Oxidative Unfolding of the Rubredoxin Domain and the Natively Disordered N-terminal Region Regulate the Catalytic Activity of Mycobacterium tuberculosis Protein Kinase G*

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Mycobacterium tuberculosis escapes killing in human macrophages by secreting protein kinase G (PknG). PknG intercepts host signaling to prevent fusion of the phagosome engulfing the mycobacteria with the lysosome and, thus, their degradation. The N-terminal NORS (no regulatory secondary structure) region of PknG (approximately residues 1–75) has been shown to play a role in PknG regulation by (auto)phosphorylation, whereas the following rubredoxin-like metal-binding motif (RD, residues ~74–147) has been shown to interact tightly with the subsequent catalytic domain (approximately residues 148–420) to mediate its redox regulation. Deletions or mutations in NORS or the redox-sensitive RD significantly decrease PknG survival function. Based on combined NMR spectroscopy, in vitro kinase assay, and molecular dynamics simulation data, we provide novel insights into the regulatory roles of the N-terminal regions. The NORS region is indeed natively disordered and rather dynamic. Consistent with most earlier data, autophosphorylation occurs in our assays only when the NORS region is present and, thus, in the NORS region. Phosphorylation of it results only in local conformational changes and does not induce interactions with the subsequent RD. Although the reduced, metal-bound RD makes tight interactions with the following catalytic domain in the published crystal structures, it can also fold in its absence. Our data further suggest that oxidation-induced unfolding of the RD regulates substrate access to the catalytic domain and, thereby, PknG function under different redox conditions, e.g. when exposed to increased levels of reactive oxidative species in host macrophages.

Mycobacterium tuberculosis (Mt), the causative agent of tuberculosis, has evolved different mechanisms to monitor redox signals. The capability of Mt to sense redox stress and to maintain redox homeostasis is important for the survival of the pathogen in the human host (1–3). Recent studies have shown that redox stress responses of mycobacteria are linked to the phosphorylation of several proteins by eukaryote-like serine/threonine kinases (4, 5). Of the 11 eukaryote-like serine/threonine kinases encoded by the Mtb genome (6), protein kinase G (PknG) and E (PknE) harbor specific redox-sensitive motifs (7, 8). The soluble PknG has been proposed to promote cellular survival of mycobacteria in host macrophages by blocking their lysosomal delivery and, thus, degradation (9). Moreover, because PknG is secreted into the cytosol of host macrophages, it is a promising drug target (9). PknG is a multidomain protein consisting of four functional regions (Fig. 1A). The N-terminal ~75 residues of the no regulatory secondary structure (NORS) region have been suggested to be intrinsically disordered and to harbor a major in vivo phosphorylation site at Thr63 (8, 10, 11). Based on the crystal structure of an N-terminal truncated variant (PknG74–750) in complex with a newly detected inhibitor (AX20017, Fig. 1B), the following redox-sensitive rubredoxin-like metal binding domain (RD) makes tight interactions with the catalytic domain (8). C-terminally, the kinase domain is flanked by a tetratricopeptide repeat domain (TPRD), a structural motif typically involved in protein-protein interactions (8).

PknG can autophosphorylate itself in trans (10, 11). However, in contrast to other (mycobacterial) kinases, the autophosphorylation does not affect the kinase activity but is required for the survival of pathogenic mycobacteria within host macrophages (10). In rubredoxins, an iron atom is typically tetrahedrally coordinated by four cysteine residues (12), but other metal ions, such as cobalt, nickel, and zinc, can replace the iron...
The redox-sensitive RD of PknG contains two CXXCG motifs (Fig. 1A) that can, in vitro, coordinate a divalent metal ion, such as zinc, iron, or cadmium, in the reduced state (8, 14–16). However, it is currently unknown which metal ion is coordinated under in vivo conditions. Three crystal structures of PknG have been published, one of PknG74–750 (RD-KD-TPRD), with the RD coordinating Cd$^{2+}$ and the KD in complex with the small molecule inhibitor AX20017 (PDB code 2PZI, Fig. 1B), and two of PknG74–405 (RD-KD), with the RD coordinating Zn$^{2+}$ and the KD in complex with either an ATP analogue (ATP-γS) or ADP as well as Mg$^{2+}$ (PDB codes 4Y12 and 4Y0X) (8, 14). The structure of the RD is very similar in all three solved structures, but the orientation of the RD with respect to the kinase domain is slightly different. In the inhibitor-bound structure, the RD interacts with the N-terminal and C-terminal lobes of the kinase domain, whereas, in the ATP analogue-bound form, the RD makes contact only with the N-terminal lobe (8, 14). It was proposed that the RD regulates the intrinsic kinase activity by restricting the accessibility of the active site (8, 14). Mutation of all four cysteines in the two conserved CXXCG motifs to alanines or serines impairs the kinase activity and renders PknG insensitive to regulation by redox changes (8, 11).

Cells of the innate immune system, such as macrophages, release high concentrations of reactive oxidative species (ROS) to kill engulfed pathogenic bacteria (17). However, based on the published crystal structures and functional data for wild-type and mutant PknG proteins, the exact mechanism of the redox regulation of the kinase activity under oxidative stress conditions remains elusive. Here we present combined NMR spectroscopy, in vitro kinase assay, and molecular dynamics (MD) simulation data that show how the dynamics as well as the local and global structure of PknG change upon oxidation of the RD and that the so far uncharacterized NORS region is indeed natively unfolded and the target region for PknG autophosphorylation in trans.

