The domain architecture of the PtkA, the first tyrosine kinase from *Mycobacterium tuberculosis* differs from the conventional kinase architecture.

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Running title: Structural studies of PtkA by NMR

Abstract

The discovery that MptpA (low-molecular-weight protein tyrosine phosphatase A) from *Mycobacterium tuberculosis* (Mtb) has an essential role for *Mtb* virulence has motivated research of tyrosine-specific phosphorylation in *Mtb* and other pathogenic bacteria. The phosphatase activity of MptpA is regulated via phosphorylation on Tyr-128 and Tyr-129. Thus far, only a single tyrosine-specific kinase, protein tyrosine kinase A (PtkA), encoded by the *Rv2232* gene has been identified within the *Mtb* genome. MptpA undergoes phosphorylation by PtkA. PtkA is an atypical bacterial tyrosine kinase, as its sequence differs from the sequence consensus within this family. The lack of structural information on PtkA hampers the detailed characterization of the MptpA-PtkA interaction. Here, using NMR spectroscopy, we provide a detailed structural characterization of the PtkA architecture and describe its intra- and intermolecular interactions with MptpA. We found that PtkA's domain architecture differs from the conventional kinase architecture and is composed of two domains, the N-terminal highly flexible IDD<sub>PtkA</sub> and the C-terminal rigid KCD<sub>PtkA</sub>. The interaction studies between the two domains together with the structural model of the IDD-KCD complex proposed in this study reveals that the IDD is unstructured and highly dynamic, allowing for a “fly-casting” like mechanism of transient interactions with the rigid KCD. This interaction modulates the accessibility of the KCD active site. In general, the structural and functional knowledge of PtkA gained in this study, is crucial for understanding the MptpA-PtkA interactions, catalytic mechanism and the role of kinase-phosphatase regulatory system in *Mtb* virulence.

*Mycobacterium tuberculosis* (Mtb) (1) increasingly evolves multi-drug resistant (MDR)-(2) and extensive drug resistant (XDR)-(3) strains, causing epidemic problems on global scale (4). New therapeutics against tuberculosis (TB) are thus urgently needed. The development of new inhibitors is challenging, especially if one considers that the requirements of anti-TB drugs to
Structural studies of PtkA by NMR

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understanding of the interactions between MptpA and PtkA, impairing the development of strategies for rational design of TB-inhibitors.

Here, we present an extensive study on the structure and dynamics of the wild-type PtkA using nuclear magnetic resonance (NMR)-spectroscopy, describing the intrinsic structural architecture of PtkA at atomic resolution. We characterize the two domains that build up the PtkA structure, an N-terminal intrinsically disordered domain (IDD_PtkA) and C-terminal kinase core domain (KCD_PtkA). Additionally, we solved the three-dimensional NMR structure of KCD_PtkA. Further, using a broad spectrum of NMR techniques we characterize inter- and intramolecular interactions of PtkA, giving important insights into the key regulatory catalytic processes of tyrosine phosphorylation-dephosphorylation mediated by PtkA-MptpA.

RESULTS

Structural study of PtkA - The sequence-based analysis of PtkA (30.6 kDa, 291 amino acids) predicts a high disorder tendency for the N-terminal part comprising 80 amino acids (Fig. S1). Our NMR data confirm the prediction, showing that PtkA consists of a well-folded kinase core domain (KCD) and an intrinsically disordered domain (IDD). The 2D-(^1H,^15N)-TROSY spectrum of the full-length PtkA shows in total 231 out of 270 expected amide resonances (Fig. 1A). Two subsets of signals can be clearly observed: (i) a set of well dispersed peaks, typical for a well-folded structure, and (ii) another set of signals with high intensity, which are clustered in the center of spectrum (8.5-7.7 ppm ^1H ppm), indicative of unstructured regions within the full-length PtkA. The 2D-(^1H,^15N)-HSQC spectrum of PtkA shows only minor changes of the chemical shift in presence of ATP (Fig. 1B). Unfortunately incubation of PtkA with ATP for 24 hours leads to precipitation of the protein, which excludes phosphorylated PtkA to be used for long-term NMR experiments.

Sequence specific NMR resonance assignment of full-length PtkA – The assignment of the ^1H,^15N and ^13C backbone resonances of the full-length PtkA was obtained using a combination of standard triple resonance NMR experiments on triple (^2H,^15N,^13C) and/or double (^15N,^13C) labeled PtkA samples. We observed that the number of visible cross-peaks increases to 20% in the presence of Mg^{2+} leading to better quality of the NMR spectra. Therefore, all assignment experiments were performed in presence of 10 mM MgCl2. In addition the selectively ^15N-Val and ^15N-Phe labeled PtkA samples were prepared to confirm ambiguous assignments. We were able to assign 93.5% of the observable backbone amides (216 out of 231 detected amide resonance in 2D-(^1H,^15N)-TROSY spectrum), which is 80% of the expected amide cross-peaks. Most of the missing chemical shift assignments were restricted to three regions: Thr^{25}-Ser^{29}, Thr^{210}-Asp^{219} and Val^{257}-His^{278} (Fig. 1C), presumably due to intermediate exchange on the NMR timescale. To complete and verify the assignment of the full-length PtkA, we undertook a protein construct optimization. The assignment of the full-length PtkA was thus aided by using two PtkA truncation mutants (Fig. 1B) containing either the N-terminal 81 amino acids (Met^{1}-Pro^{81}) representing the intrinsically disordered domain (IDD_PtkA, 8.5 kDa) or the C-terminal (Val^{75}-Val^{291}) region representing the kinase core domain (KCD_PtkA, 23 kDa). The structural properties of both constructs were investigated by NMR spectroscopy.

(i) Backbone assignment of KCD_PtkA - The 2D- (^1H,^15N)-HSQC spectrum of KCD_PtkA revealed a substantial reduction of the signal overlap, and the remaining 172 well-resolved backbone amide signals fit almost perfectly with those observed in the spectrum of the full-length PtkA (with the exception of those residues which are located close the truncation site: Gly^{76}, Glu^{77}, Ser^{78}), indicating proper folding of the KCD_PtkA. Using the KCD_PtkA in separation we were able to obtain 96.5% of the backbone assignment (166 from 172 observable
amide cross-peaks, Fig. S2A), which is 76.9% of the expected amide cross-peaks. Hence, the KCD
PtkA was used for the three-dimensional NMR-based structure determination.

