The molecular basis of phosphite and hypophosphite recognition by ABC-transporters

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Inorganic phosphate is the major bioavailable form of the essential nutrient phosphorus. However, the concentration of phosphate in most natural habitats is low enough to limit microbial growth. Under phosphate-depleted conditions some bacteria utilise phosphite and hypophosphite as alternative sources of phosphorus, but the molecular basis of reduced phosphorus acquisition from the environment is not fully understood. Here, we present crystal structures and ligand binding affinities of periplasmic binding proteins from bacterial phosphite and hypophosphite ATP-binding cassette transporters. We reveal that phosphite and hypophosphite specificity results from a combination of steric selection and the presence of a P-H...π interaction between the ligand and a conserved aromatic residue in the ligand-binding pocket. The characterisation of high affinity and specific transporters has implications for the marine phosphorus redox cycle, and might aid the use of phosphite as an alternative phosphorus source in biotechnological, industrial and agricultural applications.
Phosphorus (P), a component of nucleic acids, nucleotide cofactors and phospholipids, is essential to cell structure, metabolism and signalling. In nature it is mainly present in its most oxidised state (P valence = +5) predominantly as inorganic phosphate, which is the only form that cells can utilise directly for incorporation into biological molecules. However, phosphate availability can vary significantly in natural environments and despite the presence of high-affinity phosphate uptake systems, in many ecosystems is sufficiently depleted to limit microbial growth. For example, phosphate concentrations in the oligotrophic surface waters of the vast ocean gyres can be extremely low, influencing the biogeochemistry and productivity of marine microorganisms. When phosphate is unavailable, some bacteria have evolved mechanisms to metabolise reduced phosphorus compounds to acquire P. ATP-binding cassette (ABC) transporters for the active uptake of these compounds are encoded in the genomes of a range of microorganisms, with the transporter genes usually arranged in an operon with genes encoding enzymes that catalyse oxidation of the imported species to phosphate (Supplementary Fig. 1). Phosphonates (P valence = +3) are a diverse range of organic C-P bond containing compounds ubiquitous in natural habitats. There are multiple mechanisms for their breakdown, the most common of which is the carbon-phosphorus (C-P) lyase, which converts phosphonates to a hydrocarbon and inorganic phosphate.

The crystal structure and ligand binding properties of the periplasmic binding protein (PBP) subunit from an Escherichia coli C-P lyase linked organophosphonate transporter have been reported previously, here we focus on the uptake of the alternative inorganic reduced phosphorus compounds phosphite (HPO₃H−; P valence = +3) and hypophosphite (H₂PO₃−; P valence = +1).

The concentration of phosphate and hypophosphite in many natural environments is unknown, but they have been detected in up to micromolar amounts in some aquatic systems, where they can account for up to a third of the total dissolved P. The major sources of phosphate and hypophosphite are likely anthropogenic in origin (e.g. fungicides in agriculture), but they may also be derived from geothermal and biological processes. Many bacterial species are capable of utilising phosphite as a P source by oxidising it to phosphate (assimilatory phosphate oxidation) (Supplementary Fig. 1). The delta-proteobacteria Desulfotignum phosphitoxidans can also use phosphite as an electron donor and energy source by coupling its oxidation to the reduction of sulphate, CO₂ or nitrate (dissimilatory phosphate oxidation). Phosphite is highly water-soluble and kinetically stable, and its oxidation by the NAD-phosphate oxidoreductase/phosphite dehydrogenase (PtxD) is highly exergonic (the reduction potential of phosphate/phosphite couple at pH 7 = −650 mV resulting in a ΔG = 63.7 kJ mol⁻¹) and regenerates reductant in the form of NAD(P)H.

Results

Ligand specificity of recombinant binding proteins. We over-produced and purified PBPs of three putative phosphate transporters; PtxB from the globally important marine diazotroph Trichodesmium erythraeum IMS101 (Ps_PtxB) and PtxB from the soil bacterium Pseudomonas stutzeri WM88 (Ps_PtxB), as well as a PBP from a putative hypophosphite transporter, HtxB, also from P. stutzeri WM88 (HtxB) (Supplementary Fig. 2). The binding affinity of each protein for a range of P ligands was measured by microscale thermophoresis (MST) using a RED-tris-NTA label (NanoTemper Technologies GmbH) via the C-terminal His-tag (Table 1; Supplementary Fig. 3). Dyes conjugated to the tris-NTA moiety fluorescently label proteins. Dissociation constants (Kᵅ) are derived from three independent MST experiments and errors are inexpensive and, when provided as the sole source of P, it allows PtxD to be used as a selective marker, which has resulted in its use in numerous biotechnological and agricultural applications.

Hypophosphite can also be used as a sole P source by some microorganisms. The hypophosphite/2-oxoglutamate dioxygenase (HtxA) oxidises hypophosphite to phosphate (reduction potential at pH 7 = −740 mV) coupled to the oxidative decarboxylation of 2-oxoglutarate to succinate; the phosphate produced is subsequently further oxidised to phosphate by PtxD.

The energetically favourable oxidation of phosphite and hypophosphite, yielding phosphate and NADPH, makes them attractive P sources to microorganisms under phosphate-limited conditions; however, the potentially low environmental concentration of these compounds necessitates the need for high-affinity transporters. While the genetic and biochemical basis of phosphite and hypophosphite utilisation and the PtxD and HtxA enzymes have been characterised previously, the mechanism of high-affinity ligand binding by the cognate ABC transporters is not well studied. The use of non-physiologically high concentrations (up to 1 mM) of P ligands in previous in vivo studies has hindered clear interpretation of the physiologically relevant specificity of reduced P compound transporters. Here we elucidate the molecular basis of high-affinity phosphite and hypophosphite acquisition by determining the ligand-binding affinity and structure of the PBPs of phosphite and hypophosphite ABC transporters from environmental microorganisms. Our results reveal how a ligand-protein P-... interaction coupled with steric selection forms the basis of ligand specificity, explaining how these transporters allow bacteria to scavenge these alternative sources of P with ultra-high affinity.

