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Crystal Structure of Hypothetical Fructose-Specific EIIB from *Escherichia coli*

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We have solved the crystal structure of a predicted fructose-specific enzyme IIB^{fruc} from *Escherichia coli* (*Ec*EIIB^{fruc}) involved in the phosphoenolpyruvate-carbohydrate phosphotransferase system transferring carbohydrates across the cytoplasmic membrane. *Ec*EIIB^{fruc} belongs to a sequence family with more than 5,000 sequence homologues with 25-99% amino-acid sequence identity. It reveals a conventional Rossmann-like α-β-α sandwich fold with a unique β-sheet topology. Its C-terminus is longer than its closest relatives and forms an additional β-strand whereas the shorter C-terminus is random coil in the relatives. Interestingly, its core structure is similar to that of enzyme IIB^{cellobiose} from *E. coli* (*Ec*IIB^{cel}) transferring a phosphate moiety. In the active site of the closest *Ec*EIIB^{fruc} homologues, a unique motif C*XXGXAHT* comprising a P-loop like architecture including a histidine residue is found. The conserved cysteine on this loop may be deprotonated to act as a nucleophile similar to that of *Ec*IIB^{cel}. The conserved histidine residue is presumed to bind the negatively charged phosphate. Therefore, we propose that the catalytic mechanism of *Ec*EIIB^{fruc} is similar to that of *Ec*IIB^{cel} transferring phosphoryl moiety to a specific carbohydrate.

INTRODUCTION

In bacteria, the phosphoenolpyruvate-carbohydrate phosphortransferase system (PTS) is a chief carbohydrate transport system (Deutscher et al., 2014) catalyzing the translocation of various carbohydrate molecules across the cytoplasmic membrane. A large number of carbohydrates are phosphorylated by the PTS generally consisting of cytoplasmic proteins, enzyme EI (EI) and heat stable phosphoryl carrier protein (HPr), and a membrane-associated carbohydrate-specific permease complex enzyme II (EII). In general, EII complexes are composed of hydrophilic enzymes, EIIA and EIIB, and hydrophobic integral membrane proteins, EIIC (and EIID in a mannose- and fructose-specific PTS). The catalytic function of EI, HPr and EIIA depends on a functional histidine residue transiently phosphorylated during enzyme reaction. The active sites of EIIB subunits include either a cysteine or a histidine as a catalytic residue. The phosphoryl group which will be transferred to various carbohydrates is obtained from phosphoenolpyruvate by EI. HPr becomes phosphor-HPr by accepting the phosphoryl group from EI. At the next step, the phosphoryl group from phospho-HPr is transferred to a sugar-specific EII complex. In general, EI and HPr are not selective and are shared by different PTS systems. However, there are many different types of EIIs present for uptake of different carbon sources, and they are not interchangeable. A phylogenetic analysis of the EII proteins show that the PTS permease families are classified into seven families as follows (Marchler-Bauer et al., 2015); the (i) glucose (including glucoside) (Glc), (ii) fructose (including mannitol) (Fru), (iii) lactose (including ***N***,***N***-diacetylchitobiose) (Lac), (iv) galactitol (Gat), (v) glucitol (Gut), (vi) mannose (Man), and (vii) L-ascorbate (Asc) families. In addition, PTS components directly interact with their target proteins which carry out various cellular functions such as transport proteins or transcription regulators.

An open reading frame of ***E. coli*** str. K-12 substr. MG1655 codes for a ***frwD*** gene (NCBI RefSeq: NP_418388.1) which is annotated as putative PTS EIIB^{fruc} protein (***Ec***EIIB^{fruc}) of 12.6 kDa. It belongs to the NCBI conserved domain cd05569 (PTS_IIB_fructose) which includes subunit IIB of EII of the fructose specific PTS (Barabote and Saier, 2005). A PSI-BLAST search (http://www.ncbi.nlm.nih.gov) of this sequence revealed around six hundred proteins with sequence identity above 35% (Fig. 1). Most of the homologous sequences are annotated as fructose-like specific PTS system EIIB or fused forms, EIIBC or EIIABC. The biochemical study revealed that at least 15 different EII complexes are found in ***Escherichia coli*** (Saier and Reizer, 1994). Several putative fructose-like IIB components are also detected in the ***E. coli*** PTS system. Since the molecular structure and the functional implication of this ***Ec***EIIB^{fruc} family have not been reported, we have determined the X-ray crystal structure of ***Ec***EIIB^{fruc}. Based on the crystal structure analy-