(ii) Backbone assignment of IDD
PtkA - The presence of intrinsically disordered domain hampers the unambiguous assignment in the overcrowded spectral region of the full-length PtkA. The 2D-(1H,15N)-HSQC spectrum of IDD
PtkA at pH 7.5 revealed poor signal dispersion, similar to the observation made in the spectrum of the full-length PtkA. To facilitate the backbone assignment of the IDD
PtkA, we investigated the IDD
PtkA also at low pH. In the 2D-(1H,15N)-HSQC spectrum of IDD
PtkA measured at pH 2.0, we could detect 68 well resolved signals and were able to assign all the amide backbone using a set of 3D-NMR-experiments (HNCACB, (H)(N(CA)NNH, HNHA, HNCO), Fig. S2C. A pH titration series of 2D-(1H,15N)-HSQC spectra were recorded, ranging from pH 2.0 to pH 7.5 with a 1.5 pH unit increment, to enable the backbone assignment of the 2D-(1H,15N)-HSQC-spectrum of IDD
PtkA at pH 7.5 (Fig. S2B). The detailed characterization of the IDD
PtkA was recently published by our group (22).

The resonance assignment from the KCD
PtkA (76-291) and IDD
PtkA (1-81) was used to validate the assignment of the full-length PtkA (1-291). We were able to assign 69% of the IDD (47 signals from 68 expected) and 84% of the KCD (169 from 202 expected) backbone amide signals in the full-length PtkA at pH 7.5 (Fig. 1A). The backbone assignment of PtkA has been deposited in the Biological Magnetic Resonance Data Bank (BMRB) with entry number 34204.

Three dimensional structure determination of the KCD
PtkA - The NMR solution structure of the PtkA kinase core domain was determined (PDB entry 2F2X). Due to the limited completeness of the resonance assignment of residues located in or near to the center of the protein, the conventional structure calculation with fully automated NOESY cross-peak assignment by CYANA was unsuccessful. Missing assignment within and around two central regions (e.g., Thr210-Asp219 and Val257-His278) indicate dynamics or exchange on an intermediate NMR timescale for this part of the molecule, complicating the structural characterization of the KCD
PtkA. Both regions are particularly interesting, as they contain residues involved in the catalytic regulation of PtkA. To be able to elucidate the three-dimensional structure of PtkA, homology models were used to assist the NOESY cross-peak assignment by CYANA thereby just avoiding NOE misassignments between atoms which are actually not close to each other (assigned resonances which coincide with those of the undefined region) which would otherwise disrupt the structure and cause the convergence to fail. The subsequent structure refinement was done without the additional global distance restraints (see the experimental procedure for more details). This allowed us to characterize the structural propensities of the PtkA core domain (Fig. 2). The regions with missing assignments (Thr210-Val216 and Tyr262-Val275, indicated in gray) are undefined due to the lack of restraints (thus just merely force field based) and their spread does not necessarily indicate dynamics. Six parallel β-sheets located in lobe-1 (Fig. 2B) of the KCD
PtkA build up the hydrophobic core of the KCD
PtkA. The lobe-1 is the essential part of the PtkA containing catalytic loop (D85LD-motif), lysine residues (Lys184, Lys217 and Lys270 essential for ATP binding) and the auto-phosphorylation site (Tyr262). Lobe-2 of the core domain consists of three long α-helices (designated α1-α3) and a short α-helical section which is located between α-helix α1 and α-helix α2. Lobe-2 contains two additional tyrosine residues (Tyr146 and Tyr150), with an unknown role. However, the description of the catalytic site of PtkA built up from the structure reveals that, all the three lysine residues (Lys184, Lys217 and Lys270), tyrosines (Tyr146, Tyr150 and...
Tyr\textsuperscript{262}) and the Asp\textsuperscript{85} located in the conserved DXD motif, are surrounding the autophosphorylation site (Fig. S3A). In addition, primary sequence alignment of PtkA and two putative phosphatases (from \textit{Clostridium difficile} and phosphatase from \textit{Clostridium Acetobutylicum}) revealed no sequence analogy for the N-terminal IDD, while four conserved HAD signature motifs were found to be conserved within the C-terminal domain (Fig. S3B).

\textit{Study of PtkA dynamics: heteronuclear \textsuperscript{15}N relaxation studies} - PtkA dynamics were studied using standard heteronuclear \textsuperscript{15}N-relaxation experiments. The experiments were performed on the full-length PtkA as well as on the KCD\textsubscript{PtkA} and the IDD\textsubscript{PtkA} in order to investigate whether the domains influence the dynamics of each other. The experimentally obtained relaxation rates for \textsuperscript{15}N-T\textsubscript{1}, \textsuperscript{15}N-T\textsubscript{2} and \textsuperscript{15}N\textsuperscript{(1)}H-het nOe were used to determine the Lipari-Szabo order parameter, S\textsuperscript{2} (Fig. 3A). The relaxation study of the internal motions in the full-length PtkA shows a rigid KCD and a higher flexibility of the N-terminal domain (Met\textsuperscript{1}-Glu\textsuperscript{77}, indicated by an increase of the R\textsubscript{1}-rate, a decreased R\textsubscript{2}-rate and a lower \textsuperscript{15}N\textsuperscript{(1)}H-het nOe value). The relaxation data obtained for the KCD\textsubscript{PtkA} construct are highly similar to the full-length PtkA, which indicates that there is no major effect on the local backbone dynamics of the KCD in the absence of the IDD. For the IDD\textsubscript{PtkA}, we determine \textsuperscript{15}N-R\textsubscript{1} relaxation rates of 1.78 (± 0.13) s\textsuperscript{-1} and \textsuperscript{15}N-R\textsubscript{2} rates of 3.44 (± 0.33) s\textsuperscript{-1}. The relaxation rates thus obtained for the IDD\textsubscript{PtkA} and the full-length PtkA are comparable and confirm high flexibility and disordered state at native-like conditions for the N-terminal domain of PtkA.

