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## Backbone $^1\text{H}$ , $^{15}\text{N}$ , and $^{13}\text{C}$ resonance assignments for the NOXO1 $\beta$ PX domain

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### Abstract

NOXO1 (Nox Organizer 1) is a homolog of the NADPH oxidase protein p47<sup>phox</sup>. NADPH oxidases transfer electrons from NADPH to molecular oxygen, generating the superoxide anion. NOXO1 contains an N-terminal PX (phox homology) domain and is one of several PX domain-containing proteins found in the cytosolic subunits of the NADPH oxidase complex. These PX domains bind to membrane lipids and target the protein to membranes, recruiting other cytosolic components to the membrane bound components and aiding formation of a active enzyme complex. This recruitment represents a level of regulation of these oxidases. Here we report the backbone assignments of NOXO1 $\beta$  PX.

### Keywords

PX domain; NOXO1; NADPH oxidase; NMR backbone assignments

### Biological context

The NADPH oxidases are multiprotein enzyme complexes that generate reactive oxygen species (ROS). ROS generated by these complexes are involved in numerous biological processes including host immune response, cell growth and differentiation, signaling and cell death (reviewed in Krause and Bedard 2007). These complexes include both membrane-bound and cytosolic components. Active complex requires assembly of the cytosolic components with the membrane-bound components at the plasma membrane.

Several of the cytosolic proteins in the NADPH complexes contain a Phox homology (PX) domain. PX domains bind to phosphorylated lipids, most notably the phosphatidylinositol phosphates (Sato et al. 2001). One of these PX domain-containing cytosolic proteins is the NADPH oxidase organizer protein NOXO1. NOXO1 is a 376 residue protein that acts as an organizer within the NADPH oxidase complex to recruit the activator protein, NOXA1, to the membrane-bound catalytic subunit (Banfi et al. 2003, Geiszt et al. 2003 and Takeya et al. 2003). This colocalization represents a major regulatory element of ROS generation. One additional feature of NOXO1 is the presence of four splice variants termed  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ , with NOXO1 $\beta$  being the predominant isoform (Cheng and Lambeth 2005). These splice variants result in insertions and/or deletions within the PX domain. Here, we report the backbone  $^1\text{H}$ ,  $^{13}\text{C}$  and  $^{15}\text{N}$  assignments for the NOXO1 $\beta$  PX domain (residues 1–144) and discuss additional methods used to obtain the chemical shift assignments.

## Experimental methods

### NOXO1 $\beta$ PX Expression and Purification

The sequence for human NOXO1 $\beta$  PX (1–144) was cloned into pGEX-6P-1 (GE Biosciences) and transformed into the *E. coli* strain BL21(DE3)Codon + RIPL (Stratagene). Cultures were grown at 32°C in minimal media containing 1 g/l <sup>15</sup>NH<sub>4</sub>Cl and 2 g/l <sup>13</sup>C<sub>6</sub> glucose (<sup>2</sup>H<sub>7</sub><sup>13</sup>C<sub>6</sub> glucose for <sup>2</sup>H samples) until OD<sub>600</sub> reached 0.6 and induced with 0.5 mM IPTG at 22°C overnight (<sup>1</sup>H media) or for 72 h (<sup>2</sup>H media). Stable isotopes were purchased from Isotec/ Sigma and Cambridge Isotope Laboratories. The harvested cell pellet was resuspended in lysis buffer (20 mM Tris, 20 mM NaCl, 2 mM DTT, 0.1% (v/v) Tween-20, pH 8.0) and lysed on ice by sonication (8 cycles, 4 min/cycle, level 8, 30% duty cycle, 1/2" probe, Branson Sonifier 250). The lysate was incubated with 10  $\mu$ l Benzonase (EMD) at room temperature for one hour to digest bacterial DNA. Lysates were clarified by centrifugation for 45 min at 30,000  $\times$  *g* at 4°C. Clarified lysate containing GST-NOXO1 $\beta$  PX was run over SP Sepharose resin equilibrated in SP-A buffer (50 mM NaP<sub>i</sub>, 100 mM NaCl, 2 mM DTT, pH 7.4). The fusion protein was eluted with a salt gradient (SP-B, 50 mM NaP<sub>i</sub>, 1.5 M NaCl, 2 mM DTT, pH 7.4) and eluted at ~800 mM NaCl. The combination of Benzonase treatment and use of SP Sepharose, rather than GSH Sepharose, for initial capture of the fusion protein eliminated nucleic acid contamination of the product.

