The Human Selenoprotein VCP-interacting Membrane Protein (VIMP) Is Non-globular and Harbors a Reductase Function in an Intrinsically Disordered Region

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Background: Human VIMP/SelS is a selenoprotein involved in endoplasmic reticulum-associated degradation. Results: The cytosolic domain of VIMP constitutes an extended α-helical segment followed by an intrinsically disordered region harboring redox activity.

Conclusion: VIMP is a non-globular protein with a likely reductase function.

Significance: These findings provide new mechanistic insight into the molecular function of VIMP.

The human selenoprotein VIMP (VCP-interacting membrane protein)/SelS (selenoprotein S) localizes to the endoplasmic reticulum (ER) membrane and is involved in the process of ER-associated degradation (ERAD). To date, little is known about the presumed redox activity of VIMP, its structure and how these features might relate to the function of the protein in ERAD. Here, we use the recombiantly expressed cytosolic region of VIMP where the selenocysteine (Sec) in position 188 is replaced with a cysteine (a construct named cVIMP-Cys) to characterize redox and structural properties of the protein. We show that Cys-188 in cVIMP-Cys forms a disulfide bond with Cys-174, consistent with the presence of a Cys174-Sec188 selenolsulfide bond in the native sequence. For the disulfide bond in cVIMP-Cys we determined the reduction potential to −200 mV, and showed it to be a good substrate of thioredoxin. Based on a biochemical and structural characterization of cVIMP-Cys using analytical gel filtration, CD and NMR spectroscopy in conjunction with bioinformatics, we propose a comprehensive overall structural model for the cytosolic region of VIMP. The data clearly indicate the N-terminal half to be comprised of two extended α-helices followed by a C-terminal region that is intrinsically disordered. Redox-dependent conformational changes in cVIMP-Cys were observed only in the vicinity of the two Cys residues. Overall, the redox properties observed for cVIMP-Cys are compatible with a function as a reductase, and we speculate that the plasticity of the intrinsically disordered C-terminal region allows the protein to access many different and structurally diverse substrates.

To ensure the structural fidelity of the proteins that leave the endoplasmic reticulum (ER) by vesicular transport along the exocytic pathway, ER chaperones and folding factors assist the folding of newly synthesized proteins. Still, protein misfolding is a common occurrence for both mutant and wild-type proteins. Irreversibly misfolded proteins and subunits of unassembled oligomers are degraded by the process known as ER-associated degradation (ERAD) (for recent reviews see Refs. 1, 2). This multistep process involves substrate recognition by chaperones, dislocation to the cytosol through a presumed proteinaceous and currently unknown retrotranslocation channel (the “dislocon”), polyubiquitination by E3 ubiquitin ligases and degradation by the proteasome.

Central components of the ERAD system are conserved between Saccharomyces cerevisiae and mammalian cells (3, 4). In the ER lumen these components include the abundant chaperone BiP and associated ERdj co-chaperones, members of the PDI family of thiol-disulfide oxidoreductases, as well as lectins and sugar-modifying enzymes such as the EDEM proteins, OS-9 and XTP3-B (see e.g. Ref. 5–7). In the ER membrane, dislocon candidates include the Derlin proteins as well as E3 ubiquitin ligases (1, 2). In mammalian cells, as many as 24 transmembrane E3 ligases may exist (8), although only about 10 of these have been verified to function in ERAD (1, 2). On the cytosolic side of the ER membrane the different pathways all

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The current study was performed to further our understanding of central structural and biochemical properties of VIMP. We found that the purified cytosolic domain of VIMP constitutes a non-globular molecule with α-helical and intrinsically disordered regions. A particularly well-conserved region at the C terminus encompasses the active site residues, which have redox properties compatible with a function as a reductase. The molecular insights gained here will provide a strong starting point for future cell biological studies.

EXPERIMENTAL PROCEDURES

Plasmids and Primers—Based on the sequence of the human VIMP cDNA (IMAGE2967406 (2553); GeneService), a sequence-optimized construct for Escherichia coli expression was synthesized by GeneScript and cloned into pUC57, generating the pLE345 plasmid. Based on this construct and pET-21b (Novagen), we made the pET-21b-cVIMPpopt-Cys (pLE358) plasmid for expression of cVIMP-Cys. Using pLE345 as a template, the sequence encoding residues 49–189 of VIMP were PCR amplified using the Ndel-cVIMPpopt (5′-AAAAAGCAATGTCAGAAACTGAGCGCCCGTCTGCAGC-3′) and cVIMPorient-Cys-EcoRI (5′-AAAAAGATCTCTTACGCCCCGACGGGCGCCTGCTCGGGCC-3′) primers. The latter substitution encoded the selenocysteine with a Cys codon and included a TGA stop codon following the codon for Gly-189. The resulting fragment was subcloned into the bacterial expression vector pET-21b using the Nhel and EcoRI restriction sites. The plasmid was sequenced to confirm the correct DNA sequence. The pET-21b-His6-(Mm)p97(1–199) plasmid (pLE329) for expression of mouse His6-p97(1–199) has been described before (27).