Results

The NORS Region Shows Only Local Structural Order, and the RD Can Fold in the Absence of the KD—The structural properties and dynamics of the N-terminal NORS region and of the RD in the absence of the kinase domain have not been described yet. The $^{1}$H-$^{15}$N HSQC spectrum of the two-segment protein His-PknG1–147 (NORS-RD, supplemental Fig. S1) represents almost perfectly the sum of those of each isolated functional region (His-PknG1–75 and PknG74–147, supplemental Fig. S1). This indicates that the NORS region and the RD behave rather independently and do not interact significantly. The low signal dispersion of the NORS region indicates already that it is indeed a natively disordered protein (IDP) region. To determine in more detail the secondary structure content of each region, we measured $^{13}$C$^{\alpha}$ secondary shifts (Fig. 1C) as well as $^{3}$H$_{NIN}$H$_{N}$ scalar couplings (supplemental Fig. S2A) and $^{1}$H$^{\alpha}$ secondary shifts (supplemental Fig. S2B). To characterize the backbone dynamics of the two-segment protein His-PknG1–147 (NORS-RD) and of each isolated functional region (His-PknG1–75 and PknG74–147), we recorded $^{15}$N relaxation data, including $^{1}$H-$^{15}$N NOE (Fig. 1D) as well as $^{15}$N- T$_{1}$ and $^{15}$N- T$_{2}$ data (supplemental Fig. S3A and Results). In addition, we back-calculated the $^{13}$C$^{\alpha}$ secondary shifts from the available crystal structures of PknG using the program Sparta+ (18) and compared them with the experimentally determined ones (supplemental Fig. S4).

The NORS region (approximately residues 1–75) shows strongly negative $^{13}$C$^{\alpha}$ secondary shifts for residues preceding a proline. However, the majority of the remaining residues show only a weak propensity for α-helical secondary structure (Fig. 1C, blue columns). The $^{3}$H$_{NIN}$H$_{N}$ scalar couplings for the NORS region (supplemental Fig. S2A, blue data) are mostly between 6–8 Hz, indicating that it does not stably populate α-helical or β-sheet secondary structure and may overall only transiently and locally populate more ordered states. This is consistent with $^{1}$H-$^{15}$N NOE values typical for dynamic regions, with negative values below −0.2 for the isolated NORS region (Fig. 1D, blue symbols) and values between about 0.2 and −0.4 when connected to the RD (Fig. 1D, black symbols). The region between residues 20 and 40 appears overall more dynamic in His-PknG1–75 compared with His-PknG1–147, which is also reflected in the $^{15}$N-T$_{1}$ and $^{15}$N-T$_{2}$ data (supplemental Fig. S3 A and Results). This suggests that the presence of the RD influences the dynamics of this central region of the NORS. Overall, the NORS region appears, as predicted, natively disordered. However, it may transiently populate α-helical stretches that are interrupted by several prolines (10 in total) that may induce local backbone kinks or turns (19).

The RD shows, consistent with the large chemical shift dispersion for the residues around the two CXXCG metal-coordinating motifs ($^{106}$CWNCG$^{110}$ and $^{128}$CPYCG$^{132}$), larger positive and negative $^{13}$C$^{\alpha}$ secondary shifts than the NORS region (Fig. 1C, red columns). Thus, it appears to be present in a folded metal-bound state. Because the RD interacts tightly with the KD in the crystal structure of PknG74–750 bound to an inhibitor (PDB code 2PZI, Fig. 1B), its presence might be needed to stabilize the folded state of the RD. However, the α helix (−89−92), the 3$^{10}$ helix (−101−103), and the two β strands (−125−127 and −134−136) detected in the crystal structure (schematically indicated at the top of Fig. 1C) appear to be similarly present in the solution state of the RD not connected to the kinase. Metal coordination to the cysteines in the two CXXCG motifs (−106−110 and −128−132) induces the formation of turns, which, as helical regions, show rather strong positive $^{13}$C$^{\alpha}$ secondary shifts (Fig. 1C). The RD has as many prolines as the NORS region (each 10). Besides being involved in the already mentioned helical, sheet, and turn regions, they are further present in turns or β bridges. The region including the 3$^{10}$ helix and the following CXXCG motifs (−100−140) shows in the isolated RD and connected to the NORS region $^{1}$H-$^{15}$N NOE values of $\sim$0.4−0.8 (Fig. 1D), which are typical for rather well structured regions. However, NOE values of $\sim$0−0.2 indicate that the N-terminal α-helical stretch is already more dynamic, and negative values for the N- and C-terminal ends of the isolated RD indicate even further increased backbone dynamics on the nanosecond to picosecond time scale. Because the $^{13}$C$^{\alpha}$ secondary shifts and the $^{1}$H-$^{15}$N NOE values for the reduced, metal-bound RD are almost the same for His-PknG1–147 and PknG74–147, the presence of the NORS regions has no significant influence on its structure or backbone dynamics. This can be explained by the NORS being natively disordered as
well as connected to the RD by a dynamic linker region rich in glycines (Gly76-Gly78).

