Effects of redox potential on the Inositol 1,4,5-trisphosphate receptor L3-1 loop region: Implications in the receptor regulation
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Effects of redox potential on the Inositol 1,4,5-trisphosphate receptor L3-1 loop region: Implications in the receptor regulation

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Korean Abstract

Inositol 1,4,5-trisphosphate receptor (IP₃R)은 주요한 세포내의 Ca²⁺ channel로써 Endoplasmic Reticulum (ER)의 membrane을 6번 통과하는 integral membrane protein이다. IP₃R의 cytosolic compartments와 lumenal compartments는 각각 다른 regulatory elements에 의해 직접적으로 혹은 간접적으로 그 기능이 조절되는 것으로 알려져 있다. 이중 cytosolic region은 그 크기가 lumenal region보다 월경 크고 IP₃나 Ca²⁺와 같은 중요 인자와의 상호작용을 통해 직접적으로 IP₃R의 기능을 조절하는 것으로 알려져 있으나 lumenal region이 IP₃R의 기능을 조절하는 원리에 대해선 아직 많은 연구가 되어 있지 않다. 이러한 lumenal region의 조절기전을 밝히기 위하여 이 논문에서는 IP₃R의 lumenal region 중 L3-1의 loop에 redox potential이 미치는 영향을 연구하였다. IP₃R의 L3-1 region에 redox states가 변함에 따라 conformational change가 일어날 것이라는 추정을 NMR HSQC spectrum을 통하여 할 수 있었다. 그 이외에도 ¹⁵N-NOESY-HSQC specrum, ¹H-¹⁵N heteronuclear NOE values 그리고 Hα-Hα J-coupling values등의 data를 통해서 L3-1 region의 oxidation 되었을 때는 C34와 C42 아미노산이 intramolecular disulfide bond를 형성한다는 것을 알 수 있었다. 또한 이러한 data들은 disulfide bond에 의해 C34와 C42 사이의 아미노산들이 부분적인 helix 구조를 형성함으로써 secondary structure의 성질을 뇌게 되는 것을 보여주었다. 이에 반해 L3-1이 reduction 된 상태일때는 이 부위에 존재하는 C34, C42, C65, 세개의 cysteins 모두 free thiol기를 가짐으로써 unfolding된 상태임을 알 수 있었다. 결국 이러한 데이터로부터 우리는 C34와 C42사이의 disulfide bond와 C65의 free thiol의 각각 oxidation 그리고 reduction된 형태의 L3-1에서 redox sensor로써 작용할 것이라는 제안을 하였다. 이 논문에서는 L3-1 region의 conformation이 redox states에 따라 다른 conformation을 가지는 것으로 보아 이 region의 redox potential이 IP₃R의 기능을 조절하는데 있어서 직접적인 영향을 미칠 것으로 생각되었다.

key words
IP₃R, redox, L3-1, regulation
Abstract

The Inositol 1,4,5-trisphosphate receptor (IP$_3$R) is a major intracellular Ca$^{2+}$ channel. It is made up of 6 transmembrane helices on the Endoplasmic Reticulum (ER) and regulated by regulatory elements in the cytosolic and lumenal compartments. While cytosolic regulation such as IP$_3$ is well known, lumenal regulation is still not well understood. In this paper, we studied the effects of redox potential on the L3-1 loop region, which is a luminal loop between the 5th and 6th transmembrane helices. Our NMR HSQC data showed that different redox states induced conformational change. NMR HSQC, $^{15}$N-NOESY-HSQC spectrum, $^1$H-$^{15}$N heteronuclear NOE values and $^{15}$N-$^1$H $J$-coupling values showed that the C34 and C42 amino acids formed an intramolecular disulfide bond when the L3-1 region was oxidated by GSH/GSSG. These data also suggest that the region between C34 and C42 form a partial helix, and therefore having secondary structural characters. In contrast, all cysteines in L3-1 (C34, C42 and C65) exist as free thiols and the L3-1 region remains in an unstructured state in its DTT-treated reduction form. These results allow us to conclude that C34, C42 and C65 may play a role as sensors of redox potential: C34 and C42 function as reduction sensors, while C65 acts as an oxidation sensor. In this paper, we suggest that the differences in redox potential result in a conformational difference, and that eventually the redox potential directly affects the regulation of IP$_3$R.
1. Introduction

