The cyclic process of autophosphorylation of the C-terminal tyrosine cluster (YC) of a bacterial tyrosine kinase and its subsequent dephosphorylation following interactions with a counteracting tyrosine phosphatase regulates diverse physiological processes, including the biosynthesis and export of polysaccharides responsible for the formation of biofilms or virulence-determining capsules. We provide here the first detailed insight into this hitherto uncharacterized regulatory interaction at residue-specific resolution using *Escherichia coli* Wzc, a canonical bacterial tyrosine kinase, and its opposing tyrosine phosphatase, Wzb. The phosphatase Wzb utilizes a surface distal to the catalytic elements of the kinase, Wzc, to dock onto its catalytic domain (Wzc_CD). Wzc_CD binds in a largely YC-independent fashion near the Wzb catalytic site, inducing allosteric changes therein. YC dephosphorylation is proximity-mediated and reliant on the elevated concentration of phosphorylated YC near the Wzb active site resulting from Wzc_CD docking. Wzb principally recognizes the phosphate of its phosphotyrosine substrate and further stabilizes the tyrosine moiety through ring stacking interactions with a conserved active site tyrosine.

Tyrosine phosphorylation has been long realized to be a critical signaling mechanism in higher eukaryotes (1) that is responsible for almost every aspect of cell growth, differentiation, maturation, motility, and regulated cell death. On the other hand, although the existence of tyrosine phosphorylation in bacteria has been known for quite some time (2–4), its influence on bacterial physiology has been largely ignored. However, with mounting evidence of the role of tyrosine kinase signaling in the virulence of many pathogenic bacteria (5–7), its critical role in bacterial cell signaling is gaining prominence (8–11).

The bacterial tyrosine kinase (BY-kinase) family (10–12) that is highly conserved (13) in both Gram-negative and Gram-positive species constitutes the largest family of protein-tyrosine kinases (PTKs) in bacteria. The central role of BY-kinase-mediated signal transduction in the synthesis and export of polysaccharides responsible for biofilm or capsule formation (9, 10, 12) is well established. In addition, BY-kinases also appear to participate in diverse cellular processes, including antibiotic resistance (14), lysogenization (15), and DNA metabolism (16), indicating that the extent of their influence on bacterial physiology could rival that seen for PTKs in life’s critical processes in higher eukaryotes (17).

BY-kinases comprise a periplasmic/extracytoplasmic domain, two trans-membrane helices, and a cytoplasmic catalytic domain (CD) that is structurally distinct from those found in eukaryotic PTKs. Recently solved structures of the CDs of several BY-kinases, including Etk (18) and Wzc (19) (Fig. 1) from *Escherichia coli* and CapB from *Staphylococcus aureus* (20), confirmed their unique features highlighted by an absence of the two-lobed fold characteristic of the CDs of eukaryotic PTKs. Also striking was the close structural similarity of the BY-kinases to P-loop ATPases. BY-kinase CD structures possess ATP-binding motifs reminiscent of the Walker motifs of P-loop ATPases instead of eukaryal kinase sequence motifs (21). These motifs include the following (Fig. 1): Walker A ((A/G)XXXVGK/S(T); usually only the GK(S/T) element is conserved in BY-kinases); Walker B (D/ND/DXXP, where φ is a hydro-
phobic residue; the XXP sequence is a BY-kinase-specific extension of the Walker B motif); and an additional motif that is similar to a Walker B, called the Walker A’ (PHEHEHXDXDRXR) (11, 22).

Regulation of BY-kinases occurs through the intermolecular autophosphorylation and subsequent dephosphorylation of 3–7 tyrosine residues (the tyrosine cluster (YC)) located on the C-terminal tail (C-tail) (Fig. 1B) of the CD. Based on extensive biochemical and in vivo evidence (23), it is apparent that BY-kinases cannot be classified as being in “active” or “inactive” states, as for a majority of eukaryotic kinases, dependent simply on the phosphorylation state of key regulatory residues (24–27). It is also clear that the overall level of YC phosphorylation rather than the quantitative phosphorylation state of any specific YC tyrosine is important for activity (28, 29). Thus, the current model for BY-kinase activity suggests that activity is related to the cycling between a state where the YC is highly phosphorylated (YChigh) and a state where the YC is low phosphorylated (YClow). This high molecular weight species was eliminated by treatment of the cellular envelope, desiccation, and similar environmental perturbations (10). Regulation may be proposed. An association of the CDs is followed by trans-phosphorylation and generation of a YChigh state. The YChigh state then dissociates to expose the active site (occurred in the oligomeric state) to downstream targets, such as UDP glucose dehydrogenase, a known BY-kinase target in both Gram-negative (32) and Gram-positive species (33). Generation of the YClow state of the CD occurs by the action of protein-tyrosine phosphatases (PTPs), and this reinitiates the cycle. Therefore, this cycling between YChigh and YClow states and consequently between associated and dissociated states of the CD appears key for maintaining function (10). Thus, the critical players in sustaining this YChigh/YClow cycle that occurs in the cytoplasm are the bacterial PTPs. Several of these PTPs belong to the low molecular weight protein-tyrosine phosphatase (LMW-PTP) family.

Bacterial LMW-PTPs are wholly cytosolic and eukaryotic-like in overall structure (34–37) (Fig. 2), although they lack several key features considered to be important for substrate recognition in eukaryotes, suggesting unique mechanisms of kinase docking and regulation. Functional BY-kinase/LMW-PTP pairs in bacteria include Wzc/Wzb (38) and Etk/Etp (E. coli) (39), Ptk/Ptp (Acinetobacter johnsonii) (40), and Yco6/Yor5 (Klebsiella pneumoniae) (41), to name a few that have been characterized. Despite extensive genetic and biochemical evidence for the critical role of BY-kinase/LMW-PTP interactions in bacterial tyrosine phosphorylation-mediated signaling, no information is available on the structural elements on BY-kinases that are important for their interactions with LMW-PTPs. Further, although structural work on bacterial PTPs, including the solution structure of E. coli Wzb (34), has allowed the generation of specific hypotheses on phosphotyrosine recognition by these enzymes, information on the specific nature of substrate recognition and dephosphorylation is lacking.

Generation of the YChigh state is achieved by intermolecular autophosphorylation facilitated by the oligomerization of the cytoplasmic CDs of the BY-kinases in which the YC of one subunit (substrate-acting) is inserted into the catalytic site of the neighboring subunit (enzyme-acting) in the oligomer, as in the case of eukaryotic PTKs (30). A highly conserved sequence motif (EXXRXXR) has been shown to be critical in facilitating the self-association of the CDs of the BY-kinases necessary to accomplish trans-phosphorylation. There are three specific lines of evidence for this. (i) This motif has been shown to play a central role in generating the octameric oligomerization state seen in the crystal structures of the CDs of Wzc (19) as well as S. aureus CapB (20), hinting at a general mode of oligomerization and trans-phosphorylation. (ii) Although the isolated CDs of BY-kinases are largely monomeric in solution (as will be demonstrated below), analytical gel filtration revealed the presence of a minor population of a high molecular weight species for the CD of a Wzc mutant (K540M) that cannot bind ATP and hence is incapable of generating a YChigh state. This high molecular weight species was eliminated by mutating the conserved residues of the EXXRXXR motif to alanine (19). (iii) This same triple mutation reduced the autophosphorylation levels of Wzc (in the context of full-length protein) to around 10% of wild type (19), suggesting its importance for trans-phosphorylation. Although the tendency of the isolated BY-kinase CDs to oligomerize in solution appears to be low, this low self-affinity is likely to be enhanced several fold in the context of the membrane-bound fully assembled signaling complex that involves additional interactions involving the periplasmic domains and perhaps the membrane segments (31). However, it is to be noted that isolated BY-kinase CDs have been shown to form higher order oligomers in vivo (29) using formaldehyde cross-linking. Thus, based on the current state of knowledge, the following hypothesis for BY-kinase regulation may be proposed. An association of the CDs is followed by trans-phosphorylation and generation of a YChigh state. The CDs in the YChigh state then dissociate to expose the active site (occurred in the oligomeric state) to downstream targets, such as UDP glucose dehydrogenase, a known BY-kinase target in both Gram-negative (32) and Gram-positive species (33). Regeneration of the YClow state of the CD occurs by the action of protein-tyrosine phosphatases (PTPs), and this reinitiates the cycle.

