Backbone Assignment of Phosphorylated Cytoplasmic Domain B of Mannitol Transporter II^Mtl in *Thermoanaerobacter Tengcongensis*

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Abstract The cytoplasmic domains A and B of the mannitol transporter enzyme II^Mtl are covalently linked in *Escherichia coli*, but separately expressed in *Thermoanaerobacter Tengcongensis*. The phosphorylation of domain B (TtIIBMtl) substantially increases the binding affinity to the domain A (TtIIAMtl) in *T. Tengcongensis*. To understand the structural basis of the enhanced domain–domain interaction by protein phosphorylation, we obtained NMR backbone assignments of the phospho-TtIIBMtl using a standard suite of triple resonance experiments. Our results will be useful to monitor chemical shift changes at the active site of phosphorylation and the binding interfaces.

Keywords backbone assignment, mannitol transporter Enzyme II, phosphorylated protein *Thermoanaerobacter Tengcongensis*

Introduction

The cytoplasmic phosphoryl transfer domains A and B of the mannitol transporter enzyme II^Mtl are covalently connected by a flexible peptide linker in mesophilic bacterium such as *Escherichia coli* (*Ec*),¹³ whereas they are separately expressed in typical thermophilic bacterium, *Thermoanaerobacter Tengcongensis* (*Tt*).⁴ Previous NMR studies have determined the three-dimensional structures of unphosphorylated *EcIIBMtl* (PDB code : 1VRV)⁵ and phosphorylated *EcIIBMtl* (PDB code : 1VKR).⁶ Both structures are composed of a central four beta strands, flanked by three alpha helices with identical global folds. However, there were small differences at the phosphorylation site where the phosphoryl group is engaged in multiple hydrogen bonds.⁶ Further studies on their interaction and dynamics reported that the binding affinity between *EcIIAMtl* and *EcIIBMtl* was extremely weak (K_D = ~3.7 mM).⁷ The flexible linker was important to confine the two domains within an effective distance of 46 Å, enabling a facile intramolecular domain association.⁸,⁹ *TtIIAMtl* and *TtIIBMtl* do not have a connecting linker that facilitates the association. Instead, *TtIIAMtl* employs key interfacial residues to enhance the interaction with phosphorylated *TtIIBMtl*.¹⁰ Even though the structures were very similar, phospho-*TtIIBMtl* (K_D = ~120 µM) showed 14 times stronger affinity than free *TtIIBMtl* (K_D = ~1.7 mM). To understand the differential binding according to the phosphorylation state, structural and dynamics studies are necessary. In this study, we carried out the backbone chemical shift assignment using a suite of heteronuclear triple resonance NMR experiments. We obtained the secondary structures of phospho-*TtIIBMtl* based on the backbone dihedral angles derived from chemical shifts using TALOS+ program, which were consistent with the global fold of *EcIIBMtl*.