Protein Expression and Purification—pLE358 was transformed into competent BL21(DE3) cells and plated on Luria Broth (LB) agar containing 100 μg/ml ampicillin (Amp). One colony was inoculated in 125 ml of LB media containing 100 μg/ml Amp and grown overnight at 37 °C. The overnight culture was diluted to an optical density at 600 nm (A600) of 0.1 in LB media with Amp. The culture was then propagated to A600 = 1 at 37 °C in an orbital shaker. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 37 °C and harvested by centrifugation. Cell pellets were resuspended in 10 ml of buffer A (50 mM Na-phosphate, 25 mM NaCl, pH 8.0) with 1 mM PMSF, 5 mM EDTA, and protease inhibitor mixture (Roche) per liter of culture media and frozen at −20 °C until further use. Next, cells were sonicated and subjected to centrifugation for 1 h at 27,000 × g. The filtered supernatant was applied to a Source 15S cation exchange chromatography column (GE Healthcare) preequilibrated with buffer A at 4 °C. After loading and washing with 25 column volumes buffer A, cVIMP-Cys was eluted with a linear gradient over 20 column volumes against buffer B (50 mM Na-phosphate, 1 M NaCl, pH 8.0). In this gradient, cVIMP-Cys eluted at ~300 mM NaCl. The fractions with the highest concentration of cVIMP-Cys were pooled and concentrated to A280 ≈ 2.5. As a final purification step, 250 μl of protein sample was loaded onto a Superdex 75 gel filtration column (GE Healthcare) and eluted isocratically at 4 °C in buffer C (50 mM Na-phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.0). Protein for NMR experiments was prepared by inoculation of a colony into 125 ml of non-labeled M9 minimal media containing 100 μg/ml Amp and grown at 37 °C to A600 = 2. For uniform 15N and 13C-labeling, the preculture was diluted to A600 = 0.2 in 15N,13C-labeled M9 minimal media and propagated at 37 °C. At A600 = 1 cells were induced with 1 mM IPTG for 3 h at 37 °C.

The conformational requirements of the polypeptide chain that allow dislocation from the ER are not known, but it is generally presumed that most substrates are at least partially unfolded. In this connection it is interesting to note that several ERAD substrates contain disulfide bonds, which implies a potential need for reduction prior to dislocation. Indeed, it has been shown that perturbation of the ER redox environment can influence the dislocation process so that oxidizing conditions inhibit dislocation, whereas reducing conditions most often promote the process (11, 12). Accordingly, a variety of substrates become reduced prior to degradation (11–15). Presently, the PDI-family member Erdj5 is the only known disulfide reductase involved in ERAD (16, 17). The identity of the electron donor(s) for reduction in the ER is unknown. An Erdj5-interacting flavoprotein, ERFAD, is a potential candidate (18). Moreover, VIMP has been shown to co-immunoprecipitate with another recently been shown to co-immunoprecipitate with another selenoprotein family, which among other features has in common the presence of a positively charged, glycine-rich region located close to the C terminus. Interestingly, VIMP, SelK, and the mitochondrial protein Romo1 have been found to constitute a widespread eukaryotic selenoprotein family, which among other features has in common the presence of a positively charged, glycine-rich region located close to the C terminus. Interestingly, VIMP, SelK, and the mitochondrial protein Romo1 have been found to constitute a widespread eukaryotic selenoprotein family, which among other features has in common the presence of a positively charged, glycine-rich region located close to the C terminus.
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Purification of $^{15}N,^{13}C$-cVIMP-Cys was carried out exactly as for the non-labeled protein. For expression of the p97 N-domain, pLE329 was transformed and propagated at 37 °C exactly as described for pLE358. At $A_{600} = 0.75$, protein expression was induced with 1 mM IPTG for 3 h at 30 °C and harvested by centrifugation. Cell pellets were resuspended in 10 ml of 50 mM Na-phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0 with 1 mM PMSF and protease inhibitor mixture (Roche) per liter of culture media. Next, lysozyme was added to the cells to a final concentration of 1 mg/ml. Upon incubation on ice for 1 h, the cells were sonicated and subjected to centrifugation for 1 h at 27,000 × g. The supernatant was incubated with Ni-NTA beads for 1 h at 4 °C. The beads were transferred to an empty column and washed by gravity flow with 10 column volumes of 50 mM Na-phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0. The protein was eluted with 2 column volumes of 50 mM Na-phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0. The fractions containing mouse His6-p97(1–199) (hereafter referred to as p97N) were dialyzed into buffer C overnight at 4 °C. Next, the filtered protein pool was applied onto a Superdex 75 gel filtration column and eluted isocratically in buffer C at 4 °C. Fractions containing purified p97N were pooled and concentrated for NMR spectroscopy.

Protein Concentration Determination—The concentration of the purified cVIMP-Cys protein was determined from its absorbance at 280 nm using the theoretical extinction coefficient $14,105 M^{-1} cm^{-1}$ (29). A theoretical extinction coefficient $14,150 M^{-1} cm^{-1}$ was used for p97N. 4-Acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS) Shift Assay—75 µl (4 µM) of protein was precipitated with 20% TCA for at least 30 min at 4 °C, and spun at 16,100 × g for 15 min at 4 °C. The supernatant was discarded and the pellet dissolved in 20 µl of AMS buffer (400 mM Tris, 1.6% SDS, 0.04% bromcresol purple (pH indicator with buffer point at pH 6.8, Fluka), 15 mM AMS). The pH of the solution was then increased by titration with 1.5 M Tris, pH 8.8 until a color shift appeared. Samples were boiled for 5 min prior to loading on SDS-PAGE gels.

**Redox Equilibrium between cVIMP-Cys and Glutathione**—100 µl (4.5 µM) of protein was equilibrated with different ratios of [GSH]$^2$/[GSSG] in 50 mM Na-phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.0 at 25 °C for 16 h under an argon atmosphere. The concentration of GSSG was kept at 0.1 mM in all samples, whereas the concentration of GSH was varied from 0.6 mM to 17.8 mM resulting in [GSH]$^2$/[GSSG] ratios ranging from 3.2 mM to 3160 mM. The total reaction volume was 125 µl. Upon incubation, 50 µl was quenched with 100 mM HCl and prepared for HPLC to determine the concentrations of GSH and GSSG. The samples were spun at 16,100 × g for 15 min at 4 °C, and the supernatant run on a reversed-phase C18 column (Vydac 218 TP) with 95% buffer D (0.1% TFA in ddH$_2$O) and 5% buffer E (0.1% TFA, 10% acetonitrile). The relative peak areas corresponding to GSH and GSSG were calibrated to a set of GSH and GSSG standard solutions. The concentration of the GSH standard was determined using the absorbance of Ellman’s reagent (10 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 0.5 mM K-phosphate, 1 mM EDTA, pH 7.3) at 412 nm and the extinction coefficient 14,150 M$^{-1}$ cm$^{-1}$ (29). The concentration of the GSSG standard was calculated from the absorbance at 248 nm and the extinction coefficient 382 M$^{-1}$ cm$^{-1}$ (30). From the resulting standard curves the actual concentrations of GSH and GSSG in each reaction mixture were calculated.