**Experimental Procedures**

**Expression and Purification of WzcCD and Wzb Constructs Used for Biophysical Studies**—wzcCD, wzbCD, and wzcCDACo were cloned into pET15b vectors, transformed into E. coli BL21 DE3 cells, and purified as described in detail previously (45) for WzcCD (the various constructs utilized in this study are shown in Fig. 3). All proteins contained a thrombin-cleavable 21-resid-
due N-terminal tag. This tag appeared to ensure the long term stability of the proteins for the NMR experiments and was therefore not cleaved. After purification, the Wzc\textsubscript{CD} and Wzc\textsubscript{CDAC} samples were incubated overnight with a 25-fold excess of ATP/MgCl\textsubscript{2} followed by exchange against NMR buffer (see below) to remove the excess ATP. The Wzc\textsubscript{CD}\textsubscript{ERR/A}

**Interactions between Wzb and Wzc**

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**Walker A**

**Walker A**

**Walker B**

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**Wzc\textsubscript{CD},ERR/A**

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**Figure B**

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and wzb_{C9S} mutants were prepared from wzc_{CD} and wzb, respectively, using the QuikChange kit (Stratagene), cloned into pET15b, expressed, and purified as the corresponding wild-type proteins.

Expression and Purification of Wzc_{CD} Constructs Used for Enzymatic Assays—The DNA fragment encoding for wzc_{CD} was PCR-amplified using either the wild-type E. coli (K12) DNA or the pQE30-Wzc_{CD},ERR/A plasmid (19) and cloned into a pQE30 vector between the BamHI and HindIII restriction sites to generate wzc_{CD} (or the corresponding mutants: wzc_{CD,ERR/A}, wzc_{CD,F5} and wzc_{CD,F5,ERR/A}). Wzc_{CD,F5} and Wzc_{CD,F5,ERR/A} both bearing N-terminal His_{6} tags, were produced in E. coli XII-blue cells grown in LB medium supplemented with ampicillin and tetracycline. Overexpression was induced using 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37 °C when the A_{600} reached 0.4, followed by centrifugation for 10 min at 5000 × g. The pellet was re-suspended in 5 ml of buffer A (50 mM Tris-HCl, 200 mM NaCl, 10% glycerol, and 10 mM imidazole at pH 7.5) and stored at 4 °C. Cells were lysed by sonication and centrifuged for 30 min at 14,000 × g at 4 °C. The supernatant was filtered through 0.22-μm filters and degassed extensively before use. CM5 (carboxymethylated dextran; GE Healthcare) sensor chips were covalently coated with each of the three ligands Wzc_{CD}, Wzc_{CDAC} and Wzc_{CD,ERR/A} (theoretical pI 8.9, 9.39, and 8.73, respectively). The ligands were dissolved to final concentrations of 21 μg/ml in 10 mM sodium acetate (pH 5.3) and immobilized onto CM5 chips by amine coupling on flow cells 2 (FC2), yielding responses of 951.2, 1512.8, and 490.8 for Wzc_{CD}, Wzc_{CDAC} and Wzc_{CD,ERR/A}, respectively, after deactivation of the CM5 surface with ethanolamine-HCl. As control, flow cells 1 (FC1) were activated for amine coupling and deactivated under the same conditions as FC2 in the absence of ligand.

The analytes, Wzb and Wzb_{C9S} were diluted in a buffer containing 50 mM phosphate, 137 mM NaCl, 2.7 mM KCl, 25 mM DTT, 5 mM EDTA, and 0.005% P20-surfactant at pH 7.4 (SPR buffer), to reach final concentrations ranging from 100 nM to 10 μM. Each experiment consisted of three cycles where only the SPR buffer was passed over the CM5 sensor chip, followed by 11 cycles of different analyte concentrations (with one in duplicate). In each cycle, 50 μl of SPR buffer was injected first to stabilize the base line, followed by injection of the analyte (0–10 μM) through the two flow cells at 30 μl/min for 180 s. Responses detected on FC1 were considered to result from nonspecific binding and subtracted from the binding traces before detailed analyses. Dissociation of the bound analyte was achieved by passing SPR buffer over the two flow cells at 30 μl/min for 600 s. After the dissociation phase, a solution containing 5 mM NaCl and 4 mM MgCl_{2} and another containing 10 mM triethylamine (10 μl/min for 60 s for each) were used to regenerate the CM5-ligand surface. Each experiment was recorded in duplicate, and reproducible signals were detected for every ligand-analyte pair tested.

FIGURE 1. BY-kinases, A, sequence alignment for BY-kinases. The conserved BY-kinase sequence motifs are indicated. The location of the α2 helix is shown by the horizontal black line, and the location of the EXXRXXR motif is shown by the yellow rectangle with the conserved residues (Glu, Arg, Arg) indicated by the asterisks. This alignment of BY-kinase sequences was used to generate Fig. 6. B, structure of Wzc_{CD} (Protein Data Bank code 3LA6) (19). Conserved structural elements are colored, and the side chains of conserved residues are shown in stick representations and labeled. The characteristic BY-kinase motifs (conserved residues are shown in boldface type) for Wzc_{CD} are Walker A (E535, G536, S537, G538, G539, K540, red), Walker B (E639, V640, I641, orange), and Walker C (E712, Y713, E714, blue). Also shown are helix α2, containing the conserved 508-EA15-RS-LR514 motif (yellow); the RK-cluster (Ser473–Leu496, magenta) (only Lys476 and Lys477 are seen in the crystal structure), and the C-tail (green; the YC tyrosines (TYR703, TYR705, TYR709, TYR710, TYR711, TYR712, and TYR713) are shown). The Wzc_{CD} orientations are the same as in Fig. 6.
Interactions between Wzb and Wzc

A

Wzb E. coli K12/1-147
Amsl E. amylovora/1-144
Etp E. coli (K12)/1-148
Yor5 K. pneumoniae/1-144
Ptp A. johnsonii/1-142
EpsP. R. solanacearum/1-145
YwiE B. subtilis/1-150
PtpB S. aureus/1-139
Lip1 S. cerevisiae/1-161
Stp1 S. pombe/1-156
MPTpA M. tuberculosis/1-163
PtpA S. aureus/1-154
HCPTPA H. sapiens/1-157
BPTP B. taurus/1-157

Wzb E. coli K12/1-147
Amsl E. amylovora/1-144
Etp E. coli (K12)/1-148
Yor5 K. pneumoniae/1-144
Ptp A. johnsonii/1-142
EpsP. R. solanacearum/1-145
YwiE B. subtilis/1-150
PtpB S. aureus/1-139
Lip1 S. cerevisiae/1-161
Stp1 S. pombe/1-156
MPTpA M. tuberculosis/1-163
PtpA S. aureus/1-154
HCPTPA H. sapiens/1-157
BPTP B. taurus/1-157

B

D-loop

110 GHDVWEKQTRSE TAA TLYLLE R SARQWAQLNAE QV -- -- 147
109 GHGWIQKETDS SADFEV YGML ARSAQ EKLR S -- 144
108 GQWLEKQR DPA RSDA FEHYGM ECQSEW A VXKL S -- 148
107 GHQLEKQR DPA RSDA FEHYGM ECQSEW A VXKL S -- 143
106 GHQLEKQR DPA RSDA FEHYGM ECQSEW A VXKL S -- 142
105 GEM-DOQ DPA RSDA FEHYGM ECQSEW A VXKL S -- 145
104 GEM-DOQ DPA RSDA FEHYGM ECQSEW A VXKL S -- 144
103 GEM-DOQ DPA RSDA FEHYGM ECQSEW A VXKL S -- 145

C

N

S16

C9

D115

P116

Y117

R15

L40

C14
The experimental methodology used for backbone resonance assignments of WzcCD has been described in detail elsewhere (45). Backbone resonance assignments for WzcCD were obtained using TROSY-based (in all cases) HNCO/HN(CA)CO (512, 32, and 40 complex points with sweep widths of 13.35, 30, and 13 ppm in $^1$H, $^{15}$N, and $^{13}$C dimensions, respectively); a HNCA/HN(CA)CO pair (512, 32, and 50/40 complex points with sweep widths of 13.35, 30, and 30 ppm in $^1$H, $^{15}$N, and $^{13}$C dimensions, respectively); and a HNCACB/HN(CA)CO pair (512, 32, and 40/42 complex points with sweep widths of 13.35, 30, and 13 ppm in $^1$H, $^{15}$N, and $^{13}$C dimensions, respectively) (50). No significant chemical shift changes were seen in the core residues of WzcCD due to tail truncation (to generate WzcCDAC). Additionally, eight resonances corresponding to the 21-residue N-terminal tag (that includes 6 histidines) were also assigned, and these resonances showed no significant differences in their positions between WzcCD and WzcCDAC or for each species in the presence of Wzb. The corresponding tag in Wzb was also unaffected in the presence of WzcCD or WzcCDAC.

