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Solution Structure of Selenoprotein W and NMR Analysis of Its Interaction with 14-3-3 Proteins

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Abstract
Selenium is a trace element with significant biomedical potential. It is essential in mammals due to its occurrence in several proteins in the form of selenocysteine (Sec). One of the most abundant mammalian Sec-containing proteins is selenoprotein W (SelW). This protein of unknown function has a broad expression pattern and contains a candidate CXXU (where U represents Sec) redox motif. Here, we report the solution structure of the Sec13 →Cys variant of mouse SelW determined through high resolution NMR spectroscopy. The protein has a thioredoxin-like fold with the CXXU motif located in an exposed loop similarly to the redox-active site in thioredoxin. Protein dynamics studies revealed the rigidity of the protein backbone and mobility of two external loops and suggested a role of these loops in interaction with SelW partners. Molecular modeling of structures of other members of the Rdx family based on the SelW structure identified new conserved features in these proteins, including an aromatic cluster and interacting loops. Our previous study suggested an interaction between SelW and 14-3-3 proteins. In the present work, with the aid of NMR spectroscopy, we demonstrated specificity of this interaction and identified mobile loops in SelW as interacting surfaces. This finding suggests that 14-3-3 are redox-regulated proteins.

Abbreviations: Sec, selenocysteine; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy.

Many cellular processes are known to be regulated through reduction and oxidation (redox) (1). This regulation is carried out by a set of designated proteins, oxidoreductases. The redox function of these proteins may be supported by protein-bound cofactors or involve redox-active cysteine residues. Previous research on thiol oxidoreductases has focused only on a few of these enzymes (2). Most prominent among them are proteins of the thioredoxin fold superfAMILY, such as thioredoxin, glutaredoxin, peroxiredoxin, glutathione peroxidase, and their more distant homologs (3). Thiol oxidoreductases, however, are much more widespread, and most members of this protein class have not been functionally characterized (4).

In addition to oxidoreductases containing catalytic redox-active thiols, some enzymes evolved that possess an active site selenocysteine (Sec)2 (5). Sec is known as the 21st amino acid in proteins (6). This rare amino acid is inserted into proteins contrANTRATIONALLY in response to a UGA codon. The human set of Sec-containing proteins (selenoproteomes) consists of 25 known members (7), and selenoproteomes have also been analyzed in a variety of other organisms. Several selenoproteins have recently been functionally characterized and found to be oxidoreductases (8). For example, both major cellular redox systems, the thioredoxin and glutathione systems, are dependent on selenoproteins (thioredoxin reductases and glutathione peroxidases, respectively). However, the functions of the majority of selenoproteins are not known.

We recently reported on a family of selenoproteins and their CyS homologs, designated as the Rdx family (9). Mammalian members of this family include selenoproteins SelW, SelT, SelH, SelV, and a Cys-containing Rdx12 protein. Functions are not known for any of these proteins. Through sequence analysis and structure modeling, we predicted that these proteins possess a thioredoxin-like fold, suggesting a redox nature of catalytic Sec residues.

Among Rdx proteins, the best characterized is SelW. It is a 9-kDa selenoprotein, abundant in muscle and brain (10-14). Expression of SelW is regulated by the availability of selenium in the diet. Native SelW was reported to occur in a complex with glutathione, which was attached to one of the Cys residues in the protein (10). Very little is known about SelT and Rdx12 (9), whereas recent studies revealed that SelH is a nucleolar protein that protects cells against stress associated with hydrogen peroxide (15). SelV is highly homologous to SelW but has an additional N-terminal proline-rich extension and is expressed specifically in testes (7).