Ca\(^{2+}\) is an important messenger in many signal transduction pathways and cytosolic calcium plays a variety of roles in cellular functions (1). Dysfunction of molecules involved in \([\text{Ca}^{2+}]_c\) regulation has been implicated in various abnormalities such as Alzheimer’s disease, Parkinson’s disease and cancer. \([\text{Ca}^{2+}]_c\) is modulated by either the import of extracellular Ca\(^{2+}\) or the release of Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) stores, the Endoplasmic Reticulum (ER). The Inositol 1,4,5-trisphosphate receptor (IP\(_3\)R) is a membrane-associated Ca\(^{2+}\) channel that play a critical role in mobilizing intracellular Ca\(^{2+}\) (2). If IP\(_3\)Rs on the ER are opened, \(\text{free}[\text{Ca}^{2+}]_c\) increases, because the \(\text{free}[\text{Ca}^{2+}]_c\) is in the \(\sim\) uM range, about three orders of magnitude lower than \([\text{Ca}^{2+}]_{\text{ER}}\) (3).

IP\(_3\)R is a large protein with about 2700 amino acids (4). IP\(_3\)R forms a homo- or hetero-tetramer by conjugating each other on the membrane. There are 3 isoforms of IP\(_3\)R termed IP\(_3\)R1, IP\(_3\)R2 and IP\(_3\)R3. These isotypes have common properties but are different in terms of distribution and functions (2, 5). The monomeric IP\(_3\)R polypeptide chain can be roughly divided into 3 distinct topological regions: the large N-terminal region, the C-terminal region, both of which are located in cytoplasm, and the channel domain with 6 transmembranes and loop regions. The N-terminal region can be sub-divided into smaller partitions: the suppressor domain, the IP\(_3\) binding domain and modulatory domain (6, 7)(Fig. 1).

Fig. 1. Domain structures of the IP\(_3\)R. IP\(_3\)R is a large protein with about 2700 amino acids. TM represents transmembrane region.
The suppressor domain (1-223) has many regulator binding sites, such as Homer, CaM and RACK1 (7-12). While previous reports suggested that the suppressor domain competes with IP₃ ligands for an IP₃ binding domain for IP₃R (226-578), recent papers have suggested that the suppressor domain functions not only in the regulation of IP₃ binding affinity, but also in the coupling to the channel domain (13-18). The IP₃ binding domain is located in the N-terminal (226-578), and contains R265, K578 and R511, which are required for IP₃ binding (7, 14). In addition, the structure of the IP₃ binding domain in a complex with IP₃ has been solved by X-ray crystallography (15). The modulatory domain binds to various proteins and molecules (for example, RACK1, FKBP12, CARP, CaM, Ca²⁺, ATP, etc.) for regulating the function of IP₃R channel (7).

There are many regulatory factors, such as Ca²⁺, CaM, IP₃, IRBIT and RACK1 for IP₃R. Above all, Ca²⁺ and IP₃ are the most important regulators among these various factors (2). As the regulation of IP₃R is very complicated, it is unclear how the IP₃R is modulated by the various factors. Also unclear is whether the complex formation of IP₃R with these various regulators accompany conformational changes. A previous EM study showed that Ca²⁺ binding results in conformational change and then activation of the IP₃R channel (19). Ca²⁺ is well-known as a biphasic regulator, as Ca²⁺ can be both stimulator and inhibitor of the IP₃R channel (20-22). The detailed effects of Ca²⁺ on IP₃R regulation are still controversial and remain mysterious (19, 23-28). Some papers have proposed that the negative regulation is mediated by CaM, but this is also controversial (19, 20, 29, 30).

IP₃R is also crucially modulated at the luminal side, but the regulation factors and mechanism for this still need to be investigated. Chromogranins, in the secretory granules, have been shown to bind to the L3-2 region of IP₃R and activate the channel function (31-33). Ca²⁺ is probably the most reported regulator among the luminal regulatory factors. Recent western and pull-down analyses have revealed that ERp44, a thioredoxin domain-containing protein, acts like a negative regulator for the luminal loop of IP₃R1 in a subtype and ER luminal environment dependent manner (34). It was shown that the redox potential affects the cysteine
residues of IP$_3$R1 lumenal loop and plays an important role in the interaction between ERp44 and the IP$_3$R lumenal loop.

