Published in final edited form as:

ACS Chem Biol. 2020 January 17; 15(1): 28–32. doi:10.1021/acscchembio.9b00662.

A regulatory cysteine residue mediates reversible inactivation of NAD⁺-dependent aldehyde dehydrogenases to promote oxidative stress response

Yugang Zhang¹, Miao Wang¹, Hening Lin^{*,2}

¹Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853, USA

²Howard Hughes Medical Institution; Department of Chemistry and Chemical Biology, Ithaca, NY 14853, USA.

Abstract

Aldehyde dehydrogenases (ALDHs) are a large family of enzymes that oxidize aldehydes into carboxylic acids. All ALDHs have a conserved catalytic cysteine residue, but different co-factors preference for NAD⁺ or NADP⁺. We discovered a CC motif composed of the catalytic and an adjacent cysteine, which are prone to disulfide bond formation under oxidative stress. This facilitates rapid detection of and response to oxidants, as well as protects the catalytic cysteine from over-oxidation into irreversible products. In ALDHs, the CC motif only exists in NAD⁺-dependent ones, which leads to selective inhibition of NAD⁺-dependent ALDHs under oxidative stress, diverting carbon source to the NADPH producing ALDHs. This alleviates the oxidative stress and promotes cell survival. Our findings revealed a novel regulatory mechanism for ALDHs that functions in oxidative stress response. Many enzymes with catalytic cysteine residues have proximal cysteine, suggesting that such a regulatory mechanism may be general.

Graphical Abstract

*Corresponding Author: hl379@cornell.edu.

Author contribution

Y.Z. and H.L. designed experiments and wrote the manuscript. Y.Z. and M.W. performed cloning and protein purification. Y.Z. performed kinetics measurements and yeast growth assay. Y.Z. and M.W. did the survival assay.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website

Full details of material and methods, including purification of each proteins, kinetics measurements, yeast growth assay, and yeast survival assay, as well as Fig S1-S7 and Table S1-S3.

The authors declare no competing financial interests.

Organisms live in an oxygen environment encounter oxidative stress mediated by reactive oxygen, nitrogen, or sulfur species.¹⁻³ Oxidative stress causes damage to cellular lipids, proteins and DNA, leading to aging and a myriad of human disease including cancer, cardiovascular disease, and diabetes.² A variety of signaling mechanisms exist to sense and counteract oxidative stress. Such signaling mechanism is often mediated by cysteine residues on proteins because it can form unique post translational modifications upon oxidation, including disulfide, sulphenic acid, sulphenamide, sulphinic acid, and sulphonic acid.^{1,3-5} An important way for cell to overcome oxidative stress is via the regulation of glucose flux through GAPDH. Upon oxidative stress, the active site cysteine residue of GAPDH can be oxidized and inhibits the enzyme activity⁶. The inhibition of GAPDH turns off the glycolysis and diverts glucose flux toward pentose phosphate pathway and synthesis of NADPH. NADPH serves as the reductant for NADPH-dependent thioredoxin or glutaredoxin reductase² and its production counteracts oxidative stress. After the elimination of oxidative stress, reduced glutathione and thioredoxin restore the activity of GAPDH. In this way, cell fine-tunes the flux of glucose via reversible oxidation of the catalytic cysteine residue in GAPDH⁵.

Another common strategy for redox regulation is through redox disulfide bond switches as disulfide bond formation is reversible.⁷ Redox sensitive disulfide bond is used in cysteine coordinated metal centers, especially zinc binding proteins.⁷ Upon oxidation, the release of metal ion triggers structural change and following signaling event, such as bacterial chaperone protein Hsp33.^{8,9} Similar strategy was also used in transcription factors. The formation of disulfide bond changes the DNA binding capacity of the transcription factor and triggers response, such as bacterial transcription factor OxyR.^{10,11} Some of the active sites in enzymes can also form disulfide thus regulating its own activity.⁷

Aldehyde dehydrogenases (ALDHs) are a class of metabolic enzymes that convert aldehyde into carboxylic acid. Similar to GAPDH, they also have a catalytic cysteine residue (Fig S1). In human, there are 19 different enzymes classified as ALDHs.¹² They play important roles in cancer, stem cells, metabolism, and clearance of aldehydes.^{13,14} In *Saccharomyces cerevisiae*, there are 5 isoforms of ALDHs.¹⁵ They are important for yeast to metabolize ethanol.¹⁶⁻¹⁸ ALDHs are either NAD⁺-dependent or NADP⁺-dependent, generating NADH and NADPH, respectively, via their enzymatic activity. Here we identify a regulatory cysteine adjacent to the catalytic cysteine in NAD⁺-dependent ALDHs, but not NADP⁺-dependent ALDHs. This regulatory cysteine regulates the activity of NAD⁺-dependent ALDHs through disulfide bond formation and contribute to cellular response toward oxidative stress.