Nearly complete $^{15}$N, $^{13}$C, and $^1$H resonance assignments for full-length Wzb are available from Lescop et al. (34). However, due to some differences under our conditions, the assignments of several of these resonances needed to be confirmed. Non-uniformly sampled versions of HNCO/HN(CA)CO (512, 32, and 50 complex points with sweep widths of 13, 32, and 13 ppm in $^1$H, $^{15}$N, and $^{13}$C dimensions, respectively); HNCAHB/BCA(CO)NH (512, 32, and 50 complex points with sweep widths of 13, 32, and 65 ppm in $^1$H, $^{15}$N, and $^{13}$C dimensions, respectively); and HNCA/HN(CO)CA (512, 32, and 50 complex points with sweep widths of 10, 32, and 30 ppm in $^1$H, $^{15}$N, and $^{13}$C dimensions, respectively) were collected using a 30% sampling schedule generated by the sparse.py script (51). All data were collected at 700 MHz and 25 °C and reconstructed using MDDGui software (52).

$^{13}$C,$^1$H resonance assignments for the aromatic side chains of Wzb were obtained using (H(β)Cβ(CyCaβ)Hβ, (Hβ)Cβ(CyCaCe)He) (512 and 40 complex points with sweep widths of 12 and 30 ppm in $^1$H and $^{13}$C dimensions, respectively) (53) and $^{13}$C-edited NOESY-HSQC (512, 40, and 88 complex points with sweep widths of 12, 30, and 12 ppm in the $^1$H, $^{13}$C, and $^1$H dimensions, respectively) experiments at 700 MHz and 25 °C. A mixing time of 120 ms was used for the $^{13}$C-edited NOESY-HSQC experiment.

**NMR Titrations to Determine WzcCD/Wzb Interactions**—Chemical shift perturbations in 100 μM uniformly $^{15}$N,$^2$H-labeled WzcCD or WzcCDAC (in NMR buffer, described above) were obtained by separate titration sets with $^2$H-labeled Wzb at concentrations of 25, 50, 75, and 100 μM, and $^{15}$N,$^3$H TROSY spectra were acquired at each titration point. Similarly, 100 μM uniformly $^{15}$N,$^2$H-labeled Wzb (in NMR buffer) was titrated with either uniformly $^2$H-labeled WzcCDAC or uniformly $^3$H-labeled WzcCDAC or WzcCDAC.
Interactions between Wzb and Wzc

A series of $^{15}$N,$^1$H HSQC spectra were recorded using 100 $\mu$M $^{13}$C, $^{15}$N-labeled Wzb, each with an incremental amount of potassium phosphate to obtain the following Wzb/phosphate ratios: 1:2, 1:3, 1:5, 1:10, 1:20, 1:40, 1:100, and 1:200. Titrations were performed in a buffer containing 20 mM BisTris, 50 mM NaCl, 25 mM DTT at pH 6.0 (BT buffer). The initial volume of 350 $\mu$L increased to 363.5 $\mu$L at the end of the addition, resulting in a dilution of less than 4%. All spectra were recorded at 600 MHz at 25 °C using sweep widths of 13 ppm (512 complex points) and 30 ppm (128 complex points) in the $^1$H and $^{15}$N dimensions, respectively. Recycle delays of 1.5 s were used, and 16 transients were collected per $t_1$ point. Representative spectra corresponding to the various NMR-based titrations involving WzcCD and Wzb are shown in Fig. 4. As can be seen, the spectra are of excellent quality.

Note that for all NMR-based titrations and spin relaxation experiments (described below), the last titration point contained an equimolar ratio of WzcCD (or WzcCD$_{D3C}$) and Wzb for a total protein concentration of 200 $\mu$M. Assuming a $K_d$ of $\sim$2–5 $\mu$M, we would expect $\sim$80–85% of the proteins to be in the complexed state. We did not use higher total protein concentration (i.e. saturating equivalents) to ensure sample stability during the measurements. However, no significant additional perturbations, compared with those at the equimolar ratio, were seen in the presence of saturating amounts of ligand at lower concentrations of the species being probed.

**NMR Titrations to Determine Wzb/Phosphate Interactions**—A series of $^{15}$N,$^1$H HSQC spectra were recorded using 100 $\mu$M $^{13}$C, $^{15}$N-labeled Wzb, each with an incremental amount of potassium phosphate to obtain the following Wzb/phosphate ratios: 1:2, 1:3, 1:5, 1:10, 1:20, 1:40, 1:100, and 1:200. Titrations were performed in a buffer containing 20 mM BisTris, 50 mM NaCl, 25 mM DTT at pH 6.0 (BT buffer). The initial volume of 350 $\mu$L increased to 363.5 $\mu$L at the end of the addition, resulting in a dilution of less than 4%. All spectra were recorded at 600 MHz at 25 °C using sweep widths of 13 ppm (512 complex points) and 30 ppm (128 complex points) for the $^1$H and $^{15}$N dimensions, respectively.

**NMR Titrations to Determine Wzb/C-tail Peptide Interactions**—The WTpep, MUTpep, and PHOSpep peptides were titrated into 100 $\mu$M $^{13}$N-labeled Wzb in BT buffer to the following final concentrations: 0, 200, and 400 $\mu$M. Individual samples were made for each of the Wzb-WTpep, Wzb-MUTpep and Wzb-PHOSpep complexes, and the following spectra were acquired: $^{15}$N,$^1$H HSQC (512 and 128 complex points with sweep widths of 13 and 32 ppm in the $^1$H and $^{15}$N dimensions, respectively), constant time $^{13}$C,$^1$H HSQC optimized for the aromatic region (512 and 64 complex points with sweep widths of 12 and 30 ppm in the $^1$H and $^{13}$C dimensions, respectively), and $^{15}$N,$^1$H HSQC optimized for the methyl region (512 and 115 complex points with sweep widths of 12 and 20 ppm in the $^1$H and $^{13}$C dimensions, respectively). Only $^{15}$N,$^1$H HSQC spectra were recorded for the Wzb-MUTpep complexes. All spectra were recorded at 800 MHz and 25 °C.

In all NMR-based titrations, chemical shift perturbations (Δδ$_i$) or signal attenuations (Δ$I_i$) for each residue at each titration point (indexed by $i$) were calculated using the following equations.

$$\Delta \delta_i = \sqrt{(\Delta \delta_{0,H} - \Delta \delta_{0,N})^2 + 0.11(\Delta \delta_{0,N} - \Delta \delta_{0,H})^2}$$

(Eq. 2)

$$\Delta I_i = 1 - \frac{I_i}{I_0}$$

(Eq. 3)

The 0 subscript indexes the $^1$H/$^{15}$N chemical shifts (δ) or the intensities (I) for the reference state (no ligand present).

**Spin Relaxation Measurements**—TROSY-based relaxation experiments to obtain spin-spin relaxation rates ($R_2$) (54) were recorded at 800 MHz and 25 °C for WzcCD, WzcCD$_{D3C}$, and Wzb and for their equimolar mixtures. Samples typically were 100 $\mu$M protein in NMR buffer, with the equimolar mixtures containing a 100 $\mu$M concentration of each of the constituent proteins. Samples used (and relaxation delays) are provided in Table 1. Relaxation data were acquired with sweep widths of 13.35 ppm (512 complex points) and 30 ppm (128 complex points) in the $^1$H and $^{13}$C dimensions, respectively, for WzcCD$_{D3C}$.
WzcCD, and their equimolar mixtures with Wzb and with sweep widths of 13 ppm (512 complex points) and 30 ppm (128 complex points) in the *1H* and *15N* dimensions, respectively, for Wzb and for its equimolar mixtures with WzcCD and WzcCDAC.