Table 1 Microscale thermophoresis determined dissociation constants (Kᵅ) of Te_PtxB, Ps_PtxB, Pm_PhnD and HtxB for selected P-ligands

| Ligand               | Kᵅ (μM) | Te_PtxB | Ps_PtxB | Pm_PhnD | HtxB   |
|----------------------|---------|---------|---------|---------|--------|
| Phosphate            | 46.80 ± 3.45 | 159.5 ± 36.0 | 182.2 ± 26.3 | NBD     |
| Phosphate            | 0.17 ± 0.07  | 0.24 ± 0.03  | 0.051 ± 0.004 | >10 x 10³ |
| Methylphosphonate    | 30.63 ± 2.48 | 40.64 ± 5.70 | 108.8 ± 8.3  | NBD     |
| Ethylphosphonate     | >60 x 10³    | —        | 111.9 ± 8.7  | NBD     |
| 2-Aminophosphonate   | NBD       | NBD      | NBD      | —       |
| Hypophosphite        | 2.21 ± 0.16 | —        | —        | 0.56 ± 0.11 |

Kᵅ values presented are derived from fitting three independent experiments to Eq. (1) and errors reported as ± the estimated standard deviation of the Kᵅ. NBD indicates no binding was detected up to a ligand concentration of 10 mM; — indicates Kᵅ not measured.
reported as ± the estimated standard deviation of the $K_d$. Te_PtxB, Pm_PhnD and Ps_PtxB all bound phosphate with a nanomolar $K_d$ in the range of ~50–240 nM (Table 1; Supplementary Fig. 3). In contrast, all three proteins bound methylphosphonate (MPn) and phosphate less tightly, with dissociation constants between ~30–100 and ~50–180 μM, respectively (Table 1; Supplementary Fig. 3); these values are an order of magnitude higher than those reported previously by Feingersch et al.35. The proposed MST-derived binding affinities for Pm_PhnD were similar to those reported previously by Feingersch et al.35. The proposed hypophosphate binding, HtxB, bound hypophosphate with high affinity ($K_d = 560 ± 11$ nM), with some evidence of phosphate binding in the millimolar range but no binding detected for phosphate (Supplementary Fig. 5) or methylphosphonate up to a concentration of 10 mM (Table 1). These data show that at environmentally relevant concentrations Te_PtxB, Pm_PhnD and Ps_PtxB clearly favour phosphate as a ligand, and HtxB appears to be specific for hypophosphate.

### Phosphate binding involves a P-H...π interaction.

To determine the structural mechanism of phosphate selectivity we co-crystallised Te_PtxB and Pm_PhnD with phosphate resulting in high-resolution structures (1.95 and 1.46 Å respectively) of the closed, ligand-bound complexes (Table 2). Te_PtxB and Pm_PhnD are both typical type II PBPs38, with two lobes separated by a hinge region surrounding a buried, enclosed central cavity that forms the ligand-binding pocket (Fig. 1a, b; Supplementary Figs. 6–9) (RMSD ca: ~1.7 Å). In the closed form, the amino acids that form the walls of the binding pocket pack tightly against the phosphate moiety and a single water molecule that is also buried in the binding pocket. Each oxygen atom of the phosphate contributes to an extensive hydrogen bonding network to the main chain and side chains of a cluster of conserved residues from lobe 2 (Te_PtxB; Y55, Y100, S130, T131, S132 and H160; Pm_PhnD; Y46, S126, T127, S128, H138 and D205, Figs. 1d, e and 2e, f). In Pm_PhnD, the interaction of the D205 carboxylate with the ligand suggests that, under the crystallisation conditions used (pH 8), mono-anionic (HPO$_3^{2-}$) rather than di-anionic (HPO$_4^{3-}$) phosphate is the predominantly bound form. In Te_PtxB, it is not possible to determine the protonation state of the phosphate from the pattern of hydrogen bonding but given the pH of the crystallisation experiment (pH 4.2), it is also likely that the mono-anionic form of phosphate is bound (phosphate pK$_a$ 1.5, 6.736). Both proteins contain a conserved histidine as one of the phosphate

| Data collection | Te_PtxB/phosphate (PDB: 5JVB) | Te_PtxB/MPn (PDB: 5LQ1) | Pm_PhnD/phosphate (PDB: 5LQ5) | Pm_PhnD/MPn (PDB: 5LQ8) | Ps_PtxB apo (PDB: 5O2K) |
|----------------|-------------------------------|--------------------------|-------------------------------|--------------------------|--------------------------|
| Wavelength (Å) | 0.96861                       | 0.97629                  | 0.97951                       | 0.97951                  | 0.97951                  |
| Beamline       | i24                           | i03                      | i24                           | i24                      | i04                      |
| Resolution (Å) | 47.96–1.95 (2.00–1.95)         | 65.05–1.41 (1.43–1.41)    | 44.27–1.46 (1.49–1.46)        | 44.0–1.52 (1.55–1.52)     | 79.75–2.1 (2.14–2.1)     |
| Space group    | P2                             | P1                       | P2                            | P2                       | P2                       |
| Unit cell (a, b, c, α, β, γ) | 54.34, 69.31, 66.53, 90, 93.07, 90 | 39.48, 53.70, 66.39, 84.82, 93.07, 69.74 | 46.18, 57.41, 54.57, 107.1, 90 | 45.86, 57.08, 54.17, 109.0, 106.56, 90 | 87.947, 136.640, 90.844, 90, 115.022, 90 |
| Total reflections | 125,055 (9783)                  | 302,564 (10,007)          | 162,732 (4664)                | 88,958 (3373)            | 762,654 (37,446)         |
| Unique reflections | 35,004 (2628)                  | 91,920 (3601)             | 46,137 (1859)                 | 40,089 (1854)            | 113,030 (5565)           |
| Multiplicity    | 3.6 (3.7)                      | 3.0 (2.8)                 | 3.5 (2.5)                     | 2.2 (1.8)                | 6.7 (6.7)                |
| Completeness (%) | 97.2 (98.9)                     | 95.2 (75.4)               | 97.3 (78.4)                   | 97.0 (90.4)              | 99.9 (98.7)              |
| Mean I/σ(I)     | 7.3 (1.3)                      | 9.1 (1.1)                 | 15.8 (1.1)                    | 16.2 (1.2)               | 6.3 (1.2)                |
| CC$_{merge}$     | 0.994 (0.554)                  | 0.999 (0.48)              | 0.999 (0.569)                 | 0.997 (0.679)            | 0.992 (0.535)            |
| R$_{merge}$      | 0.081 (0.825)                  | 0.044 (0.813)             | 0.035 (0.934)                 | 0.021 (0.434)            | 0.174 (1.408)            |
| R$_{free}$       | 0.074 (0.725)                  | 0.032 (0.664)             | 0.021 (0.670)                 | 0.021 (0.431)            | 0.078 (0.625)            |
| No. of non-H atoms | 3925                          | 4016                     | 2225                          | 2192                     | 12,071                   |
| Protein         | 3925                          | 4016                     | 1.01/0.230                    | 0.146/0.180              | 0.158/0.207              |
| Ligands         | 8                             | 18                       | 4                             | 2225                     | 2192                     |
| Water           | 41                            | 213                      | 129                           | 91                       | 300                      |
| Protein residues | 504 (2 chains)                 | 514 (2 chains)            | 273 (1 chain)                 | 271 (1 chain)            | 1562 (6 chains)          |
| Average B-factors | 1.48                       | 1.51                     | 1.47                          | 1.47                     | 1.47                     |
| Main chain/side chain | 40.5/45.8                   | 19.1/28.6                | 25.6/38.0                     | 29.7/40.2                | 34.9/38.6                |
| Ligands/solvent | 26.9/36.6                     | 20.8/26.8                | 16.4/33.8                     | 25.2/34.2                | - /2.76                 |
| Ramachandran    | 97.42/100                     | 97.07/100                | 98.81/100                     | 97.78/100                | 90.56/100                |
| Molprobity score | 1.17 (100th)                  | 1.01 (99th)               | 1.08 (99th)                   | 1.05 (99th)              | 1.47 (98th)              |
| Refinement      | R$_{factor}$/R$_{free}$ | 0.215/0.267              | 0.108/0.230                    | 0.0127                    | 0.0121                    |
| RMSD bonds      | 0.0106                        | 0.0125                    | 0.0127                        | 0.0127                    | 0.0121                    |
| RMSD angles     | 1.48                          | 1.51                      | 1.47                          | 1.52                     | 1.53                     |

