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NMR spectroscopic study of a Glutaredoxin1 from *Clostridium oremlandii*

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Abstract

Grx1 is a thiol-disulfide oxidoreductase from gram positive bacterium *Clostridium oremlandii* (strain OhILAs), that plays a role in maintaining the cellular redox homeostasis. Here we report the protein purification and NMR spectroscopic study of recombinant Grx1 double mutant U13C/C16S. We collected 3D NMR spectra and assigned all the backbone chemical shifts including Cα, Cβ, CO, HN, and N of Grx1. The secondary structure estimated from chemical shifts shows that Grx1 protein is well folded and consists of three α-helices and four β-strands. This NMR result is very useful for further structural and functional study of Grx1 protein.

Key words: Grx1, *Clostridium oremlandii*, backbone assignment, NMR

Introduction

The cellular redox state is a crucial parameter of multiple metabolic and signalling processes in cells. Thioredoxins (Trxs) and glutaredoxins (Grxs) are two major redox systems that maintain the cellularredox homeostasis [1]. These proteins are small thiol-oxidoreductases that function as electron carriers in reduction of oxidized cellular proteins. Trxs and Grxs share a number of characteristics in functions and structural fold, however, Grxs has

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broad choice of substrates and reaction mechanisms [2]. Grxs comprise approximately one hundred amino acid residues which utilize glutathione (GSH) as a cofactor. The Grx system is composed of Grx, GSH, NADPH-dependent GSH reductase, wherein electrons are transferred from NADPH to Grx via GSH [3]. Two classes of Grx, monothiol and dithiol, exist depending on the number of cysteine (Cys) residues at the active site motif. Dithiol Grxs usually contain CPYC active site motif while monothiol Grxs lack the C-terminal active site thiol in CGPS motif [2,4].

Clostridium oremlandii (strain OhILAs) is an anaerobic gram positive bacterium that contains 13 selenoproteins in its genomic DNA [5]. In *C.oremlandii*, methionine sulfoxide reductase A (MsrA) and glutaredoxin (Grx1) are incorporated as members of selenoproteins. Selenoproteins contain a selenocysteine (Sec) in catalytic sites that show higher activity compared to their Cys-containing homologs. Sec-containing MsrA shows 20-fold higher activity than its Cys mutant form and is inefficiently reducible by Trx, a typical reducing agent for MsrA [6,7]. Grx1 was characterized as a strong reductant of MsrA compared to its natural Cys-containing homolog glutaredoxin (Grx2) [8]. Grx2 shares high sequence similarity with Grx1 but its reducing activity is 20-fold lower than Grx1. Thus, there is an advantage for Grx1 in reduction of MsrA.

To investigate the structural advantage of Grx1 in reduction of MsrA, we have performed the NMR study of Grx1. Native Grx1 consists of 76 amino acid residues including one Sec residue U13 in its active site UPHC motif. However, Sec-containing form of Grx1 is unsuitable in massive protein production and the resolving Cys easily breaks the protein-protein interactions in Grx1-MsrA. The construct containing double mutant U13C/C16S in Grx1 was designed. Here, we report cloning, purification and the NMR backbone assignment of recombinant Grx1 protein.

Materials and Methods

Cloning, expression and purification

Grx1 (residues 1-76) from genomic DNA of *Clostridium oremlandii* was cloned into the expression vector pET21b (Novagen). The U13C/C16S double mutation was introduced into Grx1 protein for two purposes; ease of protein purification and complex trial with MsrA from *C.oremlandii*.

Grx1 was expressed with C-terminal Histag (LEHHHHHH) following at the end of protein. The recombinant plasmid was transformed to *E.coli* BL21(DE3) cells for protein overexpression. The cells were grown in M9 minimal media containing 100 μg/ml ampicilin for $^{13}C/^{15}N$ double labeling at 37 ℃ until OD_{600} reached 0.6, then Grx1 was overexpressed at 18 ℃ for 20 h by addition of IPTG to final concentration of 0.5 mM. The cells was harvested by centrifugation at 4,500 rpm for 20 min and resuspended in the binding buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 4 mM $MgCl_2$, 5 mM imidazole). The harvested cells were disrupted by sonication and the supernatant was separated by centrifugation at 13,000 rpm for 50 min. The supernatant was loaded onto HisTrap column (GE Healthcare) equilibrated with the binding buffer and Grx1 was eluted with stepwise increasing of imidazole. The fraction containing Grx1 was concentrated and applied to HiLoad 16/60 Superdex-75 (GE healthcare) equilibrated with 20 mM HEPES, pH 7.0, 100 mM NaCl. The eluted protein was concentrated to 1 mM for NMR experiments.