We previously demonstrated that *in vitro* Ald4 can use both NAD⁺ and NADP⁺ as cofactors with similar efficiency. In cells, NAD⁺ concentration is much higher than that of NADP⁺, and thus, Ald4's major physiological cofactor should be NAD⁺ instead of NADP⁺.¹⁹ When detecting yeast Ald4 activity *in vitro*, we noticed that Ald4 had no activity *in vitro* when the reaction buffer does not contain any reducing reagents (Fig 1A, see Fig S2 for the purity of recombinant Ald4 protein). Ald6, which shares over 50% sequence similarities with Ald4, had activity under similar conditions and the activity of Ald6 only mildly increase with the treatment of DTT (Fig 1A). This difference between Ald4 and Ald6 caught our attention.

Through analysis of Ald4 sequence, we found that there is a C325 residue right next to the active site cysteine C324 in Ald4, while Ald6 does not contain an adjacent cysteine (Fig S3). Therefore, we hypothesized that the catalytic and adjacent cysteine might form disulfide bond upon oxidative stress, which will inactivate the enzyme (Fig S1). And indeed, the disulfide bond formation between Ald4 C324 and C325 was detected using mass spectrometry (MS, Fig 1C & 1D). To further validate this hypothesis, we incubated Ald4 with different reducing reagents. DTT and TCEP activate Ald4 dramatically while the physiological relevant reductant coenzyme A (CoA) and glutathione activates Ald4 moderately (Fig 1).

To test whether the activation of Ald4 is physiological relevant, we tested the response of Ald4 to glutathione. The physiological concentration of glutathione is about 1 mM to 10 mM²⁰. Ald4 activity increased linearly as glutathione increased from 0 to 10 mM, indicating that Ald4 activation can be achieved in the cell (Fig 2A).

To find out whether C325 is playing an essential role in inactivating Ald4 upon oxidation, we generated an Ald4 C325S mutant. The mutant was purified to homogeneity (Fig S2) and the activity was tested. The mutant enzyme shows similar activity as wild type enzyme in fully reduced conditions (Table S1). And as expected, the C325S mutant showed activity even when no glutathione was added. The addition of glutathione did not significantly change its activity (Fig 2A). The lower activity of Ald4 C325S under 10 mM glutathione is due to the irreversible oxidation during protein purification.

ALDHs are conserved across the kingdoms of life. In human, there are 19 different ALDHs. Aldh1 and Aldh2 family contain adjacent cysteine (either before or after the catalytic cysteine) suggesting that this regulatory mechanism is conserved in human ALDHs (Fig S3). The two adjacent cysteines in Aldh1a1, C302 and C303 are exposed in the protein fold and are in the correct orientation to form disulfide bond (Fig S4)²¹. To verify this hypothesis, we expressed and purified human Aldh1a1 (Fig S2). The enzyme activity of Aldh1a1 shows similar response to glutathione as Ald4 (Fig 2B). Through MS, the disulfide bond between active site cysteine C303 and adjacent cysteine C302 was detected (Fig S5). Thus, this regulatory mechanism is likely general to all ALDH members with the adjacent cysteine.

The two cysteines adjacent can form disulfide bond upon oxidation while single cysteine is oxidized into sulphenic acid and further irreversibly into sulphinic and sulphonic acids³. The disulfide bond can be reduced easily with reductant and restore the enzyme activity. Therefore, the adjacent cysteine not only can inactivate enzyme upon oxidation but can also help to regain enzymatic activity upon removal of oxidative conditions. To test the hypothesis, we treated both Ald4 WT and Ald4 C325S with H₂O₂ to inactivate the enzyme and recover the enzyme activity with DTT afterwards. Indeed, after DTT treatment, Ald4 WT shows much higher activity than Ald4 C325S mutant suggesting Ald4 WT inactivation is reversible (Fig 3).

Yeast contains 5 isoforms of aldehyde dehydrogenase, Ald2, Ald3, Ald4, Ald5 and Ald6.¹⁵ Among them, Ald2 and Ald3 are mostly responsible for CoA generation by oxidizing 3-aminopropanal into β -alanine.¹⁸ Ald4, Ald5 and Ald6 are mostly responsible for

metabolizing acetaldehyde generated through ethanol metabolism. Therefore, Ald4, Ald5 and Ald6 play important roles in yeast survival when using ethanol as the carbon source. Both Ald4 and Ald5 preferentially use NAD^+ as cofactors in cells while Ald6 only utilizes NADP^+ . With ethanol as the carbon source, the pentose phosphate pathway, a major pathway to generate NADPH, is shut down as the substrate is not available. In this case, Ald6 is utilized as a major pathway for NADPH production.^{19,22} Instead of cysteine, Ald6 has a serine next to the active site cysteine. As the regulatory cysteine inactivates Ald4 during oxidative stress, we hypothesized that under oxidative stress, Ald6 remains active while Ald4 is inactivated, thus diverting all acetaldehyde to Ald6 for the production of NADPH. This way, yeast can survive oxidative stress better.

