Crystal Structure of *Epiphyas postvittana* Takeout 1 with Bound Ubiquinone Supports a Role as Ligand Carriers for Takeout Proteins in Insects*

Received for publication, September 26, 2008, and in revised form, November 21, 2008  Published, JBC Papers in Press, December 10, 2008, DOI 10.1074/jbc.M807467200

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Takeout (To) proteins are found exclusively in insects and have been proposed to have important roles in various aspects of their physiology and behavior. Limited sequence similarity with juvenile hormone-binding proteins (JHBPs), which specifically bind and transport juvenile hormones in Lepidoptera, suggested a role for To proteins in binding hydrophobic ligands. We present the first crystal structure of a To protein, EpTo1 from the light brown apple moth *Epiphyas postvittana*, solved in-house by the single-wavelength anomalous diffraction technique using sulfur anomalous dispersion, and refined to 1.3 Å resolution. EpTo1 adopts the unusual α/β-wrap fold, seen only for JHBP and several mammalian lipid carrier proteins, a scaffold tailored for the binding and/or transport of hydrophobic ligands. EpTo1 has a 45 Å long, purely hydrophobic, internal tunnel that extends for the full length of the protein and accommodates a bound ligand. The latter was shown by mass spectrometry to be ubiquinone-8 and is probably derived from *Escherichia coli*. The structure provides the first direct experimental evidence that To proteins are ligand carriers; gives insights into the nature of endogenous ligand(s) of EpTo1; shows, by comparison with JHBP, a basis for different ligand specificities; and suggests a mechanism for the binding/release of ligands.

Members of the Takeout (To) family have been proposed to have important roles in various aspects of insect physiology and behavior and as such may represent novel targets for insect control strategies. The original member, *To*, was discovered in *Drosophila melanogaster* as a clock-regulated gene, acting as a molecular link between circadian rhythms and feeding behavior (1, 2). In addition, *To* expression is regulated by the somatic sex determination pathway and affects male courtship behavior (3, 4). *To* has a predicted secretion signal (1) and is secreted in the hemolymph of adult males (4). At least 80 *To* genes have now been identified from seven orders of insect, including 20 from *D. melanogaster*, but few have been characterized in any detail. Due to the localization and/or expression profiles of their transcripts, some are proposed to have roles in chemosensory perception (5–8), whereas others are thought to be involved in binding terpenoids (9), including juvenile hormones (JH) (10–15).

To proteins share limited sequence similarity and a conserved N-terminal disulfide bond with juvenile hormone-binding proteins (JHBPs). These proteins specifically bind and transport JH to target tissues in the Lepidoptera, further suggesting a role for To proteins in binding hydrophobic ligands (2). There are, however, two major differences between the families: a second disulfide bond in the JHBPs, not present in To; and the presence of two conserved C-terminal sequence motifs that are unique to the To family (2). Very recently, the structure of JHBP from the moth *Galleria mellonella* (GmJHBP) has been solved, revealing an unusual fold in which a highly curved antiparallel β-sheet wraps around a long α-helix (16). Although no direct evidence of ligand binding was obtained, two hydrophobic cavities were found within the protein, and it was hypothesized that one cavity binds JH. The presence of the second cavity was not anticipated, and its role remains unclear (16).

In the light brown apple moth *Epiphyas postvittana* (Tortricidae: Lepidoptera), a common horticultural pest, we identified an abundant To protein (EpTo1) that is more highly expressed in male when compared with female antennae (17). Here we report the identification of three additional To proteins from *E. postvittana*, and in an effort to gain a better understanding of the role(s) these proteins play in insects, we have determined the crystal structure of EpTo1. Our discovery of a surrogate ubiquinone ligand, bound deeply in the EpTo1 structure, gives the first direct evidence for ligand binding in To proteins and provides key data on the control of specificity and on likely mechanisms of ligand binding and release.

* This work was supported by New Zealand’s Foundation for Research Science and Technology (contract IDs UOAX0301 and C06X0301). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental text and two supplemental figures.