NMR data acquisition and analysis

NMR measurements were performed at 25℃ on 1 mM of $^{13}C,^{15}N$-labeled Grx1 sample in 20 mM HEPES, pH 7.0, 100 mM NaCl and 10% D_2O. NMR data were collected using Bruker Avance 800-MHz NMR spectrometer (Korea Basic Science Institute, Korea). The backbone chemical shift assignments were made by three-dimensional heteronuclear correlation experiments: HNCO, HN(CA)CO, HNCA, HN(CO)CA, HNCACB, HN(CO)CACB [9]. All NMR data were processed and analyzed by TopSpin (Bruker BioSpin), NMRPipe [10] and Sparky [11] software packages. The secondary structure of Grx1 was estimated using the PECAN program [12].

Results and Discussion

Sample preparation of Grx1

The C-terminal Histag fused Grx1 protein was overexpressed in *E.coli* BL21(DE3). The Histag fused Grx1 was purified by nickel affinity chromatography (HisTrap column). The purified protein was concentrated and then applied to size-exclusion column (HiLoad 16/60 Superdex-75 column). The Grx1 protein was eluted as a protein size of 9kD and it means that Grx1 is a monomer in solution. The eluted protein has >98% purity at SDS-PAGE. The result of purification steps is shown in Fig. 1.

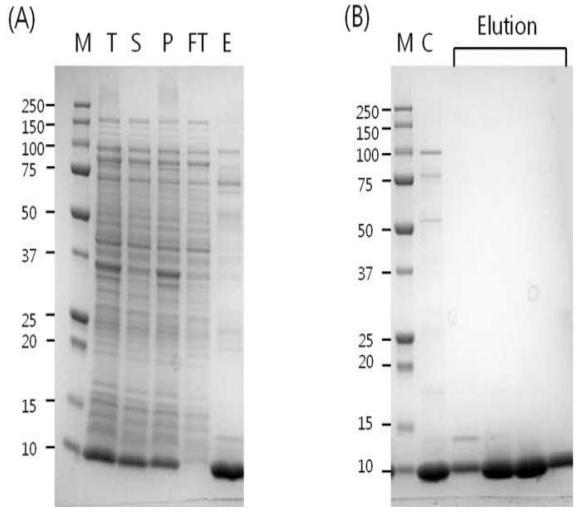

Fig. 1. Purification of Grx1. Samples from all purification steps were confirmed by the SDS-PAGE analysis. The expressed Grx1 protein was firstly purified using the HisTrap column (A) and then its eluents were applied to the Superdex 75 gel chromatography column (B). M, protein size marker; T, total fraction; S, soluble fraction; P, pellet; FT, flow-through; E, Elution; C, protein before gel filtration.

Backbone assignment of Grx1

We have assigned 95% of the expected backbone $^{1}H–^{15}N$ correlations (70 out of 74; Grx1 contains 2 proline residues) and 91% of all ^{13}CO, $^{13}C\alpha$ and $^{13}C\beta$ (205 out of 225; Fig. 2). The four missing assignments of the backbone $^{1}H–^{15}N$ correlations include M1, A2, H15 and S16. The four residues of C-terminal histag (77LEHHHHHH84) were assigned the backbone chemical shifts (Fig. 2). The assigned chemical shifts (Cα, Cβ, CO, HN, and N) of Grx1 were summarized in Table 1. NH_2 group of Asn and Gln side-chains generally produce two split HSQC cross peaks that were identified in the measured HSQC spectrum. All possible 6 set of amide side-chains peaks were identified in the HSQC spectrum (Fig. 2). Among six set of amide side-chains peaks, five set of amide side-chains produce two split HSQC cross peaks while a set of N26 side-chains produces one HSQC peak. The secondary structure of Grx1 was estimated from the assigned backbone chemical shifts by the PECAN program (Fig. 3) [12]. The chemical shifts analysis shows that Grx1 protein is well folded and consists of three α-helices and four β-strands. We could not identify two residues, H15 and S16, in the HSQC spectrum (indicated by red arrows in Fig. 3). The estimated secondary structures shows that these residues are mostly located in the boundary of the defined secondary structure. Specifically, these residues are the starting residues of predicted second helix. The secondary structure of two N-terminal and four C-terminal residues were not identified since they are located in the unstructured region. All these missing residues are expected to be partially solvent-exposed corresponding to their secondary structure estimation. This solvent-exposition and possible conformational change (the chemical exchange in NMR time scale) may contribute to the loss of sequential correlations originating from the corresponding amide protons.

Grx1 was revealed as a strong reducing protein of selenoprotein methionine sulfoxide reductase A from *Clostridium oremlandii* [8]. Our assigned backbone chemical shift of Grx1 would be very useful to define the interacting residues with selenoprotein MsrA, and to reveal the regeneration mechanism.