The Fold of the Isolated Reduced Metal-bound RD in Solution Is Overall Similar to the One Attached to the Kinase in Complex with AX20017, ADP, or ATP-γS in the Crystal State—Because the RD interacts tightly with the kinase domain in the available three crystal structures, albeit to a slightly different extent (8, 14), we further analyzed the effect of the presence of the kinase domain on the structure of the reduced, metal-bound state. Based on the superposition of the ¹H-¹⁵N HSQC spectra of His-PknG74–420 encompassing the RD, the kinase, and the RD-only protein PknG74–147 (supplemental Fig. S5), peaks for several residues are found at similar chemical shift values, e.g. those for the two side-chain amide protons of Trp¹⁰⁷ and...

![Diagram A](image1)

![Diagram B](image2)

![Diagram C](image3)

![Diagram D](image4)

![Diagram E](image5)
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Trp<sup>127</sup> of the RD. However, overall, these data did not allow us to determine in detail how similar the conformations of the RD in both proteins are. We thus recorded backbone <sup>13</sup>N-<sup>1</sup>H RDCs to compare the conformation of the RD in the isolated state with that in the available crystal structure data. Fig. 1E shows a plot of the experimentally obtained RDCs as a function of the residue sequence position compared with those back-calculated from the crystal structures of His-PknG<sub>74</sub>–750 (RD-KD-TPRD) in complex with a small molecule inhibitor (PDB code 2PZI, Fig. 1B and supplemental Fig. S6A) and of PknG<sub>74</sub>–405 (RD-KD) in complex with either ADP (PDB-ID 4Y0X, supplemental Fig. S6A) or an ATP analogue (PDB code 4Y12, supplemental Fig. S6A). The respective plots of the experimental versus calculated values for each crystal structure and a larger representation of the RD structure are shown in supplemental Fig. S6B. Overall, the conformation of the isolated RD in solution is, as already indicated by comparison of the experimental and back-calculated chemical shift data (Fig. S4), very similar to that in the presence of the kinase in the three crystal structures. The observed differences around the CXXCG motifs (residues 106–110 and 128–132) and near residue 115 between the experimental RDCs and those back-calculated based on the crystal structures can be accounted for by missing contacts to the kinase domain in the isolated RD and slightly different contacts in PDB code 2PZI versus 4Y0X and 4Y12 (supplemental Fig. S6, A and B) as well as by the fact that the RD has regions with increased backbone dynamics in solution (Fig. 1D) and in the crystal states, e.g. no coordinates for residues 116–121 in PDB code 2PZI and higher B factors in the RDs of all crystal structures (supplemental Fig. S6A).

**Deletion of the NORS and TPRD, Different Redox Conditions, and the Folding State of the Substrate Influence the Catalytic Efficiency of PknG**—To better understand the relevance of the redox-sensitive RD as well as of the NORS region for the regulation of PknG function and to complement and resolve partially contradictory results from the literature (8, 10, 11, 14), we first performed in vitro kinase assays monitoring the progress of substrate phosphorylation based on the use of radiolabeled ATP. Because PknG can autophosphorylate itself in trans in the NORS region (10), we used as substrate His-PknG<sub>74</sub>–147 encompassing both the NORS and the RD (Fig. 2A and supplemental Fig. S7, A–C). Fig. 2A shows the phosphorimaging kinase activity data using as kinases either His-tagged full-length WT PknG, the NORS deletion mutant His-PknG<sub>74</sub>–750, or the NORS and TPRD deletion mutant His-PknG<sub>74</sub>–420, all with the RD in the folded, Zn-bound state, and as substrate His-PknG<sub>1</sub>–147 with the RD either in the reduced, Zn-bound or the oxidized state. Note that a fraction of the usually higher activity of the wild-type protein arises from the additional phosphorylation of its own NORS region (supplemental Fig. S7, A–C). Compared with the wild type (Fig. 2A, blue columns), deletion of the N-terminal NORS region reduces PknG catalytic activity significantly (Fig. 2A, green columns), and additional removal of the C-terminal TPRD results in a further reduction (Fig. 2A, red columns). This is consistent with published results (10, 11, 14). Oxidized His-PknG<sub>1</sub>–147 is overall a better substrate than the protein with the RD in the reduced, metal-bound state (Fig. 2A, left versus right column of each set of differently colored columns). This suggests that PknG autophosphorylation is more efficient when the RD of the substrate is in the oxidized state, which may facilitate binding to the catalytic cleft, especially for the phosphorylation of Thr<sup>63</sup> and Thr<sup>64</sup>, which are close to the N-terminal end of the RD around residue 74. Supplemental Fig. S7B shows phosphorimaging kinase activity data from assays using as kinase either the full-length wild-type protein His-PknG<sub>1</sub>–750 or the NORS deletion mutant His-PknG<sub>74</sub>–750 with the RD either in the reduced, Zn-bound or the oxidized state and as substrate again His-PknG<sub>1</sub>–147 with the RD also either coordinating Zn<sup>2+</sup> or being oxidized. The corresponding SDS-PAGE analysis is shown in supplemental Fig. S7C. The kinase with the RD in the metal-bound state phosphorylates the substrate with the RD in the oxidized state a bit better. If the RD of the kinase is in the oxidized state, then the folding state of the RD in the substrate appears to not have a significant influence on the catalytic efficiency.