### Enzymatic Assays
- **400 µg of WzcCD (or the corresponding mutant proteins: WzcCDERR/A, WzcCDF5, and WzcCDF5ERR/A)** was incubated in the presence of 20 Ci of radioactive [*γ-32P*]ATP (3000 Ci/µmol) and 25 µm non-radioactive ATP in a buffer containing 25 mM Tris–HCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM DTT at pH 7.5 for 30 min at 37 °C. This was followed by extensive dialysis in the same buffer to remove radioactive ATP, followed by dephosphorylation using Wzb. For dephosphorylation, WzcCD (or WzcCDERR/A, or WzcCDF5, or WzcCDF5ERR/A) and Wzb were mixed in a buffer containing 100 mM sodium citrate and 1 mM EDTA at pH 6.5 and incubated for a series of times (0, 1, 2, 5, 10, 20, and 30 min) at 37 °C using several kinase/phosphatase molar ratios (5:1, 10:1, 20:1, 25:1, 30:1, and 35:1) with the quantity of Wzb being fixed at 2.85 pmol (in a reaction volume of 128 µl corresponding to a concentration of 22.3 nM). Negative controls for the same times were performed in the absence of Wzb (data not shown).

### RESULTS

#### In Vitro Interaction between WzcCD and Wzb

As a first step toward detailed characterization of the interaction between WzcCD and Wzb, we used SPR to estimate the affinity of their association. In all cases, we used WzcCD (or mutants thereof; see Fig. 3 for the various Wzc and Wzb constructs used in this study) as a ligand affixed onto the sensor chip surface and Wzb (or mutants thereof; see Fig. 3) as an analyte. First, in order to test whether the C-tail of WzcCD bearing the YC (the phosphorylated YC being the natural substrate of Wzb) is necessary for the WzcCD/Wzb interaction, we used a WzcCD construct missing the C-tail (WzcCDAC) as ligand. The sensorgram (Fig. 5A) for the WzcCDAC/Wzb interaction was biphasic, especially at higher analyte concentrations, suggesting deviations from a simple 1:1 Langmuir-type binding, making it inappropriate for an equilibrium analysis. The curvature of the association stage of the sensorgram was not sufficient (fast association) for a meaningful kinetic analysis; therefore, such an analysis was not attempted for this (or for any other) case. However, when a catalytically inactive mutant of Wzb (WzbC9S; where Cys⁹, the catalytic cysteine nucleophile, is replaced by serine) (Fig. 3) was used as the analyte, a Langmuir-type behavior (Fig. 5B) was obtained (for the WzcCDAC/WzbC9S interaction), and an equilibrium analysis yielded a *Kd* value of 5.1 ± 0.2 µM. Sensorgrams corresponding to WzcCD/Wzb (Fig. 5C) and WzcCD/WzbC9S (Fig. 5D) interactions were highly biphasic, displaying a second slow process following the initial fast association. An equilibrium analysis (data not shown) of the WzcCD/WzbC9S sensorgram at lower analyte concentrations where the non-Langmuir behavior is less pronounced (for analyte concentration below 5.0 µM) yielded a *Kd* of 2.3 ± 0.4 µM, comparable with that obtained for the WzcCDAC/WzbC9S interaction. Overall, our analysis suggests that a region on WzcCD distinct from the phosphorylated C-tail provides a majority of the binding energy for the WzcCD/Wzb interaction.

#### Binding Site of Wzb on WzcCD

Next, we attempted to ascertain specific residues on WzcCD that interact with Wzb, using solution NMR techniques. Taking cue from the SPR results that showed that the YC of WzcCD did not play a significant role in binding, we analyzed the spectral perturbations induced on a [*1H,15N*] TROSY spectrum of [*15N,2H*-labeled WzcCDAC in the presence of an equimolar ratio of full-length, uniformly [*2H*-labeled Wzb. In addition to the uniform broadening of WzcCDAC resonances due to complex formation, resulting in an increase in overall molecular mass (*15N* R₂ for apo = 26.4 ± 5.9 s⁻¹, indicative of a monomer in solution; [*15N* R₂ in the presence of Wzb at a 1:1 ratio = 41.0 ± 11.2 s⁻¹, indicating complex formation; data at 800 MHz with 100 µM WzcCDAC), specific chemical shift changes and selective resonance attenuation were seen. The largest chemical shift changes were seen on the N terminus of α1 and the C terminus of α3. Some smaller but significant changes were also seen on αα and ββ. However, an analysis of the selective signal attenuation (indicative of exchange on the intermediate time scale) was far more informative. Resonances corresponding to all of αα and most of βA, β1, β6, and β7 were completely broadened out in the presence of Wzb. As shown in Fig. 6A, the residues that display chemical...
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As expected from the SPR results, minimal spectral perturbations were seen for the YC of WzcCD. Note that the species probed by the NMR experiments (unlike the SPR results that involved the catalytically dead WzbC9S mutant) corresponds to the dephosphorylated YC. Given the efficiency of Wzb, the dephosphorylation reaction was almost complete before any NMR data could be recorded (>90% dephosphorylated in 30 min at a 1:1 ratio; data not shown). Nevertheless, some attenuations were seen in the YC, including for Tyr708 (0.52), Glu714 (fully attenuated), and Tyr715 (0.55). It is possible that this weak interaction of the dephosphorylated C-tail of WzcCD with Wzb was the cause of the second slow association event seen in the SPR analysis of WzcCD/Wzb interactions. However, as will be shown, the isolated dephosphorylated C-tail of WzcCD does not bind Wzb in trans. The weak association seen here is due to its elevated local concentration as a result of the docking interaction of Wzb with the putative oligomerization surface of WzcCD, described above. This is also the likely mechanism for substrate (i.e. phospho-YC recognition), as discussed below.

Binding Site of WzcCD on Wzb—As expected, an overall decrease in signal intensity was seen in the 15N,2H TROSY spectrum of uniformly 15N,2H-labeled Wzb in the presence of substoichiometric amounts of Wzb (1:0.25, the first point in our titration course, 1:1 being the last). In this case (i.e. for a WzcCD/Wzb ratio of 1:0.25), although no significant chemical shift changes were seen (except for Ile150; 0.19 ppm), resonances corresponding to almost all of α2 (Ala506, Ala509, Arg511, Ser512, Leu513, Arg514, Ala520, and Met521) were broadened to below the noise level (average attenuation for α2 = 0.80 ± 0.23, compared with 0.35 ± 0.29 for all resonances). Complete attenuation of some other resonances (Tyr467, Ile470, Leu473, Leu463, Ala466, Ile694, and Leu695) was seen. Notably, the side chains of these residues lie in close spatial proximity to α2, suggesting the importance of α2 in Wzc/Wzb interactions. NMR titrations with WzcCD produced similar results (Fig. 6B), again with α2 and spatially proximal regions displaying the largest specific spectral perturbations. α2 shows a degree of sequence conservation that is the highest outside the catalytic site (see Fig. 6C; also see Fig. 1A). Notably, α2 contains the conserved 508EXXRXXR514 that was discussed at length in the Introduction. It therefore appears that the Wzb docking site on WzcCD overlaps with that shown to play a role in the oligomerization and consequent trans-phosphorylation of the latter.

shift changes and selective attenuation form a continuous surface on the face opposite to that hosting the catalytic site of Wzc. In fact, no significant spectral perturbations (chemical shift changes or loss of signals) were observed at the active site. Note that 15N,2H resonances other than those belonging to the Walker B motif are largely assigned for the active site (45).

In order to obtain additional insight into the spectral perturbations, we analyzed a TROSY spectrum of WzcCD in the presence of substoichiometric amounts of Wzb (1:0.25, the first point in our titration course, 1:1 being the last). In this case (i.e. for a WzcCD/Wzb ratio of 1:0.25), although no significant chemical shift changes were seen (except for Ile150; 0.19 ppm), resonances corresponding to almost all of α2 (Ala506, Ala509, Arg511, Ser512, Leu513, Arg514, Ala520, and Met521) were broadened to below the noise level (average attenuation for α2 = 0.80 ± 0.23, compared with 0.35 ± 0.29 for all resonances). Complete attenuation of some other resonances (Tyr467, Ile470, Leu473, Leu463, Ala466, Ile694, and Leu695) was seen. Notably, the side chains of these residues lie in close spatial proximity to α2, suggesting the importance of α2 in Wzc/Wzb interactions.