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The abbreviations used are: To, takeout; JH, juvenile hormone; JHBP, juvenile hormone-binding protein; EpTo, *E. postvittana* takeout; GmJHBP, *G. mellonella* JHBP; EST, expressed sequence tag; SAD, single-wavelength anomalous diffraction; BPI, bacterial/permeability-increasing protein; CETP, cholesteryl ester transfer protein; MES, 2-[(N-morpholino)ethane sulfonic acid.
Experimental Procedures

Bioinformatics—Takeout sequences were identified within E. postvittana expressed sequence tags (ESTs) (17) using BLAST (18). ESTs encoding predicted To family members (EpTo1, EV812668; EpTo2, EV810864; EpTo3, EV812862; EpTo4, EV812824) were full-length sequenced (GenBank accession numbers: EpTo1, EU834740; EpTo2, EU834741; EpTo3, EU834742; EpTo4 EU834743). ButterflyBase, the Gene Database for Butterflies and Moths (34), was searched to retrieve Lepidoptera JHBP and To sequences. Forty-two amino acid sequences were aligned with ClustalW (19). Among the proteins included in this analysis, the Danaus plexippus BF01062B1H01.f1 sequence, which was proposed to be a To protein (32), is in fact a JHBP. Furthermore two sequences that were retrieved from a BLAST search for Manduca sexta JH29 homologues were not initially identified as To proteins. Agrius convolvuli AB294514 is referred as “Epidermal carotenoid-binding protein,” but no publication supporting this function has been released yet. EF082245 was identified from a spruce transcriptome EST project and was therefore proposed to be a protein from Picea sitchensis (Sitka spruce). Given its high similarity with To proteins, it seems more likely that this protein in fact originates from unknown moth larvae that were harvested together with the spruce sample. Phylogenetic analysis was performed using Jones-Taylor-Thornton distances and the FITCH program (20). Bootstrap analysis was performed with 1000 replicates.

Cloning, Expression, and Purification—Primers were designed to exclude the signal peptide sequence of EpTo1 (the first 20 amino acids). PCR products were cloned into pGEM-T Easy (Promega) and sequenced. Correct sequences were ligated into a pET30a vector (Novagen) to produce a His-tagged protein (His-To1). The resulting protein consists of (i) the His tag; (ii) 45 amino acids resulting from the pET30a vector; (iii) a tobacco etch virus protease recognition site (ENLYFQ); and (iv) the native EpTo1 sequence of 220 residues. Expression was induced in E. coli Rosetta-Gami2 with 0.5 mM isopropyl-β-thiogalactoside during growth in Terrific Broth medium at 30 °C. The soluble fraction was applied to a HisTrap HP column (GE Healthcare) and 20 mM Tris-HCl, pH 8.0, 100 mM NaCl as elution buffer. EpTo1 was finally concentrated to 40 mg/ml before crystallization trials. The integrity of the protein was checked by electrospray mass spectrometry. The experimental mass of EpTo1 was 24,479.7 Da, in agreement with the calculated value of 24,479.3 Da for the 220 residues of the native sequence.

Crystallization, Data Collection, and Structure Determination—EpTo1 was crystallized by hanging drop vapor diffusion at 18 °C. Drops comprised 0.7 μl of protein solution (25 mg/ml in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl) and 0.7 μl of reservoir solution (50 mM MES, pH 6.2–6.7; 20–25% polyethylene glycol 3000). Cryoprotection was by successive transfers of the crystals into mother liquor supplemented with 5, 10, 15, and 20% 3-methyl-1, 5-pentanediol. Two crystal forms (monoclinic P21 and orthorhombic P212121) were found in the same crystallization drops.