**PknG Autophosphorylation Induces Only Local Conformational Changes but No Global Folding of the NORS Region**—Complementary to the phosphorimaging kinase activity data, we monitored the phosphorylation of the NORS-RD protein His-PknG<sub>1</sub>–147 (Fig. 2B) or the NORS-only protein His-PknG<sub>1</sub>–75 (supplemental Fig. S7D) based on spectral changes in <sup>1</sup>H–<sup>15</sup>N HSQC spectra. Phosphorylation of both substrates in...
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FIGURE 2. PknG catalytic activity is sensitive to the folding state of the substrate, and autophosphorylation of the NORS region does not induce global folding. A, comparison of the phosphorylation activity of His-PknG WT (blue columns) and the PknG deletion mutants (His-PknG74–750, green columns; His-PknG74–420, red columns; all with the RD in the reduced, metal-bound state (RD/Zn)) toward the substrate His-PknG1–147 with the RD in the reduced, metal-bound (RD/Zn) or oxidized state (RD/ox) based on phosphorimaging data. The data for the wild-type enzyme include the phosphorylation in trans of its own N terminus (supplemental Fig. S7A). Kinase activity data of His-PknG wild-type and of an N-terminal truncated version (His-PknG74–750) under different redox conditions and thus with the own RD in a reduced/metal-bound or oxidized state can be found in supplemental Fig. S7, B and C. B, NMR monitoring of in vitro $^{15}$N-His-PknG1–147 phosphorylation by His-PknG74–420, both with the RD in the reduced metal-bound form (RD/Zn), based on the superposition of the $^1$H–$^{15}$N HSQC spectra of unphosphorylated $^{15}$N-His-PknG1–147 (black), and after kinase treatment for 3 h at 298 K (red) and further overnight at 310 K (green). An asterisk indicates a new peak that appears after phosphorylation. Assigned backbone amide cross-peaks that shift or show a change in signal intensity are labeled by the one-letter amino acid code and the sequence position (39). The additional label -sc indicates side chain amide protons. The NMR monitoring of the in vitro phosphorylation of $^{15}$N-His-PknG1–75 is shown in supplemental Fig. S7D.

$^{15}$N-labeled form by unlabeled His-PknG74–420 (RD-KD) results in the peak or disappearance and reappearance at new positions of several peaks of the NORS region. Based on the available assignments, this includes residues near the known in vivo phosphorylation site Thr$^{63}$ (11) as well as residues near other threonine residues known to be phosphorylated in vitro (Thr$^{23}$, Thr$^{32}$, and Thr$^{64}$) (10). Overall, His-PknG1–147 is more efficiently phosphorylated than His-PknG1–75. The appearance of some new more well dispersed peaks, e.g. around 8.7–9.3 ppm in the $^1$H dimension (Fig. 2B and supplemental Fig. S7D), suggests that autophosphorylation induces locally more ordered states but not a global folding of the NORS region.

The PknG RD Can Switch between a Reduced, Metal-bound Folded and an Oxidized, Metal-free Unfolded State—Because replacement of the cysteines of the rubredoxin-like RD to alanines or serines disables the redox regulation of PknG kinase function, and based on fluorescence data results in significant structural changes (8, 11), we also recorded $^1$H–$^{15}$N HSQC data of His-PknG1–147 (NORS-RD) under different redox conditions (Fig. 3A). When ZnCl$_2$ is added upon induction of the expression of His-PknG1–147, the black spectrum is obtained (15). This contains a subspectrum with mostly well dispersed peaks that is largely identical to the spectrum of the isolated RD (PknG74–147) that is obtained when the RP-HPLC-purified protein is refolded by adding a reducing agent, such as tris(2-carboxyethyl)phosphine, and a divalent metal ion, such as Zn$^{2+}$ (supplemental Fig. S8A) (15). As other rubredoxin motifs, the RD can also coordinate other metal ions such as Cd$^{2+}$, as in the crystal structure of PknG74–750, in complex with AX20017 (8) or Mn$^{2+}$, Co$^{2+}$, or Fe$^{3+}$, as indicated by the $^1$H–$^{15}$N HSQC data of His-PknG1–147 shown in supplemental Fig. S9. Because of the paramagnetic nature of the used metal ions, the well dispersed NMR signals of the RD are mostly not visible. However, the overall change of the spectral appearance indicates a structural transition upon metal addition (supplemental Fig. S9 and Results). Expression of PknG1–147 in minimal medium without addition of ZnCl$_2$ upon induction or addition of a rather mild oxidizing agent such as H$_2$O$_2$ together with a metal chelator such as EDTA results in the red spectrum that shows a low dispersion for all signals. Again, the spectrum of the oxidized, metal-free isolated RD (PknG74–147, supplemental Fig. S8A; see also the $^1$H–$^{15}$N NOE data in supplemental Fig. S3B) represents a subspectrum of that of oxidized His-PknG1–147 (supplemental Fig. S8B). Indicated by the low signal dispersion, the RD in the oxidized, metal-free form is, as the NORS, largely unfolded. The spectrum of a mutant of His-PknG1–147 in which all four cysteines of the two CXXCG motifs have been replaced by serines (His-PknG1–147-4C/S) looks overall similar to that of the oxidized, metal-free state (supplemental Fig. S8C). Altogether, the data indicate that the RD can switch between a reduced, metal-bound folded state and an oxidized, metal-free unfolded state (Fig. 3B). Thus, a change of the redox conditions may regulate the catalytic kinase domain by controlled unfolding or refolding of the RD, which is expected to influence the substrate access.