The remaining 75 µl of the reaction mixture was quenched with 20% TCA and modified with AMS according to the procedure described for the AMS shift assay. The AMS-modified samples were run on 18% SDS-PAGE gels and Coomassie-stained prior to quantification using ImageJ (31). The determined fraction of reduced cVIMP-Cys was plotted against the determined [GSH]$^2$/[GSSG] and fitted according to Equation 1 to obtain $K_{eq}$.

\[
\text{Reduced VIMP} = \frac{[\text{GSH}]^2/[\text{GSSG}]}{K_{eq} + [\text{GSH}]^2/[\text{GSSG}]} \tag{1}
\]

The equilibrium redox potential of cVIMP-Cys was then calculated with the Nernst equation (Equation 2) using the glutathione standard potential of $-0.240 \text{ V at pH 7.0 and } 25 ^\circ \text{C}$ (32):

\[
E_0 = E_{\text{GSH/GSSG}} - (RT/nF) \times \ln K_{eq} \tag{2}
\]

All data points were normalized to take into account that in this particular experiment a small fraction of AMS-resistant protein was observed in the sample treated with the reducing agent Tris-(2-carboxyethyl)phosphine (TCEP) (sample 1).

**Reduction of cVIMP-Cys by the Thioredoxin System**—Active recombinant rat thioredoxin reductase (TrxR) was purchased from IMCO (product TR-03-B) and recombinant human thioredoxin (Trx) was from Prospec (product Pro-569). The enzymes were diluted from the stock concentrations provided by the supplier. Measurements were performed on a Perkin Elmer Lambda 35 UV-Vis spectrometer at 25 °C. Buffers were flushed with argon and the spectrometer was blanked on an empty dry cuvette. Measurements were initiated on a cuvette containing 155 µM β-NADPH (Sigma) in buffer C (50 mM Na-phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.0). The amount of disulfide reduction was calculated from the decrease in absorbance and the extinction coefficient for β-NADPH (6200 M$^{-1}$ cm$^{-1}$). The decrease in absorbance at 340 nm was then followed over time during the subsequent addition of (in final concentrations) 0.1 µM TrxR, 25 µM cVIMP-Cys, and 0.5–1 µM Trx. At these concentrations, the assay was linearly dependent on Trx concentration.

**Analytical Gel Filtration**—Analytical gel filtration was performed by applying 220 µl of protein sample (54 µm) on a Superdex 75 column equilibrated with buffer F (50 mM Na-phosphate, 150 mM NaCl, pH 8.0) at 4 °C. The column was calibrated with a set of globular protein standards (Gel Filtration Calibration kit, Pharmacia). All protein samples were eluted isocratically at a flow rate of 0.5 ml/min. cVIMP-Cys was reduced for at least 2.5 h with 2 mM DTT and eluted from the column equilibrated in buffer F containing 2 mM DTT. Samples for AMS modification were quenched by TCA addition as soon as they eluted from the column. Subsequent SDS-PAGE analysis verified the expected redox state of each sample (data not shown). The results were analyzed by plotting the logarithm of
the molecular weight for each standard protein versus their partition coefficient $K_{av}$

$$K_{av} = \frac{V_e - V_0}{V_i - V_0}$$  \hspace{1cm} (Eq. 3)

where $V_e$ is the elution volume, $V_0$ is the void volume, and $V_i$ is the column volume. A linear fit to the data points of the standard proteins yielded the calibration curve used to calculate the apparent molecular mass of oxidized and reduced cVIMP-Cys.

**CD Spectroscopy**—Protein samples were at equal concentration (8.4 $\mu$m) buffered in 50 mm Na-phosphate pH 7.0 for the oxidized and 50 mm Na-phosphate, 1 mm TCEP, pH 7.0 for the reduced protein. For reduction, the sample was incubated in the reducing buffer for 90 min at room temperature prior to recording. Samples for AMS shift assays were quenched by TCA addition after each measurement. Subsequent SDS–PAGE analysis verified the expected redox state of each sample (data not shown).

Measurements were done in a 350 $\mu$l quartz cuvette with 1 mm light path at 5 °C. The spectra were recorded as an accumulation of 12 scans from 250 nm to 190 nm at a scan rate of 20 nm/min on a Jasco J-810 spectropolarimeter equipped with a PTC-423S temperature control device. Upon recording, all data sets were subtracted the buffer baseline and noise reduced with a Fast Fourier transform filter in the Spectra Manager software. The resulting ellipticities were normalized to concentration and number of amino acids by Equation 4,

$$[\theta]_{MRRW} = \frac{\theta \cdot MW}{10 \cdot C \cdot l \cdot N}$$  \hspace{1cm} (Eq. 4)

where $\theta$ is the ellipticity in degrees, $MW$ is the molecular weight in g/mol, $C$ is the concentration of oxidized cVIMP-Cys in g/ml, $l$ is the path length in cm, and $N$ is the number of amino acids in the sequence. $[\theta]_{MRRW}$ is short for the mean residue molar ellipticity.

