employment of *C. beijerinckii* lies in its broad substrate range (pentoses, hexoses, starch, and others) and ability to grow in inexpensive media. Many glucanases are utilized in the process of hydrolyzing various glucans into the polymers for use as the substrates in fermentation. However, the glucanases from *C. beijerinckii* are yet to be characterized. It has also been reported that addition of calcium in the fermentation media not only increases the intracellular Ca^{2+} level but also influences several key processes, such as sugar transport, butanol tolerance, and solventogenesis, as well as the activity of many enzymatic catalysts (15), which, in turn, are responsible for enhanced glucose utilization and acetone, butanol, and ethanol (ABE) productivity (16–18). Calcium supplementation also assists in the recovery of the degenerated *C. beijerinckii* (19). Taking into account these observations, we found a glucanase with the possibility of Ca^{2+} sensitivity in *C. beijerinckii* of great interest.

The LPHase enzymes hydrolyze β -1,3-glucans specifically into pentasaccharide laminaripentaose (20). β -1,3-Glucans are naturally abundant polymers, found in many fungi, bacteria, seaweeds, and plants. Thanks to their plentitude, they are the targets for industrial biofuel production. The first known structure of an LPHase (referred to as SmLPHase here onward) is from *Streptomyces matensis* DIC-108 (PDB IDs [3GD0](#) and [3GD9](#)) (21). The LPHase in many organisms, including *S. matensis*, is found as a standalone module. However, in many other examples, it also exists as multimodular proteins, particularly associated with a carbohydrate-binding module (CBM) (see, for example, references 22 and 23). The probable function of such ancillary modules is to assist the enzymatic domain in substrate recognition and efficient binding (24). PbBgl64A, the LPHase from *Paenibacillus barengoltzii*, for which the crystal structure was reported recently (PDB ID [5H9Y](#)) (23), is a two-domain protein, with a CBM56 domain in addition to the LPHase. The CBM56 moiety in PbBgl64A reportedly affects its enzyme activity, although the mechanism of its action is not known. In the case of glucanallin molecule, the presence of three domains brings about an additional level of complexity which we wished to examine.

We have studied the Ca^{2+} -binding and enzymatic properties of glucanallin. Through enzyme assays of glucanallin and its part lacking the β -crystallin domain, as well as further disabling the Ca^{2+} -binding sites, we examined the significance of β -crystallin domain in glucanallin. We demonstrate that Ca^{2+} enhances the activity of glucanallin. In addition, we have resolved the high-resolution crystal structure of the enzyme domain, which reveals the presence of some distinct features in this new member of the GH64 enzyme class. Our work shows a phenomenon of Ca^{2+} -mediated activity modulation of an enzyme indirectly through interaction with a neighboring, Ca^{2+} -binding β -crystallin domain involving the domain interface.

RESULTS

Clostrillin (β -crystallin domain) dictates the Ca^{2+} -binding nature of glucanallin. To ascertain the Ca^{2+} -sensitive nature of glucanallin, we investigated its Ca^{2+} -binding properties through spectroscopic methods. The Ca^{2+} -binding properties of the isolated β -crystallin domain (i.e., clostrillin) have been described previously (5, 25, 26).

The emission maximum of Trp for the full protein is around 338 nm and, when titrated with CaCl_2 , the intrinsic fluorescence of the protein alters only slightly, sug-

FIG 2 (A) Trp fluorescence emission spectra of glucanallin with increasing Ca^{2+} concentrations. Spectra are recorded by excitation at 295 nm. (B) Near-UV CD spectra of glucanallin upon Ca^{2+} titrations. (C) ITC thermogram of Ca^{2+} binding to glucanallin.

gesting little or no change in the Trp environment (Fig. 2A). Ca^{2+} titration does not change the near-UV circular dichroism (CD) spectrum (Fig. 2B). In the isothermal titration calorimetry (ITC) experiment, the binding is exothermic and enthalpically driven, as reflected in the negative sign of the enthalpy change (Fig. 2C). The binding data are the best fit using a two-set of sites model and the overall dissociation constant (K_d) is $22.5 \mu\text{M}$ (Table 1). The Ca^{2+} -binding affinity of glucanallin remains comparable to that of the isolated clostrillin (i.e., 4 to $8 \mu\text{M}$) (25). We conclude that in the full protein, the Ca^{2+} -binding ability of the $\beta\gamma$ -crystallin domain remains intact, although this binding does not bring about any detectable conformation change.