MD Simulations Indicate That Oxidation of the RD Increases the Accessibility of ATP in the Substrate Binding Region—The available crystal structures for PknG fragments containing both the RD and the kinase domain do not explain how oxidation of the two CXXCG motifs in the RD affects the conformation and accessibility of the catalytic domain and, thus, its activity. To complement the above structural and dynamic NMR data for the RD in different redox states, we performed six independent 250-ns MD simulations of PknG74–420 (RD-KD) with the CXXCG motifs of the RD either coordinating a metal ion (Fe$^{2+}$) or with a disulfide bond in each motif (Cys$^{106}$-Cys$^{109}$ and Cys$^{128}$-Cys$^{131}$) (supplemental Fig. S10A). Our simulations indicate that oxidation of the RD leads, on average, to a more open and better accessible ATP substrate-binding cavity (Fig. 3C). In line with the NMR data of the RD under different redox conditions (Fig. 3A and supplemental Fig. S8, A and B), we find that the RD containing the two CXXCG motifs shows overall an increased likelihood to unfold in the oxidized metal-free form, which may also favor the substrate access because of...
reduced steric clashes between the RD and the ATP binding site. To quantify the structural changes within the substrate-binding pocket, we calculated the average extension of three loops surrounding the ATP site (Fig. 3, D–F, and supplemental Fig. S10B) as well as the ATP cavity volume and solvent-accessible surface area (SASA) of residues interacting with ATP, as suggested by Scherr et al. (8). The simulations suggest that loops 1 and 3 surrounding the ATP-binding site become more extended in the oxidized states (Fig. 3, E and F), making the cavity more accessible from the bulk. Oxidation of the RD also seems to lead to a somewhat larger cavity volume and SASA of ATP-surrounding residues (supplemental Fig. S11 and data not shown). In addition to these differences in substrate accessibility, our simulations further suggest that the functionally important residue Asp276 dissociates more from the nearby Lys278 in the metal-free oxidized form relative to the
metal-bound form (supplemental Fig. S12). This, in turn, might increase the kinase activity by shifting the p\(K_a\) of Asp\(^{276}\) toward higher values, thus favoring ATP hydrolysis, in line with our kinase assay data for PknG with the RD in different redox states for a more folded/bulky substrate such as PknG1–147 if the RD is in the Zn bound state (supplemental Fig. S7, B and C).

**Discussion**

**Conformation and Dynamics of the NORS Region and PknG**

**Autophosphorylation—Intrinsically disordered proteins (IDPs) or protein regions are typically rich in polar amino acids as well as prolines and show a high net charge (20).** PknG1–75 corresponding to the NORS region contains 10 prolines and 10 negatively and seven positively charged residues as well as a high content of serines and threonines (five and 10, respectively), and, in line with this, the web program FoldIndex predicts it to be natively disordered (data not shown). Moreover, limited proteolysis of PknG resulted in a fragment lacking the first 73 residues (8). The presented NMR structural and dynamic data for the NORS region (His-PknG1–75; Fig. 1, C and D, and supplemental Figs. S2 and S3A) demonstrate that the NORS region is indeed rather unstructured and dynamic. Because the spectral appearance of the isolated NORS is about the same as that connected to the RD (Fig. 3A and supplemental Fig. S1), the two regions behave rather independently and appear not to interact. Autophosphorylation of the NORS region has been shown to play a role for the survival function of PknG (10), and some IDPs fold upon phosphorylation (20, 21). Based on the chemical shift changes of several residues near the phosphorylated threonines observed in the present NMR monitored kinase assays using as substrates His–PknG1–147 (NORS-RD, Fig. 2B) or His–PknG1–75 (NORS, supplemental Fig. S7D), phosphorylation appears not to result in global folding but only in local structural changes, and it appears not to induce interactions with the subsequent RD. Because phosphorylation locally modulates the charge as well as conformational dynamics (20, 22), it may play a role in the interaction with regulatory proteins and/or the KD or TPRD of the same PknG molecule or a neighboring one or modulate the substrate specificity. One publication describes PknG self-cleavage that results in a fragment encompassing the NORS, the RD, and the KD and another one corresponding to the TPRD as well as autophosphorylation in the TPRD (23). However, consistent with phosphorylation data from other groups using radiolabeled ATP (10, 11, 14), our data (Fig. 2 and supplemental Fig. S7) suggest that deletion of the NORS region significantly reduces the catalytic activity and that autophosphorylation only occurs when the NORS region is present and, thus, in the NORS region.