The pooled SP Sepharose peak was loaded onto GSH-Sepharose FF resin (GE Biosciences) equilibrated in GSH-A (50 mM NaP<sub>i</sub>, 800 mM NaCl, 2 mM DTT, pH 7.4). GST-NOXO1 $\beta$  PX was eluted with 15 mM reduced glutathione in GSH-A. The eluted GSH-Sepharose peak was dialyzed against 2l SP-A overnight at 4°C in the presence of 80  $\mu$ g PreScission Protease (GE Biosciences) to cleave the GST. The digested fusion protein was loaded onto Source 15S resin (GE Biosciences) equilibrated in SP-A. Cleaved NOXO1 $\beta$  PX was eluted with a salt gradient (SP-B), with the protein eluting at ~400 mM NaCl. The pooled NOXO1 $\beta$  PX was treated with Pefabloc PSC (0.1 mg/ml final concentration) for 30 min at 4°C. Final yield of purified NOXO1 $\beta$  PX was ~3 mg/l culture.

### Data Collection

NMR spectra were acquired at 293 K on a Bruker Avance DRX-600 spectrometer equipped with a triple resonance Z-gradient cryoprobe. For NMR experiments, NOXO1 $\beta$  PX was dialyzed into 100 mM NaP<sub>i</sub>, 100 mM NaCl, 0.1 mM EDTA, pH 6.9. <sup>2</sup>H<sub>5</sub> glycerol was added to 5% (v/v) after samples were concentrated. NOXO1 $\beta$  PX was concentrated to ~500  $\mu$ M for all NMR experiments. Standard pulse sequences were used to acquire 2D HSQC and 3D HNCA, HNCO, HN(CA)CB, HN(CO)CA, HN(CA)CO and HN(COCA)CB data. NMR data were processed using NMRPipe (Delaglio et al. 1995) and analyzed using NMRView (Johnson et al. 1994).

### Assignments and data deposition

Backbone assignments for NOXO1 $\beta$  PX have been made for 125 of 131 non-proline residues (Fig. 1). We have assigned 95% of HN/N, 89% of C $^{\alpha}$ , 82% of C $^{\beta}$  and 90% of C' atoms, 7/7 Gln sidechain NH<sub>2</sub> resonances 2/2 Trp indole NH resonances. <sup>13</sup>C chemical shift deviations from consensus CSI values (Wishart and Sykes 1994) and secondary structure predicted by TALOS+ (Shen et al. 2009) are shown in Fig. 2. Predicted secondary structure of NOXO1 $\beta$  PX is consistent with observed secondary structure in other solved PX domains. Backbone assignments for NOXO1 $\beta$  PX have been deposited in the BMRB with accession code 16749.

Although NOXO1 $\beta$  PX is a small domain (~16 kDa MW), perdeuteration of the protein was necessary to obtain backbone carbon assignments. With the <sup>1</sup>H/<sup>15</sup>N/<sup>13</sup>C-labeled protein,

only 152 total  $C^\alpha$  peaks and 15 total  $C^\beta$  peaks were observed in HNCA and HNCACB spectra, respectively. In contrast, the  $^2H/^{15}N/^{13}C$  NOXO1 $\beta$ PX yielded 253  $C^\alpha$  peaks and 182  $C^\beta$  peaks in HNCA and HNCACB spectra, sufficient to allow resonance assignment. We hypothesize that the poor magnetization transfer observed in the protonated protein was a result of a longer  $\tau_c$  due to the addition of glycerol to the sample buffer and the need for running experiments at a cooler temperature (293 K).