By utilizing affinity columns containing mutant versions of Rdx proteins, we found that SelW binds 14-3-3 proteins, whereas Rdx12 binds glutathione peroxidase 1 (9). The functional importance of these interactions is not known. The SelW/14-3-3 interaction is particularly interesting, since the latter proteins are a class of abundant proteins with multiple cellular functions and roles in disease (16-19). In addition, the SelW/14-3-3 interaction differs from that observed for other 14-3-3 partners, since SelW does not have canonical 14-3-3 binding motifs (R(S/X)XpSXPpRXXpXpXP, where X
denotes any amino acid residue, and pS represents a phospho-
rylated serine (20, 21). One possibility is that SelW regul-
lates 14-3-3 in a redox manner; in the oxidized form (e.g. with
a disulfide or a glutathionylated cysteine), 14-3-3 may be in-
active and require a reduced form of SelW for activation. De-
termination of the three-dimensional structure of SelW as
well as structural information on the SelW/14-3-3 interaction
would be key in understanding both the structural basis and
biological relevance of this interaction.

NMR spectroscopy is a powerful technique for structural
characterization of proteins in solution as well as for moni-
toring interactions between two or more proteins. Addition-
ally, NMR provides valuable information regarding mobility
of various parts of the protein and its residues. Here, we
used high resolution NMR spectroscopy to determine the
structure of a founding member of the Rdx family, SelW, and
examined its interactions with 14-3-3 proteins. These data re-
vealed the thioredoxin-like fold in SelW and, therefore, other
Rdx proteins and provided structural insights into the inter-
taction of SelW with 14-3-3.

Materials And Methods

Expression and Purification of SelW—Structural analysis
was carried out with a recombinant bacterially expressed
mouse SelW tagged at the C terminus with a His6 tag and
containing Cys in place of Sec13 and Ser in place of Cys10
(9). To uniformly label SelW with 15N/13C, cells were grown
in M9 minimal medium containing 99% enriched
(15NH4)2SO4 and 98% enriched 13C-labeled D-glucose (Spe-
tra Stable Isotopes). His6-SelW was overexpressed in Esche-
richia coli ER2566 (New England Biolabs) by growing cells at
37 °C until the A600 nm reached 0.6, followed by induction of
protein synthesis by the addition of 1 mM isopropyl 1-thio-
β-D-galactopyranoside for 3 h. Cells were collected by cen-
trifugation and disrupted by sonication in 25 mM phosphate
buffer, pH 7.0, containing 10 mM NaCl and 0.05% Triton X-
100 (Sigma). Additionally, 1 mM lysozyme (Sigma) and
a half-tablet of Complete Protease Inhibitor mixture (Roche
Applied Science) were added to the solution. Following
centrifugation, supernatant was applied onto a TALON Co-
IMAC-Sepharose column (BD Biosciences). The column was
washed with 20 mM imidazole in 50 mM phosphate buffer,
pH 7.0, 300 mM NaCl, followed by elution of protein with
200 mM imidazole in 50 mM phosphate buffer, pH 7.0, 300
mM NaCl buffer at 4 °C.

Expression and Purification of 14-3-3—Expression and puri-
fication of 14-3-3 was investigated by mixing 0.2-0.5 mM 15N-la-
beled SelW with unlabeled 14-3-3 at a molar ratio of 1:1.1
(SelW/14-3-3) in 25 mM phosphate buffer, pH 6.0, containing
150 mM NaCl. 15N HSQC spectra were recorded, and the
spectral changes were quantified as \( \Delta_{\text{HSQC}} = |\Delta_{\text{HSQC}}| \). As con-
trast, bovine serum albumin (Sigma) was mixed with 15N-la-
beled SelW as described above for 14-3-3, and the spectra
were recorded.