**The Role of the Redox-sensitive RD Conformation for Substrate Access to the PknG KD—**In the crystal structure of PknG74–750 (RD-KD-TPRD) in complex with the small molecule inhibitor AX20017 (PDB code 2PZI, Fig. 1B), the RD interacts with both the N- and C-terminal lobes of the kinase domain and packs on top of the ATP binding and catalytic cleft without blocking its access (8). In the crystal structures of PknG74–405 (RD-KD) in complex with ADP-Mg\(^{2+}\) (PDB code 4Y0X, supplemental Fig. S6A) or ATP-\(\gamma\)S-Mg\(^{2+}\) (PDB code 4Y12, supplemental Fig. S6A), the orientation of the RD is slightly different, and it only makes contact with the N-terminal kinase domain (14). Based on these observations, the authors suggest that the RD, in the folded metal-bound state, may regulate the catalytic activity by opening and closing the access to the substrate site (14). However, the rather small changes in the orientation of the RD relative to the kinase domain in the inhibitor-bound form and the nucleotide-bound forms appear not to be enough to explain the redox regulation of the KD by the RD. It can also not explain the inhibitory effect of AX20017 because this is a consequence of it binding to the active site. The small differences in the orientation of the RD relative to the KD in the different crystal structures arise mostly from small conformational differences because of the presence of different-size molecules in the ATP-binding region (AX20017 in 2PZI versus ADP-Mg\(^{2+}\) in 4Y0X or ATP-\(\gamma\)S-Mg\(^{2+}\) in 4Y12) as well as the presence (2PZI) or absence (4Y0X and 4Y12) of the TPRD and different crystallization conditions resulting in different crystal packing. The RD has not only dynamic regions in the isolated state in solution (Fig. 1D and supplemental Fig. S3A) but also shows, in the crystal states, rather large stretches with high B factors (supplemental Fig. S6A). Compared with the inhibitor-bound crystal structure (2PZI), the ATP-\(\gamma\)S-Mg\(^{2+}\)-bound (4Y12) and, even more, the ADP-Mg\(^{2+}\)-bound (4Y0X) forms appear to be overall less flexible (supplemental Fig. S6A). Thus, the observed variation of the orientation of the reduced, metal-bound RD in the three crystal structures and the observation that it contains dynamic regions in the solution and crystal states suggest that it has enough flexibility to allow binding of substrates. As described for other rubredoxin-like domains or zinc fingers (24, 25), the RD only adopts a defined three-dimensional fold upon metal binding, and the latter has not only a stabilizing effect, as, for example, in the protein IscU (26). Based on the presented NMR, MD, and kinase assay data of the RD in different redox states, the redox regulation of PknG kinase function is rather achieved by redox regulated unfolding and refolding of the RD, which modulates the substrate access and, thus, selectivity.

**Regulation of PknG Catalytic Activity by Its Redox-sensitive RD—**A redox regulation of the catalytic activity of PknG was initially suggested by kinase assay data for wild-type PknG and a mutant in which all four cysteines of the RD had been replaced by serines (PknG-C/S). Apparently, this mutant was devoid of catalytic activity toward a substrate corresponding to the N-terminal regions (His–PknG1–147), and that is phosphorylated in the natively unfolded NORS region (8, 10). Tiwari et al. (11) used as substrate the FHA domain containing mostly folded protein GarA, and observed the following. Deletion of the NORS and the RD together (PknG151–750) reduces the catalytic activity by \(\sim95\%\), mutation of the cysteines in either CXXCG motif to alanines (C106A/C109A = T1, C128A/C131A = T2) by \(\sim30\%\), and of both together (T1T2) by \(\sim50–75\%\). Moreover, the catalytic activity of the T1T2 mutant was not very sensitive to a shift in the redox conditions (presence of 1 mM reduced or oxidized DTT), whereas wild-type PknG showed \(\sim2.5\) higher activity under oxidizing conditions (11). The latter is contradictory to the reduction of the catalytic activity by the cysteine-to-alanine replacements, which should mimic oxidizing, metal-releasing conditions. However, addi-
tion of oxidized DTT alone may not be sufficient to induce full oxidation of the two CXXCG motifs of the RD, and thus metal release and unfolding and/or the disulfide bonds result in additional local structural order (27, 28). Lisa et al. (14) used as substrate either GarA or only a 17-mer peptide corresponding to GarA residues 14–30. Wild-type PknG shows ~45 higher activity toward the folded GarA protein than to the GarA peptide, which, with its extended conformation, is comparable with an unfolded protein stretch. In addition, the (auto)phosphorylated NORS region has been suggested to provide phosphorylated threonine-dependentanchoring sites for high-affinity interactions with the forhead-associated (FHA) domain of GarA (29, 30). Deletion of the N-terminal 137 residues, including the NORS region and most of the RD, resulted in higher activity toward the peptide but a bit lower toward the folded GarA protein (14). The latter is in contrast to the data by Tiwari et al. (11). As Lisa et al. already pointed out, the structural integrity of the deletion mutants has not been tested (14), and deletion of the RD may destabilize the kinase fold stronger than just oxidizing the RD CXXCG motifs. To complement the published kinase assay data and because PknG autophosphorylation has shown to be important for mycobacterial survival (10), we used as a substrate, as Scherr et al. (8, 10), His-PknG1–147 and as kinase either wild type His-PknG or the deletion mutants His-PknG74–420 (RD-KD) or His-PknG74–750 (RD-KD-TPRD). Based on our data (Fig. 2 and supplemental Fig. S7), the substrate with the RD in the oxidized, unfolded state is better phosphorylated. This indicates that PknG with the RD in the reduced, metal-bound, folded state may better phosphorylate substrates with extended structures, although the kinase with the RD in the oxidized, unfolded state phosphorylates the substrate His-PknG1–147 with the RD in either redox state about equally well. Because the phosphorylated threonines are located in the mostly unstructured NORS region that adopts overall a more extended peptide-like conformation, the redox state of the RD either in the substrate or the kinase is generally expected to have an overall smaller effect than for a larger folded substrate. Interestingly, in the work of Tiwari et al. (11), the PknG mutant with all four cysteines of the RD CXXCG motifs replaced by alanines still showed significant activity, whereas the one with all replaced with serines in the work by Scherr et al. (8) showed no activity. Based on our kinase assays under different redox conditions, the results from Tiwari et al. (11) make more sense because mutagenesis of the cysteines should have a similar effect as oxidative unfolding. Although there are some contradictory results regarding the catalytic activity of PknG under different redox conditions and for different mutants that arise from differences in the buffer conditions (amount of substrate and kinase, “hot” and “cold” ATP, magnesium, and manganese salts, type of reducing/oxidizing agent, and so forth) and the used substrates (GarA, GarA peptide, His-PknG1–147, or kinase-dead PknG-K181M) (8, 10, 11, 14), the data altogether suggest that deletion of the RD or mutation of the cysteines in the two RD CXXCG motifs and changes in the redox conditions affect the kinase function of PknG. Based on the provided NMR and MD data about redox induced conformational changes in PknG (Fig. 3 and supplemental Figs. S3B, S8, and S10–S12) and the functional data (Fig. 2 and Fig. S7), changes of the redox environment modulate PknG substrate selectivity more than its intrinsic catalytic efficiency.