While cryogenic probes are able to yield a marked increase in signal-to-noise relative to standard probes, they are sensitive to electrically conductive samples and yield reduced S/N in high-salt buffers. For NOXO1 $\beta$ PX solubility and stability, it was necessary for the sample buffer to contain high levels of both phosphate and salt. Sodium phosphate and sodium chloride were chosen over their potassium counterparts due to a slightly lower conductivity (Kelly et al. 2002). Voehler et al. (2006) reported that a smaller diameter NMR tube is a viable avenue for increasing S/N of high conductivity samples in a cryogenic probe, and we have used a 4 mm Shigemi tube in these experiments in order to decrease requisite  $^1H$  power and to increase S/N. Specifically,  $\sim 180 \mu l$  of the protein sample was put into a 4 mm Shigemi tube which was placed inside a 5 mm flat bottomed tube containing  $D_2O$ . Use of  $D_2O$  in the outer tube allowed spectrometer lock without  $H^N/D^N$  exchange dilution effects to the protein sample. Although the sample volume within the detect coil decreases by  $\sim 30\%$ , the S/N of data acquired in a 4 mm tube is higher than that for a sample of the same concentration in a 5 mm tube. Utilizing a smaller tube also decreased the  $^1H$  90° pulse from 15  $\mu s$  to 10.2  $\mu s$ .

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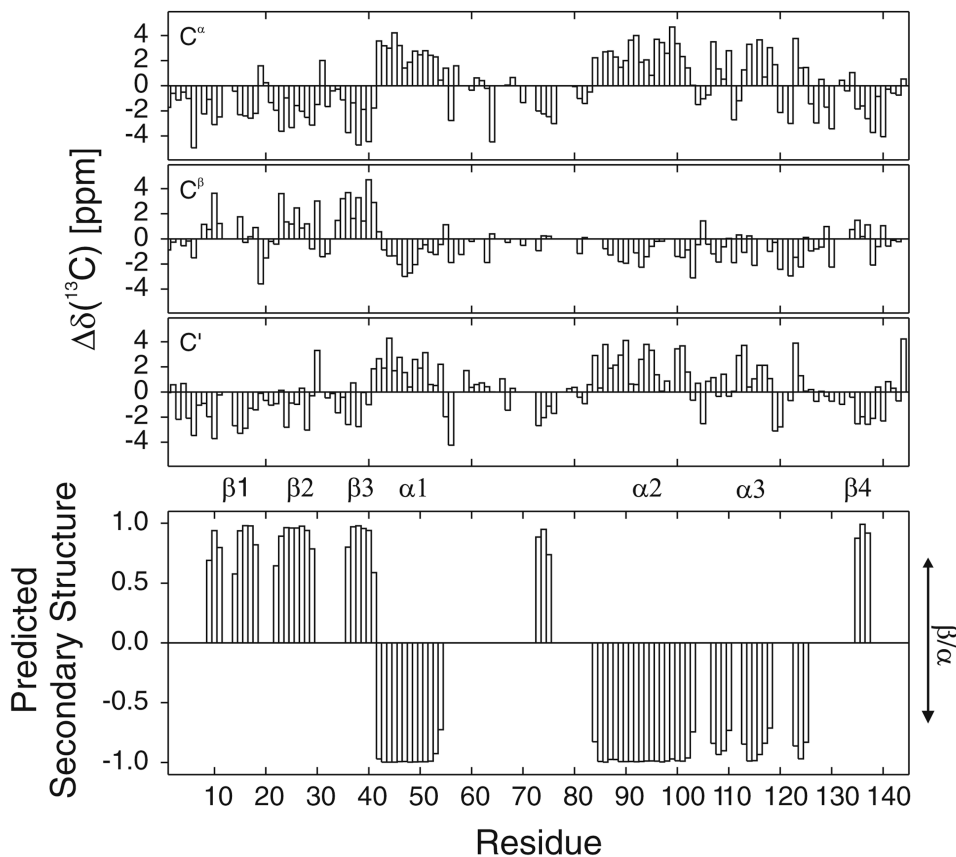
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**Fig. 2.**  $\Delta\delta(^{13}\text{C})$  for **a**  $\text{C}^\alpha$ , **b**  $\text{C}^\beta$ , and **c**  $\text{C}'$  from CSI consensus values, referenced to DSS. **d** TALOS + predicted secondary structure of NOXO1 $\beta$ PX. *Residue number* is indicated along the *x-axis* and the secondary structure is indicated along the *y-axis* with a value of +1 for  $\beta$ -sheet and -1 for  $\alpha$ -helix. The height of the bars indicate the probability of the residue being either a  $\beta$ -sheet (+1 maximum) or  $\alpha$ -helix (-1 maximum). Major PX domain structural elements are labeled