Glucanallin hydrolyzes β -1,3-glucans and yields multiple products. We checked the glycolytic activity of glucanallin (full protein) using laminarin, a β -1,3-linked linear polysaccharide with β -1,6 branches, as the substrate. The reaction products were detected on a silica-based thin-layer chromatography (TLC) sheet using diphenylamine-aniline-phosphoric acid (DPA). The major product resulting from hydrolysis by glucanallin was laminaripentaose, though laminaritetraose was produced in significant amount (Fig. 3). In addition, laminaritriose and laminaribiose could also be detected (Fig. 3). With SmLPHase, laminaripentaose was detected as the major product (20, 27), but residual levels of activity for laminaripentaose resulted in a yield of laminaritetraose after prolonged (overnight) reactions (21). The formation of laminaripentaose as a specific reaction product by SmLPHase underlies the assumption that the GH64 enzyme carries out an exotype cleavage. However, the wide cleft or groove for accommodating a large substrate is a feature of endotype glucanases (21) and is present both in SmLPHase and glucanallin.

Ca^{2+} enhances enzymatic activity significantly via a distal effect through the $\beta\gamma$ -crystallin domain. To estimate the enzymatic activity of glucanallin in quantitative terms, we performed a 3,5-dinitrosalicylic acid (DNS) assay, which involves measuring

TABLE 1 Thermodynamic parameters of Ca^{2+} binding to *C. beijerinckii* glucanallin in 50 mM Tris (pH 7.5) containing 100 mM KCl

K_d (mol^{-1})	K_d (μM) ^a	ΔH (cal mol^{-1})	ΔS (cal $\text{mol}^{-1} \text{K}^{-1}$)	ΔG (kcal mol^{-1})
$K_{d1} = 1.33 \times 10^5 \pm 0$	22.5	$-5,548 \pm 45.2$	5.14	-7.1
$K_{d2} = 1.48 \times 10^4 \pm 0$	22.5	-46.92 ± 3.58	18.9	-5.7

^a $K_d = 1/\sqrt{K1 \times K2}$, where K_d represents the overall binding affinity.

FIG 3 TLC pattern of hydrolytic products of laminarin (laminaribiose, L2; laminaritriose, L3; laminaritetraose, L4; and laminaripetaose, L5) as reaction products by glucanallin and CbLPHase.

the reducing sugars produced in the reaction through colorimetry. This approach was used in various comparative assays shown in Fig. 4 and described below. We observed significant hydrolytic activity of glucanallin upon laminarin at pH 5.0.

Since glucanallin binds Ca^{2+} , we first examined the effect of Ca^{2+} on its enzymatic activity. We repeat here that the $\beta\gamma$ -crystallin domain of glucanallin (i.e., clostrillin) is a Ca^{2+} -binding module (5). To check the impact of Ca^{2+} binding to $\beta\gamma$ -crystallin domain upon the enzyme activity, we performed the assay in the presence of 1 mM Ca^{2+} . We observed that the glucanallin (the protein with three domains) activity is enhanced >2 -fold with Ca^{2+} (Fig. 4A). On the other hand, when the same was checked in the single LPHase domain, only a slight increase was detected (Fig. 4A).

It is remarkable that although the activity of LPHase domain alone is not influenced by Ca^{2+} , the association of this domain with a $\beta\gamma$ -crystallin renders it Ca^{2+} sensitive. In order to confirm that the effect of Ca^{2+} was being mediated by the $\beta\gamma$ domain, we constructed a protein without the additional CBM (referred to as “glucanallin 2-domain” [Gluc-2D]) (Fig. 5). The Ca^{2+} -induced increase in the activity of this construct was comparable to that in glucanallin (Fig. 4B). Hence, we conclude that CBM plays little role in Ca^{2+} -induced activity enhancement of glucanallin; instead, the $\beta\gamma$ -crystallin domain is responsible for Ca^{2+} sensitivity. Moreover, in previous studies, CBMs have been demonstrated to influence the glucanase activity only upon insoluble substrates and not on soluble polysaccharides such as laminarin (24, 28–31).

Ca^{2+} binding to $\beta\gamma$ -crystallin domains is accomplished through specific N/D-N/D-X-X-S/T-S motif (see reference 5 for a description of this motif and its location in clostrillin). To ascertain the involvement of this motif in the Ca^{2+} -dependent activity enhancement of glucanallin, we examined the consequence of disabling this site in terms of the enzyme activity. Both the Ca^{2+} sites in glucanallin were disabled by mutating the fifth Thr residues (which correspond to Thr162 and Thr 203 in glucanallin) into Arg. The resultant protein was designated “glucanallin Ca^{2+} -binding disabled” (Gluc-CBD) (Fig. 5). When the assay was performed with this protein, we observed that

FIG 4 DNS reagent-based enzyme activity assessment. (A) The activities of glucanallin and CbLPHase are compared in the absence or presence of Ca²⁺ at pH 5.0. Although glucanallin exhibits a strong enhancement in activity due to Ca²⁺, the change in CbLPHase is minor. (B) The activity shown by the glucanallin two-domain protein is similar to glucanallin, while disabling the Ca²⁺-binding motif in the glucanallin-CBD construct eliminates its Ca²⁺ responsiveness. (C) The activity of the glucanallin two-domain protein was compared at pH 5.0 and 7.5. The sensitivity to Ca²⁺ is greater at pH 5.0. (D) The activities of all the four proteins were compared without or with Ca²⁺. In all of the panels, “apo” refers to the condition in which Ca²⁺ was chelated away using EGTA, whereas “holo” means the presence of Ca²⁺. The values of standard deviations were used to plot the error bars in all of the graphs.

the Ca²⁺-induced activity enhancement disappeared, and the activity was brought down approximately to the level of CbLPHase (Fig. 4B). Hence, the canonical Ca²⁺-binding site of $\beta\gamma$ -crystallin domain is responsible for Ca²⁺-induced enhancement in enzymatic activity.