| MPn methylphosphonate |
|-----------------------|
| aValues in parenthesis are for data in the high-resolution shell |
| bR$_{merge}$ = Σ|I$_i$| Σ|I$_i$| /|Σ|I$_i$| - |Σ|I$_i$| /|Σ|I$_i$| , where |I$_i$| and |I$_i$| are the observed intensity and mean intensity of related reflections, respectively |
ligands (Te_PtxB; H160 and Pm_PhnD; H158), which could help stabilise the negative charge on the phosphite. However, in Te_PtxB, one imidazole nitrogen atom acts as a hydrogen bond acceptor to a mainchain N-H (S178), suggesting that the histidine is neutral. In Pm_PhnD the equivalent interaction is made to a buried water molecule outside of the binding pocket and the hydrogen bonding network around this water molecule is not sufficient to determine the charge on the histidine.

In both structures, the hydrogen atom at the R1 position of the phosphite points towards lobe 1, packing against the face of the aromatic ring of a tyrosine residue (Te_PtxB; Y208, Pm_PhnD; Y206). This tyrosine sidechain forms a cap that is stabilised by intramolecular hydrogen bonds to residues on lobe 1 (Te_PtxB; D22, Pm_PhnD; D13 and N176), which in the closed conformation, contributes to the network of interactions that connect both lobes of the protein and bury the binding pocket from solvent (Fig. 2a, b, e, f). The distance between the R1 hydrogen and the plane of the aromatic ring of this tyrosine is ~2.6 Å, which is less than the sum of the expected van der Waals radii and, as the P-H bond also impinges upon the π system at an angle of ~140°, this...
Fig. 2 A detailed comparison of the spatial arrangement of the binding pocket and network of interactions around the ligand. The interior surface (partially transparent, atom colours) of the binding pocket and surrounding residues (sticks) in a Te_PtxB; b Pm_PhnD; c HtxB and d E. coli PhnD (PDB:3P7I). In each case, the capping residues are highlighted with a dotted surface that represents the van der Waals radii of the atoms. The external surface is also shown in each case, demonstrating how the binding pocket in reduced phosphorus binding PBPs (a–c) is buried from the external solvent and in each case is much smaller than that of the phosphonate binding PhnD from E. coli (d). The protein backbone is drawn as a cartoon, with sidechains drawn in sticks and hydrogen bonds between the capping residues indicated by black dashes. Each figure (a–d) is accompanied by a schematic (e–h) that displays the hydrogen-bonding network between the protein and the oxygen atoms of the ligand. The 2-aminoethyl group of 2AEPn is abbreviated to 2AE in h.
Interestingly, the tyrosine to alanine mutation made little difference to binding affinity, suggesting that phosphate may interact weakly with the protein in a partially open state, with few, if any interactions with the tyrosine side chain. The Y208F mutation essentially abolished phosphate binding, again showing the importance of the inter domain hydrogen bond network. Overall, this suggests that the tyrosine cap is a pre-requisite to achieve a fully closed state of the protein that engulfs the phosphate within the binding pocket and acts as a steric barrier for the binding of ligands with bulkier R groups, providing an explanation for the observed specificity of Te_PtxB and Pm_PhnD for phosphate.