NMR Spectroscopy—All NMR experiments were recorded
at 288 or 298 K on a Bruker DRX600 spectrometer equipped
with a 5-mm z-gradient TXI(H/C/N) cryogenic probe. 1H,
13C, and 15N chemical shifts were referenced to sodium
salt of 3-(trimethylsilyl)propanesulfonic acid as described in
Ref. 22. Sequence-specific backbone and side-chain as-
signments of SelW were accomplished using 15N HSQC, 13C
HSQC, HNCA, HN(CO)CA, CBCANH, CBCA(CO)NH, HBBANH,
HHBACO)NH, HCCO-TOCSY, and HCCO-COSY spectra. Aromatic
ring system assignments were obtained from two-dimensional COSY, TOCSY,
and NOEY experiments. NOE assignments were derived from two-dimensional
NOESY, three-dimensional 1H-15N
HSQC-NOE spectra and 1H-13C
HSQC-NOE spectra. Dihel-
dral angle constraints were derived from three-dimensional
HNNH spectra. NMR data were processed with the BRUKER
XWinNMR version 3.5 software, and spectral analysis was
performed using CARA version 1.4 (23). 15N-[1H]
-and integrated 15N-[1H]
-heteronuclear NOEs were calculated from two independently mea-
sured and integrated 15N-[1H] heteronuclear correlated spec-
tra acquired using enhanced sensitivity sequence employing
pulsed field gradients (24) as the ratio of the volumes with
and without [1H] saturation. Nuclear magnetic relaxation (\( T_1 \)
and \( T_2 \)) measurements of 15N were obtained by exponential
fitting of peak intensities in 15N HSQC spectra acquired with
different relaxation delays (24, 25). All NMR data reported
in this work were collected at the faculty of Natural Sciences
and Technology, Norwegian University of Science and Tech-
ology NMR center.
Table 1. Characterization of input NMR data and structural statistics for the solution structure of the reduced recombinant Sec12→Cys form of mouse SelW

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input data for structure calculation</td>
<td></td>
</tr>
<tr>
<td>NOE distance constraints</td>
<td>Total 1950, Intra 1063, Short 341, Medium 236, Long 310, Torsion angle constraints 116</td>
</tr>
<tr>
<td>CYANA target function value (Å²)</td>
<td>3.53 ± 0.26</td>
</tr>
<tr>
<td>Maximum residual distance constraint violation (Å)</td>
<td>0.4 ± 0.13</td>
</tr>
<tr>
<td>Maximal torsion angle constraint violations</td>
<td>2.75 ± 0.75</td>
</tr>
<tr>
<td>Total AMBER energies (kcal·mol⁻¹)</td>
<td>-1304.2 ± 292.7</td>
</tr>
<tr>
<td>PROCHECK Ramachandran plot analysis</td>
<td>Residues in favored regions (%) 81.6, Residues in additionally allowed regions (%) 16.3, Residues in generously allowed regions (%) 1.4, Residues in disallowed regions (%) 0.8</td>
</tr>
<tr>
<td>Root mean square deviation to the average coordinates (Å)</td>
<td>N, Cα, C′ (residues 3–88) 0.71 ± 0.12, N, Cα, C (second structure) 0.41 ± 0.10, Heavy atoms (residues 3–88) 1.12 ± 0.11, Heavy atoms (second structure) 0.86 ± 0.10</td>
</tr>
</tbody>
</table>

a The values given are the average and S.D. over the 20 energy-minimized conformers with the lowest CYANA (version 2.1.5) target function values that represent the NMR solution family.

b Concerns energy-minimized conformers.


Structure Calculation — NOE cross-peaks were identified, integrated, and assigned in the aforementioned NOESY spectra using the program CARA (23). The CALIBA (26) subroutine in CYANA 2.1 was used to convert cross-peak intensities from NOESY spectra into distance constraints. Dihedral angle constraints were derived from three-dimensional HNHA spectra and were further confirmed and supplemented with backbone torsion angle restraints obtained from secondary chemical shifts using the TALOS program (27). On the basis of this input, the structure was calculated using the torsion angle dynamics program CYANA 2.1 (28). Structure calculations started from 96 conformers with random torsion angle values, and the final 20 conformers with the lowest final CYANA target function values were energy-minimized in a vacuum using the AMBER force field (29) with the aid of the AMBER 9.0 program.