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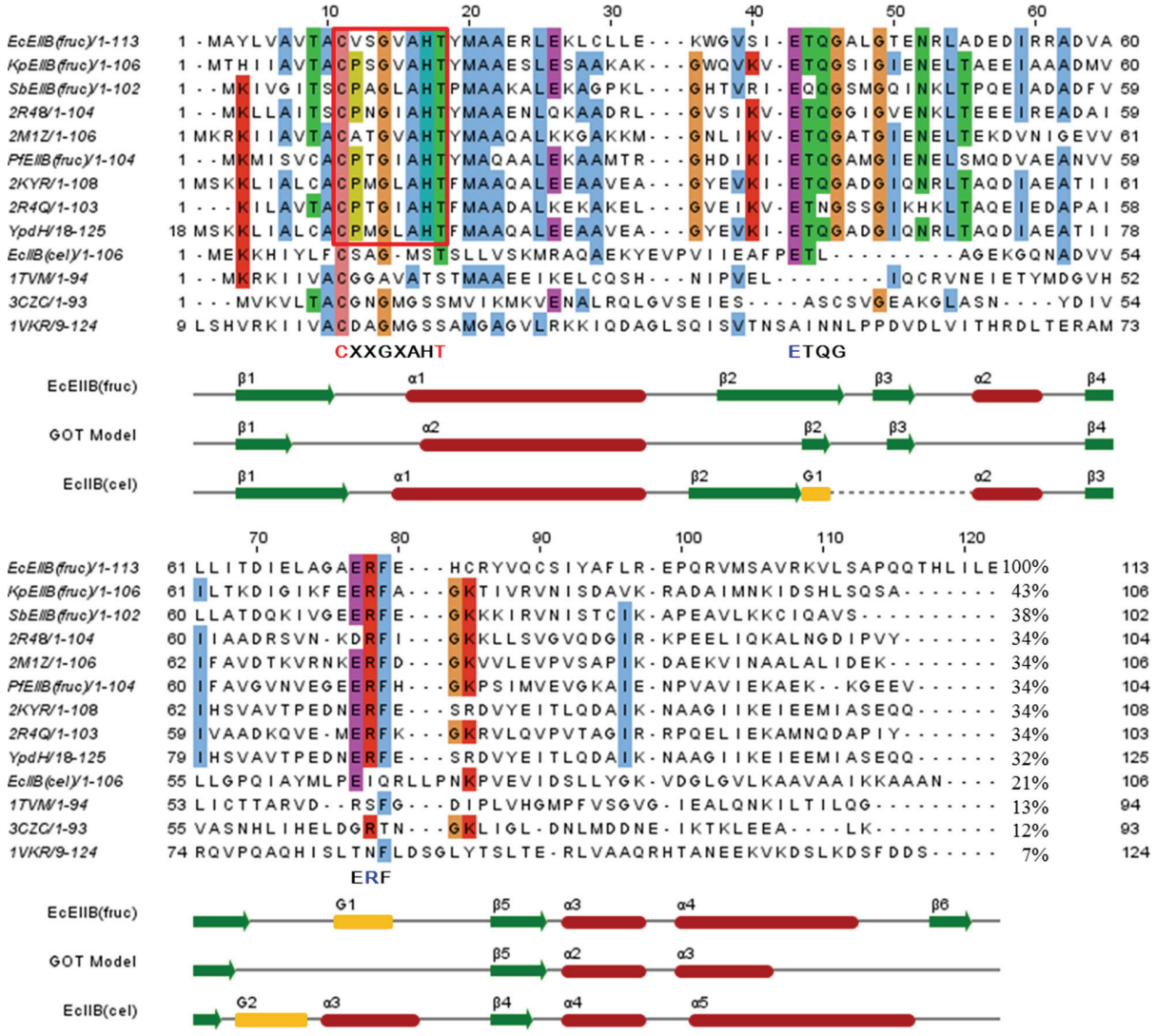