Some reports have suggested that lumenal-side regulation is involved in the conformational changes of IP$_3$R, and that this can be an important regulatory element (28, 35). In addition, Ca$^{2+}$ was suggested to induce conformational changes by affecting the lumenal loop of IP$_3$R. In this paper, we focused on the lumenal redox potential factor without Ca$^{2+}$ effects. 2 main questions that we had were: What is the nature of the conformational changes caused by the lumenal factors? Are the conformational changes induced directly by the regulatory factors or indirectly through certain accessory elements? In answering these questions, it is sometimes important to exclude the possible effects of tightly-bound accessory elements, as previously reported on the closely-related Ca$^{2+}$ channel, the ryanodine receptor (36). In this paper, we used a recombinant protein for the lumenal L3-1 region, and investigated whether the redox potential can directly affect the conformation of the region. By comparing the redox-induced conformational changes of L3-1 region, we proposed that the redox potential in lumenal side affects the thiol statuses of 2 specific cysteines in L3-1, and directly changes the conformation and dynamics of the disulfide bond-encompassed region.
Fig. 2. Structure of L3-1 region. L3-1 is a lumenal loop between the TM5 and TM6. It includes 3 cysteine residues (C34, C42, C65).
2. Materials and methods

2-1. Cloning of IP3R L3-1

The L3-1 gene sequence of Bovine IP3R was amplified using the Polymerase Chain Reaction (PCR) method. The sense primer of L3-1 has a GATGACAAGCCATGGCAAAAGATGACTTCATCTTGGAAGTAGATAGG-CTG sequence and the antisense primer of L3-1 has a sequence of AAACTCGGTACCTCACTCGACGTGCTCTTTATCTTTGCTC. PCR was performed with Ex taq (Takarakorea, Seoul, Korea) and then the product was purified with a PCR purification kit (Qiagen, Valencia, CA, USA). The purified sample was digested with Nco I and Kpn I (Takara korea, Seoul, Korea) and ligated into the His-thioredoxin vector (His-Trx, pETM20 from Dr. Ario de Marco, European Molecular Biology Laboratory, Heidelberg, Germany) using the DNA ligation kit (Takarakorea, Seoul, Korea). The ligation product was transformed into Rosetta (DE3) E. coli competent cells. Colonies with recombinant plasmids, His-Trx-L3-1, were tested through the colony PCR and small scale expression test and the solubility test. For a final confirmation, the colonies were sequenced (Macrogene, Seoul, Korea)

2-2. Expression and purification of L3-1

His-Trx-L3-1 cells were grown in LB media, with 100 ug/ml ampicillin, until the OD$_{600}$ absorbance reached 0.4 at 37 °C. When it reached 0.4, The culture flask was put on ice for 30 minutes to drop the media temperature. The protein was expressed at 20 °C after adding 1 mM I.P.T.G. This process was a modified method for improvement of solubility of the proteins. When the OD$_{600}$ became greater than 1.0, the cells were harvested by centrifugation at 4427 g for 10 minutes. The obtained pellet was
resuspended in His lysis buffer, containing 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole pH 8.0, with protease inhibitors (PMSF, aprotinin, pepstatin). The resuspended cells were sonicated and the cell lysate was obtained by centrifugation at 17000 g for 10 minutes at 4 ℃. The supernatant was incubated with Ni-NTA agarose (Qiagen, Valencia, CA, USA) resin at 4 ℃ for 15 minutes by rotation. The incubated supernatant was washed with His wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole pH 8.0) 200 ml and eluted with His elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole pH 8.0) 40 ml. We checked the concentration of the eluted protein using A$_{280}$ absorbance and then concentrated the solution using an Amicon ultra device (5 kD MWCO, Millipore, Billerica, MA). The His–Trx tag was removed by TEV protease, as His–Trx–L3–1 has a TEV cleavage site between His–Trx and L3–1. The TEV protease was produced in the laboratory using the gene provided by Dr. David Waugh of the National Cancer Institute (Frederick, MD, USA). The cleavage reaction was carried out at 4 ℃ with TEV protease, the protease inhibitor and 5 mM DTT in His elution buffer. The TEV protease exhibited activity in the His elution buffer. The extent of the cleavage was checked by monitoring 18% SDS–PAGE. The cleaved L3–1 was purified by gel–filtration with Superdex75HiLoad 16/60 (AmershamKorea, Seoul, Korea). The gel–filtration was performed in 20 mM Bis–tris 2 mM EGTA pH 6.0 buffer. The chromatogram of the gel–filtration peaks was monitored by UV absorbance at 280 nm and analyzed using the computer program Origin. The fractions were checked by SDS–PAGE.