Modeling of Rdx Proteins — Structures of mouse Rdx12 and SelT were modeled using Modeler8 version 2 based on the experimental NMR structure of mouse SelW.

Results And Discussion

General Description of the SelW Structure — Assignment of 1H, 15N, and 13C resonances for a recombinant C10S and U13C variant of mouse SelW (hereafter SelW) was achieved through standard two- and three-dimensional homo-/het-eronuclear NMR experiments. The resonance assignment was confirmed at two temperatures, 288 and 298 K. A list of NMR experiments that resulted in the assignment together with the assignment itself were deposited in the Biological Magnetic Resonance Bank under accession number 7324.

Geometrical constraints were derived from two-dimensional homonuclear and three-dimensional heteronuclear 15N- and 13C-edited NOESY. Table 1 reports distribution of constraints among four different classes according to the types of constraints and distances. Dihedral angle constraints were derived from three-dimensional HNHA spectra and were further confirmed and supplemented using the TALOS program (27). In total, 1950 NOE upper distance limit constraints and 116 angle restraints were used to calculate the three-dimensional structure of SelW using the CYANA 2.1 program, assuring high quality of the calculated structure. The structures constituting the CYANA family were further subjected to refinement using the AMBER 9.0 program (29). The coordinates of the final energy-refined family (20 conformers) together with the constraint files were deposited in the Protein Data Bank under accession code 2NPB.

Figure 1 shows a family of 20 conformers (A) and different orientations of the average three-dimensional structure (B and C) of SelW. Quality assessment for the calculated structure is reported in Table 1. The data suggest that the resulting SelW structure represents a low energy conformation with the structural data fitting well the experimentally found geometrical constraints.

The observed structural family is well defined. Average backbone root mean square deviation value with respect to the mean structure is 0.71 ± 0.12 Å. If only the protein regions characterized by the presence of secondary structure are considered, the corresponding root mean square deviation value drops to 0.41 ± 0.10 Å. Indeed, the least defined regions in the SelW family are located in two external loops (Figure 1A). Decreased resolution for these regions is due to a decreased number of geometrical constraints for residues constituting the two loops. One of the loops contains a predicted active site of SelW (e.g., the CXXU motif), whereas the second loop may be involved in substrate binding (see below). The decreased structural resolution observed for the two loops may be a consequence of increased mobility of these regions with respect to the rest of the protein. Moreover, the proposed mobility may have a physiological role (i.e., it may be required for induced fit binding between interacting partners). To examine this possibility, dynamic characterization of the protein by NMR spectroscopy was carried out (see below).

The structure of SelW consists of a four-stranded β-sheet with two extended helices and a short 310 helix, all located on one side of the β-sheet (Figure 1, B and C). Besides these secondary structures, the protein contains two external loops and a type II turn. The molecule is characterized by a β1(3–9)-α1(15–28)-β2(34–40)-β3(48–52)-β4(54–60)-α2(61–63)-α3(70–88) secondary structure pattern, wherein β1 and β2 are parallel strands forming a classical β1→α1→β2 motif, which is also observed in thioredoxin-like fold proteins (30). The axis of α2-helix is at an ~45° angle with respect to the axis of the α1-helix as a consequence of the β-sheet topology. The CXXU motif is located in the loop (residues 10–14) between β1 and α1. The second loop (residues 40–47) separates β2 and β3 strands. β3 and β4 strands form a β-hairpin with a type II turn (residues 52–54) in between. In the β3 strand, Val37 is flipped out as previously observed for Val35 in tendamistat (31).