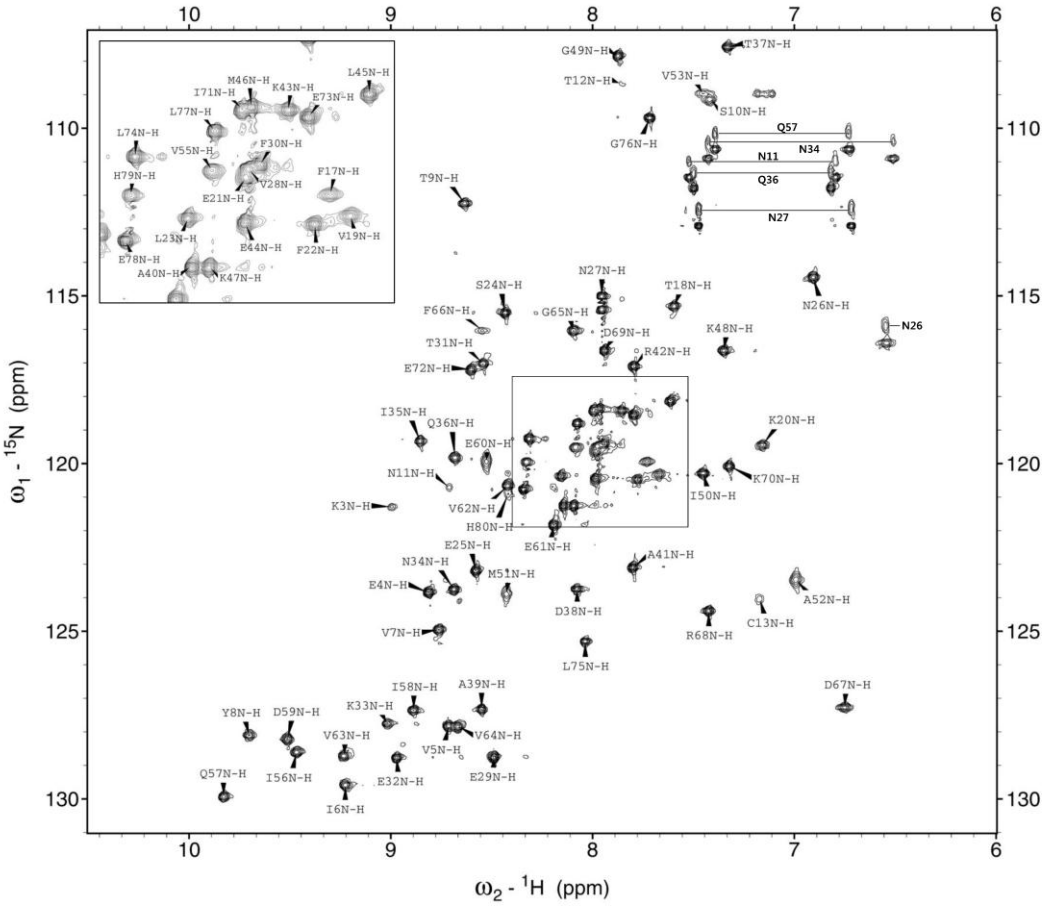

Fig. 2. ^{1}H-^{15}N HSQC spectrum of Grxl . All assigned residues are labeled and one crowded regions are magnified (insets). The assigned set of cross peaks from amide side-chains of Asn and Gln residues is indicated using a gray horizontal bar.

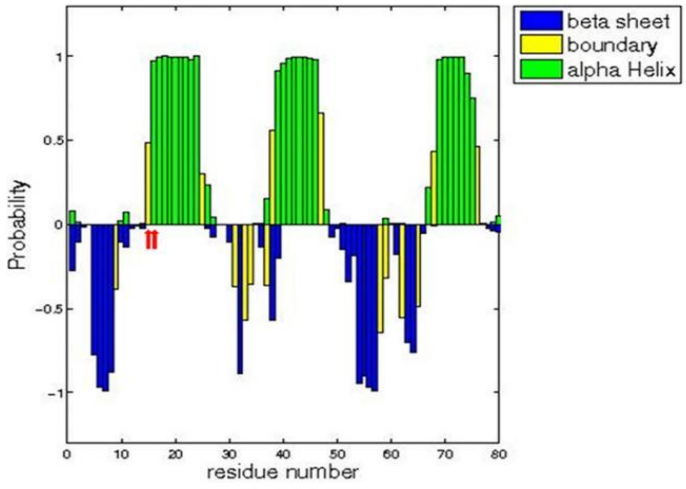

Fig. 3. The secondary structure of Grx1 was estimated from the assigned backbone chemical shifts. The probabilities of α-helix and β-sheet are represented using a positive and a negative vertical bar, respectively. The positive and negative lines indicated the boundaries of secondary structure for α-helix and β-sheet, respectively. The red arrows indicate two residues, H15 and S16, of which 1H-15N HSQC peaks were not identified.