Te_PtxB and Pm_PhnD have 2–4 orders of magnitude higher binding affinity for phosphate than phosphonates (Table 1), but whilst attempts to crystallise complexes of either protein with ethylphosphonate (EPn), 2AEPn or phosphate were unsuccessful, structures of both proteins in complex with methylphosphonate (MPn) were obtained (Table 2; Fig. 3). The overall fold of the Te_PtxB and Pm_PhnD complexes with MPn were very similar to the comparative complexes with phosphate (RMSD ca. ~0.3 Å) and since both MPn complexes were determined at high resolution (1.41–1.52 Å), the electron density for the ligand could be confidently interpreted within the binding pocket, with the MPn refining unequivocally in only one orientation (Supplementary Table 1). The only difference in chemical structure between phosphate and MPn is an exchange of the R1 hydrogen in phosphate for a methyl group in MPn. Given this similarity, it was unsurprising that the three oxygen atoms and the phosphorus atom of the MPn bind in the same position as those of the phosphate, with the methyl group of the MPn pointing in the same direction as the R1 hydrogen of the phosphate, towards the capping tyrosine residue (Y208/Y206). To accommodate the methyl group of the MPn the tyrosine moves in both structures by ~0.5 Å, compared to the complexes with phosphate, resulting in an ~16% expansion in the volume of the binding pocket (Supplementary Table 1). Despite this movement, the methyl-carbon atom of the MPn lies ~3.0 Å from the plane of the tyrosine ring in both structures. This might suggest that a C-H...π interaction is present, however the relative geometry of the two partners is sub-optimal for this type of interaction.41 Thermal ellipsoids were calculated from the anisotropic B-factors for the three structures that were better than 1.5 Å resolution (Te_PtxB and Pm_PhnD with MPn and Pm_PhnD with phosphate). These show an elongation of the ellipsoids for the tyrosine atoms in the MPn complexes, compared to those of in the phosphate complex, suggesting a degree of instability in the position of the tyrosine next to the methyl group of the MPn (Supplementary Fig. 14). Since Te_PtxB and Pm_PhnD have a significantly lower binding affinity for MPn than for phosphate (100- and 2000-fold, respectively), this suggests that the ~0.5 Å movement of the capping tyrosine may alter the network of hydrogen bonds that stabilise the closed, ligand-bound conformation. To explore this, we measured the binding affinity of the Y208A and Y208F mutants of Te_PtxB for MPn. The Ala substitution weakened the binding of MPn by a factor of 10, whilst the Phe mutation essentially abolished binding of MPn (Table 3 and Supplementary Fig. 11). This suggests that by swapping the bulky tyrosine for an alanine, the steric barrier provided by the Tyr is removed, providing space in the binding pocket for the methyl group of MPn. However, as the alanine is unable to form bridging hydrogen bonds to stabilise the closed conformation, the binding of MPn is weaker. CD shows that Y208F mutant is folded (Supplementary Fig. 10), however, we could not detect binding of MPn, suggesting that this amino-acid substitution blocks the closed ligand-bound complex from forming. Taken together, the exquisite selectivity of PtxB for phosphate can be explained not only by the favourable P-H...π interaction but also by the strongly stabilising hydrogen bonding network that engages the tyrosine cap.

Fig. 3 A comparison of phosphate and methylphosphonate binding in Te_PtxB and Pm_PhnD. a Superposition of the complexes of Te_PtxB (grey) with phosphate (white hydrogen) and methylphosphonate (MPn; yellow methyl) showing that the residues around the binding pocket are in consistent positions in each structure apart from the capping tyrosine which moves by ~0.5 Å in the MPn complex (Y208, yellow) in response to the larger van der Waals radii of the methyl group of the ligand. b Superposition of the phosphate (white hydrogen) and MPn (cyan methyl) complexes with Pm_PhnD (blue) showing a similar movement of the capping tyrosine (Y206, cyan).
Ligand binding induces a large conformational change in PtxB.

Determining an apo-structure of a PBP from this family of ABC transporters is difficult due to the inherent flexibility of the hinge region between the two domains, and it has only been achieved previously with PhnD from *E. coli* (PDB:3S4U), which was locked in an open conformation by substituting H157 with an alanine14. In our study, numerous attempts to crystallise apo forms of Te_PtxB and Pm_PhnD were unsuccessful, but a 2.1 Å resolution structure of *Pseudomonas stutzeri* PtxB (Ps_PtxB) in an open conformation was determined (Table 2). We also obtained structures of phosphate and MPn complexed Ps_PtxB (Table 4; Fig. 4) and determined the associated ligand affinities (Table 1); both the structures and binding data were similar to the other phosphate-binding proteins discussed previously. Analysis of the conformational change between the open and closed, phosphate-bound structure of Ps_PtxB using DynDom42 revealed that domain closure is induced by the estimated standard deviation of the $K_d$ NBD indicates no binding was detected up to a ligand concentration of 10 mM; − indicates $K_d$ not measured.

Table 3 Microscale thermophoresis determined dissociation constants ($K_d$) of Te_PtxB and HtxB binding pocket mutants

| Ligand         | $K_d$ (µM) |
|----------------|------------|
| Phosphate      | 46.80 ± 3.45 | 51.27 ± 1.76 |
| Phosphate      | 0.17 ± 0.07  | 134.5 ± 6.4  |
| Methylphosphonate | 30.63 ± 2.48  | 255.4 ± 20.2  |
| Hypophosphite  | 2.21 ± 0.16  | 5.6 ± 0.11    |

$K_d$ values presented are derived from fitting three independent experiments to Eq. (1) and errors reported as ± the estimated standard deviation of the $K_d$. NBD indicates no binding was detected up to a ligand concentration of 10 mM; − indicates $K_d$ not measured.

Table 4 Data collection and refinement statistics part 2

|            | Ps_PtxB/phosphate (PDB:5O2J) | Ps_PtxB/MPn (PDB:5O37) | HtxB/hypophosphate (PDB: SME4) | Pm_PtxB/phosphate (PDB: SLV1) |
|------------|-------------------------------|------------------------|--------------------------------|-------------------------------|
| Data collection |
| Wavelength (Å) | 0.92819 | 0.92819 | 0.97951 | 0.92819 |
| Beamline | i04 | i04 | i04 | i04 |
| Resolution | 3.25-1.52 (1.55-1.52) | 3.61-1.37 (1.39-1.37) | 2.81-1.52 (1.55-1.52) | 2.22-1.22 (2.16-2.12) |
| Space group | P2,2,2 | P2,2,2 | P2,2,2 | P2,2,2 |
| Unit cell (Å) | 113.21, 39.03, 63.52, 90, 90, 90 | 112.8, 39.12, 63.3, 90, 90, 90 | 40.24, 55.21, 125.2, 90, 90, 90 | 152.175, 152.175, 67.915, 90, 90, 120 |
| Total reflections | 548,602 (22,254) | 672,723 (21,736) | 303,161 (11,860) | 982,237 (39,470) |
| Unique reflections | 44,234 (2169) | 59,851 (2940) | 42,787 (2186) | 51,504 (2522) |
| Multiplicity | 12.4 (10.3) | 17.1 (10.3) | 7.1 (6.0) | 9.1 (15.7) |
| Completeness (%) | 97.28/100 | 97.3/100 | 96.48/100 | 98.09/100 |
| Mean | 11.9 (1.2) | 16.8 (1.2) | 9.8 (1.1) | 7.7 (1.4) |
| Mean intensity | 0.999 (0.510) | 1.0 (0.485) | 0.998 (0.525) | 0.996 (0.648) |
| Rmerge | 0.017 (1.774) | 0.069 (1.463) | 0.117 (1.209) | 0.36 (2.82) |
| Protein | 0.039 (0.607) | 0.022 (0.615) | 0.05 (0.553) | 0.086 (0.733) |
| Refinement |
| $R_{factor}/R_{free}$ | 0.159/0.189 | 0.138/0.177 | 0.167/0.198 | 0.218/0.264 |
| RMSD bonds | 0.0131 | 0.015 | 0.0108 | 0.0112 |
| RMSD angles | 1.48 | 1.5 | 1.45 | 1.47 |
| No. of non-H atoms |
| Protein | 1998 | 2038 | 2034 | 6010 |
| Ligands | 5 | 5 | 6 | 12 |
| Water | 193 | 225 | 175 | 123 |
| Protein residues | 259 (1 chain) | 261 (1 chain) | 255 (1 chain) | 789 (3 chains) |
| Average B-factors |
| Main chain/side chain | 17.8/26.0 | 17.1/24.1 | 18.1/24.4 | 28.7/32.2 |
| Ligands/solvent | 21.3/32.1 | 11.0/32.0 | 31.8/31.6 | 19.3/25.9 |
| Ramachandran favoured/allowed (%) | 97.28/100 | 97.3/100 | 96.48/100 | 98.09/100 |
| Molpobity score | 0.73 (99th percentile) | 1.05 (99th percentile) | 1.18 (97th percentile) | 0.78 (100th percentile) |

