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Active site phosphoryl groups in the biphosphorylated phosphotransferase complex reveal dynamics in a millisecond time scale

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ABSTRACT

The N-terminal domain of Enzyme I (EIN) and phosphocarrier HPr can form a biphosphorylated complex when they are both phosphorylated by excess cellular phosphoenolpyruvate. Here we show that the electrostatic repulsion between the phosphoryl groups in the biphosphorylated complex results in characteristic dynamics at the active site in a millisecond time scale. The dynamics is localized to phospho-His15 and the stabilizing backbone amide groups of HPr, and does not impact on the phospho-His189 of EIN. The dynamics occurs with the k_{ex} of ~500 s^{-1} which compares to the phosphoryl transfer rate of ~850 s^{-1} between EIN and HPr. The conformational dynamics in HPr may be important for its phosphotransfer reactions with multiple partner proteins.

Structured summary of protein interactions:
EIN and **HPr** bind by nuclear magnetic resonance (View Interaction).

1. Introduction

The bacterial phosphotransferase system (PTS) catalyzes a series of phosphotransfer reactions coupled with sugar transport across the membrane [1,2]. Enzyme I and HPr form the first phosphotransfer protein complex in PTS, where the phosphoryl group is transferred from the active site His189 of Enzyme I to His15 of HPr in a reversible manner [3–5]. Enzyme I comprises an N-terminal domain (EIN) which is responsible for the phosphotransfer reaction with HPr, and a C-terminal domain which binds to phosphoenolpyruvate (PEP) for the auto-phosphorylation reaction. HPr relays the phosphoryl group from Enzyme I to sugar-specific membrane transporters, Enzymes II, which translocate and phosphorylate their sugar substrates [6].

The phosphorylation states of PTS proteins modulate their cellular protein–protein interactions and regulate gene expression for sugar metabolism and chemotaxis [5]. In the absence of external sugar substrates, high phosphotransfer potential of PEP keeps cellular PTS proteins in fully phosphorylated states [7,8]. The phosphorylated PTS proteins can still associate with each other to form a transient biphosphorylated complex, which possibly helps the PTS proteins to rapidly respond to the changes in the phosphorylation states of one another. We have recently reported the structure and the binding thermodynamics of the biphosphorylated complex between EIN and HPr [9]. We found that the biphosphorylated complex maintained the same backbone fold and binding interfaces as the unphosphorylated complex structure previously determined by NMR spectroscopy albeit with reduced affinity. The structure of the unphosphorylated complex can easily accommodate a phosphoryl group between the two active site histidine side chains to accomplish the phosphotransfer reaction [10]. In other words, placing two phosphoryl groups in the active site would cause a large electrostatic repulsion in the biphosphorylated complex. In this report, we examined how the active site of the biphosphorylated complex could accommodate two phosphoryl groups in close proximity. We show that the electrostatic repulsion is relieved by local dynamics at the active site of HPr at a millisecond time scale that is comparable to the phosphoryl transfer rate between EIN and HPr.

2. Materials and methods

2.1. Cloning, protein expression and purification

EIN and HPr were cloned into a pET11a vector without tags, and overexpressed in either Luria Bertini or minimal media with $^{15}NH_4Cl$ as the sole nitrogen source. Proteins were purified by DEAE anion exchange, G75 size exclusion, and monoQ anion exchange chromatography using an AKTA purification system (GE Healthcare) as previously described [9]. The fractions containing EIN or HPr were pooled and finally exchanged into the NMR buffer (20 mM Tris, pH 7.4).

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2.2. NMR spectroscopy

NMR samples contained 1 mM EIN or HPr in 20 mM Tris, pH 7.4. HPr was phosphorylated using 50 mM PEP, 5 mM $MgCl_2$, and 10 μM Enzyme I. To phosphorylate EIN, 10 μM HPr was used in addition as Enzyme I did not directly phosphorylate EIN. ^{31}P NMR spectra were obtained using an *x,y,z*-shielded gradient quadruple resonance probe at 30 °C. $^{1}H–^{15}N$ correlation spectra were collected using Bruker Avance 600 MHz NMR spectrometer equipped with *z*-shielded gradient triple resonance cryoprobe at 37 °C. Long-range $^{1}H–^{15}N$ heteronuclear single quantum correlation (HSQC) spectra to monitor the imidazole side chains of histidines were recorded with a 22-ms dephasing delay. Titration experiments were carried out as follows: 1 mM phosphorylated HPr was prepared using 50 mM PEP, 5 mM $MgCl_2$, and 10 μM Enzyme I. Then phosphorylated EIN was added into phosphorylated HPr in a stoichiometric manner. Titration of unphosphorylated HPr was carried out in a similar manner except for the absence of PEP. NMR data were processed using the NMRPipe program and analyzed using the NMRView program [11,12].

