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Backbone chemical shift assignments for *Xanthomonas campestris* peroxiredoxin Q in the reduced and oxidized states: a dramatic change in backbone dynamics

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Abstract

Peroxiredoxins (Prx) are ubiquitous enzymes that reduce peroxides as part of antioxidant defenses and redox signaling. While Prx catalytic activity and sensitivity to hyperoxidative inactivation depend on their dynamic properties, there are few examples where their dynamics has been characterized by NMR spectroscopy. Here, we provide a foundation for studies of the solution properties of peroxiredoxin Q from the plant pathogen *Xanthomonas campestris* (XcPrxQ) by assigning the observable ¹H^N, ¹⁵N, ¹³C^α, ¹³C^β, and ¹³C' chemical shifts for both the reduced (dithiol) and oxidized (disulfide) states. In the reduced state, most of the backbone amide resonances (149/152, 98%) can be assigned in the XcPrxQ ¹H-¹⁵N HSQC spectrum. In contrast, a remarkable 51% (77) of these amide resonances are not visible in the ¹H-¹⁵N HSQC spectrum of the disulfide state of the enzyme, indicating a substantial change in backbone dynamics associated with the formation of an intramolecular C48-C84 disulfide bond.

Keywords

plant diseases; peroxidase; drug design; conformational change

Biological context

Under aerobic conditions cells are constantly exposed to reactive oxygen species, such as H₂O₂, organic peroxides, and hydroxyl radicals, which can damage cellular DNA, proteins, and lipids (Winterbourn, 2008). Aerobic organisms counter with an arsenal of antioxidant proteins including peroxiredoxins (Prxs), a family of ubiquitous, cysteine-based peroxidases that play influential roles in the turnover of peroxides including peroxynitrite (recently reviewed in Perkins et al. 2014). In eukaryotes Prxs also appear to regulate “non-oxidative-stress-related” signaling events (Rhee et al. 2012). All six known Prx subgroups – Prx1, Prx5, Prx6, Tpx, AhpE, and PrxQ (also called BCP) – require for catalysis a “peroxidatic” Cys (C_p) in a “fully folded” (FF) conformation that binds and reacts with the peroxide

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substrate to form Cys-sulfenic acid ($C_P\text{-SOH}$) and H_2O . For most Prxs, the protein then undergoes substantial rearrangement to some type of a “locally unfolded” (LU) conformation allowing the $C_P\text{-SOH}$ to form a disulfide bond with a “resolving” Cys (C_R) that can be located in various parts of the protein. Despite the importance of this FF/LU conformational change, little is still known about its thermodynamics or dynamics. Among Prxs, only the PrxQ subgroup includes members that, as monomers of ~20 kD, are amenable to facile study by NMR spectroscopy; and *Arabidopsis thaliana* PrxQ (AtPrxQ), with its C_R in helix-2, is the only PrxQ so far characterized by NMR. This latter study revealed a substantial dynamical disorder on the μs -ms timescale especially for the reduced (dithiol) form (Ådén et al. 2011).

We are initiating NMR studies of another monomeric PrxQ that undergoes a different conformational change, because it has its C_R residue in helix-3. *Xanthomonas campestris* PrxQ (XcPrxQ), from an economically important plant pathogen (Leyns et al. 1984), is 160-residues long and has relevance for drug design (Myler et al 2009) as it is representative of PrxQs from various genera of human pathogens such as *Burkholderia* (63% sequence identity), *Helicobacter* (46%), *Cryptosporidium* (42%), *Toxoplasma* (41%), *Mycobacterium* (40%), and *Acanthamoeba* (38%). Also, high resolution crystal structures for the disulfide (LU) conformation and the pseudo-dithiol (FF) conformation (Cys→Ser double variant) show that the transition from FF to LU involves a substantial unfolding of helix-3 (Liao et al. 2009). With plans to directly measure how redox state, mutations, and post-translational modifications modulate the enzyme’s structure and dynamics, and to enable chemical shift perturbation studies of ligand binding (e.g. Buchko et al. 1999), we have fully assigned the observable $^1H^N$, ^{15}N , $^{13}C^\alpha$, $^{13}C^\beta$, and $^{13}C'$ chemical shifts of native XcPrxQ in both the disulfide (LU) and dithiol (FF) states.

