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# $^{1}H$, $^{15}N$, $^{13}C$ Resonance Assignments of the Reduced and Active Form of Human Protein Tyrosine Phosphatase, PRL-1

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## Abstract

Phosphatase of regenerating liver-1 (PRL-1) is a novel target for potentially treating cancer metastases. Although its specific biochemical role in these processes has yet to be delineated, considerable evidence suggests the phosphatase activity of PRL-1 is required for promoting cancer and metastasis. PRL-1 belongs to the protein tyrosine phosphatase (PTPase) family and functions using the $CX_5R$ consensus active site motif. Like other PTPases, PRL-1 is inhibited by oxidation at its active site Cys, however, disulfide bond formation occurs unusually readily in wild-type PRL-1. Chemical shift assignments are available for oxidized wild type, but numerous, substantial changes are observed in the spectra upon reduction. Because the reduced form is active, we sought to identify a stable mutant that would resist oxidation and be useful for facilitating drug screening and development using NMR-based assays. We present here NMR assignments for a full-length, reduced and active form of PRL-1, PRL-1-C170S-C171S, that is well suited for this purpose.

## Biological Content

Phosphatase of regenerating liver (PRL) enzymes have become sought after targets for drug design because they aggressively promote cancer and metastasis. Elevated levels of the individual PRLs (PRL-1, PRL-2, and PRL-3) are found in many cancer tissues when compared with their normal counterparts, and their overexpression has been shown to increase growth rates and motility in cultured cells as well as cause tumorigenesis and metastasis *in vivo*. Additionally, elevated levels of PRL-1 and PRL-3 may play a causal role in promoting tumor cell motility and invasion (Stephens *et al*. 2005).

The PRL enzymes (20 kDa) belong to the protein tyrosine phosphatase family, which contain the signature $CX_5R$ active site motif. Dephosphorylation of substrates is accomplished by a two-step mechanism, which involves nucleophilic attack by the active site Cys on the phosphorous atom followed by hydrolysis to release the phosphate moiety from the enzyme (Sun *et al*. 2005). While the biochemical pathways by which each PRL enzyme functions remain to be determined, studies have shown that the phosphatase activity of PRL-1 is required for normal mitotic progression and that activity of these enzymes is redox regulated such that direct oxidation of the nucleophilic Cys inhibits activity (Stephens *et al.* 2005). This indicates the *reduced* form of the PRL-1 protein is the relevant target for development of anticancer therapeutics. A previously published crystal structure of wild-type PRL-1 reveals a disulfide bond forms between the active site Cys (C104) and nearby partner C49, inhibiting its activity (Sun *et al*. 2005). A second crystal structure of the reduced but inactive PRL-1 mutant (PRL-1C104S) reveals an altered organization of the active site residues that is conducive to substrate binding (Jeong *et al*. 2005; Sun *et al*. 2005). In order to conduct biologically relevant

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*in vitro* drug screening assays, identification of a stable, reduced and active form of PRL-1 is needed.

Previously, the $^{1}H$, $^{15}N$, and $^{13}C$ resonance assignments were reported for PRL-1 under nonreducing conditions (Laurence *et al*. 2004) and for PRL-2 and PRL-3 under reducing conditions (Kozlov *et al*. 2002; Zhou *et al*. 2003). Comparison of these very closely related homologues (>75% identity) reveals differences in conformation between the reduced and oxidized forms. Consequently, the majority of chemical shift positions of resonances from reduced PRL-1 are substantially altered compared to those of the previously reported oxidized form. We present here the $^{1}H$, $^{15}N$ and $^{13}C$ resonance assignments of a full-length, reduced and active form of PRL-1 that will facilitate NMR-based drug screening.

## Methods and Experiments

NMR-based structure determination of the wild-type PRL-3 enzyme under reducing conditions resulted in a disordered active site (Kozlov *et al*. 2004). Similarly, assignments for the active site residues were not possible to obtain for PRL-1 under non-reducing conditions (Laurence *et al*. 2004). The lack of data from the active site region in both cases may likely be due to dynamics caused by chemical exchange of the reducing agent with the active site thiol and disulfide bond. Because of this, mutagenesis studies were carried out to identify a fully active and stable form of PRL-1 that is less susceptible to oxidation and amenable for use in drug screening assays. The PRL-1-C170S-C171S protein meets these criteria and permitted successful assignment of the active site resonances in the reduced form. Reduced forms of PRL-1 have been identified previously by X-ray crystallography; however, in order to maintain the reduced state, numerous changes to the primary sequence were required (Sun *et al*. 2005; Jeong *et al*. 2005). The cysteine mutations are a more conservative change to the protein sequence and permitted assignments of full-length PRL-1.