The structure was solved by sulfur-SAD phasing using data collected on a Rigaku Micromax-007 HF generator, equipped with osmic optics and a Mar 345 detector. A highly redundant data set was collected to the maximum hardware resolution limit of 1.6 Å on a single P21 crystal. Data were processed with MOSFLM (21) and programs from the CCP4 package (22). The structure solution and initial model building were performed automatically with the autoSHARP package (23). The final model was obtained by cycles of manual building in Coot (24) and refinement in Refmac5 (25) against a 1.3 Å resolution data set collected on the same crystal on the BL9-2 beam line (Stanford Synchrotron Radiation Laboratory (SSRL) synchrotron, Stanford, CA). In the latter case, anisotropic individual B-factors were refined for all atoms. A 2.5 Å resolution data set was also collected in-house for the P212121 crystal form. The structure was solved by molecular replacement with Phaser (26), using the P21 structure as template. Refinement was conducted with Refmac5, with individual isotropic B-factors for all atoms and one TLS group comprising the whole protein. In both structures, Leu-104 was found to lie in the disallowed region of the Ramachandran plot (Table 1). The unusual geometry of Leu-104, supported by unambiguous electron density maps, is due to hydrogen bonding of its carbonyl group to the guanidinium group of Arg-102. From the initial experimental maps and during the course of the refinement of both crystal forms, two electron density peaks were observed simultaneously as standards. Spots were visual-
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ized under UV light (254 nm). The spots corresponding to the ligand, ubiquinone-9, and ubiquinone-10 were removed and extracted with 200 μl of chloroform/methanol (2:1 v/v) for 16 h. Aliquots were diluted 5-fold with methanol containing 0.1% formic acid and analyzed by electrospray mass spectrometry. The solutions were infused at 3 μl/min into a Thermo Finnigan LTQ-FTMS operating at a resolution of 100,000 at m/z 400. Full scan positive ion mass spectra were obtained at high mass accuracy, and selected parent ions were isolated for MS² analysis in the ion trap and also in the ion cyclotron resonance cell when the signals were sufficiently intense.

RESULTS

Identification of Four New Takeout Family Members—Four sequences that share similarities with To gene sequences were found among the 5739 EST sequences from male antennae of *E. postvittana* (17). Phylogenetic analyses of Lepidoptera JHBP/To protein sequences show that the To proteins fall into six distinct lineages, with the JHBP2s forming an outlier group within the superfamily (Fig. 1) (7, 11). Five of the six Lepidoptera To lineages (Lep1, Lep3, Lep4, Lep5, and Lep6) are supported by high bootstrap values. All lineages contain proteins from various species and the four *E. postvittana* To proteins belong to separate lineages (Fig. 1). When proteins from other insect species are added, the five strongly supported Lepidopteran lineages (Lep1, Lep3, Lep4, Lep5, and Lep6) remain separate from the eight lineages previously described (11), resulting in a complex tree with multiple lineages (supplemental Fig. 1).

Structure Determination of *E. postvittana* Takeout 1 (EpTo1)—EpTo1 was overexpressed in *E. coli* and crystallized in two crystal forms, with space groups P2₁ and P2₁₂₁ (Table 1). The structure of the P2₁ form was solved in-house by sulfur-SAD phasing, using 1.6 Å diffraction data. Despite the low Bijvoet ratio of 1.16 for Cu-Kα radiation (EpTo1 contains only eight sulfurs out of 1724 atoms, with six Met and two Cys residues), the 36-fold redundant data set allowed straightforward structure determination. Automated building in autoSHARP (23) fitted 209 out of 220 residues and refinement against a second, 1.3 Å resolution data set, collected on the same crystal at the Stanford Synchrotron Radiation Laboratory, gave a high quality final model comprising residues 5–220, with R = 0.144, R crunch = 0.182 (Table 1). The structure of the P2₁₂₁₂₁ form of EpTo1 was also solved by molecular replacement with the P2₁ structure as template and refined at 2.5 Å resolution.

Overall Structure—EpTo1 adopts an unusual α/β fold in which a central, highly curved antiparallel five-stranded β-sheet wraps around a long helix, α3, to form a cylindrical barrel ~65 Å long and ~30 Å in diameter (Fig. 2A, B). Two helices (α1 and α2) complement the β-sheet at both ends. The curvature of the β-sheet is due to a series of β-bulges that change the direction of the strands at regular intervals, dividing the individual strands into two or three fragments. At the top of the barrel (as oriented in Fig. 2A), an N-terminal arm lies across the barrel, together with a C-terminal arm and short helix α4. Finally, a β-hairpin is inserted between α1 and β1.