Methods and experiments

Cloning, expression, and purification

The full length *X. campestris* strain 17 *prxQ* gene (GI:499347373) was synthesized by GenScript (Cambridge, MA) and cloned into the NcoI and HindIII restriction sites of the expression vector pTHCm (Nelson et al. 2008) such that the expressed protein used in our studies contained no non-native residues. The expression vector was then transformed into *Escherichia coli* BL21(DE3) cells. Uniformly ^{15}N -, ^{13}C -labeled XcPrxQ was obtained by growing the transformed cells (310 K) in 750 mL of minimal medium containing $^{15}NH_4Cl$ (1 mg/mL) and D- $[^{13}C_6]$ glucose (2.0 mg/mL), NaCl (50 $\mu\text{g/mL}$), $MgSO_4$ (120 $\mu\text{g/mL}$), $CaCl_2$ (11 $\mu\text{g/mL}$), $FeCl_3$ (10 ng/mL) and the antibiotic ampicillin (100 $\mu\text{g/mL}$). When the cell culture reached an OD_{600} reading of ~0.8, it was transferred to a 298 K incubator and protein expression induced with isopropyl β -D-1-thiogalactopyranoside (0.026 $\mu\text{g/mL}$). Cells were harvested approximately four hours later by mild centrifugation, and frozen at 193 K. After thawing the frozen pellet, the cells were resuspended in 40 mL of 20 mM TrisHCl, pH 7.5 and lysed by three passes through a French Press (SLM Instruments, Valencia, CA) followed by sonication for 1 min. Insoluble cell debris was removed by centrifugation at 25,000g for 1 h with the supernatant dialyzed overnight (278 K) in 5 L of 20 mM TrisHCl, pH 7.5. XcPrxQ was purified from this solution using two chromatography

steps: High Q ion exchange (5 mL Bio-Rad Econo-Pac cartridge (Bio-Rad, Hercules, CA)) and Superdex75 gel-filtration (HiLoad 16/60 column). In the first chromatography step, approximately 5 – 10 mg of total protein was applied to the High Q column pre-equilibrated with 25 mM TrisHCl, pH 7.5 (flow rate = 5 mL/min). Oxidized XcPrxQ did not bind to the column and eluted in the flow-through, which was concentrated to ~10 mg/mL before application to a size exclusion column pre-equilibrated with 100 mM NaCl, 20 mM Tris-HCl, pH 7.1 (flow rate = 1 mL/min). To obtain XcPrxQ in the reduced state, the size exclusion buffer was made 1.0 mM in dithiothreitol (DTT) prior to the addition of the High Q fraction (which was also made 1.0 mM in DTT before loading onto the column). The major fractions contained XcPrxQ and these were pooled and concentrated to approximately 25 mg/mL for the NMR studies.

Nuclear magnetic resonance spectroscopy

The NMR data were all collected at 293 K on a double-labeled (^{13}C , ^{15}N) sample (~1.1 mM) of XcPrxQ in the oxidized and reduced states using four-channel Varian Inova-600, -750, and -800 spectrometers equipped with triple resonance probes and pulse field gradients. For the reduced state, assignment of the $^1\text{H}^{\text{N}}$, ^{13}C , and ^{15}N backbone and some of the ^{13}C side chain resonances were made from a standard two-dimensional ^1H - ^{15}N HSQC experiment and three-dimensional HNCACB, HNCA, HNCACO, HNCO, CC-TOCSY-NNH and ^{15}N -edited NOESY experiments using the Varian Biopack suite of pulse programs. A mixing time of 80 ms was used in the ^{15}N -edited NOESY experiment and the HNCACB experiment was optimized for the detection of β -carbons using a tauCC value of 7 ms. For the oxidized state, assignment of the $^1\text{H}^{\text{N}}$, ^{13}C , and ^{15}N backbone and some of the ^{13}C side chain resonances were made from a standard two-dimensional ^1H - ^{15}N HSQC experiment and three-dimensional HNCACB (^{13}C β -optimized), HNCO, and HNN experiments. An overall rotational correlation time (τ_{c}) for XcPrxQ in both states was estimated from backbone amide ^{15}N $T_1/T_{1\rho}$ ratios. Felix2007 (MSI, San Diego, CA) and Sparky (v3.115) were used to process and analyze, respectively, all the NMR data. Indirect methods (DSS = 0 ppm) were used to reference the ^1H , ^{13}C , and ^{15}N chemical shifts that were deposited into the Biological Magnetic Resonance Bank database (www.bmrb.wisc.edu) under the BMRB accession numbers 25557 (reduced) and 25566 (oxidized).