**NMR Spectroscopy**—NMR samples were in 50 mM Na-phosphate, 150 mM NaCl, 1 mM EDTA, pH 7.0, and 10% D$_2$O, 1% NaN$_3$, and 1% 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). For the backbone assignment, spectra of the reduced form of 15N,13C-labeled cVIMP-Cys (265 mm) were recorded in the presence of 10 mm TCEP, while spectra of the oxidized form of 15N,13C-labeled cVIMP-Cys (291 mm) were recorded in the presence of 5 mm GSSG. All NMR experiments were performed at 5 °C on Varian Inova 750 MHz and 800 MHz spectrometers. For sequential assignment of reduced cVIMP-Cys Heteronuclear Single Quantum Coherence (HSQC), HNCO, HNCA, HN(CA)B, CB(CA)(CO)NH, and HNN spectra were recorded. Assignment of oxidized cVIMP-Cys was based on the assignment of the reduced form and HNCA, HNCO, and HNN spectra. All pulse sequences were from Varian Biopack. All spectra were referenced to DSS, processed in nmrPipe (33), and analyzed with CCPNMR Analysis (34). HSQC spectra were recorded before and after each data acquisition to check the redox state and potential degradation of each sample. The chemical shift assignments of oxidized and reduced cVIMP-Cys have been deposited in BioMagResBank (BMRB) with the accession number 18176 and 18177, respectively.

HSQC spectra of 15N-cVIMP-Cys (50 mm) with unlabeled p97N (60 mm or 173 mm) and without p97N were recorded in the presence of 10 mm TCEP. The spectra were assigned based on the assignment of the reduced form. The intensity ratio of each peak was calculated from the height of the peak of cVIMP-Cys without p97N divided by the height of the peak of cVIMP-Cys in complex with p97N, which was marked. The weighted chemical shift difference ($\Delta \delta_{\text{weighted}}$) between cVIMP-Cys in complex with p97N and without p97N was calculated by Equation 5,

$$\Delta \delta_{\text{weighted}} = \sqrt{\left(\Delta \delta_H\right)^2 + \left(\Delta \delta_N\right)^2 / 6}$$  \hspace{1cm} (Eq. 5)

where $\Delta \delta_H$ and $\Delta \delta_N$ are the changes in $^1$H and $^{15}$N chemical shifts when p97N is added.

**RESULTS**

**Expression and Purification of cVIMP-Cys**—To analyze redox and structural properties of VIMP we chose to work with a fragment comprising the entire cytosolic region of the protein, i.e. residues 49–189 (Fig. 1A). In this construct, termed cVIMP-Cys, we added to the wild-type sequence an initiator Met and replaced the endogenous Sec at position 188 with a Cys residue. This was done due to the relatively low expression level...
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of the Sec-containing construct (cVIMP-Sec). The low expression level was observed despite the use of a system optimized for expression of human selenoproteins in E. coli that is designed to ensure the highest possible level of Sec incorporation (data not shown) (35). On the contrary, we observed good expression of cVIMP-Cys upon induction with IPTG (Fig. 1B). Given the high pI of 10.0 for cVIMP-Cys, we used cation-exchange chromatography as a first purification step. When followed by size-exclusion chromatography we obtained pure cVIMP-Cys in a yield of ~3 mg/liter of culture medium (Fig. 1B). The identity of the purified protein was confirmed by MALDI-TOF mass spectrometry. A mass of 15754.0 Da for oxidized cVIMP-Cys was obtained, in agreement with the predicted mass of 15753.9 Da (data not shown).

Cys-174 and Cys-188 Form a Disulfide in cVIMP-Cys—With the exception of SelP, human selenoproteins all contain a single Sec residue (20, 23). This residue provides redox activity as a consequence of the high nucleophilicity (20, 23). In the cytosolic domain of human VIMP, the only Cys present is found in position 174 (Fig. 1A). We reasoned that this Cys could potentially function to resolve mixed selenosulfide bridges between VIMP and its substrates, akin to the second Cys of the active site Cys-Xaa-Xaa-Cys motif in thiol-disulfide oxidoreductases (36). If so, Cys-174 should be able to form a disulfide with Cys-188 in cVIMP-Cys. This idea was tested in the experiments shown in Fig. 2, A and B. First, we investigated the mobility of purified cVIMP-Cys by non-reducing and reducing SDS-PAGE. A small but discernable mobility shift was observed with the oxidized form of the protein migrating faster than the reduced form (Fig. 2A).

As a prerequisite for determining the reduction potential of cVIMP-Cys based on the mobility difference between the oxidized and reduced forms (see below), we next used chemical modification of free cysteines with the alkylating agent AMS to exaggerate the mobility shift described above. Because modification with AMS adds 537 Da per cysteine, this procedure provides, as a result of the retarded mobility due to the addition of two molecules of AMS, a direct visualization of the fraction of molecules present in the reduced state in the sample. When pretreating the protein with the reducing agent TCEP before adding AMS, we observed the expected mobility shift (Fig. 2B). Purified cVIMP-Cys that had not been preincubated with TCEP did not shift upon AMS treatment. We concluded that the Cys174-Cys188 disulfide bond forms in the entire population of cVIMP-Cys molecules, a finding that suggests that a Cys174-Sec188 selenosulfide bond forms in the native sequence.

Reduction Potential Determination for cVIMP-Cys—The lack of purified cVIMP-Sec prevented the determination of the reduction potential for the presumed Cys174-Sec188 selenosulfide bond. Instead, we established the reduction potential for Cys174-Cys188 in cVIMP-Cys to provide an indication of the stability of the selenosulfide bond in the wild-type sequence. A priori, the method of choice for these studies was fluorescence spectroscopy, which we have previously used to determine the reduction potential for certain ER oxidoreductases (37, 38). However, we observed no significant difference between oxidized and reduced cVIMP-Cys by this method (data not shown). Therefore, we established the reduction potential for the presumed Cys174-Sec188 selenosulfide bond using a modified spectrophotometric method, which we have previously used to determine the reduction potential for certain ER oxidoreductases (37, 38).