Table 1. Assigned backbone chemical shifts (1HN, 15N, 13CO, 13Cα and 13Cβ) of Grx1

AA	HN	N	Cα	Cβ	CO	AA	HN	N	Cα	Cβ	CO
A2	-	-	48.99	16.97	171.2	**K43**	7.861	118.4	56.78	29.58	176
K3	9.001	121.3	53.94	30.77	173.2	**E44**	7.989	120.4	56.56	26.63	176.5
E4	8.813	123.8	53.52	28.25	172.5	**L45**	7.615	118.1	56.54	26.61	176.5
V5	8.713	127.8	58.69	30.34	172.4	**M46**	7.971	118.4	56.08	29.68	177.9
I6	9.226	129.6	56.67	38.5	172	**K47**	8.096	121.3	56.42	29.44	175.6
V7	8.763	125	57.42	31.17	171.6	**K48**	7.355	116.6	53.91	30.83	173.8
Y8	9.704	128.1	54.05	36.54	173.6	**G49**	7.878	107.8	42.93	-	171.2
T9	8.637	112.2	56.62	69.04	170.5	**I50**	7.456	120.3	58.28	36.72	171.9
S10	7.423	109.1	53.82	62.94	172.5	**M51**	8.432	123.9	51.48	30.02	172.1
N11	8.71	120.7	52.6	35.11	-	**A52**	7.299	123.6	48.45	18.86	171.5
T12	7.858	108.6	58.46	66.08	170.8	**V53**	7.46	109	-	-	-
C13	7.172	124.1	53.34	-	-	**P54**	-	-	59.16	32.08	172.3
S16	7.457	-	60.53	61.24	172.2	**V55**	8.086	119.5	58.63	32.6	171
F17	7.733	120	58.84	35.54	174.8	**I56**	9.473	128.6	58.28	37.98	170.6
T18	7.599	115.3	63.23	65.8	174.2	**Q57**	9.828	129.9	51.25	28.64	172
V19	7.674	120.3	63.02	28.2	173.6	**I58**	8.889	127.4	57.92	36.88	173
K20	7.162	119.4	58.2	28.74	175.8	**D59**	9.515	128.2	54.98	36.62	173
E21	7.987	119.6	56.69	26.93	175.4	**E60**	8.529	119.9	54.7	27.03	173.8
F22	7.782	120.5	58.14	36.63	175.4	**E61**	8.194	121.8	53.22	28.45	172.4
L23	8.158	120.4	-	-	-	**V62**	8.424	120.7	58.88	31.3	172.5
S24	8.438	115.5	59.48	60.06	176.3	**V63**	9.234	128.7	59.04	30.91	171.6
E25	8.582	123.2	56.01	26.25	174.2	**V64**	8.669	127.9	60.59	28.8	173.5
N26	6.912	114.4	51.11	37.49	169.8	**G65**	8.097	116	40.93	-	-
N27	7.96	115	51.91	34.31	170.9	**F66**	8.55	116.1	51.48	-	170.5
V28	7.981	119.5	59.13	29.9	173.3	**D67**	6.752	127.3	48.72	36.91	172.3
E29	8.491	128.8	53.67	27.21	173.4	**R68**	7.43	124.4	57.44	27.33	174.1
F30	7.944	119.4	52.92	40.01	170.9	**D69**	7.944	116.6	54.73	37.56	175.9
T31	8.548	117	58.78	68.13	170	**K70**	7.328	120.1	55.13	29.38	175.8
E32	8.976	128.8	51.86	29.03	172.6	**I71**	7.998	118.4	63.41	35.36	174.8
K33	9.023	127.8	51.92	31.7	170.9	**E72**	8.605	117.2	57.78	26.7	176.2
N34	8.688	123.8	49.2	36.14	174.3	**E73**	7.797	118.6	56.61	27.05	177.4
I35	8.858	119.3	61.07	35.67	173.9	**L74**	8.318	119.3	53.21	27.43	171.3
Q36	8.683	119.8	55.36	26.37	175	**L75**	8.036	125.3	54.56	27.56	176.5
T37	7.336	107.6	59.16	66.67	171.5	**G76**	7.72	109.7	44	-	172.6
D38	8.08	123.8	50.05	38.49	172.5	**L77**	8.077	118.8	50.23	-	-
A39	8.553	127.4	52.1	15.91	177.6	**E78**	8.346	120.8	53.64	27.53	173.3
A40	8.147	121.3	52.25	15.1	177.8	**H79**	8.327	119.9	52.66	26.51	172.3
A41	7.803	123.1	51.69	16.35	176.2	**H80**	8.421	120.9	-	-	-
R42	7.798	117.1	57.57	27.4	176.1						

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