INTRODUCTION

In the ER (endoplasmic reticulum), optimal redox conditions are maintained to facilitate formation of native disulfide bonds in secretory proteins. In mammalian cells, disulfide bonds are predominantly generated by the two isoforms of Ero1 (ER oxidoreductin-1): Ero1α and Ero1β. The activity of Ero1α is tightly regulated through the formation of intramolecular disulfide bonds to help ensure balanced ER redox conditions. Ero1β is less tightly regulated, but the molecular details underlying control of activity are not as well characterized as for Ero1α. Ero1β contains an additional cysteine residue (Cys262), which has been suggested to engage in an isoform-specific regulatory disulfide bond with Cys100. However, we show that the two regulatory disulfide bonds in Ero1α are likely conserved in Ero1β (Cys90–Cys130 and Cys95–Cys100). Molecular modelling of the Ero1β structure predicted that the side chain of Cys262 is completely buried. Indeed, we found this cysteine to be reduced and partially protected from alkylation in the ER of living cells. Furthermore, mutation of Cys100 – but not of Cys262 – rendered Ero1β hyperactive in cells, as did mutation of Cys350. Ero1β hyperactivity induced the UPR (unfolded protein response) and resulted in oxidative perturbation of the ER redox state. We propose that features other than a distinct pattern of regulatory disulfide bonds determine the loose redox regulation of Ero1β relative to Ero1α.

Key words: disulfide-bond formation, endoplasmic reticulum oxidoreductin-1 (Ero1), redox regulation, unfolded protein response (UPR)


Abbreviations: AMS, 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid; ATF6α, activating transcription factor 6α; BiR, immunoglobulin heavy-chain-binding protein; DTT, dithiothreitol; Dox, doxycycline; ER, endoplasmic reticulum; Ero1, endoplasmic reticulum oxidoreductin-1; Ero1α-WT, wild-type Ero1α; HEK-293 cells, human embryonic kidney cells; HERP, homocysteine-induced endoplasmic reticulum (ER) protein; NEM, N-ethylmaleimide; PDI, protein disulfide-isomerase; PERK, PKR (double-stranded-RNA-dependent protein kinase); TRAF, endoplasmic reticulum kinase; TCA, trichloroacetic acid; UPR, unfolded protein response.

1 Present address: Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2970 Hørsholm, Denmark.
2 Present address: Department of International Health, Immunology and Microbiology, University of Copenhagen, 2200 Copenhagen, Denmark.
3 To whom correspondence should be addressed (email: lellgaard@bio.ku.dk).
misfolded proteins in the ER (designated ER stress). The UPR seeks to restore ER homeostasis, for example by decreasing the ER protein load through translational arrest and in parallel up-regulating chaperones to assist folding [18]. PERK [PKR (double-stranded-RNA-dependent protein kinase)-like endoplasmic reticulum kinase], Inositol-requiring enzyme 1 (double-stranded-RNA-dependent protein kinase)-like endoplasmic reticulum kinase), Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring enzyme 1 kinase], Inositol-requiring 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Recently, we showed that overexpression in human cells of the equivalent Ero1\(\beta\) mutant (Ero1\(\beta\)-C100A/C130A) gave rise to more pronounced hyperoxidation of ERp57 relative to overexpression of Ero1\(\beta\)-WT [32], suggesting that the regulatory mechanism is shared for Ero1\(\alpha\) and Ero1\(\beta\). However, Ero1\(\beta\) contains an additional cysteine residue (Cys\(^{262}\)), which is not present in Ero1\(\alpha\). A disulfide bond between Cys\(^{100}\) and Cys\(^{262}\) was recently proposed to be present in Ero1\(\beta\) purified from Escherichia coli [7]. Moreover, Ero1\(\beta\)-C100A displayed slowed oxidation kinetics relative to Ero1\(\beta\)-WT [7], suggesting that the presence of the proposed Cys\(^{100}\)-Cys\(^{262}\) disulfide bond increases Ero1\(\beta\) activity. On this background, we decided to further investigate the interplay between intramolecular disulfide bonds and regulation of activity in Ero1\(\beta\).