Fig. 1. Multiple sequence alignment of ***Ec***EIIB^{fruc} and its homologues. Abbreviations are as follows: Predicted EIIB^{fruc} molecules from ***Escherichia coli*** str. K-12 substr. MG1655 (***Ec***EIIB(fruc)), ***Klebsiella pneumoniae*** 342 (***Kp***EIIB(fruc)), ***Streptococcus bovis*** ATCC 700338 (***Sb***EIIB(fruc)), ***Bacillus Subtilis*** Subsp. Subtilis Str. 168 (2R48), ***Listeria monocytogenes*** EGD-e (2M1Z), ***Pelosinus fermentans*** DSM 17108 (***Pf***EIIB(fruc)), ***E. coli*** K-12 (2KYR), ***Bacillus subtilis*** subsp. subtilis str. 168 (2R4Q), ***E. coli*** UTI89 (YpdH), ***Ec***IIB^{cel} (***Ec***IIB(cel)), GatB ***E. coli*** from (1TVM), PtxB from ***Streptococcus*** (3C2Z) and the cytoplasmic B domain of the mannitol transporter IIABC from ***E. coli*** (1VKR). Secondary structure elements of ***Ec***EIIB^{fruc}, the GOT model and ***Ec***IIB^{cel} are drawn below the sequences and colored red for α-helix, green for β-strand and yellow for 3_{10}-helix. The dotted lines represent the residues missing in the coordinates. The highly conserved motifs are indicated. The conserved sequences at the P-like loop motif are marked with a red box. The red characters represent the key residues for transferring a phosphoryl group. Glu38 and Arg73 forming a salt bridge are marked as the blue characters. The consensus amino acids are colored according to the Clustal X color scheme provided by the Jalview program (Waterhouse et al., 2009).

sis, molecular functions of ***Ec***EIIB^{fruc} have been inferred and discussed in this paper.

MATERIALS AND METHODS

Structure determination

Details of the preliminary X-ray study (Shin, 2008) and the structure determination (Joo et al., 2015) of ***Ec***EIIB^{fruc} had been previously published. In brief, ***Ec***EIIB^{fruc} crystals were grown using 20% (w/v) PEG MME2000 in the presence of 10 mM nickel (II) chloride hexahydrate. The X-ray dataset of ***Ec***EIIB^{fruc} was collected to 2.28 Å resolution and the crystal belonged to the primitive trigonal space group $P3_2$ with unit-cell parameters $a = b = 33.11$, $c = 154.38$ Å. Molecular replacement (MR) was performed using PHENIX (Adams et al., 2010) with highly accurate protein 3D models generated by the recently proposed method called GOT (Global-Optimization-based Template-based modeling of proteins) (Joo et al., 2015). A relatively good electron density map was obtained after automated model-building and refinement with the PHENIX AutoBuild wizard