FIGURE 2. Redox characterization of cVIMP-Cys. A, 15% Coomassie-stained SDS-PAGE gel of cVIMP-Cys under non-reducing (NR; the exact same sample loaded twice) and reducing (R; 10 mM DTT) conditions, as indicated above each lane. M denotes molecular weight marker. Note that the mobility shift observed for the reduced protein was also seen for a small fraction of the molecules in the non-reducing samples bordering the reducing samples as a result of the diffusion of the reducing agent into the neighboring lane. B, purified cVIMP-Cys was incubated either with or without 10 mM TCEP and subsequently modified with AMS. The position of oxidized (Ox) and reduced (Red) cVIMP-Cys is marked on each gel. C, equilibrium constant for the reaction between cVIMP-Cys and glutathione, 18% non-reducing Coomassie-stained SDS-PAGE gels of AMS-modified cVIMP-Cys incubated at different ratios of [GSH]2/[GSSG] (samples 2–23). The position of reduced (Red) and oxidized (Ox) cVIMP-Cys is marked. The intensity of each band was quantified using the ImageJ software and plotted as fraction reduced cVIMP-Cys versus [GSH]2/[GSSG] (mM). D, cVIMP-Cys was incubated with 0.5 mM TCEP, sample 24 (oxidized with 2 mM GSSG), and sample 25 (untreated) were not included in the plot. Neither was sample 6 (an outlier) nor sample 12 (not possible to quantify from the gel). Data points for samples 2, 14 and 23 are indicated. The following equation: Fraction reduced = [GSH]2/[GSSG]/(Keq + [GSH]2/[GSSG]) was used to fit to the data points, which yielded a Keq = 47 mM. D, cVIMP-Cys is reduced by the thioredoxin system in vitro. The absorbance of 155 μM NADPH was followed at 340 nm over time upon the addition of components of the thioredoxin system and purified cVIMP-Cys. The arrows indicate the time points where TrxR (0.1 μM), cVIMP-Cys (25 μM), and Trx (0.5 μM) were added.
NADPH functions as the electron donor to reduce thioredoxin (Trx) system to reduce cVIMP-Cys. In this system, active site. We therefore tested the ability of the cytosolic thioredoxin of VIMP-Cys obtained from analytical gel filtration on a Superdex 75 column. The apparent mass of cVIMP-Cys was calculated (yielding 41.3 kDa and 42.0 kDa for oxidized and reduced cVIMP-Cys, respectively) from the elution volumes of standard proteins (indicated by arrows). mAU, milli absorbance units. B, Far-UV CD spectra of oxidized (dashed line) and reduced (solid line) cVIMP-Cys recorded at 5 °C. The complete reduction and oxidation of cVIMP-Cys in all experiments in this figure was verified by the AMS shift assay (data not shown).

FIGURE 3. Basic structural characterization of oxidized and reduced cVIMP-Cys. A, elution profiles of oxidized (dashed line) and reduced (solid line) cVIMP-Cys from analytical gel filtration on a Superdex 75 column. The apparent mass of cVIMP-Cys was calculated (yielding 41.3 kDa and 42.0 kDa for oxidized and reduced cVIMP-Cys, respectively) from the elution volumes of standard proteins (indicated by arrows). mAU, milli absorbance units. B, Far-UV CD spectra of oxidized (dashed line) and reduced (solid line) cVIMP-Cys recorded at 5 °C. The complete reduction and oxidation of cVIMP-Cys in all experiments in this figure was verified by the AMS shift assay (data not shown).

shown). Instead, we used the AMS shift assay described above and quantified the fraction of reduced cVIMP-Cys from the intensities of the two bands representing oxidized and reduced cVIMP-Cys in Coomassie-stained gels. Although this method is unlikely to be as accurate as fluorescence spectroscopy, we nonetheless obtained reproducible results from three independent experiments. In the experimental procedure used, cVIMP-Cys was allowed to equilibrate in redox buffers of different GSH/GSSG ratios under the exclusion of oxygen. At the end of the incubation period, thiol-disulfide exchange was quenched by the addition of TCA, which at the same time precipitated the protein. After redissolving the pellet under denaturing conditions in a buffer containing AMS for modification of free cysteines, samples were resolved by SDS-PAGE. The fraction of reduced cVIMP-Cys was then plotted against experimental values for [GSH]2/[GSSG], as determined by HPLC analysis (see “Experimental Procedures”). This was done because we consistently observed that the experimentally determined [GSH]2/[GSSG] values were lower than the calculated theoretical values despite precautions to avoid air oxidation in our experiments. A prominent cause for the observed difference could thus well be oxidation of the GSH powder.

After fitting Equation 1 to the experimental data (Fig. 2C), the method gave an equilibrium constant for cVIMP-Cys and glutathione of $K_{eq} = 47$ mM. Applying the Nernst equation and the standard reduction potential of $-240$ mV at pH 7.0 and 25 °C for glutathione, this corresponds to a reduction potential of $-200$ mV for cVIMP-Cys. Based on comparative studies performed on other proteins, the reduction potential for cVIMP-Sec would be expectedly be lower than the value obtained here for cVIMP-Cys (see “Discussion”).

Oxidized cVIMP-Cys Is a Good Substrate of the Thioredoxin System in Vitro—In the cell, the stable selenosulfide in VIMP would require reduction to regenerate the reduced state of the active site. We therefore tested the ability of the cytosolic thioredoxin (Trx) system to reduce cVIMP-Cys. In this system, NADPH functions as the electron donor to reduce thioredoxin reductase (TrxR). This enzyme in turn reduces the thiol-disulfide oxidoreductase thioredoxin, which then reduces the substrate (39).

We chose to use the well-established spectrophotometric assay for the reduction of protein disulfides by Trx (40). Here, the absorbance of NADPH at 340 nm is followed upon the addition of each component of the Trx system as well as the substrate. A decrease in absorbance indicates NADPH oxidation and thus substrate reduction. Initially, 155 μM of NADPH and 0.1 μM TrxR were added to the cuvette and incubated for 5 min (Fig. 2D). Next, 25 μM oxidized cVIMP-Cys was added. This gave no change in absorbance (apart from the effect of dilution), and thus TrxR was not able to reduce cVIMP-Cys directly. Addition of 0.5 μM Trx on the other hand resulted in rapid consumption of NADPH, indicating reduction of cVIMP-Cys via Trx. As a control, addition of Trx prior to cVIMP-Cys had no effect on absorbance levels (data not shown), demonstrating that the observed decrease was dependent on cVIMP-Cys. For comparison, we investigated the reduction of human insulin and GSSG, both known substrates of Trx (40). Compared with the rate of cVIMP-Cys reduction, the three disulfides in human insulin were reduced much slower, while the rate of GSSG reduction was even slower than observed for insulin (data not shown).