FIGURE 5. SPR analysis of the interactions of WzcCD with Wzb. Sensorgrams corresponding to the interaction of WzcCD (A and B) or Wzc (C and D) at varying concentrations of wild-type Wzb (A and C) or the catalytically dead mutant of Wzb (WzbC9S; B and D) are shown. In all cases, the Wzc constructs (ligands) were affixed onto the sensor chip surface, and the Wzb constructs were used as analytes. The inset in B depicts the result of the equilibrium analysis for the WzcCD/WzbC9S interactions. Experimental data are shown as circles, and the theoretical curve corresponding to a 1:1 binding model is shown as a solid line.
near the catalytic site of Wzb (Fig. 7). The patterns of perturbations seen in the spectra of Wzb in the presence of either WzcCDAC or WzcCD were similar (Fig. 7, compare A and B). Overall, the magnitudes of the chemical shift perturbations were significantly smaller than those seen for WzcCDAC or WzcCD in the presence of Wzb, and as in those cases, an analysis of the selective attenuations was far more informative. A majority of resonances corresponding to the N terminus of Wzb, β1, α3, β3, and α4 were broadened to below the noise level. Most significantly, many resonances corresponding to the so-called P-loop that contains the CXXXYXR(T/S) ("PCVGNICRS") motif (Fig. 2), conserved in all Cys-based phosphatases (37), were significantly perturbed. However, a very interesting pattern emerged upon closer inspection of resonances corresponding to the P-loop of Wzb in the presence of WzcCDAC (Fig. 7D). Despite the absence of the YC in the WzcCDAC construct, significant spectral perturbations were noted at the catalytic site of Wzb. Residues that were broadened to below the noise level included the catalytic Cys9 (that carries out the initial nucleophilic attack on the phosphotyrosine moiety) and Arg15 (that stabilizes the transition state). Given that these changes occur in the absence of substrate (i.e. the phosphorylated YC of Wzc), they probably indicate allosteric effects at the Wzb active site upon docking interactions. The P-loop and D-loop containing key catalytic site residues are labeled. Also labeled is the so-called W-loop that, in the case of Wzb, does not contain a tryptophan. Selected residues that depict significant spectral perturbations are shown in stick representations and labeled.

**Figure 6. Analysis of perturbations in WzcCD (or WzcCDAC) spectra in the presence of Wzb.** Shown are perturbations in the "H,15N TROSY spectra of uniformly "H,15N-labeled WzcCDAC (A) or WzcCD (B) in the presence of an equimolar ratio of uniformly "H-labeled Wzb, mapped onto the WzcCD surface (Protein Data Bank code 3LA6; species probed shown in larger type). Residues that display chemical shift changes greater than the mean ± 2 S.D. (0.036 ppm for WzcCDAC; 0.044 ppm for WzcCD) are colored orange; residues that are broadened to below the noise level are colored orange. C, sequence conservation (also see Fig. 1A) in the CDs of BY-kinases plotted on the WzcCD surface using a cyan (0% conservation) to magenta (100% conservation) gradient. The EXXXXR (black), Walker A (green), Walker A' (yellow), and Walker B (red) motifs are indicated by the solid ovals.

**Figure 7. Analysis of perturbations in Wzb spectra in the presence of WzcCD (or WzcCDAC).** Perturbations in the "H,15N TROSY spectra of uniformly "H,15N-labeled Wzb in the presence of equimolar ratios of uniformly "H-labeled WzcCDAC (A) or WzcCD (B), mapped onto the Wzb surface (Protein Data Bank code 2FEK; species probed shown in larger type). Residues that display chemical shift changes greater than the mean ± 2 S.D. (0.028 ppm in the presence of WzcCDAC; 0.038 ppm in the presence of WzcCD) are colored green; residues that are broadened to below the noise level are colored orange. C, sequence conservation (also see Fig. 2A) in LMW-PTPs plotted on the Wzb surface using a cyan (0% conservation) to magenta (100% conservation) gradient. D, close-up of the Wzb active site in the presence of either WzcCDAC (left) or WzcCD (right). Significant perturbations at the active site in the absence of substrate (i.e. the C-tail in the case of WzcCDAC) indicate allosteric effects upon docking interactions. The P-loop and D-loop containing key catalytic site residues are labeled. Also labeled is the so-called W-loop that, in the case of Wzb, does not contain a tryptophan. Selected residues that depict significant spectral perturbations are shown in stick representations and labeled.
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the presence of an increasing concentration of a 16-residue peptide (WTpep) corresponding to the dephosphorylated C-terminal tail (TYr705-Lys720). Lack of any significant spectral perturbations (data not shown) confirmed the extremely low affinity in trans of the dephosphorylated C-tail of WzcCD for Wzb. Additionally, no significant spectral perturbations were seen in the presence of another 16-residue peptide (MUTpep; WTpep carrying a Y708E/Y710E/Y711E/Y713E/Y715E mutation to mimic the charge state of the fully phosphorylated YC). In vivo the YC has been found to be multiply phosphorylated (20, 23, 28), and this highly phosphorylated YC is the natural substrate of Wzb. It therefore follows that Wzb is capable of organizing several negative charges near its active site. Hence, that Wzb does not bind MUTpep is due to the fact that glutamate, although an excellent mimic for phosphoserine or phosphothreonine, is an extremely poor mimic for phosphotyrosine (55). However, significant spectral perturbations were seen in the presence of phosphate. A large number of chemical shift changes (>0.04 ppm; Fig. 8A) were seen emanating from the active site of Wzb in the presence of a 5-fold excess of phosphate. Interestingly, resonances corresponding to Cys8, Val19, Glu20, Leu40, and TYr117 only appear in the presence of a large excess of phosphate (200-fold excess under our experimental conditions), whereas additional resonances corresponding to residues Gly11, Ile13, Cys14, and Arg15 can only be seen in phosphate buffer. The appearance of additional resonances in the presence of phosphate has been noted by Lescop et al. (34) (Note that the titrations involving WzcCD, WzcCD ΔC, and Wzb, described earlier were carried out in phosphate buffer; this was to allow the analysis of spectral perturbations for several resonances at the Wzb active site that are exchange-broadened and not observable in the absence of phosphate, as described above; see “Experimental Procedures”). It therefore appears that the phosphate moiety plays a significant role in substrate recognition by Wzb. It is to be noted that Wzb has no activity toward phosphoserine or phosphothreonine, although selectivity toward phosphotyrosine is probably obtained in part by the depth of the catalytic cavity of Wzb (phosphoserine and phosphothreonine side chains are not long enough to reach the catalytic elements), and additional stabilization of the large hydrophobic tyrosine moiety near the active site is required for stable processing following phosphate recognition.

In order to ascertain the origin of the selectivity of Wzb toward phosphotyrosine, one would ideally use a peptide phosphorylated on a single tyrosine. However, such a phosphotyrosine peptide, being a natural substrate for Wzb, would be efficiently dephosphorylated before any NMR experiments could be conducted. Therefore, we monitored the spectral perturbations in Wzb in the presence of a tripeptide, EY*E (PHOSpep, where Y* represents 4-phosphomonomethylphenylaniline). The choice of this peptide incorporating an unnatural amino acid was guided by the fact that it truly mimics a phosphotyrosine (in a way that glutamate does not) in that it contains both a phosphate group and an aromatic ring. Additionally, being non-cleavable, it is stable under the enzymatic activity of Wzb. Despite the fact that the YC has been found to be multiply phosphorylated in vivo, as discussed earlier, the overall size of the Wzb catalytic cavity ensures that it cannot accommodate more than one phosphotyrosine at any given time, a condition that is effectively simulated by PHOSpep.