MATERIALS AND METHODS

Primers and plasmids

Human Ero1\(\beta\)-myc6his ([16]; a gift from R. Sitia, Milan) cloned into the pcDNA5/FRT/TO vector [23] was used as a template for QuickChange mutagenesis (Stratagene) to introduce Cys-to-Ala mutations. The following primer was used to generate the C262A mutation (only the sense strand is shown): C262A 5’-GA-CCTCATGCTAGCATCAATTTACATCTAGCCGCAAATAT-CTTTTG-3’. The C100A and C130A mutations have been described before [32]. All plasmids were sequenced to confirm their correct DNA sequence of the inserts.

Cell culture

Dox (doxycycline)-inducible Flp-In T-REx HEK-293 (Life Technologies) cell lines were generated and grown as previously described [23]. Ero1\(\beta\) expression was induced for 24 h (unless otherwise stated) using 1 \(\mu\)g/ml Dox (Sigma). For ER stress induction, cells were treated with either 5 \(\mu\)M thapsigargin (Sigma) or 2.5 \(\mu\)g/ml tunicamycin (Sigma) for 15 min at 37 \(^\circ\)C in full growth medium.

Sample preparation and AMS (4-acetamido-4’-maleimidylstilbene-2,2’-disulfonic acid) modification

Cells were treated with NEM (N-ethylmaleimide) and subsequently lysed as described elsewhere [35]. The AMS (Life Technologies) modification protocol has been described previously [35]. Reduced and oxidized control lysates were obtained from cells treated with 10 mM DTT (dithiothreitol) or 5 mM diamide (both Sigma) for 5 min at 37 \(^\circ\)C in full growth medium.

Antibodies and Western blotting

The following mouse monoclonal antibodies were used: \(\alpha\)His (Tetra-His, Qiagen), \(\alpha\)myc (9E10, Covance), \(\alpha\)\-actin (AC-15, Sigma). The rabbit polyclonal antisera used were: \(\alpha\)BiP (G8918, Sigma), \(\alpha\)ERp57 (a gift from A. Helenius, Zürich, Switzerland), \(\alpha\)HERP (a gift from L. Hendershot, Memphis, TN, U.S.A.). Western blotting was performed as previously described [24]. The shown Western blots are representative of at least two independent experiments.

Redox state analysis of Ero1\(\beta\) by TCA (trichloroacetic acid) precipitation and alkylation of free thiols

Cells cultivated to 60–80% confluency in 6 cm dishes were washed twice in PBS. They were then concomitantly lysed and precipitated by incubation in 10% (v/v) TCA for 15 min on ice. Cells were transferred to an Eppendorf tube, centrifuged (16 100 \(\times\) g, 4 \(^\circ\)C, 15 min) and the supernatant was discarded. Pellets were washed once in ice-cold acetone, centrifuged (16 100 \(\times\) g, 4 \(^\circ\)C, 15 min) and resuspended in 100 \(\mu\)l 100 mM Tris–HCl pH 7.0, 8% (v/v) glycerol, 2% (w/v) SDS, 10% dimethyl sulfoxide, 0.01% (w/v) bromocresol purple and 20 mM NEM. Samples were neutralized by drop-wise addition of 1 M Tris–HCl, pH 7.5, 2% SDS until samples turned purple (bromocresol purple changes colour between pH 5.2 and 6.8). The pellets were subsequently dissolved by sonication, incubated at RT in the dark for 1 h and the redox state of Ero1\(\beta\) was determined by non-reducing Western blotting.