The PRL-1 gene was cloned into the pET-30 Xa LIC vector as described previously (Laurence *et al*. 2004). The PRL-1 wild-type DNA was mutated to C170S-171S in two rounds using the PCR-based QuikChange method (Stratagene). The primers used to generate the C170S and C171S mutants were 5’-ggtcatagaaacaactCttgcattcaataaggatc-3’ and 5”-ggtcatagaaacaactgttCcattcaataaggctgtaactc-3’ (Integrated DNA Technologies, Coralville, IA), respectively. Capital letters indicate the mutated bases. PCR reactions were treated with DpnI (Promega) for 1.5 hours at 37°C, directly transformed into NovaBlue GigaSingles Competent cells (Novagen) and spread on LB agar plates containing 30 mg/mL kanamycin (Kan30) to select for transformed cells. Individual colonies were cultured overnight in LB Kan30 at 37° C. The resultant DNA was purified using the Wizard Plus MiniPrep System (Promega). The double mutant C170S-C171S was produced by a second round of mutagenesis. The mutated DNA was confirmed to have the correct sequence by bidirectional sequencing using the T7 promoter and T7 terminator primers (Northwoods DNA, Inc., Bemidjii, MN). The plasmid was transformed into BL21 (DE3) competent cells and selected based on kanamycin resistance. The PRL-1-C170S-C171S protein was expressed and purified using the same procedure as described previously for the wild-type protein (Laurence *et al*. 2004). Purified proteins were concentrated to approximately 1.5 mM protein in 50 mM sodium phosphate, pH 6.5 using Amicon Ultra 10 kDa MWCO centrifugal filters to generate the NMR samples used to collect resonance assignment data.

NMR samples contained 5% $D_2O$ and were analyzed in Shigemi tubes. 2D $^{1}H$-$^{15}N$ HSQCs, 3D versions of the CBCA(CO)NH, HNCACB, C(CO)NH and HNCO experiments were carried out on a Bruker Avance 800 MHz NMR spectrometer using a cryogenic, triple-resonance probe equipped with pulse field gradients. All spectra were obtained at 37°C and referenced relative to DSS (Wishart *et al*. 1995). Spectra were processed using NMRpipe (Delaglio *et al*. 1995)

and peak picked using Sparky (Goddard and Kneller). Peak lists were submitted to the PINE Server through NMRFAM, University of Wisconsin, Madison (Eghbalnia *et al*. 2005). All automated assignments were manually verified using Sparky.

Extensive studies were performed to identify the stably reduced from of PRL-1 that was used to obtain resonance assignments of the active enzyme, and as such, the data will be presented elsewhere (Skinner et al., *in preparation*). Briefly, each sample was analyzed by SDS-PAGE to verify purity and subjected to trypsin digest followed by ESI-MS to determine the redox state of the active site. A standard phosphatase activity assay with *p*-nitrophenyl phosphate (*p*NPP) was used to determine the relative activity of the C170S-C171S mutant compared to the wild-type PRL-1. Assay mixtures contained 50 mM HEPES, pH 7.5, 20 mM pNPP, and 0–10 mM DTT. Reactions were initiated by the addition of 1 mg/mL PRL-1 and monitored for absorption at 405 nm. The MS data indicate no disulfide bond formation occurs at the active site of C170S-C171S, and the activity data indicate that this mutant protein dephosphorylates pNPP in the absence of reducing agents as well as the chemically reduced wild-type PRL-1 (unpublished data).