Isotopically labeled L3–1 were produced in an M9 minimal medium with $^{15}$N NH$_4$Cl and $^{13}$C–glucose as the sole nitrogen and carbon sources depending on the labeling requirement. The culture and purification steps were similar to the unlabeled proteins.

2–3. Reduction and oxidation of L3–1
Purified IP₃R L3-1 was buffer-exchanged from 20 mM Bis-tris 2 mM EGTA pH 6.0 to 50 mM MES pH 6.0 by spin column cartridge (PIERCE, Rockford, IL, USA). The reduction of L3-1 was performed by adding a final 5 mM DTT, while the oxidation of L3-1 was carried out using the GSH/GSSG mixture system. 20 mM of GSH and 2 mM of GSSG were solubilized in the 50 mM MES pH 6.0 buffer and the pH was readjusted to pH 6.0. One tenth volume of the GSH/GSSG buffer was added to the L3-1 sample, which lacked DTT, and the sample was incubated at room temperature overnight. The extent of oxidation was analyzed by SDS-PAGE in the presence and absence of DTT in the Laemmli SDS sample buffer.

2–4. NMR spectroscopy

NMR spectroscopy was performed with samples in 50 mM MES pH 6.0 buffer. There were two types: reduced form of L3-1 (with DTT) and oxidized form of L3-1 (in GSH/GSSG buffer without DTT). The typical protein concentration was about 700 μM. The final sample had 5 % D₂O for lock purposes. The HSQC spectra were taken using a Varian Unity Inova 400 spectrometer equipped with a triple resonance Z-gradient probe. Backbone triple resonance experiments and heteronuclear NOE experiments were done on a Bruker Avance 600 MHz spectrometer equipped with a cryogenic probe. ¹⁵N–NOESY–HSQC spectra (with 80 ms mixing time) were obtained with a Bruker Avance 800 MHz spectrometer with a room temperature probe (KBSI, Ochang). HNCACB, CBCA(CO)NH, NHCO, NH(CA)CO were obtained for the backbone assignments (37). The backbone spectra were acquired with a non-linear sampling scheme to reduce the spectrometer time. A total of 550 indirect points were obtained for HNCACB and CBCA(CO)NH, which would take 1600 points in the linear sampling scheme for an equivalent resolution. For HNCO and HN(CA)CO, a total of
400 indirect dimension points were obtained. In addition, 4 step phase cycles, instead of the usual 8 cycles, were used for all of the above spectra to further reduce the time. Consequently, the 4 triple resonance spectra could be obtained in 12 hours. The non-linearly sampled spectra were processed using a Roland NMR toolkit (38) and the linearly obtained spectra were processed using NMRPipe (39). An HNHA experiment was carried out and the coupling constants were extracted as described before (40). Alanine residues were excluded from the analysis due to possible irregular relaxation behaviors of the alpha protons. Heteronuclear \(^1\text{H}-^{15}\text{N}\) NOE spectra were acquired and processed as reported previously (41, 42). All of the processed spectra were analyzed using NMRView.
3. Results