A key feature of the top of the barrel is the conserved disulfide bond, between Cys-8 and Cys-15, which joins the N-terminal arm to the first turn of the antiparallel helix α1. This disulfide bond seems likely to be critical for the structure of the opening to the ligand-binding tunnel, described below, rather than for stabilizing the overall structure. The N-terminal arm itself is somewhat flexible as the first four residues are disordered in the P2₁ crystal form. In contrast, in the P2₁₂₁₂₁ crystal form, residues 2–4 adopt well defined conformations, apparently due to crystal contacts with another molecule, and only Gly-1 is disordered. This is the only significant difference between the two structures. Structural superposition over residues 5–220 of both structures gives root mean square deviations of 0.8 and 0.3 Å for all atoms and Ca atoms, respectively.

Ligand-binding Tunnel and Bound Surrogate Ligand—The most striking feature of the EpTo1 structure is the large central tunnel, ~45 Å long: beginning with a slightly wider cavity at the top of the molecule, it extends for the entire length of the barrel (Fig. 2C). All of the main structural elements of the molecule participate in shaping the tunnel; the twisted β-sheet, α1, α2, and α3 build a hollow tube, capped at the top end by the N-terminal arm and the C-terminal α4 and at the bottom end by the β3–β4 loop and parts of α2. In the P2₁ structure, the top cavity...
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is slightly open due to the absence of the first four N-terminal residues from the model. In contrast, in the P21212 structure, residues Val-2, Leu-3, and Pro-4 cover the opening and build a completely enclosed cavity and tunnel.

The internal surface of the tunnel is almost exclusively hydrophobic, lined by the side chains of some 57 hydrophobic residues (Fig. 3). The few polar atoms at or close to the internal surface of the tunnel come from the amide and carbonyl moieties of the protein main chain, and most are engaged in interstrand hydrogen bonds of the β-sheet or interturn hydrogen bonds of α3. The only exceptions are Glu-6, Phe-189, and Ile-217. The carbonyl moiety of Phe-189 is not hydrogen-bonded but does not point into the tunnel. In contrast, the carbonyls of Glu-6 and Ile-217 are close together, both directed inwards, toward the center of the opening cavity (Fig. 3). In the P21 structure, these two carbonyls bind the only water molecule found inside the cavity (HOH 265).

In both structures, a ∼35 Å long stretch of electron density was observed inside the tunnel (Fig. 2D). The electron density suggested a ubiquinone molecule, and formal identification was achieved by TLC followed by mass spectrometry (Fig. 4). The parent ion mass found at m/z 727.56657 and the MS2 protonated parent fragments at m/z 709.44, 695.46, 667.57, 531.47, and 235.11 unambiguously identify the ligand as ubiquinone-8, which is the major isoprenoid quinone in E. coli (27). The ubiquinone-8 moiety fills almost the entire tunnel, with the quinone group positioned in the top cavity and the eight isoprenyl subunits extending all the way to the bottom. The electron density of the bound ligand is of moderate quality when compared with the rest of the structure (Fig. 2D), with B-factors for the ligand typically twice as high as those of the surrounding protein atoms.

The Conserved Takeout Motifs Form One Takeout Region—A distinctive feature of the To proteins, not present in HJBPs, is the two conserved To sequence motifs (2). In the EpTo1 structure, both motifs cluster together to form a single To region, exposed at the bottom end of the barrel (Fig. 5, B and C). Motif 1 (residues 97–113) consists of the end of β3, the β3-β4 loop, and the start of β4, whereas Motif 2 (residues 160–182) includes the long β5-α2 loop and the whole α2 helix. A network of six hydrogen bonds involving Tyr-97, Thr-109, Asp-111, Gln-113, Thr-161, Asn-162, and Phe-164 provides a tight interaction between Motif 1 and Motif 2 and confers structural integrity to the whole To region (Fig. 5B). Six hydrophobic residues from the To region (Tyr-97, Ala-99, Ile-103, Ile-108, Leu-160, and Leu-178) are buried inside the molecule and shape the end of the tunnel (Fig. 3). With the sole exception of Leu-163, whose side chain is buried between the surface of the protein and the internal tunnel, all other residues within the To region have their side chains at least partially exposed at the surface of the protein. Leu-104, Ile-105, Leu-106, Pro-107, Phe-164, Leu-170, Met-174, and Phe-177, whose hydrophobic character is very conserved for all members of the family, form a long hydrophobic groove at the surface of the molecule between Motif 1 and Motif 2 (Fig. 5D). The remaining residues of the To region are mostly polar or charged and are located on both sides of the groove.
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**Comparison with GmJHBP**—Comparison of EpTo1 with GmJHBP (16) reveals structural differences that are clearly related to ligand specificity and functional variation in the JHBP/To superfamily. With only 18% sequence identity, these two proteins are distantly related members of this family. The P2₁2₁2₁ structure of GmJHBP has been determined, and the crystal structure of EpTo1 has been determined by X-ray crystallography. The two structures are distantly related, with their respective N-terminal parts of α3 oriented at an angle of ~40° relative to each other. A similar conformation is only regained after the kink in α3 observed in the EpTo1 structure. As a consequence of this large reorganization, the tunnel is closed off at the bottom end of the EpTo1 barrel, whereas in GmJHBP, the bottom cavity is wide open to the solvent (16) (Fig. 5, C and D). The top cavity in the GmJHBP structure is also open due to reorientation of the N-terminal arm, which interacts with a symmetry-related molecule in the crystal. We find that the N-terminal arm of EpTo1 is also flexible and creates a similar opening of the cavity that leads into the long central tunnel.