## Assignments and Data Deposition

Greater than 90% of the NH, CO, Cα and Cβ resonances were assigned for PRL-1-C170S-C171S. Complete sequential backbone assignments were achieved for residues V10-Q173 with the exception of residues N27, E36, T52, E59, A111, R137 and R138 and two small regions including residues N142-K144 and R167-N169. Assignments for E36, T52, E59 and R137 are available for wild-type PRL-1, although no $^{13}C$ chemical shift information is available for R137. N27, A111 and the C-terminal regions missing in PRL-1-C170S-C171S were also not assigned in the oxidized wild type and are likely undergoing conformational exchange. More importantly, the residues comprising the PTPase active site were fully assigned for PRL-1-C170S-C171S. This information was missing in wild-type PRL-1 and should prove to be useful for NMR-based drug screening assays. More than 80% of the aliphatic side chains were assigned using the CCCONH experiment. The assignments have been deposited in the BioMagResBank under the accession number 15949 (http://www.bmrb.wisc.edu). Figure 1 shows the $^{1}H$-$^{15}N$ HSQC spectrum of PRL-1-C170S-C171S with peaks labeled.

The assignments for PRL-1-C170S-C171S correlate well with the assignments previously published for PRL-2 and PRL-3 under reducing conditions, indicating that the C170S-C171S mutant is in fact a reduced species. Comparison of PRL-1-C170S-C171S assignments to the non-reduced wild type reveals a difference in conformation between the reduced and oxidized species, which is illustrated in Figure 2a. Overall, more than 90% of the backbone amide resonances shift upon reduction, which is illustrated graphically in Figure 3. Exceptions include G123 and Y126, located at the opposite end of the central helix from the active site, and several peaks in the middle of the spectra. The backbone NH resonance of C49, which forms a disulfide bond with the active site residue C104, shifts significantly upfield by more than 9 ppm in nitrogen (Figure 3b) and 1 ppm in proton (Figure 3a) with the C170S-C171S mutation, which is also observed in wild type upon chemical reduction. The NH chemical shift of the C49 residue changes to a much greater extent than C104; nonetheless, this shift is significant with the peak moving upfield just under 1 ppm in nitrogen (Figure 3b) and 0.1 ppm in proton (Figure 3a). Cβ chemical shifts can indicate the redox state of cysteine residues (Sharma and Rajarathnam 2000), and accordingly, the Cβ chemical shifts of the C104 and C49 residues differ between the reduced and oxidized states. The Cβ shifts for C49 and C104 in non-reduced wild type are 35.21 and 36.36 ppm, respectively, while the Cβ shift for C49 is 34.01 and the Cβ peak for C104 is 29.82 ppm. The upfield shift indicates the cysteine residues are in the reduced state in PRL-1-C170S-C171S. The wild-type PRL-1 forms a disulfide bond and is reduced by dithiothreitol, which is in agreement with the Trypsin digest/MS data (not shown).

Lastly, the $^{1}H$-$^{15}N$ HSQC spectra of PRL-1-C170S-C171S overlays exceptionally well with chemically reduced wild-type PRL-1 (Figure 2b) and does not change with the addition of thiol modulating agents (data not shown). This parallels the finding that PRL-1-C170S-C171S activity also does not change with the addition of DTT (data not shown). Together, the data indicate that PRL-1-C170S-C171S represents the active form of this phosphatase, which can be used to facilitate NMR-based drug screening to identify molecules that inhibit its activity.