3-1. Effects of redox potential on the conformation of L3-1

To obtain insight into the effects of the redox potential on the conformation of L3-1, we prepared the L3-1 region in buffers with either the DTT reducing or the GSH/GSSG oxidizing system. We first investigated whether there were any mobility changes in the SDS-PAGE according to the redox potential which might suggest changes in the disulfide bond status (Fig. 3). Among the L3-1 samples prepared in the GSH/GSSG oxidizing system, the one analyzed in the SDS sample buffer without DTT (oxidized form) migrated faster than that with DTT (reduced form). In contrast, the samples prepared in the DTT buffer migrated at the same slower rates, regardless of the presence of DTT. These SDS–PAGE data suggest that there should be a disulfide bond(s) in the oxidized sample, and that the sample prepared in the DTT buffer has fully reduced cysteines. In fact, there are 3 cysteines that could form intermolecular or intramolecular disulfide bonds in the L3-1 region. The absence of any visible higher–molecular–weight species in lane 1, however, excludes the possibility of intermolecular disulfide bonds. Therefore, the changes should be due to an intramolecular disulfide bond. We further determined whether there were conformational changes between the reduced form and the oxidized form by $^{15}$N–HSQC NMR. The spectrum of the reduced L3-1 (Fig. 4A) showed a very narrow resonance dispersion in both the $^1$H and $^{15}$N dimensions, indicating that the reduced form is mostly in an unfolded state. In contrast, the spectrum of the oxidized L3-1 (Fig. 4B) showed much larger resonance dispersion, indicating that the oxidized form is in a folded state. These data suggested that L3-1 has different conformations according to whether it is in a reduced or oxidized form.
Fig. 3. Effects of the redox potential on the L3–1 gel-mobility. SDS-PAGE mobility difference in the sample, with or without DTT. Lanes 1 and 2: oxidized L3–1. Lanes 3 and 4: reduced L3–1. Lanes 2 and 4: DTT added in the SDS sample buffer. Lanes 1 and 3: without DTT or β-mercaptoethanol.

Fig. 4. Effects of the redox potential on the L3–1 HSQC spectra. A) Reduced L3–1. B) Oxidized L3–1.
3-2. Residue-specific assignments and the determination of disulfide-bonded cysteines

Since the SDS-PAGE and \^{15}\text{N}-HSQC spectrum of the GSH/GSSG oxidized L3-1 suggested that L3-1 should have an intramolecular disulfide bond(s), we performed NMR experiments. We assigned the backbone and C\(\beta\) resonances of both the oxidized and the reduced form by triple resonance NMR experiments. After the assignment, we constructed a CACB strip plot for the three cysteine residues, C34, C42 and C65. Because it is well established that the values are very different according to their disulfide bond statuses, it is possible to compare the chemical shifts of C\(\beta\). The oxidized cysteines have chemical shifts of around 40.7 ppm and the reduced cysteines have chemical shifts of around 28.4 ppm (43). Fig. 5 obviously shows that C34 and C42 are in disulfide-bonded form, with their chemical shift values at 39.5 ppm and 41.6 ppm, respectively. In contrast, the C\(\beta\) of the C65 is in a reduced form with a chemical shift value of 28.4 ppm. There were no noticeable peaks in the 28 ppm region of C34 or C42, or in the 40 ppm region of C65, indicating that there is no residual free cysteine for C34 or C42 and no residual disulfide-bonded cysteine for C65. It should be noted that we used an unusually high concentration of L3-1 (up to 780 \text{uM}) to study the structures by NMR, which could facilitate the formation of intermolecular disulfide bond(s), if this were favored at all. Overall, the population of the oxidized L3-1 sample is very homogeneous, with a single, and at the same time unique, disulfide bond involving C34 and C42.