**DISCUSSION**

The Takeout family of proteins has been established, primarily through genetic studies, as being critically involved in important aspects of insect behavior, including circadian regulation, courtship, and feeding. This was hypothesized to result from an ability to bind hydrophobic ligands.

We have shown here that EpTo1, an archetypal To protein, has the same α/β-wrap fold as the phylogenetically distant JHBP proteins, implying a common fold for the whole JHBP/To superfamily. We also provide the first direct evidence for ligand binding in this family, through the presence of the surrogate, *E. coli*-derived ubiquinone-8 molecule that occupies the ligand-binding tunnel in EpTo1. Overall, To proteins share little sequence conservation, and their phylogeny has many lineages. To proteins also have widely different pat-
terns of expression, with some being ubiquitously expressed, whereas others are tissue- and/or sex- and/or developmentally specific (3, 7–9, 11, 12). These variations, although sharing a common fold, suggest that their functions depend on the types of ligand bound and the specificity afforded by their binding cavities.

Structural comparisons give important insights into the control of specificity in this novel family of ligand-binding proteins. The α/β-wrap fold shared by EpTo1 and GmJHBP has been observed for only three other eukaryotic proteins. The mammalian lipid-binding proteins BPI and CETP each possess two α/β-wrap domains that bind and transport phosphatidylcholine (BPI) and phosphatidylcholine and cholesteryl esters (CETP) (28, 29). The third, more divergent, Aha1 protein, is not known to bind any ligand but has a role as a co-chaperone of the Hsp90 chaperone machinery in eukaryotes (30). Comparison of these proteins indicates that, within a conserved scaffold, the number, size, shape, and physicochemical properties (hydrophobicity, hydrophilicity, charge distribution) of the internal cavity(ies) can vary greatly. In GmJHBP, the conserved disulfide bond linking α3 to β5 closes off the tunnel seen in EpTo1, leaving just the cavity at the top of the barrel, as appropriate for the smaller JH ligand. In BPI and CETP, a similar disulfide exists in the N-terminal domains of each protein such that they do not have the long, narrow tunnel of EpTo1. Instead, lipids are bound in internal cavities of different size and shape whose locations in each barrel correspond approximately to the top cavities of EpTo1 and GmJHBP. Similar differences may be expected in different To proteins, enabling each of them to bind specific ligand(s) and therefore to be potentially involved in distinct biological processes.