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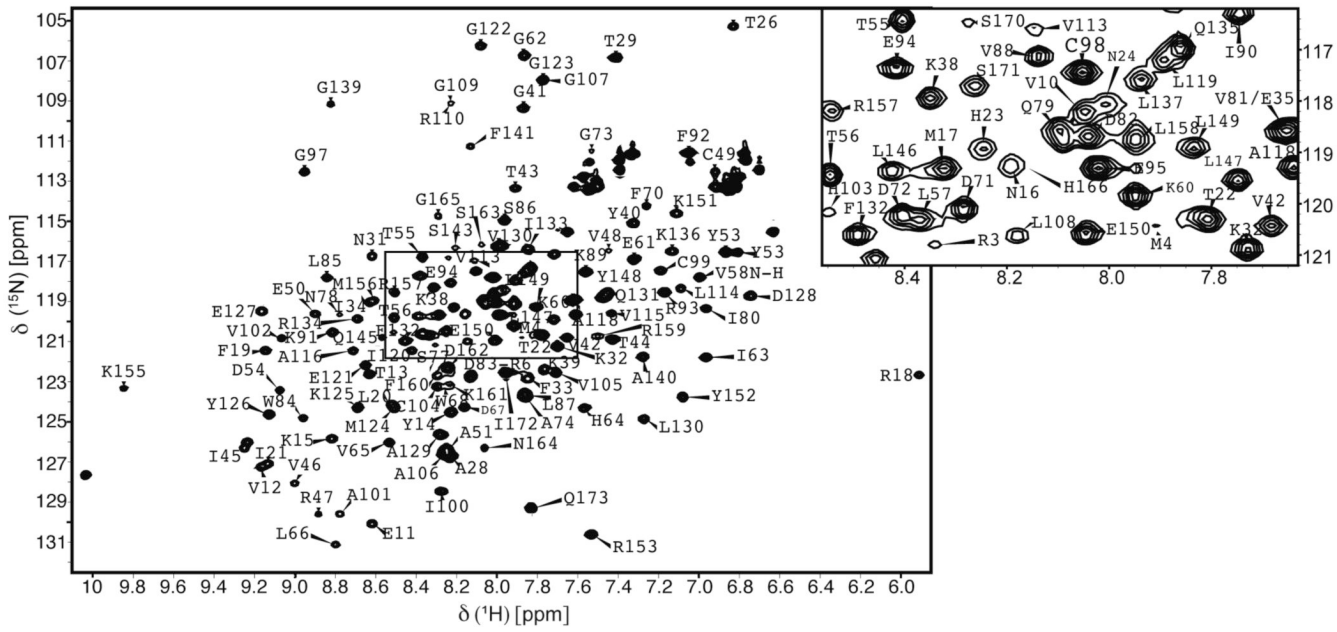


**Fig. 1.**

HSQC of 2 mM PRL-1-C170S-C171S in 50 mM sodium phosphate, pH 6.5 and 37°C. The spectrum was collected on a Bruker 800 MHz Avance spectrometer equipped with a cryogenic triple resonance probe.

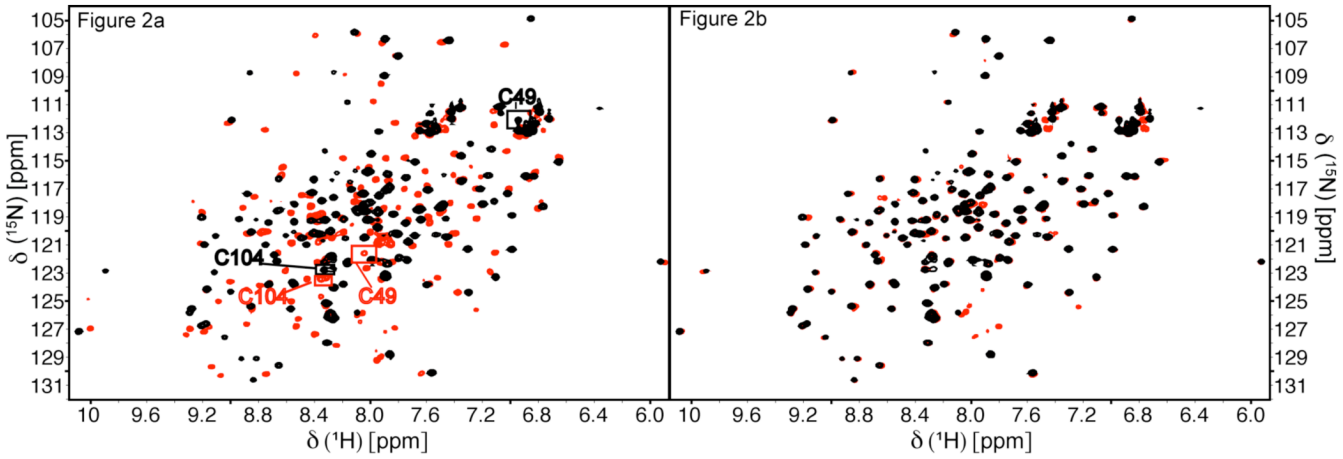


**Fig. 2.**

PRL-1 Overlays. Panel a illustrates the differences between PRL-1-C170S-C171S (black) and wild-type PRL-1 (red). The C49 and C104 residues in PRL-1-C170S-C171S are highlighted by a black box. A red box highlights the C104 and C49 residues in PRL-1-WT. Panel b displays an overlay of PRL-1-C170S-C171S (black) with chemically reduced PRL-1-WT (red). Reduction of the wild type was accomplished by the addition of 10 mM DTT. The majority of peaks in the wild type shift upon reduction to positions very similar to PRL-1-C170S-C171S. The small number of unique peaks in the spectra is likely a result of dynamic differences that arise from chemical modulation of the disulfide bond.