RESULTS

Structure homology modelling of Ero1\(\beta\) predicts Cys\(^{262}\) to be buried in the structure

The amino acid sequences of Ero1\(\beta\) are highly conserved between orthologues (Supplementary Figure S1 available at http://www.bioscirep.org/bsr/034/bsr034e103add.htm). Thus, potential roles of cysteine residues in regulatory disulfide bonds based on evolutionary conservation could not be inferred from a multiple sequence alignment. Instead, we used structure homology modelling of Ero1\(\beta\) to assess the proposed disulfide patterns in the protein (Figures 1A and 1B). The protein structure prediction software SWISS-MODEL [36] was used to predict the three-dimensional structure of Ero1\(\beta\) based on the crystal structure of inactive Ero1\(\alpha\), a mutant in essence corresponding to the OX2 form ([4]; PDB ID: 3AHR) (Figure 2A). The sequences of mature Ero1\(\alpha\) and Ero1\(\beta\) are highly similar [14] with a sequence identity of 65%. As expected from the high sequence conservation, the \(\alpha\)-helical fold in Ero1\(\alpha\) was predicted to be preserved in Ero1\(\beta\) including the four-helix bundle involved in FAD binding (Figure 2A, red-coloured \(\alpha\)-helices). The structure of the flexible region (residues 86–130) comprising the proposed Cys\(^{95}\)–Cys\(^{130}\) or the Cys\(^{95}\)–Cys\(^{100}\) disulfide bonds could not be reliably modelled (Figure 2A).

In contrast to the cysteines in the flexible region, Cys\(^{262}\) is located at the end of a conserved helix [14], which is part of the four-helix bundle (Figure 2A). Moreover, Cys\(^{262}\) is positioned close...
to a protruding \( \beta \)-hairpin, which is critical for the interaction with PDI [37]. The equivalent residue in Ero1\( \alpha \) (Ser\(^{263} \)) is completely buried. Similarly, in the Ero1\( \beta \) model, Cys\(^{262} \) is predicted to have a relative accessible surface area of 0 (as calculated by the ASAView software [38] and the GETAREA method [39]), which strongly suggests that Cys\(^{262} \) in Ero1\( \beta \) is buried in the native structure. Moreover, the side chain – SH (Cys\(^{262} \)) is predicted to form a hydrogen bond with the backbone carbonyl group of Asn\(^{259} \) (Figure 2B). This hydrogen bond seems to be part of a conserved hydrogen bond network, including hydrogen bonds from the side chain of Asn\(^{259} \) to FAD, which helps stabilize the structure in the vicinity of the bound cofactor. Finally, we also note that in Xenopus tropicalis Ero1\( \beta \), a serine residue is found in place of Cys\(^{262} \) (Supplementary Figure S1), indicating that a cysteine is not strictly necessary at this position as may have been expected if it played an important function in regulating the activity of the enzyme.

**SDS–PAGE mobility of Ero1\( \beta \) mutants suggests conservation of regulatory disulfide bonds in Ero1\( \alpha \) and Ero1\( \beta \)**

To investigate the structural importance of intramolecular disulfide bonds in human Ero1\( \beta \), we expressed Ero1\( \beta \) cysteine mutants in human cells and analysed the mobility of these mutants by non-reducing SDS–PAGE. Apart from already established stable cell lines for ectopic inducible expression of Ero1\( \beta \)-WT [23] and Ero1\( \beta \)-C100A/C130A [32], we generated three new inducible cell lines for the following mutants: Ero1\( \beta \)-C100A, Ero1\( \beta \)-C130A and Ero1\( \beta \)-C262A. As compared with Ero1\( \beta \)-WT, Ero1\( \beta \)-C100A and Ero1\( \beta \)-C262A showed similar expression levels, whereas the expression levels of Ero1\( \beta \)-C130A and Ero1\( \beta \)-C100A/C130A were lower (Figure 3A). Importantly, none of the cell lines overexpressing Ero1\( \beta \) mutants of Cys\(^{100} \) and/or Cys\(^{130} \), which turned out to be hyperactive (see below), expressed more protein than the Ero1\( \beta \)-WT-expressing cell line.