To test the hypothesis, we expressed either Ald4 WT or Ald4 C325S in Ald4 knockout (*ald4*) strain under the original promoter of Ald4 (Fig S6)^{23,24}. The expression level of the mutant and wild type Ald4 was verified with western blot (Fig S7). The resulting two strains grew similarly without H_2O_2 treatment, but the strain with Ald4 WT grew better with H_2O_2 treatment (Fig 4A). An even more pronounced difference was observed when we measured survival (instead of simple growth) under H_2O_2 treatment. Ald4 WT and Ald4 C325S yeast cells were treated with 3 mM H_2O_2 for 6 hours and then plated on regular YPD plate. Yeast expressing Ald4 WT showed better survival compared with yeast expressing Ald4 C325S (Fig 4B), indicating the importance of the adjacent regulatory cysteine in yeast survival under oxidative stress. The NADPH concentration was also measured in Ald4 WT and Ald4 C325S strains. Under normal conditions, no significant difference was observed. But under 2 mM H_2O_2 treatment, Ald4 C325S has lower NADPH concentration (Fig 4C).

Oxidation of cysteine is a common way for cells to detect and deal with oxidative stress. Compared with cysteine forming typical disulfide bond, vicinal disulfide bond is considered more sensitive to redox regulation.²⁵ The fast formation of vicinal disulfide bond prevents the irreversible oxidation of the cysteine. Here we identified the regulatory mechanism for NAD^+ -dependent ALDHs via cysteine oxidation and vicinal disulfide formation. We demonstrated the physiological function of this regulation using yeast Ald4 and Ald6. While both enzymes convert aldehyde to acetate and are important for yeast ethanol metabolism, they differ in cofactor utilization and the regulatory mechanism. Ald4 is NAD^+ -dependent while Ald6 is NADP^+ -dependent. The regulation of activity via disulfide formation between the catalytic cysteine and adjacent regulatory cysteine is only present in the NAD^+ -dependent Ald4, but not in the NADP^+ -dependent Ald6. This regulatory mechanism for Ald4 ensures the fast inactivation of Ald4 upon oxidation and the reactivation upon reduction. The inactivation of Ald4 under oxidative stress diverts the carbon flux to the NADPH-producing enzyme Ald6, which does not have the regulatory cysteine and thus is not inhibited under similar conditions. This way, yeast can survive better under oxidative stress.

The adjacent regulatory cysteine is present in human Aldh1 and Aldh2 family members. Aldh1a1 is highly overexpressed in certain types of cancer cells, stem cells, and cancer stem cells¹³. Although the exact function of Aldh1a1 is not clear, the ability of Aldh1 to metabolize retinal into retinoic acid and clear acetaldehyde together with Aldh2 may underline its function in sustaining cancer cells and stem cells¹³. In fact, the expression of

Aldh1a1 is so high that a well-designed assay, Aldefluor assay, was used to detect Aldh1a1 and the presence of stem cells or cancer stem cells²⁶. We speculate that such a regulatory mechanism may play important roles in human physiology as well.

An added value for this regulatory mechanism is that it can be used to predict the cofactor-dependence of ALDHs. Our understanding described above suggest that any ALDH containing such an adjacent regulatory cysteine should be NAD⁺-dependent. Indeed, all the ALDHs with the adjacent regulatory cysteine shown in Fig S4 (yeast Ald4, Ald5, and all the human members shown) are NAD⁺-dependent. However, the absence of this regulatory cysteine does not necessarily predict that the ALDH member is NADP⁺-dependent. For example, yeast Ald2 and Ald3 do not contain the regulatory cysteine, but they are known to be NAD⁺-dependent.

A cysteine residue adjacent to catalytic cysteine in sequence or in space are also found in other enzymes.²⁷ Atg4b, a cysteine protease essential for the initiation of apoptosis, has a cysteine close to catalytic cysteine in the structure.²⁸ Acat2, an enzyme important in ketone body biosynthesis, similarly has a cysteine close to catalytic cysteine in the enzyme structure²⁹. It is likely these enzymes are regulated similarly by oxidative stress as ALDHs. The 'CC' sequence motif can be found in many proteins in the whole proteome, suggesting the regulatory mechanism described here may broadly apply to other proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENT

We thank R. Bhawal and the Proteomics Facility of Cornell University for help with mass spectrometry, NIH SIG grant 1S10 OD017992-01 grant support for the Orbitrap Fusion mass spectrometer. pFA6a-6xGLY-3xFLAG-HIS3MX6 was a gift from M. Hochstrasser (Addgene plasmid # 20753; <http://n2t.net/addgene:20753>; RRID: Addgene_20753). The work is supported by an NIH/NIGMS grant R35GM131808.