Analytical Gel Filtration Reveals a Large Apparent Molecular Mass of cVIMP-Cys—Next we wanted to investigate structural properties and potential overall structural differences between the two redox forms of cVIMP-Cys. As a first method to do so, we performed analytical gel filtration on the oxidized and reduced protein. No significant difference was observed when comparing the elution volume of the two redox forms (Fig. 3A). The similarity in the hydrodynamic properties indicated that the overall structure did not change with the redox state. When comparing the elution volume of cVIMP-Cys to globular standard proteins, the VIMP construct was found to have an apparent molecular mass of ~42 kDa (Fig. 3A). Compared with the calculated mass of 15.8 kDa for monomeric cVIMP-Cys the surprisingly large observed apparent molecular
mass indicated a non-spherical shape of cVIMP-Cys and/or the formation of oligomers, most likely dimers or trimers.

CD Spectroscopy Displays α-Helical and Random Coil Structure—To gain further insight into the structural properties of cVIMP-Cys we used far-UV CD spectroscopy. With two distinct minima at 205 nm and 222 nm, the spectrum clearly displayed α-helical character (Fig. 3B). However, compared with the spectrum of an all α-helical protein where the peak at 222 nm is generally the most pronounced and the second band of negative ellipticity is found at 208 nm, it was clear that the cVIMP-Cys spectrum also displayed features characteristic of disordered regions. No significant change in the secondary structure content was observed between the two redox forms.

NMR Spectroscopy Indicates that cVIMP-Cys Is Partially Disordered—We next turned to NMR spectroscopy, which allowed us to gain information about redox-dependent conformational changes and structural features in cVIMP-Cys at the single residue level. We assigned H N, Nα, Cα, and C‘ chemical shifts for residues 49–71 and 124–189 in reduced and oxidized cVIMP-Cys. For reduced cVIMP-Cys we also assigned Cβ chemical shifts. The assignments of the peaks in the 1H,15N-HSQC spectrum are shown in Fig. 4. Ten peaks that were identified as not originating from side-chains could not be assigned. We did not observe any peaks from residues 72–123 suggesting that this stretch of the protein has non-optimal properties for NMR (see “Discussion”). From the poor dispersion particularly in the 1H dimension of the HSQC spectra in Fig. 4, it can be concluded that the assigned regions of cVIMP-Cys in both the reduced and oxidized states have very little structure. It is also evident by comparing the HSQC spectra of reduced and oxidized cVIMP-Cys that reduction of the disulfide has only little effect on the overall structure of the protein. However, residues 173–189 shifted significantly, indicating a local structural rearrangement in the C-terminal segment of the protein that contains the two Cys residues. In addition to the shifts observed for these backbone amides, one of the two peaks from the H119 and N119 side chain nuclei of Trp-119 and Trp-176 (Fig. 4B, inset), most likely the one belonging to Trp-176, also shifted its position between the two redox forms.

Secondary Structure Propensity Analysis—While our NMR analysis of cVIMP-Cys did not include side chain assignments or the recording of NOE spectra necessary for structure determination, it still allowed us to extract useful information about the secondary structure of the protein. For this purpose we used

FIGURE 4. HSQC spectra of oxidized and reduced cVIMP-Cys with assigned peaks. A, amino acid sequence of cVIMP-Cys. Black letters indicate residues that could not be assigned. For the assigned residues, blue and green letters indicate those that do and do not shift between the two redox states, respectively. B, HSQC spectrum of oxidized (blue) overlaid with the spectrum of reduced (green) cVIMP-Cys. The spectra were recorded on an 800 MHz spectrometer at 5 °C and pH 7.0. Assigned residues are marked with residue number and one letter code. Arrows indicate where the peaks shift. The isotope-dimension is marked on the axes. Boxes indicate zoom area I and II shown in C and D. Dashed lines indicate the pairwise arrangement of NH2-containing side chains from Asn and Gln. Inset: signals from the two Trp side chains. C and D, zoom of areas I and II. The assigned peaks are indicated with residue number and one letter code. Arrows indicate where the peak shifts to between the two redox states. Note that the spectrum of the oxidized sample contains peaks corresponding to both oxidized and reduced cVIMP-Cys, illustrating that the sample contained a mixture of the two redox forms (see for instance Gly-182 or Gly-186, panel C). The reason for this observation is currently unknown, but does not change the conclusions drawn based on these experiments.
secondary structure propensity (SSP) analysis (41). This method combines chemical shifts from different nuclei into a single score representing the expected fraction of α-helix or β-strand for each residue. The score is calculated relative to an average chemical shift of nuclei in known α-helix or β-strand structures, and ranges between 1 or −1 for the two types of secondary structure, respectively. Here, we used the chemical shifts obtained for the Cα, C′, H, and N chemical shifts of oxidized (black dots) and reduced (gray dots) cVIMP-Cys to calculate secondary structure propensities (Fig. 5). It should be noted that for reduced cVIMP-Cys, for which Cβ chemical shifts were also assigned, the inclusion of these had only a minimal effect on the SSP values. The analysis clearly indicated residues 51–69 to constitute an α-helix. For the C-terminal half of cVIMP-Cys, values close to zero were observed indicating disordered structure. However, a short glycine-rich stretch involving residues 160–172 showed a small peak with values of up to 0.23, which could indicate transient formation of a non-random structure in this region. Apart from a small difference for three residues centered on Ser-175, the SPP values did not differ considerably between the two redox states of cVIMP-Cys.