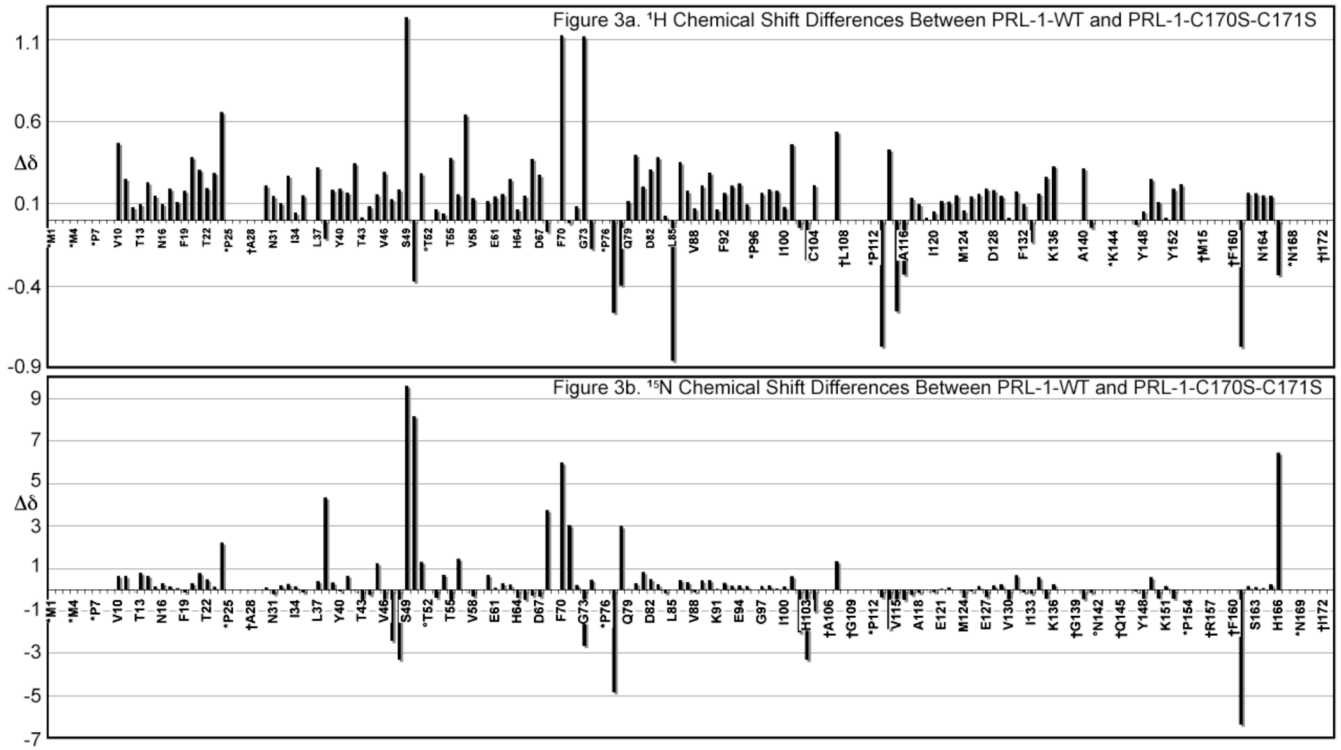


**Fig. 3.**

Chemical shift differences between PRL-1-WT and PRL-1-C170S-C171S. Panel a shows differences in proton chemical shifts and panel b illustrates changes in nitrogen chemical shifts. Δδ was calculated by subtracting δ (ppm) for PRL-1-C170S-C171S from δ (ppm) for PRL-1-WT. Residues denoted with *, º and † were either not assigned for both PRL-1 variants, assigned for wild type but not the mutant or assigned for the mutant and not wild type, respectively. Consequently, a Δδ value for these residues was not calculated and appears to be zero in the figure.