Modulation of PknG Function by ROS in the Host Cell—The presented NMR and MD structural and dynamic data suggest that the function of PknG can be modulated by oxidative unfolding of the redox-sensitive RD, which makes the catalytic cleft more accessible for substrates (Fig. 3 and supplemental Figs. S3B and S10–S12). Similar redox-sensitive regulation mechanisms involving a four-cysteine (ZnCys₄) or two-cysteine two-histidine (ZnCys₂His₂) metal center have, for example, been proposed for heat shock protein 33 (Hsp33) and the mycobacterial σ factor binding protein RslA, respectively (27, 31). Other examples are the anti-σ factors RsrR (ZnCys₄His) and ChrR (ZnCys₂His₂) that regulate the bacterial defense against oxygen and disulfide stress (28, 32, 33). Oxidative unfolding of the PknG RD in our study has been achieved using a combination of hydrogen peroxide (H₂O₂) and a metal chelator (EDTA). The degree of oxidation and metal release depend further on other conditions such as the pH or the temperature (28). For example, for Hsp33, H₂O₂ alone is also not enough to induce oxidation and metal release. Full activation of Hsp33 requires either a combination of H₂O₂ and elevated temperatures (43°C) or the stronger oxidant hypochlorous acid (HOCl) (27). Cells of the host innate immune system such as macrophages produce high concentrations of ROS, such as hydrogen peroxide (H₂O₂), superoxide (O₂⁻), or HOCl and release them into the phagosome to kill engulfed pathogenic organisms (17, 34). PknG is secreted into the host cell, where it can be localized in the cytosol and the phagosome (9). Because blocking of phagosome-lysosome fusion and, thus, mycobacterial killing affords the catalytic activity of PknG, the proposed regulation of PknG activity and/or substrate specificity by oxidative unfolding of its RD makes complete sense. Future studies have to answer the question whether PknG autophosphorylation promotes mycobacterial survival in the host by just affecting its interaction with host proteins or also by phosphorylating host proteins in the cytosol and/or phagosome. One target in the host is the protein kinase C-α, a regulator of phagocytosis and the biogenesis of the phagolysosome and the closest human homologue of PknG (34, 35). PknG has been proposed to down-regulate protein kinase C-α by stimulating its degradation and to be, in vitro, able to proteolytically cleave but not phosphorylate it (35). Future studies have to address the question whether other proteins in the host cell are targeted, which are most likely also involved in controlling phagosome-lysosome fusion, and how exactly PknG interacts with them to modulate host signaling, involving, for example, interactions of the autophosphorylated NORS region with FHA domains of human target proteins similar to those with the mycobacterial substrate protein GarA (29, 30).

Experimental Procedures

Cloning and Mutagenesis—Expression plasmids (pET-15b) for His-tagged PknG1–750 (wild-type, NORS-RD-KD-TPRD, Fig. 1A) as well as PknG1–147 (NORS-RD) and PknG74–750 (RD-KD-TPRD) were kindly provided by the group of Prof. Dr. Jean Pieters from the Biozentrum of the University of
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Basel. The expression plasmids pET-15b::PknG1–75 and pET-15b::PknG74–147 were obtained by a mutagenesis-based approach as described earlier (15). The quadruple mutant His-PknG1–147–4C/4S, in which Cys106, Cys109, Cys128, and Cys131 are replaced by serines and the expression plasmid His-PknG74–420 (RD-KD) that was derived from the one for His-PknG74–750 by introducing a stop codon at position 421 were prepared using the QuikChange site-directed mutagenesis method (Stratagene, La Jolla, CA).