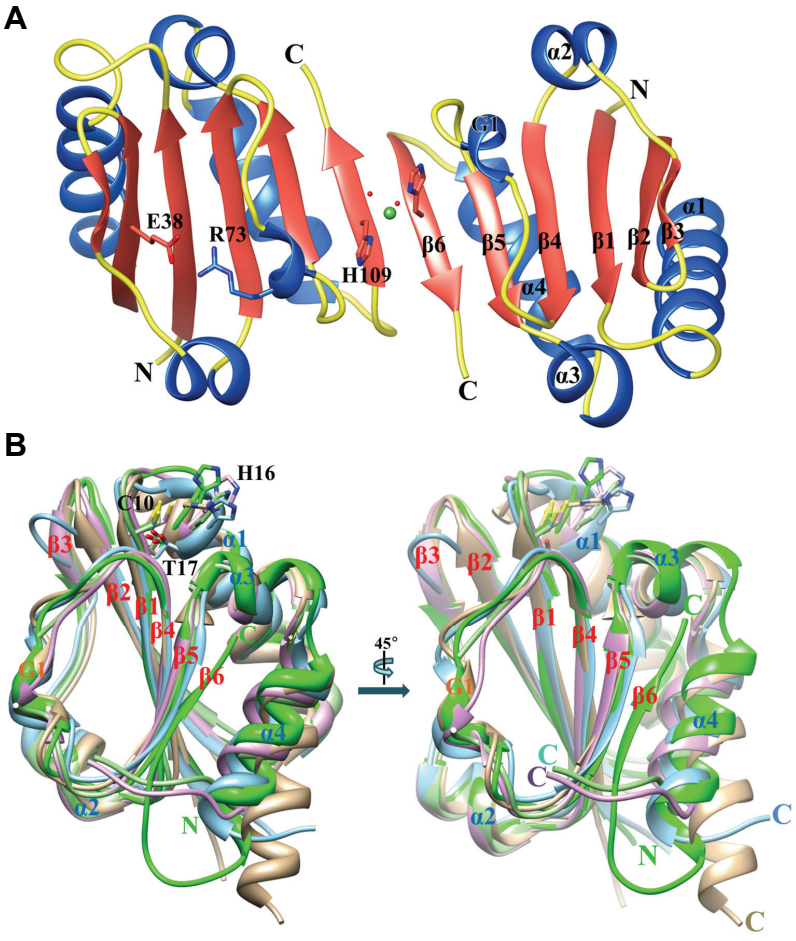

Fig. 2. (A) The crystal structure of dimeric *Ec*EIIB^{fruc}. The secondary structural elements of *Ec*EIIB^{fruc} are labeled. The two water molecules (red) and two histidine residues coordinating to nickel ion (green) are drawn. Glu38 and Arg73 forming a salt bridge with a distance of ~2.7 Å are also drawn. (B) The superimposed crystal structures of *Ec*EIIB^{fruc} (green) and its closest homologues, 2R48 (Plum), 2R4Q (Light green), 2M1Z (Sky blue) and 2KYR (Tan), in two different views related by a 45° rotation. The secondary structural elements of *Ec*EIIB^{fruc} are labeled. Some C-terminals are labeled according to their ribbon colors. The potential active site residues, cysteine, histidine and threonine residues are drawn with a stick model.

(Afonine et al., 2012). The electron density map clearly showed the presence of two protomers of *Ec*EIIB^{fruc} in the asymmetric unit. Interestingly, they form a dimer bridged by a nickel ion contained in the crystallization solution. The final model exhibited good stereochemical geometry and was refined to R- and R-free values of 22.5% and 29.9%, respectively (Joo et al., 2015). The atomic coordinates and structure factors of the *Ec*EIIB^{fruc} structure have been deposited in the RCSB Protein Data Bank under the accession code 4TN5.

RESULTS AND DISCUSSION

The crystal structure of *Ec*EIIB^{fruc} has been determined at the resolution of 2.4 Å (Supplementary Fig. S1). The *Ec*EIIB^{fruc} structure includes all residues of the original sequence. The structure of the *Ec*EIIB^{fruc} protein is composed of a central six-stranded parallel open twisted β-sheet, which is flanked by three α-helices (α1, α3, α4) on the concave side and two (α2, G1) on the convex side (Fig. 2A). In the asymmetric unit, the crystal structure shows an artificial dimer seemingly being triggered by one nickel ion coordinated to two neighboring His109 residues located on the C-terminus of each protomer (Fig. 2A). This coordination may induce a loop-to-strand conversion of the C-terminal loop resulting in the formation of the last β-strand β6. As a result, a twelve stranded β-sheet composed of two contiguous β-sheets is formed in the dimer. However, *Ec*EIIB^{fruc} is a monomer as indicated in the monodisperse peak with molecular weight of 13 kDa from Dynamic light scattering (DynaPro 99, Proterion Corporation, USA) (Shin, 2008) and this dimeric form is not found in the other crystal structures of its closest homologues as mentioned below. It is noteworthy that the addition of zinc ions produced same kinds of crystals. However nickel ions improved the diffraction limits of crystals (Shin, 2008). In the crystal structure, two histidine residues and two water molecules coordinated the nickel ion. During the refolding step, various metals were tried but a dimeric size was not detected in size exclusion chromatography and dynamic light scattering. Furthermore, the CD spectrum showed the same secondary structure contents regardless of the presence of metal ions including zinc and nickel ions (Oganesyan et al., 2005). However, the metal induced dimerization of *Ec*EIIB^{fruc} cannot be thoroughly excluded and its biorelevance should be further studied inside bacterial cells.