3. Results

3.1. Model of the active site of the biphosphorylated complex

To estimate how close the two phosphoryl groups would be apart in the biphosphorylated complex, we first modeled the active site structure of the biphosphorylated EIN–HPr complex. The solution structure of an unphosphorylated EIN–HPr complex has been previously determined by NMR, and the active site is shown in Fig. 1A [10]. The active site His189 of EIN accepts a phosphoryl group at its Nε2 atom which is buried in the unphosphorylated state. Phosphorylation of EIN requires a change in the side chain χ_2 angle of His189 from g^+ to g^- conformation to expose the Nε2 atom to solvent to accommodate the incoming phosphoryl group [13]. In this conformation, Nε2 of His189 from and Nδ1 of His15 from HPr are brought together so that minimal structural displacement at the active site can easily accomplish successful phosphotransfer reactions. When we added two phosphoryl groups to the active site of the complex in this conformation, the distance between Nε2 atom of His189 of EIN and Nδ1 atom of His15 of HPr was measured as ~6.9 Å, and the distance between two phosphorus atoms was ~5.9 Å (Fig. 1B). Considering that there are two negative charges on each phosphoryl group at pH 7.4, large electrostatic repulsion is anticipated between the neighboring phosphoryl groups at the active site. To examine whether the electrostatic repulsion causes one or both of the phosphoryl groups to move away from each other, we first monitored the chemical shift change of the phosphoryl groups upon complex formation using ^{31}P NMR spectroscopy.

3.2. Chemical shift changes of phosphoryl groups from ^{31}P NMR

We used ^{31}P NMR to directly monitor the phosphoryl groups at the active site before and after the complex formation. Figs. 2A and 2B show the ^{31}P NMR spectra of phosphorylated EIN and HPr. The ^{31}P NMR signals at 2.02 ppm and −1.16 ppm originate from inorganic phosphate and PEP, respectively, at pH 7.4 and 30 °C (referenced to phosphoric acid at 0 ppm). The ^{31}P NMR signal of Nδ1-phosphorylated HPr appeared at −3.77 ppm, and that of Nε2-phosphorylated EIN appeared at −5.15 ppm. When the biphosphorylated complex was formed, the ^{31}P NMR signal of the phosphorylated HPr split into two peaks at −3.13 ppm and −4.68 ppm, with significant line-broadening (Fig. 2C). This is characteristic of an

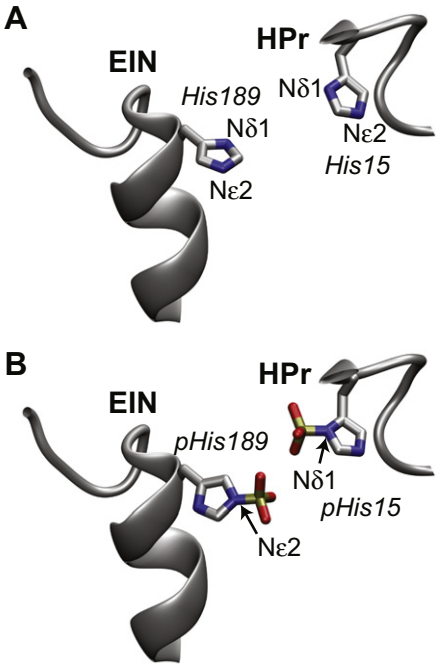

Fig. 1. (A) The three-dimensional structure of the active site of the unphosphorylated complex between EIN and HPr determined by NMR spectroscopy (PDB code: 3EZA), [10] and (B) a model structure of the active site of the biphosphorylated complex. Only the side chains of active site His189 in EIN and His15 in HPr were drawn in stick model. The side-chain atoms are *color-coded* according to atom types: carbon (*gray*), nitrogen (*blue*), oxygen (*red*), and phosphorus (*yellow*).

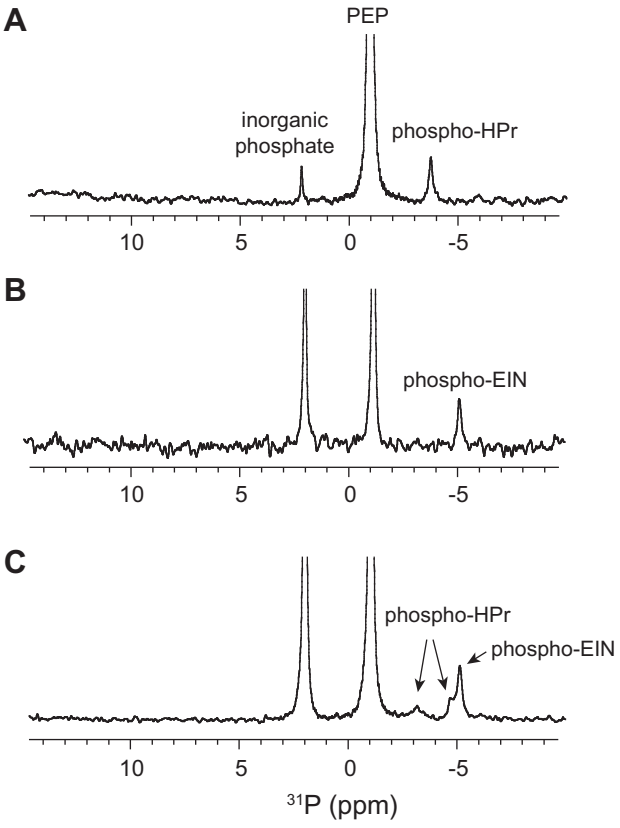

Fig. 2. ^{31}P NMR spectra of (A) 1 mM of phosphorylated HPr, (B) 1 mM of phosphorylated EIN, and (C) 1 mM of phosphorylated HPr with 1 mM of phosphorylated EIN. 10 μM of Enzyme I, 5 mM $MgCl_2$ as well as 50 mM PEP were used to keep the HPr and EIN in fully phosphorylated states during the measurement.