3-3. Identification of the L3-1 affected by oxidation

We compared the \^{15}\text{N} HSQC NMR spectra of the reduced and oxidized L3-1 to identify the regions with different conformations in each forms.
Fig. 5. Identification of the disulfide bonded cysteine residues. The chemical shift values of the CBCA strip is shown on the right. The reference chemical shift values are displayed on the left.
The center of the reduced and oxidized spectra mostly matched, indicating that the residues corresponding to these peaks are in a similar environment in both states (Fig. 2 and Fig. 6). The assignment data showed that the newly appearing peaks on the oxidized spectrum were almost exclusively from the residues between C34 and C42, the two disulfide-bonded cysteines (Fig. 6). Therefore, our data suggest that the disulfide bond induces conformational changes in the region encompassed by the bond.
Fig. 6. Identification of shifted residues in the reduced and oxidized L3-1. Black peaks indicated reduced L3-1 and red peaks showed oxidized L3-1. New peaks, formed by the oxidation, are mostly by the residues between the C34 and C42 (labeled with numbers).
To determine whether oxidation induces some characteristics of the secondary structures at the region between C34 and C42, we carried out a $^{15}$N-NOESY experiment. $^1$H-$^1$H projection of $^{15}$N-NOESY–HSQC spectrum indicated the difference between the reduced L3–1 form and oxidized L3–1 (Fig. 7A). While the spectrum of the oxidized form showed many cross peaks between the backbone amide protons, revealing a folded structure, the spectrum of the reduced form showed few cross peaks. The lower part of Fig. 7A suggests that the reduced L3–1 is an almost entirely unstructured form, consistent with the previously mentioned narrow dispersion of the HSQC spectrum. In contrast to reduced L3–1, oxidized L3–1 appears to have some properties of secondary structures, because the upper part of Fig. 7A showed cross peaks. In addition, the hypothesis that secondary structures have been formed is supported by the presence of continuous $d_{\text{NN}}$ peaks in the strip plot of the $^{15}$N–NOESY–HSQC spectrum (Fig 7B). Fig. 7B showed the plot from C34 to C42, between the two disulfide bonded cysteines. As the $d_{\text{NN}}$ peaks also showed linear stretches, we proposed that this region between C34 and C42 has at least some of the properties of an $\alpha$-helix. In addition, we experimented further to confirm the property of secondary structures, by 3-bond HN–Ha J-coupling values, which are the most suitable method to define the backbone torsion angles and, consequently, to interpret secondary structure properties (Fig. 7C). The 3-bond HN–Ha J-coupling values of oxidized L3–1 were obtained with the HNHA spectrum. The average coupling constant outside of the L3–1 region was 6.52 Hz, expressed on the mean baseline as dashed line. As shown in Fig. 7C, only C34–C42 residues have the smallest coupling constant with values of J coupling < 5 Hz. We suggest that the C34–C42 residues with the smallest J coupling values should have properties of helices based on the fact that residues with typical helices have J coupling values < 5 Hz. Nevertheless, it is noteworthy...
that the 35 and 38 residues, located within the proposed disulfide-bonded region, have larger J coupling values than 5 Hz (7.49 and 8.35 Hz, respectively). Therefore, the residues in the C34–C42 region seem to show mostly helical properties, with two exceptions that are possibly in extended conformation.

3–5. Dynamic difference between reduced and oxidized L3–1

Our data suggested that the disulfide-bonded region C34–C42 obtained helical secondary structure properties upon oxidation. The dynamic property of L3–1 is verified by a heteronuclear $^1$H–$^{15}$N NOE experiment, which is a method that identifies fast motions in protein. We acquired the $^1$H–$^{15}$N NOE data for both oxidized L3–1 and reduced L3–1 (Fig. 8). In the reduced L3–1 $^1$H–$^{15}$N NOE data (Fig. 8A), we showed very fast internal motion, because the whole of the $^1$H–$^{15}$N NOE values was less than 0. This also signifies that the reduced L3–1 region is flexible. In contrast, the C34–C42 residues among the oxidized L3–1 (Fig. 8B) were more rigid than in the reduced form, with $^1$H–$^{15}$N NOE values beyond 0.5. Such results are consistent with previous NOESY and HNHA data. Although the disulfide bonded region form helical secondary structures in oxidized L3–1, the rest of the molecule remains in an unstructured form with fast motion.
Fig. 7. Secondary structural characters of the oxidized L3-1. A) \(^{15}\text{N}-\text{NOESY}-\text{HSQC}\) spectrum showing the \(^{1}\text{H}-^{1}\text{H}\) plane of the spectrum. The upper spectrum is oxidized L3-1 and the area with cross peaks is indicated with the dashed ellipse. The lower spectrum is reduced L3-1. B) A strip plot of the \(^{15}\text{N}-\text{NOESY}-\text{HSQC}\) spectrum of the C34–C42 residues in the oxidized L3-1. C) \(\text{H}_\text{N}-\text{H}_\alpha\) J-coupling values obtained from the HNHA spectrum for the oxidized L3-1. The dotted line is the mean value of all the \(\text{H}_\text{N}-\text{H}_\alpha\) J-coupling values.
Fig. 8. $^1$H-$^{15}$N heteronuclear NOE values of A) reduced and B) oxidized L3-1. The values were obtained using the peak intensities of the reference and irradiated spectra of each sample.
Discussion