*Ec*EIIB^{fruc} belongs to a sequence family with more than 5000 sequence homologues having 25-99% amino-acid sequence identity obtained by a PSI-BLAST search (Fig. 1). The 3D structure alignment search with Dali (Holm and Rosenstrom, 2010) reveals that the crystal structure of *Ec*EIIB^{fruc} determined in this study, along with the closest homologues (Z-scores above 14), 2R48 (mannose-specific EIIB from ***Bacillus subtilis***), 2R4Q

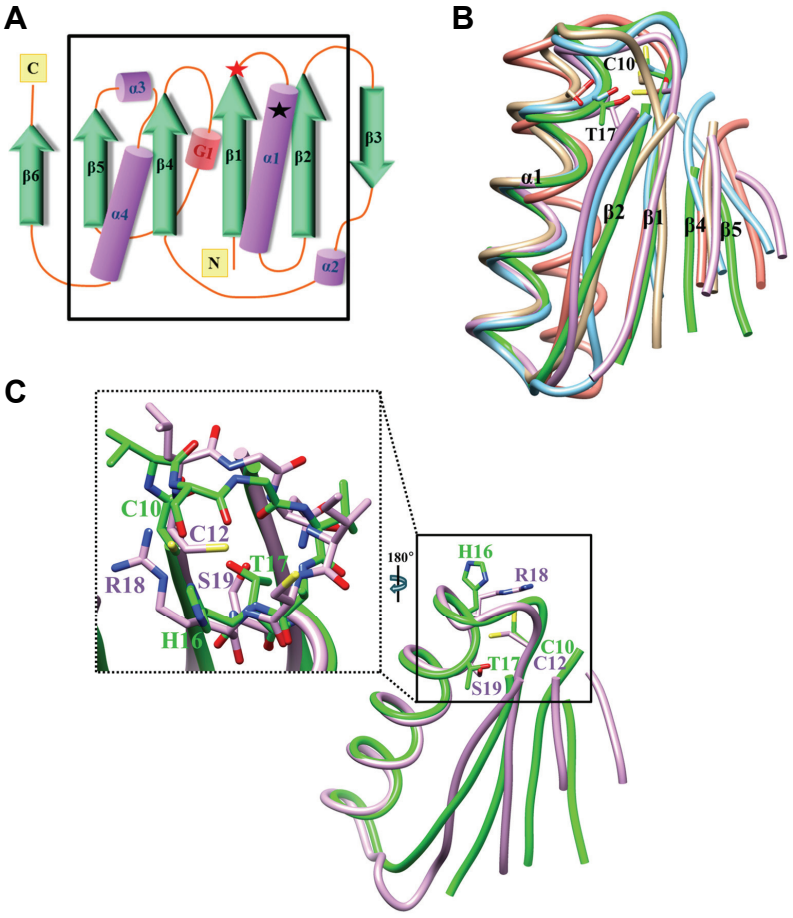

Fig. 3. (A) Topology diagram is shown. α-helices are represented by purple cylinders, β-strands by green arrows, and 3_{10}-helix by a red cylinder. Secondary structure elements of helices and strands are labeled. Cys10 and Thr17 are represented by red and black stars, respectively. The boxed region represents the topology overlapped with ***Ec***IIB^{cel}. (B) The superimposed core structures of ***Ec***EIIB^{fruc} (green) and ***Ec***IIB^{cel} homologues, 1IIB (Plum), 1TVM (Tan), 1VKR (Sky blue) and 3CZC (Salmon). The secondary structural elements of ***Ec***EIIB^{fruc} are labeled. The potential active site residues, cysteine and threonine residues are drawn with a stick model. (C) The superimposed core structures of ***Ec***EIIB^{fruc} (green) and the low-molecular-weight PTPase (plum) from ***Bos taurus*** (PDB ID: 1PHR). The potential active site residues of both proteins are labeled and drawn with a stick model. Their catalytic loops are depicted in the solid box. The zoomed view represents the detailed view of the P-like loop of ***Ec***EIIB^{fruc} and the P-loop of low-molecular-weight PTPase. The residues of consensus sequence are drawn with a stick model.

(fructose-specific EIIB from ***B. subtilis***), 2M1Z (hypothetical protein lmo0427 from ***Listeria monocytogenes***) and 2KYR (fructose-like enzyme IIB from ***E. coli***), forms a unique fold family not found in other protein structures. It is composed of β1, α1, β2, β3, α2, β4, G1, β5, α3 and α4. The salt bridge formed by Glu38 and Arg73 (Fig. 2A) from two highly conserved sequence motifs, ^{38}ETQG^{41} and ^{72}ERF^{74} (Fig. 1), seems to stabilize the overall architecture of the family. The root-mean-square (rms) deviations of matching Cα atom positions of ***Ec***EIIB^{fruc} compared with its closest homologues are 0.727 Å^2 (70 Cα atoms of 2R48) 0.965 Å^2 (71 of 2R4Q), 1.090 Å^2 (86 of 2M1Z) and 0.989 Å^2 (80 of 2KYR), respectively. It is noteworthy that the C-terminus of ***Ec***EIIB^{fruc} is longer than its closest homologues and forms an