These results provide definitive evidence that the $\beta\gamma$ domain in glucanallin functions as a mediator of Ca²⁺-dependent modulation. It is evident from the TLC assay that the addition of Ca²⁺ made no change in the nature of products (Fig. 3). Also, the presence of two extra domains causes no difference in the nature of reaction products of glucanallin and CbLPHase (laminaribiose, L2; laminaritriose, L3; laminaritetraose, L4; and laminaripentaose, L5) (Fig. 3). The sizes of the products also remain unaltered by pH (at pH 5.0 and 7.5) (Fig. 3).

Glucanallin is optimized for use in an acidic environment. In the endeavor to develop improved methods for biofuel production, the uses of various *Clostridium* strains and many glucanases are being explored. It has been found that the acidic environment is a preferred condition for greater efficiency of the biofuel production (32, 33).

To study the suitability of glucanallin for this process, we checked its enzyme activity in the pH range of 4.0 to 9.0 and found that pH 5.0 corresponds to the maximum

FIG 5 Schematic representation of four protein constructs used in this work (B to E), along with the parental molecule (A). (A) Natural protein encoded by the gene corresponding to *C. beijerinckii* glucanase. (B) Glucanallin was prepared and studied. It lacks the N-terminal signal sequence presented in the naturally translated protein. (C) Glucanallin two-domain protein consists of the β - γ -crystallin and LPHase domains. (D) Glucanallin-CBD differs from glucanallin only in that the β - γ -crystallin Ca^{2+} -binding motifs in the former have been disabled. (E) CbLPHase, a single-domain protein.

activity. To examine the effect of pH upon the enzymatic activity, we performed various experiments at two pH values: 5.0 and 7.5. The effect of Ca^{2+} is more pronounced at pH 5.0 than at pH 7.5 in all of the constructs (Fig. 4C).

We studied the suitability of glucanallin to acidic conditions in terms of its thermal and chemical stability. At the acidic pH (pH 5), CbLPHase is observed to be more stable than at pH 7.5, as it completely unfolds in 3.3 M guanidinium chloride (GdmCl) (Fig. 6). During the unfolding transition at pH 7.5, precipitation of partially unfolded species is observed, which leads to the absence of data points between 1 and 3 M in Fig. 6B. This precipitation does not happen when the unfolding transition is observed at pH 5.0 (Fig. 6E). Glucanallin shows increased stability at pH 5.0 in thermal unfolding observed through far-UV CD spectroscopy (Fig. 6C and F). At pH 5.0, glucanallin loses its secondary structural features at 70°C compared to 60°C at pH 7.5, indicating a significant difference in stability. Based on these results, we may conclude that glucanallin and CbLPHase both are optimized to function in the acidic environment, which is the best condition for ABE fermentation by *C. beijerinckii* (32, 33).

Structural features of CbLPHase. To gain insights into the structural underpinnings of the function of glucanallin and the relationship between its constituent domains, we attempted to crystallize the full protein. X-ray diffraction data were collected at 1.86-Å resolution, and the structure was determined. The Matthews coefficient (34) and solvent content calculated for the full protein (65 kDa) correspond to 1.39 and 11.6%, respectively, for one molecule in the asymmetric unit, which was very unlikely. Upon structure solution, it was realized that the crystallized portion (based on electron density) was restricted to the region from residue 215 to 589 that corresponds to the LPHase (GH64) domain (hence it is called CbLPHase). The N-terminal part (~210 residues), encompassing the putative CBM and β - γ -crystallin domains, was cleaved

FIG 6 Equilibrium unfolding studies of glucanallin and CbLPHase at pH 7.5 and 5.0. To evaluate the chemical stability, Trp fluorescence emission maxima were plotted against increasing concentrations of GdmCl for glucanallin at pH 7.5 (A), CbLPHase at pH 7.5 (B), glucanallin at pH 5.0 (D), and CbLPHase at pH 5.0 (E). During the unfolding of CbLPHase at pH 7.5, precipitation of partially unfolded species led to the absence of data points between 1 and 3 M in panel B. The far UV-CD spectra at selected temperatures were also plotted to compare the secondary structural changes for glucanallin at pH 7.5 (C) and glucanallin at pH 5.0 (F).

during the crystallization process. Incidentally, the full glucanallin is quite stable in buffered solutions and remains intact for at least 30 days at 4°C. When calculated for the enzyme domain (LPHase, 42 kDa) alone, the resulting values for Matthews coefficient (34) and solvent content were 2.17 and 43.4%, respectively, for one molecule in an asymmetric unit.