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Experimental Methods

Sample preparation - The gene TtIIB^Ml (385-477) was PCR-amplified from *Thermoanaerobacter tengcongensis* genomic with the following primers: TtIIB^Ml forward (5’- GTGCCACGGTTCTAAATGGATAACCCCTG CCGAAACTC), and TtIIB^Ml reverse (3’- GAGTTTCGCCAGGTTACATTTTGAAGCC GCGTGCCAC). The PCR product was digested with NcoI (Thermo Fisher Scientific) and XhoI (Thermo Fisher Scientific) restriction enzyme and subcloned into the modified pET-32a vector with N-terminal 6xHis-Trx tag separated by a TEV cleavage site. The phosphoryl residue, Cys-395, was mutated to Ser using the QuickChange kit (Agilent Technologies), and the new construct sequence was verified by DNA sequencing. The plasmid were introduced into *Escherichia coli* strain BL21star(DE3) (Invitrogen) cells for expression. Cells were grown in minimal medium supplemented by 15NH4Cl and 13C6-glucose, induced with 1 mM isopropyl-D-thiogalactopyranoside at an A600 of 0.7, and harvested by centrifugation after overnight of induction. The cell pellet was resuspended in 50 ml (per liter of culture) of 20 mM Tris, pH 7.4, 200 mM NaCl and 1 mM phenylmethylsulfonyl fluoride. The suspension was lysed by three passages through Emulsiflex (Avestin, Canada) after homogenizing and centrifuged at 24,000 g for 20 min at 4°C. The supernatant fraction was filtered and loaded onto a HisTrap nickel-Sepharose column and the fusion protein was eluted with a 100-ml gradient of imidazole (10–500mM). The protein was then dialyzed against 50 mM Tris-HCl, pH 8.0, 4mM β-mercaptoethanol and digested with TEV protease. The cleaved 6xHis-Trx tag was removed by loading the digested proteins over a nickel-Sepharose column. Relevant fractions were purified by Superdex 75 gel filtration column (GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 7.4, 200 mM NaCl and then on a monoS cation exchange column (8ml; GE Healthcare) with a 160-ml gradient of 1M NaCl. Phosphorylation of TtIIB^Ml was conducted as previous study. Phosphorylated proteins were finally concentrated in 20 mM Tris, pH 7.4. NMR samples contained 1mM [U-15N/13C]-phospho-TtIIB^Ml (C395S) in 20mM Tris, pH 7.4 and 90% H2O /10% D2O.

NMR experiments and structure calculation and secondary structure prediction - NMR spectra were collected at 298K on Bruker AVANCE 700MHz spectrometers equipped with a z-shielded gradient triple resonance cryoprobe. Spectra were processed using the NMRPipe program and analyzed using the PIPP/CAPP/STAPP program, or NMRView. Sequential assignment of 13C, 15N-labeled phospho-TtIIB^Ml protein was using 2D 15N-HSQC spectrum and 3D triple resonance through-bond scalar correlation experiments such as 3D HNCO, HNCACO, HNCA, HN(CO)CA, CBCA(CO)NH experiments. Overall secondary structure and the backbone dihedral torsion angles were predicted from TALOS+. Backbone assignment of phospho-TtIIB^Ml – Since the phosphoryl groups on cysteine were labile and slowly lost to buffer by acid-catalyzed dephosphorylation reactions, the active site Cys395 of TtIIB^Ml was mutated to serine which could be permanently phosphorylated as a stable form. TtIIB^Ml(C395S), consists of 92 amino acid residues with molecular weight of 10.6 kDa. We carried out a suite of heteronuclear triple resonance NMR spectroscopy. The 1H, 15N and 13C assignment obtained for phospho-TtIIB^Ml (C395S) are listed in Table 1. Backbone assignments for amide resonances were obtained for 82 residues out of 89 residues (92%), excluding three proline residues. The backbone amide resonances with missing assignment are between F462 and V467 that corresponds to a flexible loop in phospho-*EcIIB^Ml*. Overall secondary structure and the backbone dihedral torsion angles were predicted from TALOS+ based on combined HN, 15N, Cα, Cβ and CO backbone chemical shifts.
Backbone assignment of phosphorylated IIBMtl of T. tengcongensis

and conformational changes in the flexible loop. In comparison with amide peaks of the free TtIIBMtl, the residues near the active site phosphorylated Ser395 showed large chemical shift perturbations. The assignments are annotated with residue types and numbers in Figure 1. In summary, 92% of Cα, 93% of Cβ and 90% of CO were assigned and listed in Table 1.