\textit{Hydrogen-Deuterium exchange studies} - Hydrogen-deuterium (H-D) exchange studies were used to study the solvent accessibility of the amide protons of PtkA in the presence and absence of the IDD. The exchange rate (k\textsubscript{ex}) of the backbone amides of the full-length PtkA and the KCD\textsubscript{PtkA} were determined (Fig. 3B). Fifteen minutes after reconstitution of the freeze-dried full-length PtkA sample into D\textsubscript{2}O, only 33% of the backbone amide cross-peaks were observed in comparison to the signals in H\textsubscript{2}O. All amide signals corresponding to the residues of IDD disappeared, which is typical for very flexible dynamic regions and thus well in agreement with the results from our heteronuclear relaxation studies. In contrast, 10% of the amide signals of residues located in the KCD were still visible even after 19 hours. These very slowly exchanging backbone amide protons correspond to the residue: Val\textsuperscript{82}-Asp\textsuperscript{85}, Leu\textsuperscript{167}-Arg\textsuperscript{172}, Val\textsuperscript{176}-Arg\textsuperscript{172}, Val\textsuperscript{176}-Thr\textsuperscript{182}, Leu\textsuperscript{193}-Phe\textsuperscript{196}, Ala\textsuperscript{206}, Leu\textsuperscript{225}, Val\textsuperscript{236}-Asp\textsuperscript{240}, Leu\textsuperscript{285}-Val\textsuperscript{291}, suggesting involvement of those residues in the hydrogen bond network of highly protected secondary structure elements in the KCD. Furthermore, the correlation between the secondary structure elements and H-D exchange rate reveals that the KCD is inherently stable and rigid. At the same time, different dynamic properties of the α-helices in the KCD were observed. Slow exchanging amide protons from residues in loop region between β-sheet β1 and α-helix α1 and residues in the α-helix α\textsubscript{4-6} suggest potential exposure to the hydrophobic part of the protein, while α-helix α\textsubscript{1-3} and α\textsubscript{6-7} fluctuate in solution. Moreover, for PtkA without the IDD, slight differences in the backbone amide hydrogen exchange behaviors were observed after 3h of reconstitution in D\textsubscript{2}O (Fig. S4). Signals corresponding to the residues: Val\textsuperscript{144} and Ala\textsuperscript{152}, located in the α-helix (α\textsubscript{2}) containing Tyr\textsuperscript{146} and Tyr\textsuperscript{150}, Phe\textsuperscript{161} located in the α\textsubscript{2}-α\textsubscript{3} loop and Val\textsuperscript{256} located near to the Tyr\textsuperscript{262}, were missing in the full-length PtkA but still detectable in the spectrum of KCD\textsubscript{PtkA}. We speculate that these minor differences observed may play an important role during the autocatalytic regulation of PtkA, involving tyrosine phosphorylation (7). Hence, the dynamic nature of the intrinsically disordered N-terminal domain may play a role in modulating the behavior and arrangement of the important helices involved in catalysis.
**Intramolecular hydrogen bonding** - A temperature series was performed to study intramolecular hydrogen bonding based on the amide proton temperature dependence. We determined the temperature coefficient (Tcoeff. in ppb/K, Fig. 3C) from the amide chemical shift values by acquiring a series of 2D-(1H,15N)-HSQC spectra measured at different temperatures, from 301 K to 283 K with a 3° increments (Fig. S5). We analyzed the full-length PtkA as well as the KCD<sub>PtkA</sub> and IDD<sub>PtkA</sub>, to examine the involvement of the amide protons in the formation of rigid or transient hydrogen-bonded structure elements. Amide protons with a temperature coefficient more negative than -4.5 ppb/K (indicated by the dotted line in Fig. 3C) are generally not hydrogen bonded (23). Many values obtained for amide protons of residues located in the KCD are larger than -4.5 ppb/K, which clearly suggests their involvement in the hydrogen-bond formation, which is in agreement with the presence of abundant secondary structural elements in this domain. On the other side, for the IDD, nearly all values are below -4.5 ppb/K, indicating that the residues in this domain are generally not hydrogen-bonded. Only Asn<sub>48</sub> (-3.7 ppb/K), Gly<sub>49</sub> (-3.6 ppb/K) and Asn<sub>60</sub> (-4.0 ppb/K), which show a temperature coefficient larger than -4.5 ppb/K were observed, indicating a potential involvement of those residues in transient hydrogen bonding.

**PtkA intramolecular IDD-KCD interaction** - The intramolecular domain-domain interactions were examined by comparing the chemical shifts observed in 2D-(1H,15N)-HSQC spectra of the full-length PtkA, IDD<sub>PtkA</sub> and KCD<sub>PtkA</sub> (Fig. S6A-B). The spectra of the full-length PtkA and the KCD<sub>PtkA</sub> indicate that the folding of the KCD is not affected by truncation of the IDD. The comparison of the amide chemical shift of the IDD in the full-length PtkA and the IDD<sub>PtkA</sub> spectra show the same chemical shift for the most of the cross-peaks, except for the residues located proximate to the truncation site as well for Asn<sub>48</sub> (Fig. S6A), suggesting weak domain-domain interactions. To study whether such interactions actually occur, we performed NMR-titration experiments between 15N-labeled KCD<sub>PtkA</sub> and unlabeled IDD<sub>PtkA</sub> (up to molar ratio 1:2) and vice versa (up to molar ratio 1:3) at 298 K and pH 7.5 in presence and absence of ATP (10 mM). The 2D-(1H,15N)-HSQC spectra recorded during the titration series revealed no significant CSPs (Fig. S6C-D). Based on these results, we assume that interactions between the IDD<sub>PtkA</sub> and KCD<sub>PtkA</sub> in trans (IDD and KCD are separated by mutational deletion), if present, are transient and rather weak. Nevertheless, information based on the amide chemical shift is primarily limited to the protein backbone and may thus be insufficient to detect weak interactions. Paramagnetic relaxation enhancement (PRE) NMR spectroscopy has been efficiently used to study weak and transient interactions. To detect these potential weak inter-domain interaction of PtkA, we used the site-directed spin label (SDSL)-approach. With SDSL, we analyzed long-range PtkA domain-domain interactions in cis (native state in full-length PtkA). The nitroxide radical, MTSL, was covalently attached to the conserved cysteine (Cys<sub>61</sub>) residue located on the IDD. Further, to investigate the presence of residual structure in the IDD (Fig. S1), two single cysteine PtkA mutants were designed including mutations on alanine 10 (PtkA/C61A/A10C) and serine 41 (PtkA/C61A/A10C). The point mutations were introduced (i) far away from the potential predicted secondary structure element such that it does not disturb the fold (A10C) and (ii) a second mutant (S41C) within the folded region in order to interrupt the formation of the secondary structural element. After spin labeling with MTSL, 2D-(1H,15N)-HSQC spectra of the paramagnetic and diamagnetic protein (after addition of ascorbic acid) were acquired. In all spectra of the protein containing a paramagnetic center, PRE (paramagnetic relaxation enhancement)-induced line broadening effects (LBE, Fig. 4A) were observed. The intensity ratio analysis of paramagnetic and diamagnetic (I<sub>para</sub>/I<sub>dia</sub>) PtkA resolved the mostly affected regions (Fig. 4B).
all the three investigated PtkA-constructs, the same regions in KCD were affected (Ipara/Idia \( \leq 0.4 \), Glu\(^{114}\)-Gly\(^{134}\), Asp\(^{162}\), Thr\(^{188}\)-Ile\(^{192}\), Ile\(^{205}\)-Gly\(^{212}\), Leu\(^{231}\)-Met\(^{237}\), Trp\(^{260}\) and Ile\(^{282}\)) suggesting a preferential position of the IDD relative to the core domain and the possibility of transient long-range domain-domain interactions. To visualize the IDD-KCD interaction, a set of experimental restraints from the spin-labeled complexes was used for the calculation of the model shown in Fig. 4C.

Analysis of this model shows the presence of multiple orientations for the IDD within the IDD-KCD complex suggesting a dynamic nature for this interaction. Further, the ensemble of similar orientation indicates that the IDD transiently interacts with the KCD and masks the ATP-binding site (Fig. 4C, right).