**VIMP Binds p97N with Only Minor Effects in the Disordered C Terminus**—The redox function of Sec indicates that orthologs of selenoproteins containing a Cys in place of the Sec also serve a function as thiol-disulfide oxidoreductases (42). Identification of such Cys/Sec pairs in homologous proteins has recently been used to uncover new thiol-disulfide oxidoreductases by data base mining (42). To identify potential Cys-containing orthologs of VIMP and thus gain further insight into this family of proteins, we performed a multiple sequence alignment for VIMP from a wide range of species (supplemental Fig. S1). The alignment identified three distinct subgroups (I–III) of VIMP proteins defined by sequence differences relating to the proposed redox-active residues. The proteins in subgroup III indeed contain a Cys in place of the Sec (see supplemental data for a more detailed discussion of the alignment).

Recently, a sequence motif with the approximate consensus R-X₅-A-A-X₂-R (where X denotes any residue) has been defined as the core of a p97-binding motif called a VIM (for VCP-interacting motif) (43–45). The VIM region has a highly predicted propensity for α-helix, and has experimentally been demonstrated to be helical in complex with p97 (44, 45). In human VIMP, this minimal VIM covers residues 78–88 (supplemental Fig. S1). Based on sequence conservation, the VIM in VIMP might well comprise additional important residues such as an arginine directly following the two alanines, and a Met-Leu-Gln-Glu motif directly following the last arginine of the minimal VIM (supplemental Fig. S1). In the alignment, the region around the VIM is the best conserved.

In the context of the current data, we wanted to investigate whether the binding of VIMP to p97 would, in addition to the VIM, involve the disordered C-terminal region. Previously, it has been demonstrated that VIMP does not bind to p97 devoid of the so-called N-domain (22). Moreover, the N-domain has been shown to bind directly to the VIM of different proteins via a central cleft between two subdomains (44, 45).

Titration of 15N-labeled cVIMP-Cys with the N-domain of (p97N) resulted in sequence-specific and concentration-dependent changes in the ¹H, 15N HSQC spectra (Fig. 6A), demonstrating an interaction between the two proteins. Specifically, the peaks from residues 50–71 of cVIMP-Cys (50 μM) showed significant line broadening, which resulted in an ~85% decrease in signal intensity at 60 μM p97N and complete loss of several signals at 173 μM p97N (Fig. 6B). In addition, small yet distinct changes in chemical shifts occurred (Fig. 6C). These spectral changes clearly show that the environment of residues 50–71, quite closely corresponding to the predicted helix (Fig. 5), changed upon the formation of a VIMP-p97N complex, either as a result of an interaction between these residues and p97N or caused by long-range structural changes in VIMP induced by p97N. The VIM region of cVIMP-Cys is located in the segment of the protein that was not observed in the ¹H, 15N HSQC. Consequently, based on these data we cannot verify that p97 interacts with this particular motif, although we find it highly likely based on all available information.

The unstructured C-terminal part of cVIMP-Cys was less affected by the binding of p97N than the N-terminal part. Only a few residues in the C-terminal part of cVIMP-Cys showed significant changes in chemical shifts (Fig. 6C). In contrast, sequence-dependent line broadening was observed, although the effect was much smaller than in the N-terminal region of cVIMP-Cys (Fig. 6B). Residues ~163–181 were broadened the most, which suggested the formation of some transient structure between this region of cVIMP-Cys and the N-domain of p97. However, the data gave no indication for the formation of a compact stable structure in the C-terminal region of cVIMP-Cys upon binding of p97N.

**DISCUSSION**

The current study reveals fundamental redox and structural properties of human VIMP. In cVIMP-Cys we identified a disulfide bond between Cys-174 and Cys-188 with a reduction
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FIGURE 6. cVIMP-Cys interacts with the N-domain of p97. A, HSQC spectra were recorded of $^{15}$N-cVIMP-Cys (50 $\mu$M) in the absence of p97N, and in the presence of 60 $\mu$M and 173 $\mu$M p97N. Four selected peaks from the HSQC spectra are shown as an overlay of 50 $\mu$M $^{15}$N-cVIMP-Cys (gray), 50 $\mu$M $^{15}$N-cVIMP-Cys with 60 $\mu$M p97N (blue), 4-fold decrease in the base contour level, and 50 $\mu$M $^{15}$N-cVIMP-Cys with 173 $\mu$M p97N (red, 8-fold decrease in the base contour level). The plotting at different contour levels of the spectra is necessary to see peaks in all three spectra. Arrows indicate the direction of the chemical shift change. $B$, plot of intensity ratio ($I_{\text{base}}/I_{\text{sample}}$) as a function of residue number. $I_{\text{base}}$ denotes the intensity of peaks in the sample with either 60 $\mu$M p97N (blue) or 173 $\mu$M (red) p97N, and $I_{\text{sample}}$ refers to the intensity of peaks in the sample without p97N. $C$, change in peak positions reported as the weighted chemical shift difference induced by addition of p97N ($\Delta\delta_{\text{weighted}}$) is plotted as a function of residue number for peaks in the samples with 60 $\mu$M p97N (blue) and 173 $\mu$M p97N (red). $\Delta\delta_{\text{weighted}}$ is given in parts per million (ppb). Peaks that were poorly defined or completely absent are not included in the plots in panels $B$ and $C$ (thus the lack of data points for e.g., most of residues 50–71 in the sample with 173 $\mu$M p97N). Signals for residues 72–123 were not observed even in the absence of p97N (see text for details).

During the process of reduction, a mixed selenolsulfide will be formed with the substrate. This bond would then be resolved by Cys-174 to form the Cys174-Sec188 selenolsulfide, which must be reduced for the enzyme to function in another round of substrate reduction. The TrxR/Trx and glutathione reductase/glutaredoxin systems comprise the two cellular pathways for reduction of proteins in the cytosol (49). Here, we have shown that VIMP is a good substrate for the Trx system in vitro. While not ruling out a role of the glutathione system, the data point to the thioreductin system as a prime candidate for further cellular studies.