Electrostatic Surface Potential — The electrostatic surface potential of mouse SelW is shown in Figure 1D. The data reveal that electrostatic charges are rather smoothly distributed over the protein surface. Indeed, the surface lacks strong...
positive or negative patches. Nevertheless, a small positively charged region is found in the $^{31}$ helix (residues 61-63). Both external loops (Tyr$^9$-Ser$^{10}$-Gly$^{11}$-Ala$^{12}$-Cys$^{13}$-Gly$^{14}$-Tyr$^{15}$ and Glu$^{39}$-Gly$^{40}$-Thr$^{41}$-Pro$^{42}$-Asn$^{43}$-Val$^{44}$-Thr$^{45}$-Gly$^{46}$-Phe$^{47}$) (indicated by circles in Figure 1D) lack charged amino acids (with the possible exception of Cys in the CXXU motif). This observation suggests that the interaction between SelW and its physiological partner(s) takes place without the assistance of electrostatic forces. Moreover, it should be noted that three of the four loop ends contain aromatic amino acids. They might work as anchoring points for the loops in relation to the protein backbone core. Furthermore, these residues are often flanked by glycines (i.e. Gly$^{14}$, Gly$^{40}$, and Gly$^{46}$) and a serine (Ser$^{10}$), which might offer greater backbone flexibility.
and support loop mobility. Altogether, these residues may confer both rigidity to the structural elements and increased flexibility to the loops, resulting in an optimal structural network for the predicted catalytic site of SelW.

**Mobility Studies**—As discussed above, the two external loops in the solution structure of SelW reveal a markedly lower resolution with respect to the rest of the protein that could be explained by increased loop mobility. To examine this possibility, we elucidated dynamic behavior of SelW in a ps/ns time scale through analysis of $^{15}$N backbone $T_1$ and $T_2$ and heteronuclear $^{15}$N-$^{1}$H NOE values. Figure 2 reports these parameters as a function of residue number. It is evident that the vast majority of residues exhibit $T_1$, $T_2$, and NOE values characteristic of nonmobile amino acids in a protein of the SelW size. However, the data cannot sufficiently describe the residues of the two external loops (i.e. residues 10-14 and 40-47). Intensities of the corresponding cross-peaks in $^{15}$N HSQC spectra are significantly lower with respect to those of other residues; therefore, the errors for relaxation times and heteronuclear NOEs are significantly higher. The significantly decreased intensity of the relevant cross-peaks in $^{15}$N HSQC spectra suggests that the loop residues are more mobile than the rest of the molecule. For the rigid part of SelW, the $T_1/T_2$ ratio was 4.37, which, assuming a spherical geometry of the particle, corresponds to an overall rotational correlation time $\tau_c = 5.8$ ns (25).

**Structural Comparison of Mouse and Bacterial SelW Proteins**—During the course of this study, an unpublished crystal structure of a distant bacterial SelW homolog appeared in the Protein Data Bank (accession code 2FA8). Structural comparison of mammalian and bacterial proteins is important from both functional and evolutionary points of view. Structure-assisted alignment of these two sequences (i.e. Protein Data Bank codes 2NPB and 2FA8) shows 18.2% identity in amino acid sequences. Interestingly, in pairwise alignments, BLAST and FASTA do not recognize these proteins as homologs with sufficient $E$ values. Nevertheless, mouse SelW and bacterial SelW-like proteins show an overall conservation of both structure and the predicted active site $\text{CX}X\text{X/C/U}$ motif. These proteins also conserve the $\beta$-hairpin (residues 48-56 in mouse SelW) as well as positively charged residues in the $\alpha$ helix. Side-by-side views of mouse and bacterial SelW structures are shown in Figure 3 (this analysis was performed using the coordinates of one bacterial SelW monomer in the crystal structure of the tetramer). The calculated backbone root mean square deviation value was 1.43 Å, indicating very similar three-dimensional structures in mammalian and bacterial SelWs. In turn, close structural similarity suggests that the proteins may exhibit similar functions in bacteria and mammals.