**PtkA auto-phosphorylation and regulation of its catalytic activity**—The catalytic activity of PtkA is regulated via auto-phosphorylation of tyrosine residues in presence of phosphate donor ATP or GTP (18). For the transfer of the phosphoryl group (PO\(_3\)^{2-}) from ATP to the target protein and the formation of the physiological substrate XATP\(^{1-}\), the kinase requires divalent cations (e.g. X = Mn\(^{2+}\), Mg\(^{2+}\)). Three tyrosine residues (Tyr\(^{146}\), Tyr\(^{150}\) and Tyr\(^{262}\)) are present in the sequence of PtkA and represent potential sites for auto-phosphorylation. In particular, Tyr\(^{262}\) was considered as the target for phosphorylation during auto-catalysis (18). PtkA itself represents a target protein for phosphorylation by the *Mtb* endogenous eSTPK (20). We investigated (i) the auto-phosphorylation of PtkA, (ii) the effect of Mg\(^{2+}\), (iii) interactions between PtkA in non-phosphorylated and phosphorylated state with MptpA, (iv) dephosphorylation of PtkA by MptpA and (v) PtkA phosphorylation by serine/threonine kinase (PKA, Fig. S11) (22).

(i) **PtkA auto-phosphorylation** - The auto-phosphorylation activity of full-length PtkA was previously measured and confirmed using a Luciferase assay (16). Furthermore, the auto-phosphorylation site in PtkA resides on the KCD and not on the IDD. In order to test the influence of the IDD on the auto-phosphorylation of PtkA we performed a luminescence assay using a KCD\(_{PtkA}\) construct alone and in presence of the IDD\(_{PtkA}\) and normalized the activity to that of PtkA, which was set to 100% (Fig. 5C and Fig. S12). The results thus obtained suggest that the KCD\(_{PtkA}\) is five times more active than that of the full-length PtkA. Activity of the KCD\(_{PtkA}\) measured together with isolated IDD (IDD\(_{PtkA}\)) shows a decrease in activity compared to the KCD\(_{PtkA}\) alone, suggesting that the IDD has an inhibitory role towards the kinase activity. The IDD in PtkA presents several potential PTM (phosphorylation) sites. Surprisingly, the activity of the PtkA in which the IDD was phosphorylated (PtkA*-P) showed four times more activity than that of its non-phosphorylated form (PtkA*) (Fig. 5C). These results strongly suggest that the IDD transiently binds to the KCD and masks the substrate binding site and inhibits the kinase activity. Using 1D-\(^{31}\)P-NMR spectroscopy, we monitored the auto-phosphorylation reaction of PtkA in presence and absence of the IDD after overnight incubation of PtkA (100 \(\mu\)M) with ATP (10 mM) at pH 7.5, 25°C (Fig. 5D). In both cases, an additional signal at – 0.98 ppm indicative for the phosphorylated state of PtkA, was observed. MALDI-MS confirmed that PtkA was one time phosphorylated (Fig. S10).

(ii) **PtkA interactions with Mg\(^{2+}\)** - To explore the effect of Mg\(^{2+}\) on the structural propensities of PtkA, we performed NMR studies in the presence and absence of MgCl\(_2\). Addition of MgCl\(_2\) (10 mM) into NMR-buffer highly improves the quality of the 2D-(\(^{1}\)H,\(^{15}\)N)-HSQC spectrum of PtkA, resulting in 20% more detectable amide cross-peaks. Comparison of the chemical shift of the full-length PtkA obtained in presence and absence of MgCl\(_2\) allowed us to delineate strong Mg\(^{2+}\)-induced CSPs. The most affected resonances come from residues of the KCD. Additional experiments using KCD\(_{PtkA}\) showed similar effect of Mg\(^{2+}\) (Fig. S7).

(iii) **PtkA-MptpA interface** - In addition to previously reported MptpA-PtkA interaction
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studies (16), for which the interaction was only mapped on the MptpA protein, we performed a set of NMR titrations for mapping the interaction interface on PtkA. For these studies, $^{15}$N-labeled full-length PtkA as well as $^{15}$N-labeled KCD PtkA were titrated with unlabeled MptpA (up to molar ratio 1:4) in presence or absence of 5 mM ATP. Similar to the interactions studies from the MptpA, we obtained a number of small but evident CSPs and were consistent for both (PtkA and KCD PtkA) the constructs (Fig. S8A, B). The largest number of chemical shift changes were observed for the residues surrounding the catalytic-loop (D$_{85}$LD), located between β-sheet (β1) and α-helix (α1). Additionally, residues located in the α-helix (α5) and β-sheet (β3) that envelops the auto-phosphorylation site are also affected indicating their involvement in the binding interfaces with MptpA. Furthermore, mapping of the CSPs onto the structure of PtkA shows slight differences of the affected KCD area in presence and absence of IDD. The α2-α3 loop located in the lobe-2 of KCD seems to be more involved in the interactions in absence of IDD. Interestingly, the α3-helix which contains the two tyrosine residues Tyr$_{146}$ and Tyr$_{150}$ also showed similar differences in the hydrogen-deuterium exchange experiments measured in presence and absence of IDD.

(iv) Dephosphorylation of PtkA by MptpA - To study the dephosphorylation of PtkA by MptpA, we synthesized peptides derived from PtkA, representing the auto-phosphorylation site in (i) non-phosphorylated (D$_{264}$-K$_{270}$/Tyr$_{262}$) and (ii) phosphorylated Tyr$_{262}$ (D$_{264}$-K$_{270}$/pTyr$_{262}$) state. 1D-$^{31}$P-NMR of the PtkA (D$_{264}$-K$_{270}$/pTyr$_{262}$) peptide shows a signal at -0.17 ppm indicating phosphorylation of Tyr$_{262}$ (Fig. 5E). We monitored this signal of the PtkA-peptide (D$_{264}$-K$_{270}$/pTyr$_{262}$) both upon addition of and in the absence of the phosphatase MptpA (Fig. S9). Dephosphorylation of PtkA peptide (D$_{264}$-K$_{270}$/pTyr$_{262}$) is indicated by the disappearance of the signal in the 1D-$^{31}$P NMR spectra at -0.17 ppm and appearance of one additional signal at 2.29 ppm corresponding to the inorganic phosphate (P$_i$). The aromatic signals (Hδ and Hε) of Tyr$_{262}$ reappear at a different chemical shift upon dephosphorylation by MptpA. In addition, 2D-(H,$^{13}$C)-HSQC spectrum of the aromatic region of PtkA peptide (D$_{264}$-K$_{270}$/pTyr$_{262}$) acquired in the absence and presence of MptpA shows clear CSPs corresponding to the Tyr$_{262}$ side chain aromatic hydrogens (Hδ and Hε). These studies confirm that MptpA can potentially dephosphorylate isolated phosphorylated peptide derived from PtkA.