The structure of CbLPHase was determined by molecular replacement using the crystal structure of LPHase from *Streptomyces matensis* DIC-108 (SmLPHase) as a template (PDB ID 3GD0) (21). Similar to SmLPHase, CbLPHase is composed of a barrel domain comprising mainly β -strands and a mixed (α/β) domain comprising helices and β -strands (Fig. 7A). The barrel domain has a core structure made up of 13 strands (β 1 to β 4, β 7 to β 9, β 12, β 13, β 15, β 16, and two C-terminal strands, β 22 and β 23) (Fig. 7A). This core region is flanked by four more strands (β 5, β 6, β 10, and β 11). This part of the domain also contains two 3_{10} helices. The mixed α/β domain comprises six α -helices (α 1 to α 6), which are covered from the exterior face by four strands of a β -sheet (β 18- β 21). Strands β 14 and β 17 occupy the other side of the α/β domain, facing the β -barrel domain. In addition, the α/β domain region has four 3_{10} helices (η 3 to η 6). CbLPHase takes the form of a crescent, with a wide-open groove present for ligand binding (Fig. 7A). This wide groove is located between the β -barrel domain and the mixed α/β -domain and the ligand-binding cleft in it is lined by negatively charged residues (Fig. 7B). Most residues lining the ligand-binding pocket are conserved between CbLPHase and SmLPHase (Fig. 7C). Based on the structural considerations, mutagenesis, and biochemical studies, Glu154 and Asp170 were identified to be the catalytic residues in SmLPHase (21). Glu154 serves as the proton donor, and Asp170 serves as a basic catalyst. The corresponding amino acids in CbLPHase were identified as Glu354 and Asp370. The residues Thr156, Asn158, and Trp163 in SmLPHase, which constitute the subsite +5, are represented by the residues Ala356, Thr358, and Trp363 in CbLPHase (Fig. 7C). At this site of SmLPHase, the glucan substrate is positioned

FIG 7 (A) Cartoon representation of the crystal structure of CbLPHase solved at 1.86-Å resolution. Secondary structures were rendered using the DSSP plug-in in PyMOL. (B) Electrostatic surface representation of the crystal structure showing the wide groove of the ligand-binding cleft. A triple-helical polymer curdlan is modeled to highlight that the groove can accommodate complex helical polymers. The coordinates for the triple-helical curdlan were downloaded from PolySacDb (63, 64). (C) Stereo representation of the superposition of SmlPHase in ligand-bound (blue, PDB ID 3GD9) and unbound (yellow, PDB ID 3GD0) forms with CbLPHase (purple) highlighting the catalytic residues.

through its reducing end after diffusing into the cleft and is further stabilized by several polar and hydrogen bonding interactions (21). A major part of CbLPHase structure has similarities to thaumatin-like proteins (TLPs), which are food allergens from fruits, such as banana (PDB ID 1Z3Q) (35), apple (PDB ID 3ZS3), and cherry (PDB ID 2AHN) (Fig. 8A). TLPs are ~200 to 220 residues long, which corresponds to ca. 53 to 59% of the CbLPHase structure (374 residues). These proteins share a 14 to 16% sequence identity and a root mean square deviation (RMSD) range of 2.6 to 2.8 Å (36). On the basis of structural comparison with thaumatin-like food allergens (35), we can divide the topology of CbLPHase into four distinct regions, I to IV (Fig. 8B). Regions I and II of CbLPHase correspond to TLPs structure (Fig. 8A and B).

Structural comparison with other LPHases. CbLPHase shows prominent structural differences with the ligand-bound (PDB ID 3GD9) and unbound (PDB ID 3GD0) forms of SmlPHase in the noncatalytic region. The structure of CbLPHase superposes with the structure of SmlPHase with an RMSD of 2.03 Å over 321 C α atoms (Fig. 8C). The segments of CbLPHase and SmlPHase that have substantial overall structural differences are highlighted in a zoomed inset view (Fig. 8C). The β -sheet of the mixed α/β -domain is twisted in CbLPHase with respect to SmlPHase (Fig. 8C). Strands β 5 and

FIG 8 (A) Structural comparison of CbLPHase with different TLPs. TLP from banana is shown in gray (PDB ID 1Z3Q), that from cherry is in cyan (PDB ID 2AHN), and that from apple is in teal (PDB ID 3Z53). (B) Structure of CbLPHase showing four distinct regions (in blue, pink, red, and yellow). The topology diagram of CbLPHase shows the corresponding regions with highlighted colors. (C) Structural overlap of SmLPHase (yellow, PDB ID 3GD0) and CbLPHase (green, 5H4E) to highlight the differences between two structures. (D) Structural comparison between CbLPHase (PDB ID 5H4E) and PbBgl64A (PDB ID 5H9Y). Region III is different in CbLPHase, whereas region IV is missing in PbBgl64A. PbBgl64A was crystallized with laminarhexaose (23).