additional β-strand β6 whereas the shorter C-termini of the other members are unstructured (Fig. 2B). As a result, the central β-sheet of ***Ec***EIIB^{fruc} has the topology of 6↑5↑4↑1↑2↑3↓ instead of 5↑4↑1↑2↑3↓ found in the other members. The Dali search revealed that the topology of the six-stranded β-sheet, 6↑5↑4↑1↑2↑3↓, is unique and not detected in other protein structures. Although this additional β-stand is stabilized in our crystal form by an inter-molecular antiparallel β-bonding and chelation of a nickel ion by the neighboring two His108 (Fig. 2A), it is not yet clear whether the last β-strand is an intrinsic structure or an artificial result. In fact, the predicted secondary structure of the C-terminus of ***Ec***EIIB^{fruc} calculated with PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) (Buchan et al., 2013) is a loop. Therefore, the state of the C-terminus in solution and the possibility of a transition from disordered to ordered or ***vice versa*** related to its function are still under investigation. The next homologue group of ***Ec***EIIB^{fruc} is DNA-binding response regulators with Z-scores above 8. However, this family has a different β-sheet topology from ***Ec***EIIB^{fruc} and contains unique active site residues. The structure of the EIIB subunit of fructose permease (EIIB^{Lev}), another homologue of ***Ec***EIIB^{fruc}, also has a different β-sheet topology (3↑2↑4↑1↑5↑6↑7↓) with the active site residue, His15 (Schauder et al., 1998).

Interestingly, the Dali search revealed that the core β-sheet topology, 2↑1↑4↑5↑, of ***Ec***EIIB^{fruc} is closer to that of enzyme IIB^{cellobiose} from ***E. coli*** (***Ec***IIB^{cel}, PDB: 1IIB) (van Montfort et al., 1997) with the Z-score of 7.2 (Fig. 3A). It contains a β-sheet with the topology of 2↑1↑3↑4↑ lacking two β-strands at each end of the sheet. ***Ec***IIB^{cel} homologues comprises a protein family with the enzyme GatB of the galactitol-specific phosphoenolpyruvate-dependent phosphotransferase system from ***E. coli*** (1TVM), the cytoplasmic B domain of the mannitol transporter IIABC from ***E. coli*** (1VKR), and PtxB from ***Streptococcus*** (3CZC). They have the β-sheet topology of 2↑1↑3↑4↑ with a conserved cysteine (Cys10) and a threonine/serine (Thr17) located at the active site constructed by a P-like loop (Fig. 3B) as mentioned below (Su et al., 1994). The cysteine residue functions ***via*** a cysteine-phosphate intermediate and the threonine residue assists the binding of phosphate (Ab et al., 1997).