MPn methylphosphonate

$a$Values in parenthesis are for data in the high-resolution shell

$b$Ramachandran = $\Sigma l_{a/b} - l_{a/b} - l_{a/b} - l_{a/b}$, where $l_{a/b}$ and $l_{a/b}$ are the observed intensity and mean intensity of related reflections, respectively

Interaction between the phosphate and the protein, but also by a combination of a steric requirement for a hydrogen at the R1 position of the ligand and a strengthening network of hydrogen bonds around the binding pocket that stabilise the closed, phosphate-bound state.
and closed conformations shows that lobe 2 (residues 96–84 and 202–255, 1.55 Å RMSD cα) shows some differences between the open and ligand-bound states. The positions of Y94, which hydrogen bonds to one of the phosphite oxygen atoms in the closed structure, and D16, which hydrogen bonds to and stabilises the capping tyrosine in the closed structure, move ~7 Å and ~4.5 Å (cα–cα), respectively, on ligand binding. Y94 resides on the hinge region, the conformation of which alters substantially during domain closure, whilst D16 sits on a flexible loop that latches over the binding pocket in the closed conformation. In addition, the B-factors of the core residues in lobe 2 are much lower in the closed, ligand bound, conformation compared to those in the open structure, implying lower overall flexibility when phosphite is bound to the protein (Supplementary Fig. 15).

HtxB binds hypophosphite using a similar P-H...π interaction. The structure of HtxB in complex with hypophosphite (Table 4) shows that the protein adopts the same closed conformation as seen in the complex of Te_PtxB with phosphite (RMSD cα 1.86 Å) (Fig. 1c, f). The ligand-binding pocket is very similar, with two of the hypophosphate oxygen atoms (O1 and O2) adopting the same position and making the same interaction as the equivalent oxygen atoms in the Te_PtxB/phosphite complex (Fig. 1f, h). However, H160, Y55 and S130, which all make interactions with O3 of the phosphite in Te_PtxB are replaced in HtxB with F158, W52 and N128, respectively. This has the effect of removing the hydrogen bonding potential around the O3 site, reducing the volume of the binding pocket by ~36 % (Fig. 2c, g; Supplementary Table 1) and providing steric selectivity for hypophosphite (two oxygen substituents) over phosphite (three oxygen substituents). Consistent with this, we observed only negligible levels of phosphite binding to HtxB in our MST assays (Table 1). Conversely, hypophosphite binds to Te_PtxB with low micromolar affinity (Ka ~2 μM), indicating that presumably its smaller size can be accommodated within the Te_PtxB binding pocket, albeit with suboptimal interactions.

The changes in the ligand-binding pocket also extend to the capping residue, Y208, which in Te_PtxB provides the π system for the P-H...π interaction. In HtxB, a buried ion pair occurs in this region of the structure (D206 from lobe 1 and R178 from lobe 2), which connects the two lobes of the protein when it is in the closed conformation (Fig. 1f). Nevertheless, in HtxB the P-H...π interaction between the ligand and the protein is conserved by the presence of a tryptophan residue, W52 (Fig. 1h). The aromatic ring system of this tryptophan is positioned adjacent to the hydrogen at the R2 position of hypophosphite and is in the appropriate orientation for a P-H...π interaction to occur (~2.6 Å, 140°), in the same way as seen in the phosphate-binding proteins (Fig. 1f). Replacing this tryptophan with a phenylalanine or tyrosine substantially reduces the binding affinity of HtxB for hypophosphite by 50-fold and 250-fold, respectively (Table 3; Supplementary Fig. 11). The smaller size of both sidechains would clearly alter the packing within this region of the binding pocket and may not provide a large enough platform for the R2 hydrogen of the hypophosphite to pack against. The lower binding affinity in the tyrosine mutant may be due to its hydroxyl group interfering with Phe158 on the adjacent side of the binding pocket, possibly blocking closure of the protein around its substrate. Changing the tryptophan to an alanine results in the complete abolition of binding, suggesting the packing interactions between the tryptophan, nearby residues and the ligand are critical for high-affinity binding of hypophosphite. Therefore, we have demonstrated that in both phosphite and hypophosphite binding proteins, positioning the π electron system of an aromatic amino acid adjacent to the P-H atom of the ligand is a conserved molecular mechanism governing specificity of the PBPs for reduced phosphorus ligands.