Unlike in the case of phosphate, where a large number of chemical shift changes were seen, suggesting chemical exchange on the fast time scale (as expected from a $K_d$ value of $630 \pm 118 \mu M$ obtained by fitting the chemical shift changes against phosphate concentration), PHOSpep generated significant changes in intensity without any major chemical shift changes. In addition, several peaks showed doubling, with one peak reducing in intensity and the other increasing in intensity with increasing PHOSpep concentration, indicative of slow exchange on the chemical shift time scale (Fig. 8B). Fitting the changes in intensity with PHOSpep concentration yields a $K_d$ of $261 \pm 65 \mu M$, suggesting an affinity that is comparable with that seen for phosphate alone. This reinforces the fact the majority of the binding affinity of WzcCD for Wzb is provided by docking interactions that do not involve the YC. A point to note is that the slow exchange regime seen here, although unusual for the weak affinity, is however not unprecedented (56, 57). This apparent anomaly could be due to an unusual $k_{on}$ or the result of a slow conformational change following the binding event. Here we analyze these effects only qualitatively in the context of YC recognition; a more detailed analysis, not relevant in the present context, is left for future studies. Overall, the spectral perturbations seen in Wzb in the presence of PHOSpep are similar to those seen with phosphate alone. However, PHOSpep induces a larger degree of spectral perturbations, in relative terms, on helix a5 of Wzb compared with phosphate (Fig. 8, compare A and C). This helix includes two aromatic residues, Phe124 (minimal perturbation, although the flanking residues Thr123 and Ala125 have attenuations of 0.40 and 0.46, respectively) and TYr128 (attenuation $= 0.43$; in fact, the stretch between TYr128 and Leu131 shows an average attenuation of 0.51), which are highly conserved in bacterial LMW-PTPs (see Fig. 2A). The perturbations in these regions and the suggestion that the D-loop of TYr117 contributes to substrate recognition open the possibility that a reorientation of a5 increases base-stacking interactions with the phosphotyrosine moiety. As stated earlier, the $^{13}$N,${}^1$H resonance corresponding to TYr117 can only be seen at high phosphate concentration. In order to probe the role of TYr117, and possibly of Phe124 and TYr128, in substrate stabilization, we analyzed $^{13}$C,${}^1$H HSQC spectra, focusing specifically on the aromatic groups (Fig. 9A). The aromatic resonances corresponding to Phe124 were weak and could not be analyzed. However, significant attenuations were seen for the $\delta$- and $\epsilon$-resonances corresponding to TYr117 in the presence of PHOSpep, although none of the resonances corresponding to TYr128 displayed any significant perturbations. No detectable perturbations for any of the aromatic resonances were seen in the presence of WTpep (Fig. 9B). Therefore, it seems likely that TYr117 does play a role in substrate recognition, although no definitive conclusion can be reached for the role of Phe124. As alluded to earlier by Lescop et al. (34), Leu40 in the so-called W-loop superimposes well on the specific resi-
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due in eukaryotic LMW-PTPs that have been shown to play a central role in substrate recognition. Although the $^{15}$N,$^1$H resonances of Leu$^{40}$ are not visible in the absence of phosphate, the methyl positions of Leu$^{40}$ are significantly attenuated in the presence of PHOSpep (Fig. 9C) but not WTpep (Fig. 9D). Thus, our results provide evidence supporting the conjecture (34) that the tyrosine moiety of the substrate phosphotyrosine is stabilized through ring-stacking interactions with a conserved D-loop aromatic residue, and additional stability is imparted through hydrophobic interactions with the W-loop.

In Vitro Dephosphorylation of Wzc$_{CD}$ by Wzb and Role of the EXXXR Motif—In order to ascertain the functional relevance of the NMR-determined binding site for Wzb on Wzc$_{CD}$, we determined the influence of mutations on the conserved EXXXR motif of Wzc$_{CD}$ on its ability to interact with Wzb and to be dephosphorylated by it. For this purpose, we chose a Wzc$_{CD}$ construct bearing a E508A/R511A/R514A triple mutation (Wzc$_{CD}$,ERR/A; see Fig. 3). It has been previously shown that E. coli cells complemented with this mutant produced 70% less colanic acid when compared with those carrying wild-type Wzc (19). As shown by the sensorgram in Fig. 10A (the scale is the same as in Fig. 5, C and D), Wzc$_{CD}$,ERR/A appears to bind very weakly to Wzb. In addition, wild-type Wzc$_{CD}$ is dephosphorylated more rapidly by Wzb compared with Wzc$_{CD}$,ERR/A (i.e. ~94% compared with ~69% after a 30-min incubation for a 20:1 ratio) (Fig. 10B). However, not surprisingly, our attempts to quantitatively analyze the dephosphorylation kinetics and obtain $K_m$ and $k_{cat}$ values were unsuccessful, given the multiply (and heterogeneously, as mentioned above) phosphorylated YC. A somewhat meaningful set of results could be obtained assuming the presence of two sets of sites on the YC: one set that could be dephosphorylated by Wzb in a highly efficient fashion and a second set that is dephosphorylated with lower efficiency (data not shown). This behavior is somewhat evident by the biexponential behavior displayed by the curves shown in Fig. 10B. To obtain more quantitative $K_m$ and $k_{cat}$ values, we created Wzc$_{CD}$ and Wzc$_{CD}$,ERR/A mutants that contain a single phosphorylatable tyrosine on the C-tail. These mutants (Fig. 3), named Wzc$_{CD}$,F$_5$ and Wzc$_{CD}$,ERR/A,F$_5$, contain a single tyrosine (Tyr$^{715}$) in the YC, with all other C-tail tyrosine residues mutated to phenylalanine (Y705F, Y708F, Y710F, Y711F, and Y713F). Tyr$^{715}$ from each Wzc$_{CD}$ subunit was found to engage the active site of a neighboring subunit in the crystal structure (19) of the Wzc$_{CD}$ oligomer. Hence, retaining Tyr$^{715}$ was the obvious choice for the enzymatic assays. The dephosphorylation data corresponding to these mutants could be fitted to sin-

FIGURE 8. Substrate recognition by Wzb. A, residues that display significant spectral perturbations in the presence of phosphate are mapped onto a ribbon representation of Wzb. Residues that display chemical shift changes greater than 0.04 ppm (mean + 2 S.D.) in the presence of 5 mol eq of phosphate are colored green. Residues for which the corresponding amide resonances can only be seen in the presence of 50 mM phosphate, 500 mol eq. B, although no significant spectral perturbations in $^{15}$N,$^1$H HSQC spectra of Wzb can be seen in the presence of either WTpep or MUTpep, major perturbations are seen in the presence of PHOSpep (data for 2 and 4 mol eq shown). A small subset of Wzb resonances that are attenuated in the presence of PHOSpep are circled and labeled. One-dimensional slices through resonances corresponding to Arg$^{17}$ and Ala$^{55}$ demonstrate the appearance of new peaks (indicated by the asterisk) in the presence of increasing concentrations of PHOSpep, indicative of slow exchange on the chemical shift time scale. C, spectral perturbations from B (for 4 mol eq of PHOSpep) are mapped onto a ribbon representation of Wzb. Residues for which the backbone amide resonances display attenuations of more than 0.38 (averaged over two independent measurements, greater than the mean + 3 S.D.) in the presence of the peptide are colored green. Residues that are not observable due to exchange broadening (Cys$^9$, Val$^{10}$, Leu$^{40}$, and Tyr$^{117}$) are colored orange. Key residues discussed in this work are labeled. Helix $\alpha 5$, which shows an increase in spectral perturbations (in relative terms) in the presence of PHOSpep compared with phosphate (A), is also labeled.

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gle-exponential functions (e.g., for a 35:1 kinase/phosphatase ratio, the dephosphorylation rate is 1.81 ± 0.06 min⁻¹ for WzcCD,F5 and 0.28 ± 0.20 min⁻¹ for WzcCD,ERR/A,F5) (Fig. 10C). Analysis of the enzyme kinetics in a Hanes-Woolf framework (Fig. 10D) confirmed that the decreased dephosphorylation rate was indeed the result of the reduced ability of WzcCD,ERR/A,F5 to form an enzyme-substrate complex with Wzb. On the other hand, the \( k_{\text{cat}} \) for WzcCD,F5 is only ~3.5-fold higher than that for WzcCD,ERR/A,F5. One may therefore conclude that mutations in the conserved EXXRXXR motif on the \( \alpha_2 \) helix of WzcCD lead to a reduction in its affinity for Wzb, resulting in a decrease in the efficiency of dephosphorylation.