Assignments and data deposition

The expectation that XcPrxQ is monomeric in solution (Liao et al. 2009) is corroborated by the elution of both the disulfide and dithiol forms of the enzyme from the gel filtration column with an identical retention time of ~89 min characteristic of a ~17 kDa protein under our conditions (data not shown). Also, the isotropic overall rotational correlation times (τ_{c}) inferred from ^{15}N spin relaxation times were the same for XcPrxQ in the oxidized and reduced states, 10.6 ± 0.1 ns at 293 K, a value consistent with an ~17 kDa protein. Finally, the reasonably efficient magnetization transfer through the side chain carbons in the three-dimensional NMR experiments used to make chemical shift assignments in both the reduced and oxidized states would be unlikely with a protonated protein if XcPrxQ formed ~35 kDa dimers (Yee et al., 2002).

The features in the ^1H - ^{15}N HSQC spectrum for reduced (dithiol) XcPrxQ (Fig. 1A) are characteristic of a folded protein (Yee et al. 2002): cross peaks of near uniform intensity that are dispersed in both the proton and nitrogen dimensions. Out of the 152 expected backbone amide resonances ($160 - (7 \text{ Pro} + \text{Met}1)$), 149 were assigned (98%) to cross peaks in the ^1H - ^{15}N HSQC spectrum. The three unassigned residues, Y40, S44, and G47, could not be unambiguously assigned to the three remaining unassigned amide cross peaks due to poor magnetization transfer to carbon resonances in the three-dimensional backbone assignment experiments. The chemical shifts for the β -carbons of the two catalytic cysteine residues C_P (C48) and C_R (C84) are 32.2 and 26.9 ppm, respectively. Such chemical shifts are consistent with cysteine residues in the Cys-thiol/thiolate state (Sharma and Rajarstnam, 2000) expected in the presence of the reducing agent DTT.

For oxidized XcPrxQ, the wide chemical shift dispersion of the cross peaks in both the proton and nitrogen dimensions in the ^1H - ^{15}N HSQC spectrum (Fig. 1B), is similarly characteristic of a folded protein (Yee et al. 2002). However, relative to the ^1H - ^{15}N HSQC spectrum for reduced XcPrxQ (Fig. 1A), the intensity of the cross peaks are not as uniform and approximately half (51%, 77/152) of the expected amide resonances are missing under conditions that are identical except for the absence of the reducing agent DTT. Collecting the reduced ^1H - ^{15}N HSQC spectrum at 283 K instead of 293 K did not increase the number of observable amide resonances. Of the remaining amide cross peaks observed for disulfide XcPrxQ, many overlay with amide cross peaks for reduced XcPrxQ, allowing a rapid tentative assignment based on the assignments made for the dithiol form. The analysis of the HNCACB (β -optimized) and HNN data collected on oxidized XcPrxQ confirmed the tentative assignments and also allowed the assignment of the remaining amide cross peaks (Fig. 1B). In the disulfide XcPrxQ spectrum, a second set of less intense cross peaks were observed for residues A4 – L6 (blue in Fig. 1B) reflecting a second minor orientation at the N-terminus, a region that is disordered in both crystal structures (Laio et al. 2009). The amides of C48 and C84 are not observed in the ^1H - ^{15}N HSQC spectrum for oxidized XcPrxQ, and therefore, the presence of the disulfide bond cannot be confirmed from cysteine β -carbon chemical shifts.

While there are some intensity differences in corresponding amide resonances in the reduced and oxidized forms of XcPrxQ, most of the chemical shift differences are small, less than 0.1 ppm (Fig. 2A). This suggests that in the regions of the protein where amide resonances are observed in both states, the structure is rather similar. To see which parts of the protein correspond to the amide resonances missing in the ^1H - ^{15}N HSQC spectrum of oxidized XcPrxQ, we have mapped them onto the primary amino acid sequence of XcPrxQ (Fig. 2B). Interestingly, all five amide resonances with average chemical shift perturbations greater than 0.1 ppm – S20, G22, A57, F91, and L119 (Fig. 2A) – are adjacent to or just one residue away from a segment with missing amide resonances suggesting that a structural change is associated with at least some of the missing amide resonances. Such a structural difference is indeed seen in comparing the XcPrxQ LU disulfide (3GKK) and FF pseudo-dithiol (3GKM) crystal structures (Fig. 2C). Though much of the structure changes minimally, substantial rearrangements occur in the length and orientation of helix-3 (circled in Fig. 2C), which contains the resolving cysteine, C_R, and extends from V77 – Q87 in the pseudo-

dithiol structure (blue), but only from C84 – Q87 in the disulfide structure (gold), with electron density absent from K78 – D81 consistent with that region being disordered (Laio et al. 2009).