$\beta 6$ present in the barrel domain of CbLPHase have no equivalent strands in SmLPHase (PDB IDs 3GD0 and 3GD9) (Fig. 7A and 8C). Sequence-based alignment revealed an insertion of the sequence ISDND (residues 268 to 272) in the corresponding region, which could have given rise to this structural feature. Further, the region corresponding to $\beta 11$, $\beta 12$, and $\alpha 2$ of SmLPHase is less ordered in CbLPHase and exists as a loop (Fig. 8C). This region interacts with $\alpha 1$ helix, which also differs structurally between the two proteins. Other than these structural differences in the noncatalytic areas, no structural difference between CbLPHase and SmLPHase was observed with respect to the positions of catalytic residues (Fig. 7C and 8C).

What makes glucanallin different from PbBgl64A is the presence of an additional domain (β -crystallin) between the CBM and LPHase domains. Structural superposition of CbLPHase and the LPHase region of PbBgl64A yields an RMSD of 1.23 Å over 301 C α atoms (Fig. 8D). It has been shown that PbBgl64A acts primarily on helical polymer curdlan and less efficiently on branched polymers like laminarin (23). Both curdlan and laminarin are polysaccharides in which glucose molecules polymerize through β -1,3 bonding. They both form a triple-helical polymeric structure; however, laminarin has β -1,6 branching, while curdlan is unbranched.

Unlike SmLPHase, PbBgl64A structure lacks strands and an α -helix corresponding to the region IV (Fig. 8D). It thus has a relatively smaller groove for substrate binding

compared to CbLPHase (Fig. 8D). In the crystal structure of PbBgl64A, two chains of laminarihexose molecule were captured (Fig. 8D). This mode of binding is suggestive of the triple helical structure of polymeric substrates (23). The groove observed in CbLPHases hints of a similar nature: it is quite wide and appears to accommodate higher-order structures of helical polymers (Fig. 7B and 8D).

The structures of different LPHases have subtle differences in regions neighboring or flanking the catalytic site. These differences could be linked to accommodating diverse structural complexities associated with the conformational states of β -glucan polymers. Further exploration of the ligand-bound structures and analysis of structural transitions upon ligand binding would be worthwhile.

DISCUSSION

The glycoside hydrolases are a diverse set of enzymes with a vast range of substrate specificity. Befitting the diversity in their substrate nature and action mechanism, they are classified into 165 enzyme families (CAZy database [12]). One aspect of the diversity in glycoside hydrolases is their recruitment of various ancillary modules, such as different kinds of CBMs. We observe that, in a subset of this diverse group, this organization is expanded to another level of complexity by addition of a β - γ -crystallin domain. We propose to christen the occurrence of such an architecture " β - γ -crystallination." The carbohydrate-active enzymes are a major, but not the only, group whose members have a β - γ -crystallin domain appended to them. A variety of other enzymes too exhibits this phenomenon.

In many of the β - γ -crystallinated glycoside hydrolases (from families 5, 16, 19, 28, 54, 64, 76, and 81) which harbor functional N/D-N/D-X₁-X₂-S/T-S motif of Ca²⁺ binding, the role of β - γ -crystallin may be linked to Ca²⁺. On the other hand, its function has also taken a course independent of Ca²⁺ binding, as in GH29 fucosidases and the putative GH54 α -L-arabinofuranosidase B family protein, which lack this motif (37). In the case of glucanallin, we found this protein to be Ca²⁺ sensitive and responsive in terms of the enzymatic function. We demonstrate that Ca²⁺ binding to a β - γ -crystallin domain modulates the activity of its neighboring LPHase domain. Some changes in the interface between CbLPHase and the β - γ -crystallin domains may be proposed as a plausible mechanism underlying this phenomenon. Despite our best efforts, we could not crystallize the protein with all three domains, and hence the correct determination of the residues involved could not be accomplished.

The members of the GH64 enzyme family remain one of the less-studied sets of enzymes. Only a few GH64 enzymes have been characterized so far (20–23, 38). Before this work, only two of GH64 enzymes have been structurally characterized (i.e., SmLPHase and PbBgl64A). Previously, the nature of products obtained from SmLPHase led to an approximate characterization of the GH64 enzymes as exotype glucanases, despite the presence of a wide cleft or groove in them. In the case of glucanallin/CbLPHase, laminaritriose and laminaribiose have been detected as reaction products along with laminaripentaose and laminaritetraose, suggesting a possibility of endotype cleavage as well. The recent work on PbBgl64A has led to the proposition of a model for triple-helical glucan recognition that explains how the enzyme cleaves the polymeric substrate as laminaripentaose in an exotype manner (23). This model also opens up a possibility of accommodating the substrate in the catalytic groove for endomode hydrolysis producing a wide range of polysaccharides. The observation made in glucanallin/CbLPHase lends itself to a similar explanation. The additional depth in the groove facilitated by the IV region in CbLPHase and SmLPHase may be useful in accommodating polysaccharides with branched chains. The smaller size of this groove in PbBgl64A is perhaps responsible for its higher activity upon curdlan than upon laminarin.