**In Vitro Dephosphorylation of WzcCD by Wzb and Role of the Wzb Residues Leu⁴⁰ and Tyr¹¹⁷**—In order to test the importance of the W-loop Leu⁴⁰ and the D-loop Tyr¹¹⁷ in the Wzb catalyzed dephosphorylation of WzcCD in vitro, we created Wzb mutants WzbL₄₀A, WzbY₁₁₇A, and the double mutant WzbL₄₀A/Y₁₁₇A. Although the activity of WzbL₄₀A appeared to be marginally lower than that of wild-type Wzb, WzbY₁₁₇A (and the WzbL₄₀A/Y₁₁₇A double mutant) was significantly less efficient in dephosphorylating WzcCD (see Fig. 11). This suggests that although Leu⁴⁰ may play a secondary role in interacting with the phosphotyrosine substrate, as suggested by the NMR studies described above, its involvement is less critical than that of Tyr¹¹⁷.
DISCUSSION

Maintenance of the natural equilibrium between the YC\text{high} and YC\text{low} states of Wzc, a direct result of the activity of the cytosolic LMW-PTP Wzb, appears to be critical for the production of colanic acid and survival under stress (43). Here, we have used a variety of methods to ascertain the structural elements that mediate Wzc/Wzb interactions that are central in regulating the BY-kinase signaling pathway. Using SPR measurements, we have found that a majority of the binding energy for the interactions of Wzc with Wzb is provided by elements external to the phosphorylated YC of the former. Perturbations in solution NMR spectra reveal that the Wzb binding surface on Wzc involves a region that does not include any of the Wzc catalytic elements but involves the \( \alpha \) helix bearing a motif (E\text{XX}R\text{XX}R) that is highly conserved in BY-kinases, in addition to elements that are spatially proximate to it. In fact, most of the perturbations, including and around the E\text{XX}R\text{XX}R motif, involve extensive line broadening rather than chemical shift perturbations. We attribute these effects to the direct binding event that occurs on the intermediate exchange regime on the chemical shift time scale. The chemical shift perturbations, indicative of fast exchange, occur peripheral to the binding site and likely

![Figure 10](image1.png)

**Figure 10.** Dephosphorylation of wild-type and mutated Wzc<sub>CD</sub> by Wzb. A, sensorgram of the interaction of Wzc<sub>CD,ERR/A</sub> (ligand) with Wzb<sub>C9S</sub> (analyte) (the scale is the same as in Fig. 5, C and D). B, time course of Wzb-catalyzed dephosphorylation of Wzc<sub>CD</sub> (black) or Wzc<sub>CD,ERR/A</sub> (red). Data for a kinase/phosphatase ratio of 20:1 is shown as a representative example. C, time course of Wzb-catalyzed dephosphorylation of Wzc<sub>C9S</sub> (black) or Wzc<sub>CD,ERR/A</sub> (red). Data for kinase/phosphatase ratios of 35:1 (solid lines) and 5:1 (dashed lines) are shown as representative examples. D, Hanes-Woolf fits using either Wzc<sub>CD,F5</sub> (black) or Wzc<sub>CD,ERR/A,F5</sub> (red) as substrates for Wzb.

![Figure 11](image2.png)

**Figure 11.** Dephosphorylation of Wzc<sub>CD</sub> by wild-type and mutated Wzb. Mutation of Tyr<sup>117</sup> on the Wzb D-loop to alanine significantly reduces its ability to dephosphorylate Wzc<sub>CD</sub>, whereas mutation of the W-loop Leu<sup>40</sup> to alanine has only a minor effect. Also shown are data for the L40A/Y117A double mutant. Data for the catalytically dead Wzb mutant (Wzb<sub>C9S</sub>) and wild-type Wzb are also shown for comparison. A, autoradiogram obtained after incubation of phosphorylated Wzc<sub>CD</sub> with Wzb and mutants thereof for a 10-min period. B, residual phosphorylation after 10-min (black) and 25-min (red) incubation periods. The intensities are normalized against the 10-min data for Wzb<sub>C9S</sub>. The dotted lines serve as guides for the residual phosphorylation levels when using wild-type Wzb.
represent conformational changes occurring upon Wzb binding. A more detailed parsing of these effects can only be achieved upon the availability of an atomic resolution model of the WzcCD-Wzb complex and a detailed analysis of the changes in dynamics upon complex formation. The critical role of the EXXRXXR motif in binding Wzb is confirmed by binding studies and biochemical assays, which reveal that mutations in this motif greatly reduce the ability of WzcCD to interact with and be dephosphorylated by Wzb. As discussed above, many lines of data indicate that the EXXRXXR motif plays a key role in the formation of asymmetric oligomers of WzcCD, allowing the YC of one molecule to access the catalytic site of another, a necessary condition to allow the intermolecular autophosphorylation. Our present results add a new wrinkle to this model, suggesting that not only does Wzb generate YC_{low} states from the YC_{high}, states through its enzymatic activity, but it also probably prevents the reassociation (and consequent phosphorylation) of the WzcCD monomers. One may speculate that the purpose of this protection is to allow the active site of WzcCD, which is occluded in the oligomeric state, to be accessible to downstream targets, such as Ugd (32). The studies presented here involve the isolated CD of Wzc. It is quite possible that conformation of WzcCD and its interactions with Wzb may be modulated by periplasmic events involving the ectodomain of Wzc and indeed its trans-membrane segments. Transmission of extracytoplasmic events through the membrane to modulate the activity of cytoplasmic kinase domains has been extensively investigated in eukaryotic receptor tyrosine kinases, most notably the epidermal growth factor receptor (58, 59). In addition, cytoplasmic events, including post-translation modifications, such as phosphorylation, could also modify the interactions between WzcCD and Wzb. This mode of regulation has been seen in eukaryotic tyrosine phosphatases. For example, in T-cells, the hematopoietic PTP docks onto the mitogen-activated protein kinase (MAPK), ERK2, using a conserved D-site sequence (60). The hematopoietic PTP stays bound to ERK2 even after dephosphorylating the phosphorylated tyrosine on the activation loop of ERK2 and sequesters the inactive kinase to the cytosol (61). Detachment occurs upon phosphorylation of a D-site serine on hematopoietic PTP by protein kinase A (PKA) (62). This is an intriguing scenario, especially because it has recently been shown that Etp, the cognate LMW-PTP for Etk, is phosphorylated on a D-loop tyrosine by a yet unknown cellular kinase that is neither Etk nor Wzc (63). However, we have not yet found compelling evidence that the corresponding tyrosine (Tyr^{117}) in Wzb is phosphorylated in the cellular milieu.

As mentioned previously, the interaction of Wzb with WzcCD does not seem to require the phosphorylated YC. The use of motifs for docking interactions external to the substrate moieties to enhance affinity and specificity has been well documented in eukaryotic phosphatases (64, 65). Our results further demonstrate that the catalytic site of Wzb, especially the P-loop bearing the conserved CXXXRRXS motif, undergoes conformational changes upon docking interactions with WzcCD that lacks the YC. This contrasts with interactions on the WzcCD side that do not seem to display any discernable allosteric effects at its active site. Thus, docking interactions outside the Wzb catalytic site appear to trigger conformational changes in the catalytic P-loop, perhaps priming it for catalysis. Wzb undergoes significant conformational changes in the presence of phosphate, with most of these changes localized on the catalytic P-loop, on the D-loop, and, to a lesser extent, on the W-loop. Stehle et al. (66) have recently solved the structure of MptpA from Mycobacterium tuberculosis in the phosphate-free form. This structure, which represents the first LMW-PTP structure in the absence of phosphate, provides a means to compare the conformational changes expected upon phosphate binding given that the crystal structure of MptpA bound to a phosphomimetic chloride ion exists (67). In this study, Stehle et al. (66) show that MptpA adopts an open structure in the absence of phosphate with the D- and P-loops more distant from each other, whereas the presence of chloride induces a more closed conformation, greatly reducing the distance between the two loops. The perturbations seen in the Wzb D- and P-loops in our case are consistent with these observations. We further note the presence of extensive conformational exchange in the P-loop in the absence of phosphate, as reflected by the missing resonances. This conformational sampling is greatly reduced in the presence of phosphate, a fact that has also been previously demonstrated (34). Thus, the phosphate group of the phosphotyrosine moiety appears critical in substrate recognition by Wzb. The tyrosine moiety is stabilized largely by ring-stacking interactions with the D-loop tyrosine (Tyr^{117}) and hydrophobic interactions with a W-loop leucine (Leu^{40}). Notably, the Tyr^{117} position is strictly conserved in bacterial LMW-PTPs (Phe^{120} in Bacillus subtilis; see Fig. 2A), whereas the Leu^{40} position, although less strictly conserved, always contains a large hydrophobic residue. The central role of Tyr^{117} in stabilizing the substrate, in what is likely an optimal orientation for catalysis, is confirmed by the fact that the phosphotyrosine phosphatase activity of Wzb is significantly reduced in a Y117A mutant. Considering all of the results presented here, it seems that docking interactions between Wzc and Wzb bring the phosphorylated YC close to the catalytic site of Wzb. Dephosphorylation by Wzb proceeds due to the high local concentration of the substrate, phosphorylated YC, despite the low inherent affinity of Wzb for the isolated phospho-YC. Thus, YC dephosphorylation by Wzb may therefore be appropriately described as proximity-mediated (68).