Missing amide cross peaks in ^1H - ^{15}N HSQC spectra of proteins typically identify regions undergoing motion or chemical exchange in the intermediate timescale (ms to μs) (e.g. Buchko et al. 1999; Ådén et al. 2011) and are often associated with regions involved in binding and catalysis (e.g. Müller et al. 1992). A mapping of the amide resonances missing in the ^1H - ^{15}N HSQC spectrum of oxidized XcPrxQ onto the crystal structure of pseudo-dithiol XcPrxQ (Fig. 2D) reveals that the extensive set of missing resonances (red) are all surrounding the region (helix-3) that is the focal point of the FF/LU conformation change. The disappearance of ~50% of the amide resonances in the ^1H - ^{15}N HSQC spectrum of reduced XcPrxQ upon the formation of a single intramolecular C_P-C_R disulfide bond indicates the protein has undergone a drastic change in backbone dynamics even for the nearby residues that do not apparently change much in their average conformation. This is fascinatingly reminiscent of, but opposite to, the large changes in dynamics observed for AtPrxQ. For AtPrxQ nearly 50% of the backbone amide peaks in the ^1H - ^{15}N HSQC spectrum were absent when in the *reduced* state (Ådén et al. 2011). As noted in the introduction, the AtPrxQ C_R is located in helix-2 rather than helix-3, and so it is plausible that these enzymes truly do experience very different structural and dynamics changes in their FF/LU transitions. Although further analysis is beyond the scope of this assignments report, these results make clear that different Prxs can behave very differently and that the chemical shift assignments presented here for XcPrxQ in the dithiol and disulfide states provide a basis for what will be very informative studies that provide residue-level insights into the interplay of Prx function, structure, and dynamics.

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Fig 1a

Fig 1b

Fig. 1.

^1H - ^{15}N HSQC spectra of dithiol and disulfide forms of XcPrxQ. (A) ^1H - ^{15}N HSQC spectrum for dithiol XcPrxQ (~1.5 mM) collected at a proton resonance frequency of 750 MHz, 293 K, in 100 mM NaCl, 20 mM Tris, 1 mM DTT, pH 7.0, with labels for assigned amide cross peaks (purple), unassigned amide cross peaks ("?"), tryptophan NεH groups (cyan and with an "r"), and amide side chain resonances (dashed red horizontal lines). An "x" marks the D3 cross peak that is below the contour limit, and resonances for D96 (9.08 and 133.4 ppm) and R123 (9.06 and 134.1 ppm) are not shown. (B) Same as A but for

oxidized XcPrxQ (~1.5 mM) collected at a proton resonance frequency of 800 MHz, 293 K, in 100 mM NaCl, 20 mM Tris, pH 7.0. A second set of minor cross peaks, labeled with a (2) and colored blue, are observed for residues A4, V5, and L6.

Fig. 2.

Mapping dithiol versus disulfide XcPrxQ chemical shift changes onto the sequence and structure. **(A)** Plot of the root-mean-square combined chemical shift perturbation in the ^1H and ^{15}N resonances ($\Delta_{rms} = [((^1\text{H}^N)^2 + (^{15}\text{N}/5)^2)/2]^{1/2}$) between dithiol and disulfide states of XcPrxQ. Residues with $\Delta_{rms} > 0.1$ ppm are identified. **(B)** Amino acid sequence of XcPrxQ highlighting in red residues having missing amide cross peaks in the ^1H - ^{15}N HSQC spectrum of disulfide XcPrxQ. Five residues missing resonances not colored red are the C_P and C_R residues forming an intramolecular disulfide bond (black) and the three residues not assigned in the dithiol spectrum (grey). Shown above the sequence are the α -helices (gold eclipses) and β -strands (cyan rectangles) identified in the crystal structure of disulfide XcPrxQ (3GKK) following the authors' labeling scheme (Liao et al. 2009). The pink α -helix, α_3 , corresponds the extended helix observed in the crystal structure of reduced XcPrxQ (3GKM) **(C)** Superposition of the crystal structures of pseudo-dithiol (3GKM, blue) and disulfide (3GKK, gold) XcPrxQ using the program Superpose (Maiti et al., 2004). The C_P and C_R disulfide is shown (sticks) and the residues are labeled. The region most altered during FF/LU conformation change, α_3 , is highlighted (dashed cyan ellipse). **(D)** The

missing amide resonances in the ^1H - ^{15}N HSQC spectrum of disulfide *XcPrxQ* mapped, in red, onto a cartoon representation of the crystal structure of pseudo-dithiol *XcPrxQ* (3GKM).