The study by Han et al. reports increased solventogenesis in response to 0.5 mg/ml CaCl₂ in media (15). Many proteins involved in processes such as heat shock response, transcription, DNA repair, carbohydrate metabolism, and sporulation showed altered expression in response to increased Ca²⁺ (15). In addition, Ca²⁺ also increased the

activity of important enzymes involved in solventogenic processes, such as coenzyme A transferase, acetate kinase, and acetoacetate decarboxylase. Glucanallin is another enzyme which we report to have increased activity due to the higher concentration of Ca^{2+} in the media. Arguably, though a significant part of Ca^{2+} -driven increase in solventogenesis is due to enhanced glucose uptake, other changes also contribute to it. Furthermore, it has been seen in many plants, that upon pathogen encounter, the glucanase activities increase ca. 1.5- to 2-fold as a result of their defense response (39, 40). Thus, the Ca^{2+} accentuated (2-fold) activity of glucanallin may be physiologically significant.

Our study demonstrates that the ancillary domains increase the stability as well as the activity of glucanallin in Ca^{2+} -dependent manner. On the other hand, in phosphatidylinositol phospholipase C from *Lysinibacillus sphaericus*, the $\beta\gamma$ -crystallin domain increases the enzyme stability but not the activity (41). The role of such associations yet needs to be understood completely and should be explored in the biological context. The promiscuous domains in proteins have been implicated in protein-protein interaction and signaling pathways (42, 43). Domain promiscuity is also correlated with an inherent capability of the domains to be used in various contexts (44), which could be a reason behind the recruitment of $\beta\gamma$ -crystallins in numerous proteins. The occurrence of a single domain, yet a monomeric, $\beta\gamma$ -crystallin is rare. In a few studies, a $\beta\gamma$ -crystallin domain has been known to stabilize the domain it is associated with (44–48). Domain-domain interface dictates the properties of many two or multidomain $\beta\gamma$ -crystallins that includes the lens β - and γ -crystallins (47, 49). A remarkable instance of the domain-derived modulation was observed in abundant perithecial protein (APP), a nonlens homolog from *Neurospora*. In APP, a canonical Ca^{2+} -binding site of $\beta\gamma$ -crystallin domain is rendered cryptic owing to interactions with residues from an Ig-like domain (50). As already mentioned, a similar phenomenon is possibly responsible for effecting the Ca^{2+} -dependent activity modulation in glucanallin. However, given the apparent diversity among the nonlens $\beta\gamma$ -crystallins (8), a general rule cannot be expected for all of them and their functional landscape is likely to be rather variegated.

The significance of this widespread phenomenon $\beta\gamma$ -crystallination should be studied in other instances also. Again, there is no universal mechanism guaranteed; however, this endeavor would reveal the diverse paths and purposes involved in the recruitment of new domain. The widespread occurrence of the $\beta\gamma$ -crystallin domain in prokaryotes and their diverse forms foretell their involvement in a diverse set of functions. Since the study of Ca^{2+} signaling in bacteria is still in its incipient phase (51), the recognition of $\beta\gamma$ -crystallins as potential mediators of Ca^{2+} -regulated processes may be a significant advance.

MATERIALS AND METHODS

Cloning, overexpression, and protein purification. The DNA encoding the full glucanallin protein (NCBI accession no. YP_001309930; refseq: [WP_012059029](#)) was cloned from the genomic DNA of *C. beijerinckii* NCIMB 8052 (obtained from DSMZ, Germany). The sequence corresponding to the 30 residues of N terminus was omitted in order to exclude the signal sequence. The remaining 1,680-bp insert was cloned into pET28a vector (Novagen) at the BamHI site. From this pET28a-glucanallin plasmid, the DNA fragment of 1,104 bp corresponding to the LPHase domain (named CbLPHase) was subcloned further. The cloning vector used was pET28b, and the insert was ligated at NheI and BamHI sites. For subcloning Gluc-2D, pET28a vector was used, and the 1,413-bp region corresponding to the $\beta\gamma$ -crystallin and LPHase domains was inserted between NheI and BamHI. The four constructs used in this study are illustrated in Fig. 5.