The multiple sequence alignment shown in Fig. 1 indicates that these two residues are also conserved among *Ec*EIIB^{fruc} homologues. Since these residues are critical in transferring a phosphoryl group in *Ec*IIB^{cel} homologues, Cys10 and Thr17 of *Ec*EIIB^{fruc} may function in a similar way.

As for *Ec*IIB^{cel} homologues, the phosphorylation site of EIIB is located on this conserved cysteine residue at the N-terminal end of the P-like loop. This cysteine receives the phosphoryl group from EIIA and transfers it to the specific carbohydrate when bound at the catalytic site of EIIC. The catalytic activity of the cysteine is aided by the P-like loop structurally similar to that of the phosphate binding P-loop of the phosphotyrosine protein phosphatase (PTPase) superfamily (consensus sequence C***XXXXX***R(S/T)) (Su et al., 1994). The P-loop forms an anion-binding motif together with the helix dipole of helix α1 and thus provides a favorable environment to generate deprotonated cysteine as a nucleophile and to accommodate a negatively charged phosphoryl group. The conserved arginine residue in the consensus sequence supports the catalytic process. However, the P-like loop of *Ec*IIB^{cel} homologues lack the conserved arginine residue at the corresponding position of the P-loop of the PTPase superfamily and thus the insufficient positive charge is thought to be compensated by their partner molecules (Lei et al., 2009).

As mentioned above, the architecture of the core structure of *Ec*EIIB^{fruc} is quite similar to the overall structure of *Ec*IIB^{cel} together with the conserved residues, Cys10 and Thr17. When the core structural elements of *Ec*EIIB^{fruc} are superimposed with those of *Ec*IIB^{cel} homologues (Fig. 3B), the rms deviations of aligned Cα atoms are 0.822 Å^2 (25 Cα atoms of 1IIB) 0.929 Å^2 (28 of 1TVM), 1.030 Å^2 (32 of 1VKR) and 1.253 Å^2 (10 of 3CZC), respectively. However, unlike *Ec*IIB^{cel}, *Ec*EIIB^{fruc} homologues contain a conserved histidine His16 which may assist accommodating a negatively charged phosphoryl group. The spatial position occupied by His16 in the P-like loop is almost equivalent to that of the functional arginine of the P-loop of the PTPase superfamily (Fig. 3C). Consequently, the *Ec*EIIB^{fruc} family has a unique consensus sequence of C***XXGXAHT*** at the P-like loop. Interestingly, the crystal structures of the nucleotide binding subunit B of A_1A_0 ATP synthase (Sankhala et al., 2014) and human PIR1 (Tadwal et al., 2012) revealed that they also contain a catalytic histidine residue in their P-loops which interacts with a phosphate moiety.

In summary, we have determined the crystal structure of *Ec*EIIB^{fruc}. The Dali search revealed that its core structure resembles that of *Ec*IIB^{cel}. Though the overall fold is quite similar to different IIB enzymes and other fold families, *Ec*EIIB^{fruc} homologues still consist of an independent subfamily due to their unique topological connection. Based on the sequence alignment and structural comparison of its homologues, a unique motif C***XXGXAHT*** comprising a P-loop like architecture is also inferred. Therefore, we proposed that the conserved cysteine on this loop may be deprotonated to act as a nucleophile to transfer phosphoryl moiety to a specific carbohydrate. The bigger size of *Ec*EIIB^{fruc} homologues together with the presence of extra catalytic histidine over *Ec*IIB^{cel} suggests that though their core molecular function is similar, their biological partners and substrates may be different. A further study is going on to find a biological partner and substrate of *Ec*EIIB^{fruc}.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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