DISCUSSION

Non-canonical bacterial kinases possessing tyrosine kinase activity play an important role in the cellular regulation of bacteria. Those kinases possibly belong to various protein families with multiple functions as there is no sequential consensus between them. Besides PtkA from Mycobacterium tuberculosis (18), other kinases, such as DivL of Caulobacter crescentus (24), WaaP of Pseudomonas aeruginosa (25) or PutA of Salmonella typhimurium (26), were found and termed as odd PTKs (19). How widespread such tyrosine kinases are in prokaryotes is still unclear, but the existence of atypical tyrosine kinases within one species suggests the existence of additional members of the same subfamily. The presence of homologs in the entire operon (similar to that found for the protein tyrosine kinase PtkA of Mtb) in multiple Actinomycetes species like Rhodococcus, Corynebacterium, Gordonia or Amycoliciococcus suggests that these proteins, even with limited homology, are protein-tyrosine kinases. Furthermore, as many as 924 unique PtkA orthologs were identified in all the domains of life (19).

The tyrosine phosphorylation and dephosphorylation represents a significant part of the survival regulation of Mtb (27). The interaction of Mtb protein-tyrosine phosphatase MptpA with the host defense machinery plays an important role in the mycobacterial virulence (17). Moreover, PtkA was recently shown to play a central role in promoting the growth of Mtb in macrophages (21), further underlining the significance of the
interaction studies between MptpA and its cognate kinase PtkA and its role in *Mtb* regulation. Both enzymes represent potential candidates for the rational drug design, where inactivation of either one of the partners or their interaction may inhibit the pathogen survival. Furthermore, the PtkA-MptpA operon conserved among numerous *Actinobacteria* species with limited identity for PtkA (39-76%) is in contrast to *Mtb* complex, where this operon is highly conserved, with 99.7-100% protein identity (19). This additionally indicates the important role of this protein in the *Mtb* physiology.

We characterized the structure of PtkA and investigated its interactions with MptpA in detail using NMR spectroscopy. The structural architecture of PtkA deviates from other bacterial kinases, as it contains an N-terminal intrinsically disordered domain, which is linked to the well-folded kinase core domain. The KCD is the catalytic center of PtkA containing (i) catalytic loop (D\textsuperscript{85}LD-motif) located between \(\beta\)-sheet (\(\beta\)1) and \(\alpha\)-helix (\(\alpha\)1) secondary structure and (ii) the autophosphorylation site (Tyr\textsuperscript{262}) located near to the protein hydrophobic core. The PtkA conformation is stabilized by binding of a divalent cation Mg\textsuperscript{2+} which may regulate the substrate binding. Moreover, MptpA binds to the KCD of PtkA and dephosphorylates the kinase. The dynamic properties of both domains are very different and suggest the possibility of an inter-domain controlled regulation of PtkA activity. The IDD is unstructured and highly dynamic, allowing for a “fly-casting” like mechanism of transient interactions with the rigid KCD. This interaction thereby regulates the accessibility of the KCD active site (which is involved in auto-phosphorylation). The decrease of the PtkA activity in presence of IDD suggests an inhibitory effect of the disordered domain during the auto-phosphorylation. Spin label studies indicated the existence of residual long-range transient interactions of the IDD with the KCD. Based on those results, we made a model of the IDD-KCD structure which shows that the preferred position of the IDD is located near to the catalytic site. Our studies show that the presence of the IDD does not affect the fold of the KCD and has only minor effects on the solvent accessibility of the KCD. The changes in dynamics are restricted to the upper lobe of the KCD (\(\alpha\)-helix (\(\alpha\)3)) which harbors two tyrosines whose role and phosphorylation status is unclear. The kinase activity was observed in presence as well as in absence of IDD, which is in agreement with the results obtained in 1D-\textsuperscript{31}P NMR spectra, where in both cases the phosphorylation of PtkA was detected. However, our activity studies show that the IDD has an inhibitory effect on the PtkA enzymatic activity. This suggest that the IDD masks the region of Tyr\textsuperscript{262}, thereby restricting the accessibility of this key residue involved in the auto-phosphorylation of PtkA. Based on the above observations, we propose two conformational states for PtkA: (i) open state where the IDD is away from the auto-phosphorylation site, increasing the accessibility of Tyr\textsuperscript{262} for the phosphorylation and (ii) closed state where the Tyr\textsuperscript{262} is masked by the IDD. Multi-site phosphorylation of PtkA by serine/threonine kinase, PKA, induces conformational changes of IDD which abolish IDD movement and promote the open state of PtkA, where the Tyr\textsuperscript{262} is accessible to the phosphorylation. However, the exact catalytic mechanism behind the modulation of the PtkA activity can be a subject for further investigation.

Based on our findings, we propose the following model to explain the potential function for this unusual IDD in PtkA, in the context of overall regulation of *Mtb* virulence (Fig. 6). MptpA is secreted from the mycobacterial cytosol to the cytosol of infected macrophages by an unknown translocation mechanism. During the phagosome-macrophage fusion MptpA migrates into the macrophage cytosol. The phosphorylation state of MptpA at this point is unclear, however, we know that activated PtkA catalyzes the
phosphorylation of MptpA, thereby increasing its activity (17) which possibly plays a key role in the translocating MptpA (Fig. 6, left inset) to the cytosol of infected macrophages. Moreover, multisite phosphorylation of IDD of PtkA shifts the balance between the “open” and “closed” states of the IDD towards a more “open” state. As a consequence of this PTM of the IDD, PtkA might now possess enhanced activity towards phosphorylating MptpA and might as well get anchored to the Mtb cell-wall (Fig. 6, right zoom). Phosphorylation driven membrane anchoring has been previously observed for protein kinase C (28). In summary, the structural and functional knowledge of PtkA gained in this study, is crucial for understanding the MptpA-PtkA interactions, catalytic mechanism and in general the role of kinase-phosphatase regulatory system in Mtb virulence.