As previously observed [33], the monomeric form of exogenous Ero1\( \beta \)-WT migrated as two distinct redox species (Red and Ox) when cells were treated with NEM to alkylate free thiols in situ prior to lysis (Figure 3B, lane 2 and Figure 3C, lane 3). However, upon TCA precipitation with subsequent NEM treatment, monomeric Ero1\( \beta \)-WT migrated as one redox species (Figure 3D, lane 4). TCA precipitation rapidly quenches thiol–disulfide exchange reactions and denatures proteins, enabling alkylation of thiols buried in the native structure [40]. When cells are in situ NEM-treated, approximately 20% of the cellular protein thiols have been shown to be inaccessible to NEM [41]. Such NEM inaccessibility is thought to be a consequence of these thiols being buried in the native structure [40]. We therefore suggest that inefficient alkylation of (a) free thiol(s) buried in the structure of Ero1\( \beta \) gives rise to rearrangement of disulfide bonds upon denaturation, leading to the appearance of the Red Ero1\( \beta \) redox form (Figures 3B and 3C). Conversely, when all free thiols are efficiently alkylated, Ero1\( \beta \)-WT is preserved as a single redox species visible on SDS–PAGE gels (Figure 3D).

The SDS–PAGE mobility of the Ero1\( \beta \) variants on non-reducing gels (Figure 3D) is consistent with Ero1\( \beta \) having a similar pattern of disulfide bonds as Ero1\( \alpha \) (Figures 1B and 1C). We were able to detect a relatively small migration shift between Ero1\( \beta \)-WT and Ero1\( \beta \)-C100A (Figure 3D, lanes 4–5) suggesting that Cys\(^{100} \) is not engaged in a long-range disulfide bond. In contrast, a larger shift was observed upon mutation of Cys\(^{300} \) (Figure 3D, lanes 6–7) consistent with removal of the longer-ranging Cys\(^{90} \)–Cys\(^{130} \) disulfide bond. No redox species of Ero1\( \beta \)-C100A co-migrated with Ero1\( \beta \)-C130A (Figure 3D, lanes 5–6), suggesting that the Cys\(^{90} \)–Cys\(^{130} \) disulfide bond is intact in Ero1\( \beta \)-C100A.
Figure 3  SDS–PAGE mobility of Ero1β variants suggests that Ero1α and Ero1β share their sets of regulatory disulfide bonds

(A) Expression of His- and Myc-tagged Ero1β variants was induced with Dox for 24 h and cells were NEM treated to alkylate-free thiols. Equal amounts of protein from lysates were analysed by reducing SDS–PAGE and Western blotting using αHis (Ero1β) and αActin (loading control) to compare expression levels of Ero1β variants. (B,C) Cell lysates were obtained as in (A). The SDS–PAGE mobility of the Ero1β variants was analysed under non-reducing (Non-red) or reducing (Red) conditions by αmyc or αHis Western blotting. The open and filled circles indicate the previously described OX and Red redox forms of Ero1β WT [33], respectively, and vertical hairlines denote removal of lanes. Asterisk denotes a background band. (D) Expression of Ero1β variants was induced as in (A). Cells were subjected to TCA precipitation to rapidly quench thiol-disulfide exchange reactions and to denature cellular proteins. Precipitates were redissolved in a buffer containing NEM to alkylate free thiols. Subsequently, the SDS–PAGE mobility of the Ero1β variants was analysed under non-reducing conditions by αHis Western blotting. Section signs (§) indicate possible Ero1β mixed-disulfide dimeric species and the asterisk (*) denotes a background band.

Figure 4  Hyperoxidation of ERp57 is intensified by removal of regulatory disulfide bonds in Ero1β

(A–C) Where indicated, expression of Ero1β variants was induced with Dox for 24 h. Prior to lysis, cells were treated with NEM to alkylate free thiols. After cell lysis, cysteines present in disulfides were reduced and decorated with AMS. Such AMS modification of active-site cysteines originally present in the oxidized state gives rise to slower SDS–PAGE mobility compared with the (NEM-decorated) pool of ERp57 containing reduced active-site cysteines. The cellular redox state of ERp57 was visualized by Western blotting. DTT and Diamide (Dia) treated-cells were used to show the mobility of fully oxidized (Ox) and reduced (Red) ERp57. A vertical hairline denotes removal of lanes.
A fraction of Ero1β is present as a disulfide-bonded homodimer in human cells [33] and when expressed in bacteria [7]. Moreover, Ero1β engages in heterodimeric mixed-disulfide species with PDI and ERp44 in human cells [42]. The possible dimeric species involving Ero1β-WT and Ero1β-C262A were similar (Figure 3D, lanes 3–4), suggesting that Cys3262 is not involved in formation of mixed-disulfide dimeric species. Notably, Ero1β-C262A did not migrate slower than Ero1β-WT (Figure 3D, lanes 3–4), suggesting that Cys3262 is not engaged in a long-range disulfide bond. Instead, Ero1β-C262A was present exclusively as the ox redox species in lysates from cells treated in situ with NEM (Figure 3C, lane 2), and as a single redox species co-migrating with Ero1β-WT in lysates from cells subjected to TCA precipitation (Figure 3D, lane 3). This clearly suggests that non-native ex vivo disulfide shuffling in lysates of in situ NEM-treated cells observed for Ero1β-WT (Figures 3B and 3C) depends on the presence of Cys3262, and that this residue is inaccessible to NEM in the native structure.