Figure 3A shows the side chains of all aromatic residues in the mouse SelW structure. Of eight aromatic residues, six (9, 15, 19, 47, 48, and 66) form a cluster right behind the predicted active site of SelW. This cluster is situated too far from the $\text{CX}X\text{X/C(U)}$ motif (average distance is $\sim$15 Å) to have a direct role in the redox reaction. However, it might be relevant for binding of interaction partners and/or maintaining the

**Figure 2.** $^{15}$N relaxation data and $^{15}$N-$^{1}$H heteronuclear NOE for reduced SelW. The error bars indicate S.D. determined from replicate measurements. Secondary structure elements of the protein are reported at the top.
recently, the structures of B21 loops in this class of proteins lack charged residues. The N-terminal part begins with \( \beta_2(43-47)-\alpha_3(48-51) \) for Rdx12, and its \( \alpha_3 \)-helix is positioned such that it covers the N-terminal upper part of the protein. The SelT model includes \( \beta_1(1-3)-\alpha_1(9-23)-\beta_2(29-32)-\alpha_3(36-40)-\beta_3(43-47)-3\alpha_3(48-51)\alpha_3(58-76) \) secondary structure pattern. The N-terminal part begins with the \( \beta_1 \) strand, resulting in shorter \( \beta \) strands in the \( \beta_1-\alpha_1-\beta_2 \) motif with respect to SelW. Additionally, SelT contains a 70-amino acid insertion consisting of four predicted helices between \( \beta_2 \) and \( \beta_3 \) strands. The function of this insert is not known, but it is located in the modeled structure near the CXXU motif.

Our structural analysis shows that the predicted active sites of SelW, Rdx12, and SelT are located in the first external loop between \( \beta_1 \) and \( \alpha_1 \) elements. Besides this feature, another common structural element in all Rdx proteins is the aromatic cluster behind the active center, suggesting a common function of this cluster in these proteins. Additionally, two external loops in this class of proteins lack charged residues.

**Structural Characterization of Other Rdx Family Members** —The three-dimensional structure of SelW allowed us to model structures of other members of the Rdx family. Figure 4A shows SelT, Rdx12, and SelW structures, together with the structure of reduced thioredoxin (for comparison). The four proteins contain several common structural elements, including the overall \( \beta-\alpha-\beta-\beta-\alpha \) secondary structure core. However, in thioredoxin, an additional \( \alpha \)-helix is inserted between the second and third \( \beta \) strands. This helix is part of the overall thioredoxin fold. Thus, Rdx family proteins possess a thioredoxin-like fold as recently defined (30).

The Rdx12 structural model is similar to the structure of SelW, with the exception that Rdx12 has a 24-amino acid N-terminal extension upstream of the \( \beta_1 \) strand as well as 9 amino acids downstream of \( \alpha_2 \)-helix. The secondary structure of Rdx12, \( \beta_1(25-28)-\alpha_1(35-49)-\beta_2(53-56)-\beta_3(65-69)-\beta_1(72-76)-3\alpha_3(77-79) \) and \( \alpha_4(87-105) \), closely mimics that of SelW. \( \beta \) strands in the \( \beta_1-\alpha_1-\beta_2 \) motif appear to be shorter in Rdx12 than in SelW, and its \( \alpha_2 \)-helix is positioned such that it covers the N-terminal upper part of the protein.

The SelT model includes \( \beta_1(1-3)-\alpha_1(9-23)-\beta_2(29-32)-\beta_3(36-40)-\beta_4(43-47)-3\alpha_3(48-51)-\alpha_3(58-76) \) secondary structure pattern. The N-terminal part begins with the \( \beta_1 \) strand, resulting in shorter \( \beta \) strands in the \( \beta_1-\alpha_1-\beta_2 \) motif with respect to SelW. Additionally, SelT contains a 70-amino acid insertion consisting of four predicted helices between \( \beta_2 \) and \( \beta_3 \) strands. The function of this insert is not known, but it is located in the modeled structure near the CXXU motif.