**EXPERIMENTAL PROCEDURE**

**Cloning, expression and purification of PtkA** - The pET151/D-TOPO plasmid encoding wild-type PtkA (Rv2232, Met1-Val291) was transformed into E. coli BL21 (DE3) cells for expression. The PtkA subdomains IDD<sub>PtkA</sub> (Met<sub>1</sub>-Leu<sub>81</sub>) and KCD<sub>PtkA</sub> (Gly<sub>76</sub>-Val<sub>291</sub>) were generated by polymerase chain reaction (PCR). Wild-type PtkA-DNA was used as a template for DNA replication. The PCR product was amplified into the pRS-45-vector using NedI and BamH1 as restriction sites. The efficiency of the insertion was confirmed via nucleotide sequencing. The PtkA-DNA-plasmid generated via PCR was transformed into E. coli BL21 (DE3) for protein expression. The expression of recombinant PtkA was performed in LB-medium for unlabeled protein and in M9-medium for 15N/13C uniformly labeled PtkA. Cell cultures (supplemented with 1 mM ampicillin) were grown at 37 °C with aeration (120 rpm) to an optical density (OD) at A<sub>600nm</sub> ~ 0.7, incubated at 0 °C for 15 min and induced using 1 mM isopropyl 1-thio-β-D-galactopyranoside for overexpression. Triple (²H, ¹³C, ¹⁵N) labeled PtkA was expressed in rich growth medium solution labeled with stable isotopes (²H (> 95%), ¹³C, ¹⁵N) (Silantes). After adaptation of the cells to 70% and 100% D<sub>2</sub>O at A<sub>600nm</sub> ~ 0.5, the cells were washed twice in PBS/D<sub>2</sub>O and resuspended in E.coli-OD<sub>2</sub> CDN Silantes medium at A<sub>600nm</sub> ~ 0.7. The protein expression was induced at A<sub>600nm</sub> ~ 1.0 using 1 mM isopropyl 1-thio-β-D-galactopyranoside. After incubation overnight for unlabeled or ¹⁵N/¹³C uniformly labeled PtkA and 12 hours for ²H, ¹³C, ¹⁵N labeled PtkA at 16 °C with aeration (120 rpm), the cells were centrifuged (4.000 × g, 45 min, 4 °C) and the cell pellet was resuspended in lysis buffer (300 mM NaCl, 50 mM Tris-HCl/pH 8.0, 10 mM 2-Mercaptoethanol), supplemented with complete protease inhibitor (1 tablet/100 mL EDTA-free, Roche). The cells were disrupted for 15 min using M-110P microfluidier (15,000 PSI). The supernatant was separated from cell residues by centrifugation (18.000 × g, 45 min., 4 °C). The soluble protein fractions were analyzed by SDS-PAGE. Fractions containing pure protein were pooled and stored at -80 °C or immediately used for further experimental procedures.

**Expression and purification of MptpA** - The plasmid pET16bTEV containing the MptpA sequence (Rv 2234, Met<sub>1</sub>-Ser<sub>163</sub>) was transformed into BL21 (DE3) pLysS E coli cells, expressed and purified as described in detail previously (17). The expression and purification of MptpA sequence (Rv 2234, Met<sub>1</sub>-Ser<sub>163</sub>) was transformed into BL21 (DE3) pLysS E coli cells, expressed and purified as described in detail previously (17). The expression and purification of MptpA sequence (Rv 2234, Met<sub>1</sub>-Ser<sub>163</sub>) was transformed into BL21 (DE3) pLysS E coli cells, expressed and purified as described in detail previously (17).

**Peptide synthesis** – The solid-phase peptide synthesis was carried out by standard Fmoc chemistry. The C-terminal residue was manually attached to a 2-chlorotrityl chloride resin. Peptides
were purified by reversed-phase HPLC and characterized using electrospray ionization mass spectrometry and analytical HPLC.

Luciferase Assay – The autophosphorylation activity of the protein was determined using a Kinase-Glo® luminescent kinase assay (Promega). 20 µM PtkA was added to the assay buffer (300 mM NaCl, 50 mM Tris-HCl/pH 8.0, 10 mM DTT) containing different amounts of ATP (1, 2.5, 5, 7.5, 10 µM, Fermentas) in presence of 10 mM MgCl₂. After overnight incubation at 25 °C, the assay mix (50 µL) was applied on to a 96-well plate (white, E&K Scientific EK-25075) and the luciferase mixture (50 µL) was added. After incubation for 2 h, the luminescence was measured using a Veritas™ Microplate Luminometer.

Auto-phosphorylation Reaction – The auto-phosphorylation reaction of PtkA was performed using unlabeled 100 µM protein and 10 mM ATP in 50 mM HEPES-NaOH/pH 7.5 buffer containing 300 mM NaCl, 10 mM DTT and 10 mM MgCl₂. After incubation overnight at 25 °C, MALDI-MS as well as 1D-31P-NMR were measured.

Dephosphorylation/Phosphorylation Reaction – The auto-phosphorylated PtkA (see above) was incubated overnight with MptpA at a concentration ratio of 1: 0.5 and 1:1 and analyzed by 1D-31P-NMR and MALDI-MS. The dephosphorylation assay with PtkA phosphorylated peptide (Asp²⁶⁴-Lys²⁷⁰/pTyr²⁶²) was performed in a 3 mm NMR tube containing 50 mM HEPES-NaOH/pH 7.5 buffer containing 300 mM NaCl, 10 mM MgCl₂, 90% H₂O / 10% D₂O and 3 mM peptide. NMR spectra were acquired directly after addition of 40 µM MptpA.

NMR Spectroscopy – NMR experiments were performed at 298 K on Bruker spectrometers (600-, 700-, 800-, 900- or 950- MHz) equipped with TXI-HCN cryogenic probes. The protein samples (0.1-0.3 mM) were measured in NMR buffer (50 mM HEPES-NaOH/pH 7.5, 300 mM NaCl, 10 mM DTT, 10 mM MgCl₂, 90% H₂O / 10% D₂O) using 3 mm NMR tubes. The spectrometer was locked on D₂O.

Resonance Assignment experiments - For the backbone assignment of PtkA, a set of 3D triple resonance experiments were collected including HNHA, HNCO, HNCA, HN(CO)CA, HNCACB, HN(CO)CACB experiments on either double and/or triple labeled protein. The side chain assignment of the aliphatic resonances was obtained using HCCCONH and CCONH experiments. 2D HBCBCGCDHD and HBCBCGCDCEHE spectra and an aromatic 13C-filtered TOCSY spectrum were used for the assignment of the aromatic side chain resonances. For assignment and nOe-based distance restraints 3D (1H, 1H, 15N)-NOESY-HSQC (mixing time, 120 ms), aromatic and aliphatic 3D (1H, 1H, 13C)-NOESY-HSQC (mixing time, 75 ms and 120 ms) in H₂O were collected.

Heteronuclear relaxation experiments – 15N-relaxation experiments were performed at 298 K on a 700 MHz spectrometer. The R₁ longitudinal 15N relaxation rates were obtained from a series of experiments acquired with varying delays of 100, 200, 400, 600, 800, 1200, 1600, 2000, 2400 and 3000 ms. R₂ transverse 15N relaxation rates were determined from a series of spectra using following delays: 16.96, 33.92, 50.88, 67.84, 84.80, 101.76, 118.72, 135.68 ms. The 15N{1H}het-nOes values were obtained from the analysis of peak intensity ratio (I_{nOe}/I_{nonOe}) from the recorded spectra with and without saturation of amide protons. The order parameter, S², was determined using TENSOR2 (29).

Hydrogen-Deuterium exchange – For the H/D exchange experiment, 150 µL (200 µM) protein in NMR buffer was lyophilized and dissolved in the equivalent volume of deuterium oxide (D₂O). A series of 2D-(1H,15N)-HSQC spectra were recorded immediately after the addition of D₂O, for a duration of 15 minutes within the first hour and subsequently for 30 minutes, for a total duration of 19 h. As a reference for the spectra evaluation a 2D-(1H,15N)-HSQC spectrum of the protein (200 µM) in NMR buffer (with 90% H₂O / 10% D₂O) was measured before. The H/D exchange rate for each residue was.
determined by fitting the amide peak intensities to exponential decays over the time for each residue \( I(t) = I(0) \cdot \exp(-k_{ex} \cdot t) \). Where \( I(t) \) and \( I(0) \) are the intensities at the given time \( t \) and \( t = 0 \) and \( k_{ex} \) is the rate constant of the hydrogen-deuterium exchange reaction.