Structural comparisons with phosphonate binding PBPs. A phylogenetic tree of members of the phosphonate/phosphite/phosphate periplasmic substrate binding protein family (KEGG orthology K02044; InterPro Family IPR005770; Pfam12974) shows they cluster into five main sub-groups based on primary sequence (Fig. 5a). These groups are: (1) C-P lyase-linked phosphonate transporters (PhnD); (2) phosphite dehydrogenase (PtxD)-linked phosphite transporters (PtxB); (3) the PhnD C-P lyase enzyme; (4) phosphite transporters (HtxB); and (5) transporters we have referred to as 'hybrid' as they share sequence features with both PtxB and HtxB. We have determined the structure of three proteins from group 2 (Te_PtxB, Pm_PtxB and Ps_PtxB) and proteins from groups 3 (Pm_PhnD) and 4 (HtxB). From structure-based sequence alignments of representative members of these groups it is clear that the residues imparting phosphite and hypophosphite specificity are strictly conserved (Fig. 5b). The only
having homologues of PhnYZ, which have been shown to oxidise
Pseudomonas aeruginosa ligand14, 15, 35. This D/T/E cap is conserved in many other
contributing to the low nanomolar binding af
Ec_PhnD (Fig. 2d, h). These changes result in Ec_PhnD having
conserved, the capping residues that confer phosphite speci
whilst the residues that interact with the ligand oxygen atoms are
bond acceptors to the amino group of the 2AEPn (Fig. 2d),
a larger binding pocket required for phosphonate binding,
other proteins from this family with known structures are C-P
lyase-linked phosphonate binding PhnDs (yellow), HtxB homologues (green) and a putative 'hybrid' class of transporters (red). Proteins characterised in
this study are marked with an asterisk and the scale bar represents the number of substitutions per site. 
 Sequence alignment of reduced phosphorus compound binding PBPs, coloured as in a. 2-Aminoethylphosphonate-specific cap residues are highlighted yellow, phosphate-specific cap residues are highlighted grey (PtxB) or purple (PhnD) and hypophosphate-specific cap residues are highlighted green. Conserved residues that form H-bonds with ligand oxygen atoms are highlighted in blue. The position in the alignment is shown above the sequence and residue numbering for each protein at row ends. For residue numbering, the red (or orange, see below) value corresponds to the residue numbering of the structures, as the predicted N-terminal signal peptides were not present in recombinant proteins, and the black number in parentheses is the residue number of the unprocessed proteins. The second residue (following the initiator Met1) in the recombinant proteins used in this study is shown in red and bold in the sequence; in the case of proteins not
directly studied here, the equivalent residues/residue numbers are shown in orange. For full details of the proteins included in this figure see Supplementary Table 3.

**Prochlorococcus ecotypes with two phosphite transporters.**

Prochlorococcus is abundant in low phosphate ocean regions but
cannot grow on phosphonates12, 35, 43 despite some ecotypes
having homologues of PhnYZ, which have been shown to oxidise
2AEpn in vitro14, 45; therefore, phosphite could be an important P
source to this globally significant oceanic bacteria. The gene
encoding Pm_PhD in *P. marinus* MIT9301i is not responsive to
P-limitation and is not genomically linked to known phosphite or
phosphonate utilisation genes12, 43, 46, nevertheless, we and
others35 have shown that it binds phosphite with high affinity. Some *P. marinus* ecotypes also have a *ptxABCD* operon, encoding a PtxABC-transporter and a phosphite dehydrogenase12, indicating that these bacteria have a second phosphite uptake system. Based on protein phylogeny (Fig. 5a and ref. 12), these two types of phosphite binding protein not only branch separately from C-P lyase-linked PhnD and HtxB homologues, but also from each other, indicating that they represent phylogenetically separate phosphite transporters. Conversely to the phosphite-specific PtxB proteins discussed above, the *P. marinus* MIT9301 PtxB (Pm_PtxB) has previously been reported to have similar low micromolar binding affinities for phosphite, MPn and EPn, measured by ITC35. To try to explain this, we determined the structure of the Pm_PtxB/phosphite complex (Table 4), which shows that Pm_PtxB has the same overall fold as Te_PtxB, with complete conservation of the amino acids that coordinate the ligand, including the P-H...π interaction with Y206 (Supplementary Fig. 16). We calculated the volume of the binding pocket in Pm_PtxB and found that it is 23–29% larger than those of the
phosphite-specific PBPs (Supplementary Table 1). Comparison of the Pm_PtxB binding pocket with that of Te_PtxB (RMSD ca: 0.75 Å) and Pm_PhnD (RMSD ca: 1.52 Å) shows that this expansion is due to Ile20, which is conserved in Pm_PhnD (Ile11) and a leucine (Leu20) in Te_PtxB, as it adopts a different rotamer that forms a small hydrophobic cavity next to the capping tyrosine that is absent in the other proteins (Fig. 6). This seems to be due to several sequence changes in the second shell of amino acids within lobe 1 that create space for the Ile to rotate (Fig. 6).

We were unable to measure the dissociation constant of Pm_PtxB for P-ligands here as the protein showed evidence of aggregation in the MST assay. Thus, from the structural similarities with Te_PtxB we can speculate that Pm_PtxB is most likely specific for phosphite, however, due to the extra space in the binding pocket could also bind simple phosphonates, in agreement with the previously reported ITC binding data.

The unknown substrate of the Prochlorococcus PhnYZ system, encoded downstream of the ptxABCD genes, may also be a ligand of Pm_PtxB. Therefore, some Prochlorococcus ecotypes appear to have two independent phosphite transporter systems, and may also be able to take up some simple phosphate species. The PhnDCE transporter is ubiquitous in Prochlorococcus and is also present in numerous marine Synechococcus strains, but its in vivo function is unknown as only ecotypes that have PtxABCD can grow on phosphite as a sole P source. Further studies are therefore required to ascertain the biological role of phosphite uptake by the PhnDCE transporter and the in situ substrates of the PtxABCD transporter in this important primary producer.

Discussion
We have determined structures of PBPs from reduced phosphorus compound ABC transporter systems in complex with phosphite and hypophosphite. The structures reveal that ligand specificity, measured here by thermophoresis, arises from a combination of steric considerations and the interaction between a ligand P-H hydrogen atom and an aromatic π system in the binding pocket. In phosphite binding PBPs a strictly conserved tyrosine residue provides the π system, and in HtxB an invariant tryptophan makes the equivalent interaction with hypophosphite. We identified similar P-H…π interactions in 12 small-molecule crystal structures within the Cambridge Structural Database (Supplementary Table 2; Supplementary Fig. 17), however, such interactions with reduced phosphorus species could not be identified in protein structures in the PDB. It thus appears that, although C-H…π, N-H…π and cation-X…π bonds are well characterised in proteins, the structures reported here represent the first observations of P-H…π interactions in protein/ligand complexes.