All of the recombinant proteins were translated with 6×His tags artificially added to their N termini. Heterologous expression was carried out in *E. coli* BL21(DE3). For glucanallin, the culture was induced with 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside) in the log phase and incubated at 37°C for 4 to 5 h postinduction. In case of CbLPHase, the culture was induced using 0.25 mM concentration of IPTG, and postinduction incubation was performed at 18°C for ~12 h. The cells were harvested by centrifugation at 6,000 × *g* for 10 min. The harvested cells were resuspended in the lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl [pH 8.0]) containing 1 mM phenylmethylsulfonyl fluoride. The suspension was incubated with 1 $\mu\text{g}/\text{ml}$ lysozyme on ice for 30 min, followed by sonication and centrifugation. The resulting supernatant was loaded onto a Ni-nitrilotriacetic acid (Ni-NTA) column (manually packed using Qiagen Ni-NTA-agarose resin) preequilibrated with three-column volumes of lysis buffer. The column was washed with washing buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM

imidazole; pH 8.0). Proteins were eluted using 50 mM NaH_2PO_4 buffer, pH 8.0, containing 300 mM NaCl with an imidazole gradient of 50 to 500 mM. After the purity of protein was checked by using SDS-PAGE, the pooled fractions were dialyzed against the same buffer, which was devoid of imidazole. Gel filtration chromatography was used for the final stage of purification with Sephacryl S-200 columns from GE Healthcare. Buffers were decalcified by passing through a Chelex-100 resin column (Bio-Rad, Hercules, CA), whereas the protein was decalcified by incubation for 45 to 60 min with 10 μM EDTA, followed by buffer exchange in a 3-kDa-cutoff Amicon stirred cell (Millipore).

Generation of glucanallin-CBD mutant and its purification. The Ca^{2+} -binding sites of glucanallin were disabled by site-directed mutagenesis PCR. The Thr162 and Thr203 residues were converted to Arg. The success of mutation was confirmed by DNA sequencing. The following primers were used: NDWMTS (Thr162), forward primer TTCAAATGATTGGATGAGATCACTTAAAGTTCCAA and reverse primer TTGGAACTTTAAGTGATCTCATCCAATCATTTGGAA; and for NDKMTS (Thr203), forward primer AATGATGCTAATGATAGTCTGTTAAATTT and reverse primer AAATTTTAAACAGATCTATCATTAGCATCATT. The overexpression of Gluc-CBD was carried out by induction with 0.5 mM IPTG, followed by postinduction incubation at 37°C for 4 to 5 h. The purification steps were similar to those for other proteins.

Crystallization. The purified protein in 50 mM Tris buffer (pH 7.5) containing 100 mM KCl and 5 mM CaCl_2 was used to set up screens. Commercial screens from Crystal Screen HT, Index HT (Hampton Research) were used to set up crystallization in 96-well format in three subwell Greiner plates by using a sitting-drop vapor diffusion method. Protein concentrations of 5, 8, and 10 mg/ml were used to mix 1 μl of protein with 1 μl of reservoir solution within each of the subwells, respectively. Diffraction quality crystals were obtained in condition F-12 (0.2 M sodium chloride, 0.1 M HEPES [pH 7.5], 25% [wt/vol] polyethylene glycol 3350) of an Index Screen (Hampton Research) at 4°C. All crystallization experiments were performed using our in-house high-throughput crystallization facility.

Structure determination. Data were collected in-house, using a Rigaku rotating anode generator equipped with a MAR-345dtb detector. A total 375 frames were collected with an oscillation width of 0.5°. Data were processed and scaled using HKL2000 (52). Protein was crystallized in a space group $P2_1$ and had a solvent content of 43.4%. The CCP4 suite of programs version 6.0 (53) was used for merging and conversion of reflection formats. The structure was determined by molecular replacement using a model, based on the crystal structure of LPHase from *Streptomyces matensis* DIC-108 (PDB ID 3GDO) with ~30% sequence identity as a search template (54). The model obtained from the initial cycles of MR-ROSETTA (in the PHENIX suite) was further modified manually to get the model, which led to successful MOLREP solution. ArpWarp (55) was used for automated model building. The manual model building was performed in COOT (56). Iterative cycles of refinements were performed using PHENIX (57). A total of 5% of the total data were used for R_{free} calculations. The final model was obtained with an R_{work} and R_{free} values of 16.1 and 21.4%, respectively (Table 2). The final refined model was validated using MOLPROBITY appended in PHENIX Suite (58). All of the crystal structure figures were rendered in PyMOL (The PyMOL molecular graphics system). For structural superposition, SSM inbuilt in COOT was used. The topology diagram was rendered using PDBsum and the secondary structure prediction was made by DSSP using PyMOL plug-in.

Molecular spectroscopy. Tryptophan fluorescence emission spectra were recorded on a Hitachi F-7500 spectrofluorimeter (Hitachi, Inc.). Protein samples (500 μl ; 0.1 to 0.2 mg/ml) in 50 mM Tris-HCl (pH 7.5) and 100 mM KCl were used for most measurements unless specified otherwise. The excitation wavelength was 295 nm, and emission spectra were recorded in the wavelength range of 300 to 450 nm. Spectra were recorded in the correct spectrum mode using an excitation and emission bandpass of 5 nm each. Far- and near-UV CD spectra were recorded on a Jasco J-815 spectropolarimeter in a quartz cuvette with an appropriate path length. For thermal unfolding measurements, spectra were recorded at from 25 to 90°C using a Jasco Peltier system attached to the sample holder for temperature control. Data obtained were plotted and fit using Origin 6.0 software.