In conclusion, we have, for the very first time, presented a comprehensive analysis of the interactions between the catalytic domain of the BY-kinase Wzc with its cognate phosphatase, Wzb. We have identified elements within the catalytic domain of Wzc that are crucial for the recognition of Wzb. In addition, our studies provide insight into the recognition by Wzb of the phosphorylated YC of Wzc, its natural substrate. These results represent a major step forward in obtaining a comprehensive understanding of the regulation of the BY-kinase signaling pathway by bacterial PTPs.

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Interactions between Wzb and Wzc

41. Prenta, R., Jarraud, S., Vincent, C., Doublet, P., Duclos, B., Etienne, J., and Cozzone, A. J. (2002) Isolation and characterization of a protein-tyrosine kinase and a phosphotyrosine-protein phosphatase from Klebsiella pneumoniae. Comp. Biochem. Physiol. B 131, 103–112

42. Whitfield, C. (2006) Biosynthesis and assembly of capsular polysaccharides in Escherichia coli. Annu. Rev. Biochem. 75, 39–68

43. Obadia, B., Lacour, S., Doublet, P., Baubichon-Cortay, H., Cozzone, A. J., and Grangeasse, C. (2007) Influence of tyrosine-kinase Wzc activity on colanic acid production in Escherichia coli K12 cells. J. Mol. Biol. 367, 42–53

44. Mao, Y., Doyle, M. P., and Chen, J. (2006) Role of colanic acid exopolysaccharide in the survival of enterohaemorrhagic Escherichia coli O157:H7 in simulated gastrointestinal fluids. Lett. Appl. Microbiol. 42, 642–647

45. Temel, D. B., Dutta, K., and Ghose, R. (2012) Sequence-specific 1H, 13C and 15N assignments of the catalytic domain of the Escherichia coli protein tyrosine kinase, Wzc. Biomol. NMR Assign. DOI 10.1007/s12104-012-9448-0

46. Muratore, K. E., and Cole, P. A. (2007) A lock on phosphotyrosine signaling. ACS Chem. Biol. 2, 454–456

47. Boggs, P. T., Donaldson, J. R., Byrd, R. H., and Schnabel, R. B. (1989) ODRPACK software for weighted orthogonal distance regression. ACM Trans. Math. Software 15, 348–364

48. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) NMRPipe. A multidimensional spectral processing system based on UNIX pipes. J. Biomol. NMR 6, 277–293

49. Johnson, B. A. (2004) Using NMRView to visualize and analyze the NMR spectra of macromolecules. Methods Mol. Biol. 278, 313–352

50. Salzmann, M., Pervushin, K., Wider, G., Senn, H., and Wüthrich, K. (1998) TROSY in triple-resonance experiments. New perspectives for sequential NMR assignment of large proteins. Proc. Natl. Acad. Sci. U.S.A. 95, 13585–13590

51. Lemak, A., Gutmanas, A., Chitayat, S., Karra, M., Farès, C., Sunnerhagen, M., and Arrowsmith, C. H. (2011) A novel strategy for NMR resonance assignment and protein structure determination. J. Biomol. NMR 49, 27–38

52. Orekhov, V. Y., and Jaravine, V. A. (2011) Analysis of non-uniformly sampled spectra with multi-dimensional decomposition. Prog. Nucl. Magn. Reson. Spectrosc. 59, 271–292

53. Yamazaki, T., Forman-Kay, J., and Kay, L. E. (1993) Two-dimensional NMR experiments for correlating 13C/1H and 1H/31P chemical shifts of aromatic residues in 13C-labeled proteins via scalar couplings. J. Am. Chem. Soc. 115, 11054–11055

54. Zhu, G., Xia, Y., Nicholson, L. K., and Sze, K. H. (2000) Protein dynamics measurements by TROSY-based NMR experiments. J. Magn. Reson. 143, 423–426

55. Anthis, N. J., Haling, J. R., Oxley, C. L., Memo, M., Wegener, K. L., Lim, C. J., Ginsberg, M. H., and Campbell, I. D. (2009) β integrin tyrosine phosphorylation is a conserved mechanism for regulating talin-induced integrin activation. J. Biol. Chem. 284, 36700–36710

56. Zuideweg, E. R. (2002) Mapping protein-protein interactions in solution by NMR spectroscopy. Biochemistry 41, 1–7

57. Latham, M. P., Zimmermann, G. R., and Pardi, A. (2009) NMR chemical exchange as a probe for ligand-binding kinetics in a theophylline-binding RNA aptamer. J. Am. Chem. Soc. 131, 5052–5053

58. Arkhipov, A., Shan, Y., Das, R., Endres, N. F., Eastwood, M. P., Wemmer, D. E., Kuriyan, J., and Shaw, D. E. (2011) Architecture and membrane interactions of the EGF receptor. Cell 152, 557–569

59. Endres, N. F., Das, R., Smith, A. W., Arkhipov, A., Kovacs, E., Huang, Y., Pelton, J. G., Shan, Y., Shaw, D. E., Wemmer, D. E., Groves, J. T., and Kuriyan, J. (2013) Conformational coupling across the plasma membrane in activation of the EGF receptor. Cell 152, 543–556

60. Bardwell, J. L. (2006) Mechanisms of MAPK signalling specificity. Biochem. Soc. Trans. 34, 837–841

61. Pettiford, S. M., and Herbst, R. (2003) The protein tyrosine phosphatase H2PT regulates nuclear translocation of ERK2 and can modulate megakaryocytic differentiation of K562 cells. Leukemia 17, 366–378

62. Saxena, M., Williams, S., Taskén, K., and Mustelin, T. (1999) Crosstalk between Camp-dependent kinase and MAP kinase through a protein tyrosine phosphatase. Nat. Cell Biol. 1, 305–311

63. Nadler, C., Koby, S., Peleg, A., Johnson, A. C., Saddala, K. C., Sathiyanamorthy, K., Smith, B. E., Saper, M. A., and Rosenshine, I. (2012) Cycling of Etk and Etp phosphorylation states is involved in formation of group 4 capsule by Escherichia coli. PLoS One 7, e37984

64. Reményi, A., Good, M. C., and Lim, W. A. (2006) Docking interactions in protein kinase and phosphatase networks. Curr. Opin. Struct. Biol. 16, 676–685

65. Virshup, D. M., and Shenolikar, S. (2009) From promiscuity to precision. Protein phosphatases get a makeover. Mol. Cell 33, 537–545

66. Stehle, T., Sree ramulu, S., Löhr, F., Richter, C., Saxena, K., Jonker, H. R., and Schwab, H. (2012) The apo-structure of the low molecular weight protein-tyrosine phosphatase A (MptpA) from Mycobacterium tuberculosis allows for better target-specific drug development. J. Biol. Chem. 287, 34569–34582

67. Madhurantakam, C., Rajakumara, E., Mazumdar, P. A., Saha, B., Mitra, D., Wilker, H. G., Sankaranarayanan, R., and Das, A. K. (2005) Crystal structure of low-molecular-weight protein tyrosine phosphatase from Mycobacterium tuberculosis at 1.9 Å resolution. J. Bacteriol. 187, 2175–2181

68. Rainey, M. A., Callaway, K., Barnes, R., Wilson, B., and Dalby, K. N. (2005) Proximity-induced catalysis by the protein kinase ERK2. J. Am. Chem. Soc. 127, 10494–10495