In the case of EpTo1, the endogenous ligand(s) is (are) not known. The surrogate E. coli ubiquinone-8 moiety within the cavity is bound as a result of excellent shape complementarity, with no specific hydrogen bonding observed between the quinone group and the protein atoms. Ubiquinone-9 and ubiquinone-10, carrying respectively one and two additional isoprene subunits, are the main isoprenoid quinones found in insects (31). These longer compounds do not fit within the EpTo1 cavity, however, ruling out a possible role for EpTo1 as a ubiquinone transporter in E. postvittana. On the other hand, the long internal tunnel of EpTo1 seems inappropriate for the specific binding of short chain compounds such as JHs. With only two hydrogen bond acceptors, which are in any case confined to the entrance cavity, the binding tunnel of EpTo1 is well suited for elongated and strongly hydrophobic compounds. Isoprenoids such as, carotenoids, triterpenoids, or even long chain fatty acids could therefore be suitable ligands for EpTo1. Many of these compounds are potential precursors for signaling molecules and other essential secondary compounds in insects, suggesting a potential route by which members of the To family, acting as carriers, could be involved in various insect physiologies and behaviors. Indeed the fact that EpTo1 is more highly expressed in male when compared with female antennae (17) suggests a role in a sex-specific function such as courtship or reproduction.
Access of the ligand to the internal tunnel of EpTo1 presumably occurs via the entry cavity at the top of the barrel. In GmJHBP, the N-terminal arm was proposed to act as a lid to enclose JH inside this top cavity (16). In EpTo1, the N-terminal arm is also flexible, but the opening observed in the P2₁ structure is too narrow to allow a large molecule like ubiquinone to enter the cavity (supplemental Fig. 2A). In both EpTo1 crystal forms and in GmJHBP, however, both the N-terminal arm and the C-terminal helix have significantly higher B-factors than the rest of the structure (Table 1). This suggests that this helix, which is very exposed to the solvent and has only minimal contact with the adjacent β5, also has some flexibility. Simultaneous movements of both the N-terminal arm and the C-terminal helix could create a much broader opening at the top of the barrel and facilitate access of large compounds to the cavity (supplemental Fig. 2B).

At the bottom end of the barrel, the two To motifs cluster in a single region, with conserved residues forming a hydrophobic groove on the protein surface. Having established that To proteins are ligand carriers, we propose that this exposed hydrophobic patch could be involved in a targeting process, the nature of which remains unknown. We further propose that this region is likely to play a role in ligand release. Once deeply buried inside the tunnel, it seems unlikely that a simple passive mechanism could trigger release of a long hydrophobic ligand from EpTo1. In GmJHBP, the bottom cavity is wide open to the solvent as a result of the different conformation of this part of the barrel, corresponding to the To region. The individual structural elements of the “closed” To region of EpTo1 are largely preserved, however, in this “open” state. Despite a shift of α2 and of the β3-β4 loop, and straightening of α3, the only real structural change is in the conversion of the β5-α2 loop of To Motif 2 into a second helix in GmJHBP.

Such conformational changes in the To region could allow the ligand to be released from the long cavity via the bottom of the protein, although it would require the disruption of the six hydrogen bonds linking Motif 1 and Motif 2. In the context of a direct interaction between the To region and a cellular target, as proposed above, such a mechanism could be achieved and would permit the ligand to be released at the target side. A similar tunneling mechanism has been proposed for lipid transfer by CETP, in which the lipids transit the internal cavity through different entry and exit ports in each barrel (29). It would not be possible for GmJHBP, however, because the disulfide bond linking α3 to β5 constrains the internal cavity, dividing it into two parts and not allowing passage between them. The smaller JH ligand presumably enters and exits via a simple lid on the top cavity.

FIGURE 5. The Takeout region of EpTo1 and structural comparison with GmJHBP. A, EpTo1 and GmJHBP are displayed with the same orientation after three-dimensional superimposition. The disulfide bonds are displayed in stick mode and highlighted by black circles. The internal cavities are shown in light blue. In EpTo1, the ubiquinone-8 moiety is drawn in pink. B, bottom views of the barrels, highlighting the conserved To region of EpTo1, with Motif 1 (in green) and Motif 2 (in orange). The six hydrogen bonds linking Motif 1 and Motif 2 are represented by blue lines. In GmJHBP, the structure adopts an alternative, open, conformation at the bottom part of the barrel. For comparison, the same color code is used for the elements of GmJHBP corresponding to Motif 1 and Motif 2 of EpTo1. The second disulfide bond, conserved in JHBPs but absent in To proteins, links α3 to the external strand of the β-sheet (black circle). C, surface representation of the To region of EpTo1, with the same orientation and color code as in B, showing the groove located at the interface between the two motifs. In GmJHBP, the arrow indicates the wide opening of the bottom cavity to the solvent. D, the same view as C with the hydrophobic residues mapped onto the protein surface shown in purple.
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