Secondary structure of phospho-TtIIBMtl - Secondary structural information of phospho-TtIIBMtl was calculated by using TALOS+ program16 based on the 1H, 15N, Cα, Cβ and CO chemical shifts. The height of the bars indicates the probability of the secondary structure prediction (Fig. 2 upper panel). The secondary structure prediction showed that phospho-TtIIBMtl (C395S) is comprised of four β strands and three α-helices. Residue L390–S395 (β1), F418–A426 (β2), V436–T439 (β3) and K455–V459 (β4) constitute the β strands, and S401–E415 (α1), E441–V450 (α2) and F462–L475 (α3) constitute the α-helices. Phospho-EcIIBMtl is composed of central four beta strands flanked by three alpha helices. The results of backbone torsion angles (φ, ψ) are presented in Ramachandran plot (Figure 2 lower panel), 73 residues are in most favorable regions, and six residues are in allowed region, and three residues are in generously allowed regions. Clearly, the secondary structure prediction and torsion angle prediction data suggest that phospho-EcIIBMtl and phospho-TtIIBMtl (C395S) share a common backbone scaffold. Our results demonstrate that phospho-EcIIBMtl and phospho-TtIIBMtl exploit different strategy to accomplish an efficient binding to their partner IIA proteins. Small differences at the binding interface as well as variable dynamic nature are likely responsible for the disparate binding properties.

Figure 1. 1H–15N HSQC spectra of 13C/15N-labelled phospho-TtIIBMtl in 20mM TrisHCl pH 7.4, 298K. The backbone amide cross peaks are annotated by residue names and numbers. An expansion of the boxed region is provided in the upper left corner.
| Residue | HN  | N   | CA  | CB  | CO  |
|---------|-----|-----|-----|-----|-----|
| THR     | 7.835 | 112.5 | 60.8 | 70.3 | 174.29 |
| LEU     | 8.21 | 125.21 | 53.25 | 41.95 | 175.09 |
| PRO     | 62.83 | 32.76 | 427 VAL | 8.504 | 126.2 | 65.62 | 31.59 | 177.62 |
| LYS     | 9.405 | 119.8 | 57 | 33.78 | 173.92 |
| LEU     | 8.21 | 125.21 | 53.25 | 41.95 | 175.09 |
| SER     | 9.005 | 114.99 | 52.12 | 19.02 | 177.02 |
| ASN     | 8.278 | 113.23 | 54.7 | 37.61 | 175.8 |
| GLN     | 7.475 | 115.79 | 63.75 | 37.62 | 175.88 |
| SER     | 9.005 | 114.99 | 52.12 | 19.02 | 177.02 |
| ALA     | 8.579 | 124.94 | 55.7 | 38.21 | 174.47 |
| ASP     | 8.26 | 124.24 | 59.91 | 34.33 | 182.42 |
| HIS     | 8.893 | 125.19 | 55.03 | 38.21 | 174.47 |
| LEU     | 8.256 | 115.12 | 63.14 | 30.91 | 176.06 |

**Table 1.** Backbone HN, N, CA, Cβ, CO chemical shifts of phospho-T1IBmtl (unit: ppm)
Backbone assignment of phosphorylated IIB<sup>Mt</sup> of <i>T. tengcongensis</i>

| Residue | Backbone Assignment | ϕ (°) | ψ (°) | Δυ (°) | Δς (°) | ω (°) |
|---------|---------------------|-------|-------|--------|--------|-------|
| 464     | ASP                 | 471   | 8.313 | 121.36 | 62.25  | 39.24 |
| 465     | ASN                 | 472   | 8.756 | 120.92 | 67.3   | 31.45 |
| 466     | THR                 | 473   | 7.825 | 118.26 | 59.37  | 32.44 |
| 467     | VAL                 | 474   | 7.632 | 114.57 | 60.55  | 63.31 |
| 468     | TYR                 | 475   | 7.033 | 121.48 | 55.29  | 41.86 |
| 469     | ILE                 | 476   | 7.622 | 119.06 | 55.77  | 31.45 |
| 470     | GLU                 | 477   | 7.893 | 127.72 | 58.02  | 33.71 |

**Figure 2.** Predicted secondary structure (upper panel) and the backbone derived Ramachandran plot of phospho-TtIIBMt (lower panel). Predicted secondary structure (blue positive bar, beta-sheet; red negative bar, helix) for all residues are shown in the lower panel. The height of the bars reflects the probability of the neural network secondary structure prediction, and schematic representations of secondary structure are displayed above the prediction scores. (upper panel). Backbone torsion angles (ϕ, ψ) were predicted using TALOS<sup>+</sup> with backbone chemical shifts and plotted upon the Ramachandran plot (lower panel).

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