Several selenoproteins such as thioreductin reductase and methionine-R-sulfoxide reductases have orthologs containing a Cys in place of the Sec (see e.g. (50, 51)). Studies on such proteins have shown that efficient catalysis is not dependent on the Sec, and that each type of ortholog has evolved specific characteristics of the active-site sequence to optimize activity. The VIMP proteins in subgroup III (see supplemental Fig. S1) provide another example of such orthologs where the active site Sec has been replaced by a Cys.

The SSP analysis predicted secondary structural properties for cVIMP-Cys with the exception of residues 72–123, which were not detected in the NMR spectra (Fig. 5). However, the Protein Data Bank contains an entry for a crystal structure for the fragment of human VIMP comprising residues 52–122 (PDB ID: 2Q2F). This structure is unpublished and has therefore not been subjected to peer review. Still, it strongly corroborates our findings from the CD and NMR analyses (see below). The fragment shows an elongated structure with two $\alpha$-helices constituting residues 52–69 and 71–122, respectively. Based on this structure and the current data we propose an overall structural model of VIMP where the transmembrane region is followed almost directly by the extended $\alpha$-helical structure, which is in turn followed by an intrinsically disordered region (Fig. 7).

The notion that cVIMP-Cys is extended rather than being globular with a classical hydrophobic core is supported by fluorescence spectroscopy, which showed an emission maximum at 356 nm (data not shown), a value typically observed for solvent-exposed Trp residues. In the $\alpha$-helical crystal structure, the first helix closely matches the predictions of the SSP analysis. The second long helix could well be stabilized by dimer formation since residues 80–120 are strongly predicted by the SSP analysis (PairCoil2 (52) and MultiCoil (53) algorithms to form a dimeric $\alpha$-helical structure, as also recently noted elsewhere (25). Moreover, the backbone amides of this long helix could not be detected by NMR spectroscopy. This observation clearly demonstrates that
residues 72–123 adopt some structure. The reason that no NMR signals are observed could be: (i) that the putative coiled-coil region has very slow rotational diffusion, or (ii) that the coiled-coil region engages in a conformational exchange process (e.g. monomer-dimer equilibrium) on the milli-second time scale. Both scenarios would result in fast transverse relaxation and consequently signals with very low intensities.

In the HSQC spectra, dilution of VIMP from 265 μM to 32.5 μM did not result in any change (data not shown). A change would have been expected if exchange between monomeric and dimeric VIMP were the reason for the missing peaks at 265 μM. If slow tumbling was causing the missing peaks it would be expected that increasing the temperature, which also increases the tumbling rate, would give sharper peaks and that more peaks would appear. In contrast, we observed fewer peaks when increasing the temperature (data not shown). It is thus likely that the reason for the missing peaks is some conformational exchange process other than a monomer-dimer equilibrium.

The C-terminal half of eVIMP-Cys, residues 123–189, showed clear indications by a number of the applied techniques to be intrinsically disordered. The overall structure of the cytosolic region is based on findings in this study, as well as the crystal structure of a VIMP fragment comprising residues 52–122 (PDB ID: 2Q2F; unpublished). This cytosolic region consists of an α-helical region (blue) harboring the putative VIM (residues 78–88; pink), and an intrinsically disordered region (residues 123–189; green). The expected selenosulfide bond formed by Cys-174 and Sec188 is indicated. Numbers denote residue positions.

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FIGURE 7. An overall structural model for VIMP. The ER (orange) and transmembrane regions (gray) are based on predictions using TMHMM (21, 22). The overall structure of the cytosolic region is based on findings in this study, as well as the crystal structure of a VIMP fragment comprising residues 52–122 (PDB ID: 2Q2F; unpublished). This cytosolic region consists of an α-helical region (blue) harboring the putative VIM (residues 78–88; pink), and an intrinsically disordered region (residues 123–189; green). The expected selenosulfide bond formed by Cys-174 and Sec188 is indicated. Numbers denote residue positions.

We thus speculate that residues 163–189 support the interaction with a cellular redox regulator (e.g. thioredoxin) and contain substrate recognition elements. Indeed, the observed effect of p97 binding on residues 163–181 (Fig. 6B) could reflect the capability of this region for protein-protein interaction. The conformational plasticity offered by residues ~123–162 would then provide adaptability for interacting with substrates of different shapes. A similar mode of operation has been observed for the E3 ligase from yeast, San1, where conserved regions of around 20 residues are responsible for substrate binding, while intervening regions of disorder were proposed to provide flexibility for binding a variety of substrates (58).

Though other redox functions are possible, the redox properties of VIMP would presumably allow it to reduce particularly stable disulfides in ERAD substrates that were not reduced prior to retrotranslocation. Such stable disulfides could for instance be present in small compact domains formed in large multidomain proteins prior to misfolding of other regions of the molecule. Ongoing experiments are directed at investigating these ideas in detail.

Acknowledgments—We thank R. Hartmann-Petersen for helpful suggestions, E. Arnér (Karolinska Institutet, Stockholm) for valuable advice on setting up the NADPH/TrxR/Trx assay, K. S. Jensen for initial help on the project, H. Meyer for providing the p97N expression plasmid, Z. Nikrozi for technical assistance, and all members of the Ellgaard laboratory for critical reading of the manuscript.

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It is interesting to note that the C-terminal region encompassing Tyr163-Gly189 is well conserved irrespective of the sequence subgroup to which it belongs (supplemental Fig. S1), and despite the observation that intrinsically disordered regions are typically not well conserved throughout evolution (56, 57). We thus speculate that residues 163–189 support the interaction with a cellular redox regulator (e.g. thioredoxin) and contain substrate recognition elements. Indeed, the observed effect of p97 binding on residues 163–181 (Fig. 6B) could reflect the capability of this region for protein-protein interaction. The conformational plasticity offered by residues ~123–162 would then provide adaptability for interacting with substrates of different shapes. A similar mode of operation has been observed for the E3 ligase from yeast, San1, where conserved regions of around 20 residues are responsible for substrate binding, while intervening regions of disorder were proposed to provide flexibility for binding a variety of substrates (58).

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