Crystal structure of Ero1α, a flavoenzyme responsible for protein disulfide generation in human cells

Many secretory and membrane proteins form disulfide bonds in the endoplasmic reticulum (ER), under the assistance of numerous thiol-disulfide oxidoreductases. The major players in this oxidative reaction are protein disulfide isomerase (PDI) and Ero1, both highly conserved from yeast to mammals. Ero1 generates disulfide bonds *de novo* in conjunction with a flavin adenine dinucleotide (FAD) cofactor and transfers them to PDI. Importantly, since the generation of each disulfide bond by Ero1 is accompanied by the production of one molecule of hydrogen peroxide (H_2O_2), a potential reactive oxygen species (ROS) source, Ero1 activity must be strictly regulated in living cells. Recent studies have suggested that human Ero1, called Ero1α, has four regulatory cysteines (Cys94, Cys99, Cys104, and Cys131) and modulates its own oxidative activity through disulfide rearrangement among these cysteines: an active form contains the Cys94-Cys99 disulfide while an inactive form possesses the Cys94-Cys131 and possibly Cys99-Cys104 disulfides [1,2]. A similar feedback regulation mechanism is likely to operate in yeast Ero1p [3].

In this work, we determined the crystal structures of active and inactive forms of human Ero1α at 2.35 and 3.07 Å resolutions, respectively, using beamline **BL44XU** [4]. The entire human Ero1α chain was found to have a single globular fold highly rich in α-helices, in which five intramolecular disulfide bonds were identified (Cys35-48, Cys37-46, Cys85-Cys391, Cys208-Cys241, and Cys394-Cys397) (Fig. 1). The catalytic core region of human Ero1α constitutes the four-helix bundle scaffold with an interior electron-accepting cofactor, which is a hallmark of disulfide bond-generating enzymes widely distributed from bacteria to eukaryotes [5]. The detailed structure of the FAD-proximal site revealed that a protein disulfide bond is generated *de novo* by the cooperation of the Cys394-Cys397 pair and bound FAD (Fig. 2). We thus propose that a charge transfer (CT) complex and a C(4a) covalent adduct between flavin and a thiolate anion of Cys397 are transiently formed as obligatory steps for disulfide bond generation (Fig. 2).

Notably, all of the regulatory cysteines were contained in the highly flexible loop lacking electron density (called a 'regulatory loop'), and therefore their locations could not be specified in active Ero1α (Fig. 3(a), left). It is conceivable that the intrinsically flexible nature of this loop is essential for electron shuttling from PDI to the FAD-proximal site of Ero1α. In sharp contrast, inactive Ero1α exhibited significant electron density derived from the Cys94-Cys131 disulfide and its neighboring segment (Fig. 3(a), right). This was most likely due to the increased constraint caused by the disulfide linkage between Cys94 and Cys131, which could be the primary reason for the impaired intramolecular electron shuttle in Ero1α. In agreement with this notion, the insertion of a three- or six-glycine repeat into the site immediately after Cys131 significantly restored the PDI oxidation activity of inactive Ero1α, probably because of the increase in the mobility of the regulatory loop. Thus, human Ero1α modulates its oxidative activity by properly positioning regulatory cysteines within an intrinsically flexible loop, and by finely tuning the electron shuttle ability of the loop through disulfide rearrangements (Fig. 3(b)).

Another central issue concerning the Ero1α-PDI oxidative system is how Ero1α specifically and effectively oxidizes PDI among the nearly twenty ER-resident PDI family member proteins. Although PDI and ERp57 assume a similar overall fold and three-dimensional arrangement of four thioredoxin-like domains, the latter was reported to be a poor and presumably nonphysiologic substrate of Ero1α.

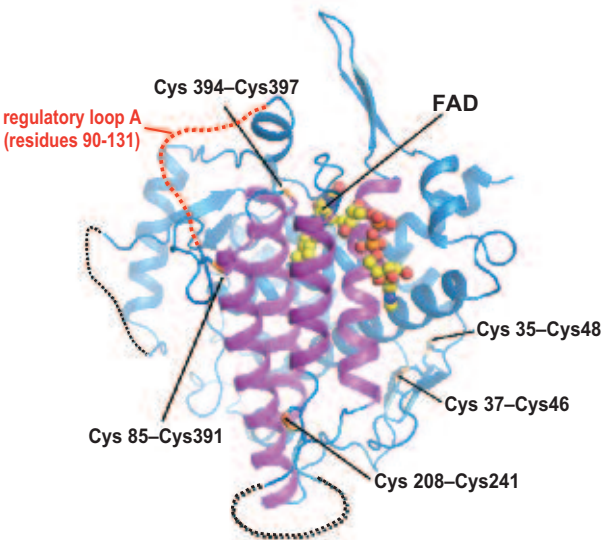

Fig. 1. Overall structure of human Ero1α. The crystal structure of the active form of human Ero1α is represented by a ribbon diagram. Loop segments that could not be modeled owing to the lack of electron density are shown by dotted lines. The regulatory loop including Cys94, Cys99, Cys104, and Cys131 is illustrated by a red dotted line. Structural and active-site disulfides characterized in this study are represented by sticks. The four-helix bundle constituting the catalytic core region of Ero1α is shown in magenta. The FAD molecule is represented by balls, in which carbon, nitrogen, oxygen, and phosphorus atoms are shown in yellow, blue, red, and orange, respectively.