Isothermal titration calorimetry. Macroscopic Ca^{2+} binding affinities were determined on an isothermal titration calorimeter (VP-ITC; Microcal, Inc.). Protein samples and calcium chloride solutions were prepared in Chelex-purified 50 mM Tris buffer (pH 7.5) and 100 mM KCl. Then, 1.6 ml of a 100 μM protein solution was degassed and used for titration at 30°C with 10 mM CaCl_2 as the ligand. Next, a 4- μl injection volume of the ligand was used for each titration. Blanks were obtained by titrating the buffer with the same concentration of calcium chloride solution. The quantity of heat released was fitted using nonlinear curve fitting with different models using the software Origin (v6) supplied by MicroCal.

Equilibrium unfolding. Equilibrium unfolding of the protein was performed in GdmCl. Increasing concentrations of denaturant GdmCl (from 8 M stock) were added to the protein solution (protein concentration, 0.1 mg/ml) to achieve the required concentrations (ranging from 0 to 6 M) of denaturant in two conditions (with 10 μM EDTA or with 3 mM CaCl_2), followed by incubation overnight at room temperature to achieve equilibrium. For equilibrium unfolding transitions, >70 data points were acquired (59). The unfolding transitions were followed by monitoring the intrinsic fluorescence at 295-nm excitation, and data fitting was performed using various models.

Enzyme activity assay. For the TLC-based assay, the prospective enzyme substrate laminarin (purchased from Megazyme) at a concentration of 10 mg/ml was incubated with ~10 μM protein in a reaction volume of 50 μl . After 48 h of incubation at 37°C, 10 μl of the reaction mixture was spotted onto a silica-based TLC sheet. The sheet was developed with *n*-butanol/acetone/water (2/1/1 [vol/vol/vol]). To detect the products, DPA reagent (2 g of diphenylamine, 2 ml of aniline, 100 ml of acetone, 10 ml of orthophosphoric acid) was used (60, 61). After spraying DPA reagent, the color was developed by subjecting plates to ca. 120 to 150°C for 15 min. The enzymatic activity was also measured through colorimetry using DNS reagent. The hydrolysis of β -1,3 glycosidic bonds in β -1,3-glucans yields sugars

TABLE 2 Crystallographic data and refinement statistics

Parameter	Result for glucanallin
Crystallographic data	
X-ray source	Cu-K α rotating anode
Space group	$P2_1$
Cell dimensions	
<i>a</i> (Å)	45.76
<i>b</i> (Å)	72.84
<i>c</i> (Å)	56.46
β (°)	103.85
Resolution range (Å) ^a	25.00–1.86 (1.93–1.86)
Total observations	228,390
Unique reflections	28,957
Completeness (%)	95.3 (83.6)
<i>R</i> _{merge} (%)	4.5 (22.8)
Avg <i>I</i> / σ (<i>I</i>)	40.6 (6.0)
Redundancy	7.9 (7.1)
Refinement	
Resolution (Å)	23.64–1.86
No. of reflections	28,955 (2,695)
<i>R</i> _{work} (%)	16.1 (19.49)
<i>R</i> _{free} (%)	21.4 (27.74)
Monomers/AU	1
No. of atoms	
Protein	2,969
Water	460
RMSD	
Bond length (Å)	0.007
Bond angle (°)	1.005
Mean B value (Å ²)	
Protein	23.9
Water	31.1
Ramachandran favored (%)	97.05
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	1.6
Clash score	1.35
PDB ID	5H4E

^aValues in parentheses are for the highest-resolution shell.

with reducing ends. The quantity of reducing sugars produced is estimated using a DNS assay (62). The enzyme reaction was performed in a 1-ml volume with 2 mg/ml laminarin and 10 mg/ml of protein. Two buffer systems—50 mM sodium acetate (pH 5.0) and 50 mM HEPES–100 mM KCl (pH 7.5)—were used for different pH studies. In the reactions with calcium, 1 mM CaCl₂ was added. The reaction was carried out at 37°C with incubation for 15 h. The enzyme reaction was stopped by adding 1 ml of DNS reagent, and the reaction tubes were placed in boiling water for 10 min. Next, Rochelle salt (KNaC₄H₄O₆) was mixed into the sample immediately, and it was allowed to cool to room temperature. The development of color was estimated by reading the absorbance at 540 nm using a UV-Vis spectrophotometer. All of the reactions were set up in triplicates.

Data availability. The atomic coordinates and structure factors (code 5H4E) were deposited in the Protein Data Bank (<http://www.pdb.org/>).

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