Our structural analysis shows that the predicted active sites of SelW, Rdx12, and SelT are located in the first external loop between \( \beta_1 \) and \( \alpha_1 \) elements. Besides this feature, another common structural element in all Rdx proteins is the aromatic cluster behind the active center, suggesting a common function of this cluster in these proteins. Additionally, two external loops in this class of proteins lack charged residues.

**Figure 3.** Backbone structure of reduced SelW that shows aromatic amino acid side chains. Numbering corresponds to the indicated aromatic amino acids. A, mouse SelW (Protein Data Bank code 2NPB); B, bacterial SelW-like homolog (Protein Data Bank code 2FA8).

**Structural Comparison of the Rdx Family and Structurally Related Sec-containing Proteins** —Recently, the structures of Cys versions of two thioredoxin-like selenoproteins, Sep15 and SelM (Figure 4B), were determined (32) (Protein Data Bank codes 2A4H and 2A2P, respectively). These proteins are structurally related to Rdx proteins in having an overall thioredoxin-like fold and lacking the thioredoxin helix following the \( \beta_1-\alpha_1-\beta_2 \) structural motif. The second loop appears to be shorter in Sep15 and SelM, similar to that in the Rdx family. One difference between Rdx proteins on one side and Sep15 and SelM on the other is that the \( \alpha_1 \)-helix in the latter proteins is divided into two smaller \( \alpha \)-helices with a kink in between.

All proteins shown in Figure 4 lack charged and hydrophobic residues within the two external loops, which appears to be a characteristic feature of these proteins. On the other hand, thioredoxin, Sep15, and SelM, in contrast to Rdx proteins, do not have the aromatic cluster. Since the fold of all of these proteins is roughly the same, we can exclude the role of aromatic residues in maintaining protein topology.

**Interaction between SelW and 14-3-3** —Our previous studies revealed that 14-3-3 proteins bind SelW in pull-down and immunoprecipitation experiments (9). To characterize the structural basis of this interaction, we examined binding between these two proteins by NMR spectroscopy. The interaction between SelW and 14-3-3 was monitored through spectral changes in \( ^{15} \text{N HSQC} \) spectra of labeled SelW upon the addition of unlabeled 14-3-3. This way, the changes occurring with the heteronuclear \( ^{1} \text{H}^{15} \text{N} \) spectrum of SelW were not hampered by additional peaks arising from 14-3-3 and could be reliably deciphered.

\( \beta \) and \( \gamma \) forms of 14-3-3 were investigated as both N- and C-terminal His-tagged proteins. Interestingly, although 14-3-3 containing a C-terminal His tag was folded, the same protein containing an N-terminal His tag was not and, therefore, was not suitable for structural studies. Consistent with this observation, the \( ^{15} \text{N HSQC} \) spectrum of SelW was not changed upon the addition of the N-terminally tagged 14-3-3\( ^\gamma \), indicating that the unfolded 14-3-3 does not interact with SelW.
In contrast, the addition of either β or γ forms of 14-3-3 containing C-terminal His tags to the labeled SelW resulted in the same significant changes in the SelW heteronuclear spectrum. 15N HSQC spectra of SelW alone and SelW in the presence of the 14-3-3γ isoform (molar ratio 1:1.1) are shown in Figure 5(A and B). A simple visual comparison shows that although the general pattern of 15N HSQC SelW spectra in both cases remained the same, the spectra bear significant differences. Some cross-peaks disappeared from the 15N HSQC SelW spectrum upon the addition of 14-3-3 (significant increase in the signal line widths), and other peaks shifted, whereas the positions of the majority of the cross-peaks remained unchanged. Such changes in the NMR spectra are indicative of interaction occurring between these proteins. Our results, therefore, confirm previous data (9) on the interaction between SelW and 14-3-3.