Temperature series – The 2D-(1H,15N)-HSQC spectra were recorded at 3° intervals ranging from 283 K to 301 K on a 700 MHz spectrometer.

Paramagnetic Spin Labeling - Single-cysteine mutation of PtkA (which contains one wild-type cysteine) were engineered using quick change site-directed mutagenesis protocol (Invitrogen). The wild-type cysteine was mutated to alanine (C61A). This point-mutated DNA was used as a template for preparing single-cysteine mutations at Ala\(^{10}\) (C61A/A10C) and Ser\(^{41}\) (C61A/S41C). All mutations were confirmed by DNA sequencing of the entire open reading frame (Eurofins). Protein mutants were expressed in competent \( E. coli \) cells (BL21 (DE3), NEB), grown in M9 minimal media enriched with \(^{15}\)N-ammonium chloride and purified as described above. The side-directed spin labeling (SDSL) was carried out using the MTSL (1-oxy-2, 2, 5, 5-tetramethyl-D-pyrroline-3-methyl-methane-thiosulfonate) spin label. Protein was dissolved in the DTT-free, 50 mM HEPES/NaOH (pH 7.5) labeling buffer, containing 300 mM NaCl, 10 mM MgCl\(_2\) and concentrated to 500 \( \mu \)M using Vivaspin concentrators (MWCO 10 kDa, GE Healthcare). For spin labeling, a 10-fold molar access of MTSL over cysteine was added. MTSL stock solution was prepared by dissolving 10 mg MTSL in 50 \( \mu \)L of DMSO. After addition of MTSL to the protein solution, the reaction mixture was incubated in the dark overnight at 25 °C (wild type) and 4 °C (C61A/A10C, C61A/S41C). To remove excess of spin label, the labeling buffer was exchanged (50 mM HEPES-NaOH, pH 7.5, 300 mM NaCl, 10 mM MgCl\(_2\)) using PD-10 desalting column (Sephadex G-25 Medium, GE Healthcare). The modified protein was concentrated to 300-500 \( \mu \)M (Vivaspin concentrators, MWCO 10 kDa) for NMR. The spin label was reduced by the addition of 5 mM ascorbate from a freshly prepared stock solution of 500 mM, to yield an approximately 10-fold molar excess of reducing agent over the spin label.

NMR data analysis – All spectra were processed using Topspin version 3.2 (Bruker Biospin) and analyzed using SPARKY version 3.114 (30).