Based on these results, we propose that the regulatory disulfide bonds in Ero1α (Cys94–Cys131 and Cys99–Cys104) are conserved in Ero1β (Cys90–Cys130 and Cys95–Cys100) and that Cys3262 constitutes a poorly accessible free thiol in the native structure. The deduced disulfide pattern in the ox redox form of Ero1β is shown in Figure 1(B).

**Removal of either of the regulatory disulfide bonds increases the activity of Ero1β in cells**

We next wanted to assess the relative contribution of the proposed disulfide bonds (Cys90–Cys130 and Cys95–Cys100) to the regulation of Ero1β activity. First, we analysed the cellular redox state of Ero1α (Cys94–Cys131 and Cys99–Cys104) that Ero1β (Cys90–Cys130 and Cys95–Cys100) and that Cys3262 constitutes a poorly accessible free thiol in the native structure. The deduced disulfide pattern in the ox redox form of Ero1β is shown in Figure 1(B).

 Tight regulation of Ero1α activity is important to maintain balanced ER redox conditions [23–25]. We propose that the regulatory disulfide bonds in Ero1α and Ero1β are conserved (Figures 1B and 1C). This conclusion is based on several lines of evidence, including molecular modelling of the Ero1β structure (Figure 2), SDS–PAGE mobility analysis of Ero1β mutants (Figures 3B–3D) and ER redox (Figure 4) and ER stress readouts (Figure 5). Overall, the findings that overexpression of Ero1β mutants devoid of Cys100 and/or Cys130 induces the UPR, hyperoxidizes ERp57 and that Ero1β-C100A/C130A hyperoxidizes an ER-localized glutathione sensor [32], indicate that the underlying mechanism is likely to involve an oxidizing perturbation of the ER redox environment, which in turn results in protein misfolding and therefore activation of the UPR.

In a previous study [7], Ero1β-C262A purified from E. coli displayed a prominent slow-migrating redox species when compared with Ero1β-WT by non-reducing SDS–PAGE, indicating the loss of a long-range disulfide. Furthermore, analysis of tryptic fragments supported the presence of a Cys100—Cys262 disulfide bond. Finally, the Ero1β-C100A mutant was less active in vitro than Ero1β-WT, suggesting that the presence of the proposed Cys100—Cys262 disulfide bond positively regulates the activity of Ero1β.

Here, we expressed Ero1β (and mutants thereof) in its native environment in the ER of human cells and reached the conclusion that a disulfide bond between Cys100 and Cys262 is not likely to form. Thus, mutation of Cys100 rendered Ero1β hyperactive and overexpression of Ero1β-C262A showed effects comparable to Ero1β-WT overexpression. We also provide two-fold evidence that Cys262 is a solvent inaccessible residue in the native structure of Ero1β. First, a fraction of Ero1β-WT molecules rearrange into a redox species that migrates as the Red form upon in situ NEM treatment in a Cys262-dependent manner, suggesting that NEM cannot gain access to Cys262 under native conditions. Secondly, a homology model of Ero1β based on the crystal structure of Ero1α places Cys262 in a non-solvent exposed site in a highly conserved α-helix. Collectively, these findings strongly support the conclusion that Cys262 does not engage in an intramolecular disulfide bond with Cys100. To verify the proposed
activity relative to Ero1α apparently loose redox regulation of Ero1α. Active site sequence of Ero1α (Asp–Lys–Cys396) substantially increases the oxidase activity of Ero1α as has previously been achieved for Ero1α by mass spectrometry, indicating that overexpressed Ero1α is present solely as the OX redox species. Unfortunately, the redox state of endogenous Ero1α assessed by SDS–PAGE mobility under non-reducing conditions is currently unknown. Moreover, we currently do not know why Ero1β migrates 5–7 kDa faster than Ero1α on non-reducing SDS–PAGE gels [23], even though the predicted molecular mass of mature Ero1β is only 1–2 kDa smaller than the corresponding mass of mature Ero1α. Since deglycosylation of Ero1β gives rise to a more pronounced mobility shift on SDS–PAGE gels as compared with Ero1α [16], the presence of N-linked glycans cannot explain the unexpectedly large difference in SDS–PAGE mobility between Ero1α and Ero1β.