Phosphite is a useful P source for biotechnological, industrial and agricultural applications. PtxD has been introduced to Arabidopsis, tobacco and rice, allowing the use of phosphite as a dual fertilisation and weed control system. PtxD and phosphite are also used as selectable markers in bacteria and algae; to provide competitive advantage in industrial fermentation processes; and in NADH-dependent biocatalysis for in situ cofactor regeneration. So far these applications have only exploited PtxD, relying on non-specific phosphite uptake.
that requires media phosphate concentrations of ≥0.1 mM. As phosphate is cheap it can be provided at high concentrations, but our binding data suggest that introducing a high-affinity transporter would reduce the phosphate requirement in these applications. Furthermore, some technologies require that phosphate uptake is highly specific. For example, a recent study by Hirota et al. showed the use of phosphate-dependent growth as a biocontainment strategy, which requires a phosphate transporter that does not transport phosphate. The authors introduced PtxD and the Ralstonia sp. 4506 PtxABC transporter into an E. coli strain devoid of its native phosphate acquisition systems, resulting in a strain that grew on phosphate, but also phosphate when the ligand was supplied at 1 mM. This concentration is several orders of magnitude higher than environmental phosphate concentrations, and based on the micromolar KD for phosphate binding to PtxB, determined in our study (Table 1), it is not surprising that phosphate supported growth. In the same study, addition of the P. stutzeri WM88 HtxBCDE hypophosphate transporter to the same E. coli strain did not allow growth on phosphate. On the contrary, our binding studies which show that HtxB does not bind phosphate (Supplementary Fig. 5), but did allow growth on phosphate, which we would not have anticipated. Hirota et al. showed that the affinity of the HtxBCDE hypophosphate transporter is clearly enough to support uptake in vivo when phosphate was supplied at 1 mM, but not when lowered to 5 µM, which still exceeds the highest known environmental phosphate concentrations. We did not detect significant phosphate binding by recombinant HtxB, which, from our structural evidence, is consistent with the architecture (Fig. 1f) and size (Supplementary Table 1) of the HtxB binding pocket. This suggests that for HtxB to bind phosphate the protein must undergo a conformational change or the mode of binding of the ligand would have to be different to that with hypophosphate.

In summary, we have determined the molecular basis for high affinity and specific phosphate and hypophosphate uptake in transporters from globally important and abundant terrestrial and marine microorganisms. The structures of two distinct types of phosphate-binding protein and a hypophosphate-binding protein reveal that a P-H...π interaction ensures high-affinity binding of specific ligands, representing a universal mechanism in these PBPs for recognising ligands with a P-H bond. The initial encounter with phosphate or hypophosphate in the bacterial periplasm is the first step in assimilating these potentially important P species, which are increasingly recognised as important constituents of the global phosphorus redox cycle1-12 that may influence the biogeochemistry and productivity of the oligotrophic oceans and terrestrial ecosystems13-23.

Methods

**DNA manipulation.** Primers and plasmids used in this study are provided in Supplementary Tables 4 and 5 respectively. *Escherichia coli* (M109 competent cells (Promega, UK) were used for all standard cloning steps. The *Trichodesmium ptxB* gene (Tery_0366, Te_ptxB) minus predicted N-terminal signal peptide coding sequence (nucleotides 1-81 = amino acids 1-27, SignalP 4.1 Server24) and stop codons was amplified from *Trichodesmium erythraeum* LMS101 (Feuerli-Beubot et al.25) obtained from NCMC, USA) genomic DNA using Q5 DNA polymerase (New England Biolabs, UK) and primers Te_ptxB-F and Te_ptxB-R and cloned into the NdeI and XhoI sites of pET21a+ (Novagen, UK). The *Prochlorococcus marinus* MIT 9301 pafs (P9301_12511, Pm_pfas) and ptxB (P9301_12511, Pm_ptxB) and *Pseudomonas stutzeri* WM88 (Ps_ptxB and htxB genes with their N-terminal signal peptide coding sequences26 (Pm_pHdxN = nucleotides 1-72, amino acids 1-24; Pm_pHdxN = nucleotides 1-63, amino acids 1-21; Ps_pHdxN = nucleotides 1-69, amino acids 1-23; htxB = nucleotides 1-99, amino acids 1-33) and stop codons removed were synthesised with codons optimised for expression in *E. coli* (Integrated DNA Technologies, Inc., USA) and sub-cloned into pET21a+ as above. The Quick Change II Site-Directed Mutagenesis Kit (Agilent Technologies, USA) was used to generate genes encoding point mutants. All genes were sequence verified (GATC Biotech) and confirmed to be in-frame with the C-terminal His6-tag.

**Protein production and purification.** Plasmids were introduced into *E. coli* BL21 (DE3) (Invitrogen, UK) for protein production. Cultures were grown in LB broth with 100 µg ml⁻¹ ampicillin shaking (250 rpm) at 37 °C. At an optical density of 600 nm of ~0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM to induce expression and the cultures were incubated for a further 16 h at 18–20 °C. Cells were harvested by centrifugation at 12,000 × g for 20 min at 4 °C and suspended in binding buffer (25 mM HEPES (4-(2-hydroxy-1,1-benzyl)-1H-pyrazol-5-yl)-[1,1′-biphenyl]-4-carboxylic acid) pH 7.5, 0.5 M NaCl, 5 mM imidazole, EDTA-free protease inhibitor (Roche, UK)). Cell suspensions were sonicated on ice (6 × 30 s bursts with 30 s intervals between) and insoluble debris removed by centrifugation at 45,000 × g for 20 min at 4 °C. Proteins were purified by immuno-affinity chromatography on a 5 ml Chelating SepharoseTM Fast Flow resin column (GE Healthcare, UK). Bound protein was washed with binding buffer containing 20 mM imidazole and then eluted into 25 mM HEPES pH 7.5, 400 mM imidazole, 0.1–0.2 M NaCl. Proteins were further purified on a 22 ml Superdex S200 Increase column (GE Healthcare) in 25 mM Tris/HCl 0.2 M NaCl pH 7.4 and eluted as single peaks. Peak fractions were pooled and concentrated to 2.5 ml and then was buffer exchanged into 25 mM Tris/HCl pH 7.4 with 0–0.2 M NaCl using a PD-10 desalting column (GE Healthcare). Proteins were used immediately or stored at 4 °C.