Protein Expression and Purification—Protein expression and purification of His-PknG1–147, His-PknG1–75, and PknG74–147 were carried out as described previously (15). All proteins were overexpressed in Escherichia coli BL21 (DE3) cells (Novagen). Following induction with isopropyl β-d-1-thiogalactopyranoside, cells were grown for 16 h at 15 °C, whereas, for the expression of PknG1–147–4C/4S, cells were grown for 2 h at 37 °C. For in vitro kinase assays, His-PknG1–750, His-PknG74–750, and His-PknG74–420 were expressed in rich medium (LB). For NMR measurements, His-PknG1–75, His-PknG1–147, and PknG74–147 were expressed in 15N- or 15N-13C-enriched M9 minimal medium (36) supplemented with 1× BME vitamin solution (Sigma) and trace elements as described previously (15). His-PknG74–420 was expressed in 15N-enriched M9 minimal medium containing 70% D2O. Cells were harvested by centrifugation and sonicated, and the supernatant, after centrifugation, was loaded on a gravity flow column filled with nickel-nitrilotriacetic acid-agarose beads (Qiagen). Fractions containing significant amounts of PknG1–750, PknG74–750, or PknG74–420 were pooled, concentrated, and further purified by size exclusion chromatography using a 200 pg Superdex™ HiLoad™ 16/600 column equilibrated in 20 mM Tris (pH 7.5), 150 mM NaCl, 0.05% NaN3, 5% D2O, and 10 μM unlabeled, catalytically active His-PknG74–420. Consecutive spectra were acquired at 298 K on a Bruker Avance 500 MHz spectrometer equipped with a cryogenic probe. Overnight incubation of the NMR sample at 310 K was done using a thermostated water bath.

NMR Sample Preparation (without Kinase Assays)—The protein concentration of the 15N- and 15N-13C-labeled samples of His-PknG1–75, PknG74–147, and His-PknG1–147 in 20 mM Tris (pH 7.5) and 150 mM NaCl (95% H2O, 5% D2O) ranged from 0.1–0.8 mM. The sample of 15N-13C-PknG74–147 (0.2 mM) for measuring residual dipolar couplings contained ~17 mg/ml PF1 phages (ASLA Biotech). The protein concentration of 15N-D-PknG74–420 in 20 mM Tris (pH 7.5), 500 mM NaCl, 10 mM tris(2-carboxyethyl)phosphine, 0.5 mM MgCl2, and 0.5 mM ATP was 0.1 mM.

NMR Spectroscopy—NMR spectra were acquired at 298 K on Bruker Avance 500, 600, and 900 MHz spectrometers; the 500 and 900 MHz ones were equipped with cryogenic probes. The data were processed with NMRPipe (37) and analyzed using NMRView (38). Assignments for 13C, 15N, and 1H nuclei were based on three-dimensional constant-time HNCA, CBCANH, CCONH-TOCSY, and HNCO spectra as described previously (39). The 13C and 1H secondary shifts were calculated as the difference between the measured chemical shift value and the random coil value for the respective amino acid (40). 1H_HNCO coupling constants were obtained from three-dimensional HNHA spectra (41).

Information about the backbone dynamics were derived from 15N relaxation data, including T1 (spin-lattice relaxation), T2 (spin-spin relaxation), and 1H-{15N} NOE. 15N-1H residual dipolar couplings were obtained from the analysis of 15N-1H-IPAP-HSQC data (42). The maximal 1D_N, H for PknG74–147 was 15.7 Hz.

Molecular Dynamics Simulations—Full atomistic molecular models of PknG74–420 with the RD in the Fe2+ and oxidized states were constructed based on the crystal structure of PknG74–750 in complex with the inhibitor AX20017 (PDB code 2PZI) (8). The loops missing in the crystal structure were modeled using ModLoop (43). The models were solvated in a water box with Na+ /Cl− ions, mimicking a 100 mM salt concentration. The molecular systems comprising ~62,500 atoms were simulated in an isothermal-isobaric (NPT) ensemble at T = 310 K and p = 101.3 kPa for 250 ns using 2-fs integration time steps and treating long-range electrostatic effects using the particle mesh Ewald approach. Three MD simulations for
each state, in total 1.5 μs, were performed in NAMD 2.9 (44) using the CHARMM27 force field (45) and force field parameters for the Fe²⁺-4Cys center obtained from the literature (46). Visual Molecular Dynamics (47) was used for analyzing the MD trajectories, and cavity volumes were calculated using the fppocket package (48). The average extension of three loops surrounding the ATP-binding site, loop 1 (residues 94–105), loop 2 (residues 292–297), and loop 3 (residue 298–310), were calculated from the averaged Cα distances between residues $d_{\text{Loop1}}$: Thr²⁹⁵ and Gly¹⁰¹, Asn⁹⁶ and Ser¹⁰², Pro⁹⁷ and Lys¹⁰³, and Val²⁹⁸ and Arg¹⁰⁴; $d_{\text{Loop2}}$: Ile²⁹⁹ and Gly²⁹⁵, Asp²⁹³ and Ala²⁹⁶, and Lys²⁹⁴ and Val²⁹⁷; and $d_{\text{Loop3}}$: Ser²⁹⁸ and Phe³⁰³, Arg⁷⁷ and Gly⁷⁷, Ile³⁰⁰ and Tyr³⁰⁵, and Asn¹⁰¹ and Leu³⁰⁶.

Author Contributions—S. A. D. designed and coordinated the study, helped acquire and analyze the NMR data, and wrote the paper. M. W. designed, performed, and analyzed the NMR and kinase assay data shown in Figs. 1–3 and supplemental Figs. S1–S9 and helped write the manuscript. Q. L. designed, performed, and analyzed the molecular dynamics simulation data shown in Fig. 3 and supplemental Figs. S10–S12. V. R. I. K. designed and coordinated the molecular dynamics part of the study and wrote the corresponding part of the paper. All authors reviewed the results and approved the final version of the manuscript.

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