Structure calculation – Structure calculations were performed using the software packages CYANA (31-33) and ARIA/CNS (34-36). A conventional structure calculation with fully automated NOESY cross peak assignment failed due to the limited completeness of the resonance assignment (about 75%), especially due to the fact that the missing assignments reside in or near the center of the protein. Generally, a completeness of more than 90% (33) would suffice for a reliable automated NOESY assignment. The NOESY cross-peaks originating from not assigned regions may be misinterpreted to distances between atoms with coinciding assigned resonances, consequently disrupting the structure and causing the convergence to fail. To circumvent this problem, we made use of initial homology models to assist the initial iterative structure calculation with automated NOESY cross-peak assignment by CYANA. These structures were built by SwissModel (37) using a selection of homologous structures (eg. PDB codes: 3mc1, 3sd7, 4ex6, 2ah5, 2nyv, 2yy6, 2hc, 2hi0, 2hd0, 2hdz, 4eel, 3l5k, 2ifr and 3klz) which have a proper range (around residues 80-289) and sufficient sequence identity (above 20%). For the regions that are consistent with the TALOS-N secondary structure prediction (38), very ample CA-CA distance restraints (up to 20 Å) were extracted which merely define the global shape of the molecule, thereby just avoiding NOE misassignments between atoms which are actually not close to each other. The unambiguous assigned nOes in the 3D-(1H, 1H, 15N)-NOESY-HSQC (aliphatic and aromatic) and 3D-(1H, 1H, 15N)-NOESY-HSQC spectra were validated and inspected by using of Sparky 3.114 (30) and subsequently used for the
structure refinement (without the additional global distance restraints). The chemical shift tolerances were set to 0.015 and 0.025 ppm for the bound protons and other protons respectively, and 0.20 ppm for the heavy atoms. In addition to nOe data, hydrogen bond distances and dihedral angle restraints for the secondary structure elements (based on TALOS-N predictions and confirmed with the Swiss-Models and initial structure calculations), as well as $^1$D (HN) Residual Dipolar Couplings (RDCs) and $^3$J (H$^N$H$^\alpha$) coupling constant restraints were included in the structure calculation with CYANA (100 structures per iteration, 15000 refinement steps). The final bundle of 20 best (lowest energy) structures was used as input for a refinement in explicit water with CNS 1.1 (39) using the ARIA 1.2 setup and protocols (40). The $^3$J(H$^N$H$^\alpha$) coupling constants were obtained via a three-dimensional H$^N$H$^\alpha$-HMQC experiment (41,42) and directly included in the structure calculation by using the karplus relationship. $^1$D (HN) Residual Dipolar Couplings were measured in Pf1 bacteriophages (6 mg/mL, strain LP11-92, ASLA Biotech) as alignment medium at 700 MHz and 298 K. The $^1$D (HN) were extracted from IPAP-(15N,$^1$H)-HSQC (43) spectra. RDCs were included in the structure calculation as direct susceptibility anisotropy restraints (SANI) and examined using the program PALES (44). The rhombicity and axial coefficients for the final alignment tensor were: R=0.2 and D=4.6 with a correlation coefficient R=0.97. Moreover, $^{15}$N relaxation data ($T_1/T_2$ values) were included as diffusion anisotropy restraints in this final refinement stage. The values for the anisotropy (1.2) and rhombicity (0.3) of the rotational diffusion tensor from the final structure bundle were determined using the program Tensor 2 (29). The impact of the diffusion restraints on the structure refinement is however negligible as the anisotropy of the molecule turned out to be moderate and smaller than the suggested minimal value of 1.5 (45). The overall rotational correlation time ($t_c$=12.9 ns) is in agreement with the value predicted for a monomeric protein ($t_c = 14.4$ ns at 298 K) by the program HYDRONMR (46).
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FIGURE 1. A, Backbone assignment of the full-length PtkA. 2D-(1H,15N)-TROSY spectrum of the full-length PtkA, acquired at 950 MHz, 298 K and pH 7.5. The spectral region with a large signal overlap (between 7.6 ppm and 8.6 ppm in the 1H- and 107 ppm and 129 ppm in the 15N-dimension) is highlighted with a box. B, PtkA construct optimization. (right) 2D- (1H,15N)-HSQC spectra of KCD PtkA (76-291, 23 kDa) and (left) IDD PtkA (1-81, 8.5 kDa) acquired at 600 MHz, 298 K and pH 7.5. C, Primary amino acid sequence of the full-length PtkA (Rv2234, Met1-Val291). The region of the intrinsically disordered domain (IDD) is indicated by blue dotted line. Residues with missing backbone amide assignment are highlighted with a gray background.
FIGURE 2. NMR solution structure of KCD PtkA. A, Structural statistics for the ensemble of the 20 best NMR structures of PtkA. B, Ribbon representation of the lowest energy structure (top) and the bundle of the 20 lowest energy structure (bottom) rotated by 180°. Lobe-1 and -2, catalytic motive (D85LD) and the auto-phosphorylation site (circle) are highlighted. The overall structure of KCD PtkA consists of six parallel folded β-sheets and nine α-helix secondary structures. Regions with the missing assignment (Thr210-Val216 and Tyr262-Val275) are colored in gray. The figure was generated by PyMOL.
FIGURE 3. A, PtkA dynamics before and after truncation of one of the domains. (top—cylinders indicate α-helices, arrows β-sheet) Schematic representation of NMR chemical shift based TALOS+ prediction of the secondary structure elements of KCD_{PtkA}. Plot of the determined order parameter (S^2) for the full-length PtkA and KCD_{PtkA}.
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construct as a function of residue number (S² was generated using TENSOR2 software (29) based on the three experimentally measured relaxation parameters: T₁, T₂ and ¹⁵N-het nOe). Plot of the R₁-, R₂- relaxation rates and heteronuclear ¹⁵N{¹H}-het nOe as a function of residue number of the full-length PtK (black) and KCDₚₖ (red) determined at 700 MHz and IDDₚₖ (blue) measured at 600 MHz, 298 K, in 50 mM HEPES-NaOH buffer (pH 7.5) containing 300 µM protein, 300 mM NaCl, 10 mM DTT, 10 mM MgCl₂, 10% D₂O / 90% H₂O. B, Hydrogen-deuterium exchange rate (kex, s⁻¹) of full-length PtK (black) and KCDₚₖ (red) as a function of residue number. The missing exchange rates could not be determined due to: rapidly exchanging amides hydrogens (directly after solvation in D₂O), unassigned or overlapping cross-peaks, residue being proline. C, Determined temperature coefficient [ppb/K] of full-length PtK (black), KCDₚₖ (red) and IDDₚₖ (blue) as a function of residue number. The error estimate is derived from the Gaussian noise in the peak intensity.
FIGURE 4. Structural model for the IDD-KCD interaction in PtkA. A, Overlay of the 2D-(1H,15N)-HSQC spectra of paramagnetic (orange) and diamagnetic (black) full-length PtkA: PtkA C61A/A10C, MTSL-labeled residue C10 (left), PtkA C61A/S41C, MTSL-labeled residue C41 (middle), wild type PtkA, MTSL-labeled residue C61 (right). Spectra were measured at 900 MHz, 298 K in 50 mM HEPES-NaOH buffer (pH 7.5) containing 300 mM NaCl, 10 mM MgCl₂, 10 % D₂O/90 % H₂O. MTSL was reduced with ascorbic acid. The signals broadened out beyond detection due to the paramagnetic center are labeled. The C61A mutation is highlighted with circle. B, Normalized intensity ratios (Ipara/Idia) of amide cross-peaks vs amino acid sequence, determined for wild type PtkA (C61-MTSL), PtkA C61A/A10C (C10-MTSL) and PtkA C61A/S41C (C41-MTSL). Missing data points correspond to peak overlap or unassigned residues. The error estimate is derived from the Gaussian noise in the peak intensity. C, PRE-based model representing the transient interaction between IDD and KCD. Blue part corresponds to the N-terminal IDD, grey indicate KCD: (top) schematic representation, (bottom) cartoon representation showing spin labeled position (orange: A10C, S41C and C61) and the catalytic site (red: D₈₅LD, Y262).
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FIGURE 5. PTKA auto-phosphorylation and regulation of its catalytic activity. A, Schematic representation of the PTKA regulatory system: (i) auto-phosphorylation of tyrosine residues in PTKA and (ii) dephosphorylation by MptpA. B, Model depicting the role of IDD in regulating the catalytic activity of PTKA. The dynamic character of IDD modulates the accessibility of Tyr262 generating an open (active protein) and closed (inactive protein) state of PTKA. Multi-site PTM of IDD promotes the open state of PTKA, due to the phosphorylation-induced conformational changes of the IDD, increasing the accessibility of Tyr262 for auto-phosphorylation. C, Luciferase assay of PTKA. The activity of the full-length PTKA was set to 100%. * indicate auto-phosphorylated protein. The error bars reports the standard deviation. D, 1D-31P-NMR spectra of: ATP in buffer (bottom), full-length PTKA (middle, blue) and KCDPTK construct (top, black) after overnight incubation with ATP at room temperature. Signal indicating phosphorylation of PTKA is marked with black arrow and red box. Signal from inorganic phosphate results from the hydrolysis of ATP. E, 1D-31P-NMR spectra of PTKA phosphorylated peptide D264-K270/pTyr262 (blue) and after incubation with MptpA (black).
FIGURE 6. Potential role of PtkA and their unusual IDD in the Mycobacterium tuberculosis virulence. (left) role of the MptpA in the Mtb virulence. MptpA interacts with the host endogenous signaling pathway upon binding to the H subunit of the vacuolar proton pump (V-H^+ATPase) and dephosphorylation of the human Vacuolar Protein Sorting 33B (VPS33B), being a part of the homotypic fusion and protein sorting (HOPS)-complexes. Dephosphorylation of VPS33B suppresses the phagosome-lysosome fusion and promotes Mtb persistency. The mechanism of migration of MptpA from the cytosol of Mtb to the macrophage cytosol through the mycobacterial cell-wall is unknown. (right) schematic representation of Mtb including its complex cell-wall structure (according to (47)) and the role of PtkA and its unusual IDD. IDD of PtkA regulates the accessibility of the auto-phosphorylation site which is located in the KCD. Long-range transient interactions between those domains generate an open and close state of PtkA, which control the protein auto-activity. PTM of IDD promotes the open state of PtkA increasing its ability to auto-phosphorylation. High active PtkA regulates the phosphorylation of MptpA, thereby modulating MptpA activity (17) and possibly contributing to its translocation through the mycobacterial cell-wall. On the other side, the presence of IDD in the PtkA structure can play a role in the subcellular relocalization of PtkA to the cell membrane. The translocation of PtkA can be triggered by the auto-phosphorylation of the KCD or post-translational multisite phosphorylation of IDD. (right corner) The dynamic behavior of IDD in response to change in pH (22).
The domain architecture of the PtkA, the first tyrosine kinase from *Mycobacterium tuberculosis* differs from the conventional kinase architecture.

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