**Thermophoresis.** The binding affinity of proteins for P ligands was determined using Microscale Thermophoresis (MST) with a Monolith NT.115 instrument (NanoTemper Technologies, Germany). Protein (100 µM) was labelled with RED-tris-NTA dye (NanoTemper Technologies) according to the manufacturer’s instructions in 50 mM HEPES pH 7.4, 250 mM NaCl, 0.05% Tween-20. A volume of 10 µl of 100 mM labelled protein was mixed with 10 µl of ligand in 50 mM HEPES pH 7.4, 250 mM NaCl, 0.05% Tween-20. 4 µl of protein–ligand mixture was loaded into Premium grade capillaries (NanoTemper Technologies) and thermophoresis was measured at 22 °C for 22 s with 20% LED power and 40% infrared laser power. Data from three independent measurements were combined and analysed using the MO.Affinity Analysis software version 2.1 (NanoTemper Technologies), fitted to a single binding site model (Eq. 1) where [I] is the concentration of ligand and data plotted using Igor Pro version 7.04 (Wavemetrics Inc., USA).

\[
\text{f(I)} = \frac{[I][\text{protein}}{K_D + [I] + [\text{protein}}[\text{protein}} + K_D)
\]

Equation 1 shows the single binding site model used to determine dissociation constants.

**Isothermal titration calorimetry.** ITC experiments were performed on a Nano ITC (TA Instruments, USA) at 22 °C. Purified Te_pxtB (200 µM) was exchanged into a buffer containing 50 mM HEPES, 250 mM NaCl, 0.05% Tween-20, pH 7.4 using a PD-10 desalting column. The ligand was dissolved in the same buffer, and the protein concentration was titrated to 2 mM. Each titration consisted of 12 injections of 16 µl of ligand into a 160 µl protein sample. Control experiments were carried out by injecting ligand into the buffer, and the resulting heat of dilution was subtracted from the binding isotherm data. The first injection was ignored in the final analysis. The raw ITC data were processed using NanoAnalyse Data Analysis V 3.7.0 (TA Instruments) and fitted to a single-site binding model. Data were plotted in Igor Pro version 7.04 (Wavemetrics Inc.).

**Circular dichroism spectroscopy.** Spectra were recorded with a JASCO-810 spectrometer (JASCO, UK). Protein (0.1 mg ml⁻¹) was in 5 mM sodium phosphate buffer, pH 7.4 at 25 °C. Spectra were recorded in a cuvette with a 0.1-cm path length. Spectra were recorded continuously, from 250 to 190 nm (50 nm s⁻¹, 1 nm increments, 4 s response, 6 accumulations).

**Crystallisation and structure determination.** Te_pxtB (10 mg ml⁻¹) was co-crystallised with 5 mM sodium phosphate or methylphosphonic acid (MPm) (pH 7.0) by sitting-drop vapor diffusion (200 nl: 200 nl, 290 K) in 0.2 M NaCl, 0.1 M phosphate-citrate buffer pH 4.2 and 20% (w/v) PEG 8000 or 0.2 M magnesium chloride, 0.1 M sodium acetate pH 5 and 20% (w/v) PEG 6000, respectively. Pm_pHdxN (7 mg ml⁻¹) was co-crystallised using the same method, but in 0.1 M SPG (succinic acid, sodium dihydrogen phosphate and glycine) buffer pH 8 and 25% (w/v) PEG 1500 or 0.1 M MMT (DL-malic acid, MES and Tris base) buffer pH 8 and 25% (w/v) PEG 1500, for the phosphate or MPn complexes, respectively. Apo Ps_pxtB (11 mg ml⁻¹) was crystallised using the same method, but in conditions containing 0.1 tri-sodium citrate pH 5.5 and 20% (w/v) PEG 3000 and in the presence of 5 mM ethylphosphonate (EPn), no electron density for which was observed in the resulting structure. Crystals of Ps_pxtB (11 mg ml⁻¹) in complex with phosphate and MPn were grown at pH 5 and pH 4, respectively, from 0.1 M MMT buffer (DL-malic acid, MES and Tris base) and 25% (w/v) PEG 1500. HptB (7 mg ml⁻¹) was co-crystallised with 3 mM sodium hypophosphate using the same method, but in 0.2 M sodium/potassium tartrate, 0.1 M bis-tris propane pH 8.5 and 20% (w/v) PEG 3350. For all the above proteins, thin, rod-shaped
The apo structure of Ps_PtxB was determined by molecular replacement using a Pm_PhnD/phosphite and Ps_PtxB/MPn) were revalidated using the same methods as those used with the Te_PtxB/phosphite study are given in Supplementary Table3. The authors declare that the data support the findings of this study are available from the corresponding author upon reasonable request.

Data availability. Atomic coordinates and structure factors for the reported crystal structures have been deposited in the RCSB Protein Data Bank (www.pdb.org) with accession codes: SIVB (Te_PtxB/phosphate), SLQ1 (Te_PtxB/MnP), SLQ5 (Pm_PhnD/phosphate), SLQ8 (Pm_PhnD/MnP), 5O2K (apo Ps_PtxB), 5O2J (Pm_PhnD/MPn), 5O37 (Ps_PtxB/MPn), 5ME4 (HtxB/hypophosphite) and 5LV1 (Pm_PtxB/phosphite). Uniprot accession codes of proteins described in this study are given in Supplementary Table3. The authors declare that the data support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
A.H. and T.S.B. conceived the study. C.B., N.B.P.A., T.S.B., P.J.B. and C.N.H. designed the experiments. D.P. performed experiments and analyzed data. C.B., N.B.P.A., C.N.H. and A.H. conducted the statistical analysis. B.S. assisted with NMR spectroscopy. C.B., B.S. and A.H. prepared crystals. C.B. collected and processed X-ray diffraction data, determined structures and performed structural analysis. D.P. and A.H. generated expression plasmids. N.B.P.A., B.S., A.A.B. and C.N.H. conceived the study. C.B., N.B.P.A., C.N.H. and A.H. performed structural analysis. B.S. assisted with binding data analysis, diffraction data processing and structure determination for Pn_PtxB. P.J.B. performed structural analysis. A.H. performed bioinformatics. C.B. and A.H. wrote the paper. N.B.P.A., T.S.B., P.J.B. and C.N.H. edited and proofread the manuscript. All authors read and approved the final paper.

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