IP_3R functions as a regulator of the Ca^{2+} that is mainly stored in the intracellular Ca^{2+} stores, such as ER. In this paper, we investigated the effects of the redox potential on IP_3R, particularly on the L3-1 region, to determine whether the redox potential is directly responsible for regulation of IP_3R. Our research focused on the L3-1 region only, because L3-2 does not have any cysteine residues, although L3-2 also exists in the ER luminal side. In addition, we studied the structural aspects of L3-1 to elucidate the details of the link between the redox potential and the regulatory mechanism. Because there have been no structural reports on the L3-1 region, we studied the effects of the redox potential on the conformation of L3-1. The C34 and C42 residues formed intracellular disulfide bonds by the GSH/GSSG system. Oxidation induced a significant conformational change within C34–C42, inducing secondary structural characters. As shown, the HSQC spectrum of the oxidized form, which showed a much larger dispersion of peaks than the reduced form, indicated that it undergoes large micro-environmental changes although the affected region did not form a formal helix. Such a difference between redox potentials suggest that the disulfide bond could sequester the C34–C42 region, therefore hiding the important epitopes for interaction with other regulators. On the other hand, the oxidation–induced helical secondary character could act as a novel binding site for other regulatory proteins. In a previous paper (34), it was reported that the reduced L3-1 region interacts with ERp44, and that oxidized L3-1 cannot bind to ERp44. Given that ERp44 was discovered by pull-down experiments with the reduced form of L3-1, we speculate that the same experiment with oxidized L3-1 could reveal novel luminal–side regulators that act in the oxidized environment. In addition, we showed that the dynamic properties changes remarkably from very fast internal motion to
reduced motion by oxidation of C34–C42 residues. As there is a growing amount of evidence that protein motions can directly affect protein functions (44–46), it would be very interesting to see if the observed dynamical differences also affect the regulator binding or channel-opening process, independently of the loop conformational changes. Higo et al. suggested that the luminal interchain disulfide bond functions as an important regulating mechanism of the IP$_3$R tetramer (34). It should be noted that our data do not exclude the possibility of an interchain disulfide bond in the tetrameric IP$_3$R channel. We suggest that the free thiol of C65 could be available for an interchain disulfide bond in an oxidizing condition or in the presence of other factors. As the C34 and C42 make an intrachain disulfide bond, C65 should be the most probable residue for the interchain disulfide bond. Therefore, we can also propose the possibility that the interchain disulfide bond could be an important regulator. It is noteworthy that the mutation of C65 completely abolishes the activity of tetrameric IP$_3$R (34). Therefore, we can hypothesize that the roles of the free thiol group of C65 might be different from those of C34 and C42. C34 and C42 might act together as a ‘reduction sensor’ as the disulfide bond is reduced, when the ER luminal environment becomes more reducing, whereas C65 could function as an ‘oxidation sensor’, as it is available for an interchain disulfide bond in a more oxidizing surrounding. As the major redox states of ER lumen is more oxidizing than cytosol, we prepared the L3-1 oxidized form using a GSH/GSSG redox system (47, 48). We suggest that the greatest possible effects of redox potential is the disulfide bond formation in the L3-1 region. However, we do not exclude the possibility that other redox potential–dependent modifications (sulfoxidation or nitroxylation) could also occur. In those cases, the inability of the disulfide–bonded conformation could be a regulatory factor of IP$_3$R. Taken together, we conclude that the redox potential can regulate the IP$_3$R conformation directly by inducing the intramolecular disulfide bond and secondary structural characters.
Fig. 9. Schematic model for the regulation of the IP$_3$R by redox potential. The disulfide bond formed between C34–42 residues indicated a partial helix-like secondary structure. The redox potential showed different secondary structures between the reduced and oxidized L3–1.
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