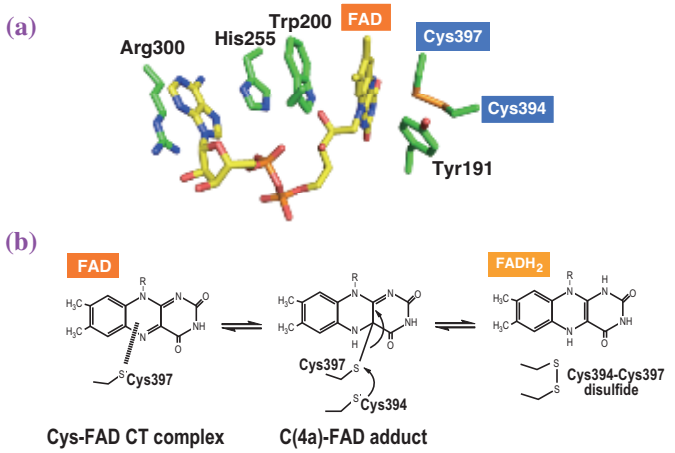

Fig. 2. FAD-binding mode of human Ero1α. **(a)** Structure of the FAD-binding site in human Ero1α. Residues involved in aromatic ring stacking and van der Waals contact with the FAD moiety are numbered and shown in stick representation. **(b)** Proposed chemical scheme of *de novo* disulfide bond generation by the cooperation of the Cys394-Cys397 pair of human Ero1α and FAD.

Taking into account that the b'-domain of PDI uniquely contains a hydrophobic pocket, we constructed two chimeric proteins in which the b'-domain was mutually replaced between PDI and ERp57. Interestingly, the ERp57-based chimera possessing the PDI b'-domain in place of the ERp57 b'-domain was oxidized by Ero1α as effectively as wild-type PDI. Conversely, the PDI-based chimera that had the ERp57 b'-domain substituted for the PDI b'-domain exhibited an extremely slow oxidation by Ero1α. The surface plasmon resonance measurements also demonstrated that the replacement of the b'-domain between PDI and ERp57 strongly affected the affinity for Ero1α of these two enzymes. Taken together, we conclude that the PDI b'-domain is a key functional element that determines the affinity and reactivity between PDI and Ero1α. The present findings elucidate the molecular basis of regulated and specific PDI oxidations by Ero1α.

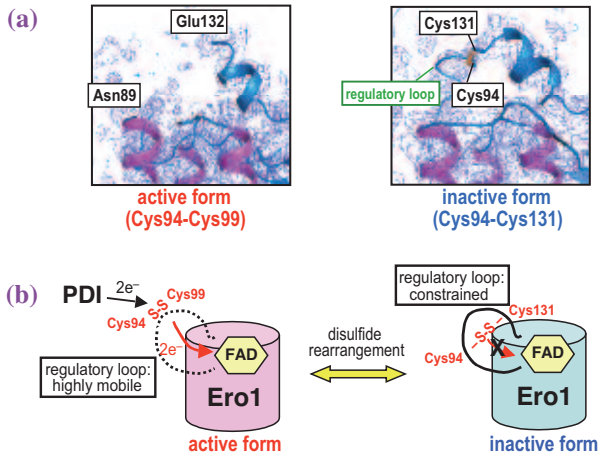

Fig. 3. Structural basis of Ero1α regulation. **(a)** Local conformational changes upon disulfide rearrangement within the regulatory loop. The Cys94-Cys131 disulfide formed in inactive Ero1α is shown in stick representation. The electron density map is shown at the 1.0 contour level. Note that electron density is completely invisible for the regulatory loop (residues 90-131) in the active form, but not in the inactive form. **(b)** Proposed model of Ero1α regulation through the internal disulfide rearrangement within the regulatory loop. The difference in combination pattern between the regulatory cysteines finely tunes the electron shuttle ability of the loop.

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References

[1] C. Appenzeller-Herzog *et al.*: EMBO J. **27** (2008) 2977.
[2] K.M. Baker *et al.*: EMBO J. **27** (2008) 2988.
[3] C.S. Sevier *et al.*: Cell **129** (2007) 333.
[4] *K. Inaba, S. Masui, H. Iida, S. Vavassori, R. Sitia and M. Suzuki: EMBO J. **29** (2010) 3330.*
[5] K. Inaba: Genes Cells **15** (2010) 935.