The fact that SelW does not interact with the unfolded N-terminally tagged 14-3-3 indicates that the observed interaction between SelW and 14-3-3 is specific. To further examine this interaction, we monitored changes occurring in 15N HSQC SelW spectrum upon the addition of bovine serum albumin. The latter is a 66.4-kDa protein and therefore has a size comparable with that of 14-3-3 (homodimer is a 56-kDa protein). Supplemental Figure 1 shows the 15N HSQC spectrum of SelW in the presence of bovine serum albumin at a molar ratio of 1:1.1 (SelW/bovine serum albumin). Comparison of this spectrum with that of SelW alone (Figure 5A) identified no differences, indicating that these two proteins do not interact in solution. Overall, our data strongly suggest that the SelW-14-3-3 interaction is both specific and probably physiologically relevant.

Taking advantage of the SelW NMR assignment, we also determined the identity of SelW residues that are involved in this interaction by comparing chemical shifts of the corresponding nuclei in respective spectra (i.e. reference spectrum versus the spectrum following the addition of the target compound). Residues whose nuclei show either change in chemical shift or broadening of NMR signals are involved in the...
interaction between two molecules, whereas other residues, for which no spectral changes are observed, are neither coupled to nor directly involved in it (33, 34). In our work, both shifts of cross-peaks and broadening of NMR signals beyond detection were observed upon the addition of γ-14-3-3 to SelW. Figure 6 shows chemical shift differences for the corresponding amidic proton nuclei as a function of residue number. The chemical shift difference for residues whose cross-peaks in the 15N HSQC spectrum broadened beyond detection and which, therefore, were located at the interacting interphase, was attributed to 50 Hz (a maximal value reported in Figure 6) for visualization purposes. The analogous behavior was also observed for β-14-3-3. As can be seen in Figure 6, SelW contains three regions characterized by perturbation of NMR properties of amino acids (i.e. involved in the interaction with 14-3-3): residues 8-22, 38-51, and 58-64. These regions are mapped on the SelW structure and differently colored in Figure 1E. Our data demonstrate that two external loops (residues 9-15 and 40-48) and a short 310 turn (residues 60-63) of SelW are involved in binding of 14-3-3.

We hypothesize that the proposed increased mobility of these loops is functionally relevant and is required for interaction of SelW with its physiological partners. Our results complement those previously reported regarding this interaction through biochemical studies (9). Taken together, it appears that SelW and 14-3-3 proteins interact in solution via the formation of an intermolecular complex. Having the thioredoxin-like fold and CXXC(U) motif in SelW, such interaction could be redox-sensitive. The most plausible scenario for this reaction would involve intermolecular reduction of the oxidized 14-3-3 protein by SelW. Cys13 (the residue that replaces the natural Sec13) is located in the first external loop and might play the role of the attacking residue in the reduction reaction, whereas Cys10 may be a resolving residue. The exact mechanism of the interaction between SelW and 14-3-3 should await determination of the structure of the intermolecular complex, in progress in our laboratories.

Conclusions—In conclusion, this study reports the first solution structure of mammalian SelW that was calculated through high resolution NMR spectroscopy. The structure revealed a thioredoxin-like fold, a β-α-β-β-α secondary structure core, andaCXXU(SXXC in the mutant) motif in an exposed loop in a position similar to the redox-active CXXC site in thioredoxin.

The SelW structure permitted interpretation of the sequences of homologous proteins and prediction of their structures. The NMR-derived structure also suggested a role for SelW in redox regulation. Analysis of protein dynamics revealed rigidity of the backbone except for two external loops. These loops appear to participate in the interaction of SelW with 14-3-3 proteins. A specific interaction with folded 14-3-3 protein was found to involve the conserved active site CXXU residues, suggesting that the interaction could depend on the redox state of SelW.
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The atomic coordinates and structure factors (code 2NPB) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ: http://www.rcsb.org/.

References


Supplementary Figure 1. 2D 15N HSQC spectra of reduced SelW in the presence of BSA (molar ratio 1:1.1).