As Ero1α and Ero1β likely share their sets of regulatory disulfide bonds, features other than a distinct pattern of disulfide bonds must determine the loose redox regulation of Ero1β relative to Ero1α. Mutation of the Cys894–Phe–Lys–Cys897 inner active site sequence of Ero1α to the Ero1β sequence (Cys903–Asp–Lys–Cys906) substantially increases the oxidase activity of Ero1α [7]. This suggests that Asp903 in Ero1β contributes to the apparently loose redox regulation of Ero1β relative to Ero1α.

As previously proposed [22], the loose regulation of Ero1β activity relative to Ero1α could be explained by a higher reduction potential of the regulatory disulfide bonds in Ero1β. The high expression of Ero1β in the pancreas and salivary gland indicates a specific role of the protein in secretory tissues. Accordingly, oxidative folding of pro-insulin is impeded in pancreatic islet cells derived from Ero1β-compromised mice, an effect that is not exacerbated by concomitantly compromising Ero1α function [45]. However, increasing disulfide-bond formation by exogenous Ero1α expression stimulates oxidative folding of pro-insulin [46]. These observations suggest that the loose regulation of Ero1β activity could have evolved to optimally support the high demand of disulfide bonds in secretory tissues.

**AUTHOR CONTRIBUTION**

Henning Hansen and Lars Ellgaard designed and supervised the experimental work. Henning Hansen, Cecilie Søltoft, Jonas Schmidt, Julia Birk and Christian Appenzeller-Herzog performed the experiments. Henning Hansen and Lars Ellgaard wrote the paper, and Christian Appenzeller-Herzog contributed to revision of the paper.

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SUPPLEMENTARY DATA

Biochemical evidence that regulation of Ero1β activity in human cells does not involve the isoform-specific cysteine 262

Henning G. HANSEN*, Cecilie L. SØLTOFT*, Jonas D. SCHMIDT*, Julia BIRK†, Christian APPENZELLER-HERZOG*† and Lars ELLGAARD*3

*Department of Biology, University of Copenhagen, 2200 Copenhagen, Denmark
†Department of Pharmaceutical Sciences, University of Basel, 4056 Basel, Switzerland

Supplementary Figure S1 is on the following page.
Figure S1  Evolutionary conservation of Ero1β

A multiple sequence alignment of Ero1β orthologs was performed with Muscle [1] using the following UniProt entries. *Xenopus tropicalis* (frog; F6ULN4); *Gallus gallus* (chicken; E1C917); *Anolis carolinensis* (lizard; G1KAL4); *Monodelphis domestica* (opossum; F7CL82); *Mus musculus* (mouse; Q8R2E9); *Canis familiaris* (dog; F1Q091); *Homo sapiens* (human; Q86YB8); *Danio rerio* (zebrafish; E7F2A8); *Oryzias latipes* (rice fish; H2L719); *Takifugu rubripes* (pufferfish; H2TT03). Black boxes indicate amino acid identities and grey boxes show amino acid similarities when found in at least seven of the nine sequences. The human sequence is shown in boldface, cysteine residues are shown in red colour and amino acid position of the cysteine residues in the human sequence is indicated above the alignment.

REFERENCE


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