References

- (1). D'Autréaux B; Toledano MB ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat. Rev. Mol. Cell Biol* 2007, 8, 813. [PubMed: 17848967]
- (2). Valko M; Leibfritz D; Moncol J; Cronin MT; Mazur M; Telser J Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol* 2007, 39, 44–84. [PubMed: 16978905]
- (3). Paulsen CE; Carroll KS Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. *Chem. Rev* 2013, 113, 4633–4679. [PubMed: 23514336]
- (4). Winterbourn CC; Hampton MB Thiol chemistry and specificity in redox signaling. *Free Radic. Biol. Med* 2008, 45, 549–561. [PubMed: 18544350]
- (5). Brandes N; Schmitt S; Jakob U Thiol-based redox switches in eukaryotic proteins. *Antioxid. Redox Signal* 2009, 11, 997–1014. [PubMed: 18999917]
- (6). Peralta D; Bronowska AK; Morgan B; Doka E; Van Laer K; Nagy P; Grater F; Dick TP A proton relay enhances H₂O₂ sensitivity of GAPDH to facilitate metabolic adaptation. *Nat. Chem. Biol* 2015, 11, 156–163. [PubMed: 25580853]
- (7). Cremers CM; Jakob U Oxidant sensing by reversible disulfide bond formation. *J. Biol. Chem* 2013, 288, 26489–26496. [PubMed: 23861395]

- (8). Winter J; Ilbert M; Graf PC; Ozcelik D; Jakob U Bleach activates a redox-regulated chaperone by oxidative protein unfolding. *Cell* 2008, 135, 691–701. [PubMed: 19013278]
- (9). Jakob U; Eser M; Bardwell JC Redox switch of hsp33 has a novel zinc-binding motif. *J. Biol. Chem* 2000, 275, 38302–38310. [PubMed: 10976105]
- (10). Zheng M; Aslund F; Storz G Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 1998, 279, 1718–1721. [PubMed: 9497290]
- (11). Choi H; Kim S; Mukhopadhyay P; Cho S; Woo J; Storz G; Ryu SE Structural basis of the redox switch in the OxyR transcription factor. *Cell* 2001, 105, 103–113. [PubMed: 11301006]
- (12). Jackson B; Brocker C; Thompson DC; Black W; Vasiliou K; Nebert DW; Vasiliou V Update on the aldehyde dehydrogenase gene (ALDH) superfamily. *Hum. Genomics* 2011, 5, 283–303. [PubMed: 21712190]
- (13). Tomita H; Tanaka K; Tanaka T; Hara A Aldehyde dehydrogenase 1A1 in stem cells and cancer. *Oncotarget* 2016, 7, 11018–11032. [PubMed: 26783961]
- (14). Chang PM-H; Chen C-H; Yeh C-C; Lu H-J; Liu T-T; Chen M-H; Liu C-Y; Wu ATH; Yang M-H; Tai S-K; Mochly-Rosen D; Huang C-YF Transcriptome analysis and prognosis of ALDH isoforms in human cancer. *Sci. Rep* 2018, 8, 2713. [PubMed: 29426835]
- (15). Navarro-Avino JP; Prasad R; Miralles VJ; Benito RM; Serrano R A proposal for nomenclature of aldehyde dehydrogenases in *Saccharomyces cerevisiae* and characterization of the stress-inducible ALD2 and ALD3 genes. *Yeast* 1999, 15, 829–842. [PubMed: 10407263]
- (16). Boubekour S; Bunoust O; Camougrand N; Castroviejo M; Rigoulet M; Guerin B A mitochondrial pyruvate dehydrogenase bypass in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem* 1999, 274, 21044–21048. [PubMed: 10409655]
- (17). Tessier WD; Meaden PG; Dickinson FM; Midgley M Identification and disruption of the gene encoding the K(+)-activated acetaldehyde dehydrogenase of *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett* 1998, 164, 29–34. [PubMed: 9675847]
- (18). White WH; Skatrud PL; Xue Z; Toyn JH Specialization of function among aldehyde dehydrogenases: the ALD2 and ALD3 genes are required for beta-alanine biosynthesis in *Saccharomyces cerevisiae*. *Genetics* 2003, 163, 69–77. [PubMed: 12586697]
- (19). Zhang Y; Lin Z; Wang M; Lin H Selective Usage of Isozymes for Stress Response. *ACS Chem. Biol* 2018, 13, 3059–3064. [PubMed: 30346689]
- (20). Meister A Glutathione metabolism and its selective modification. *J. Biol. Chem* 1988, 263, 17205–17208. [PubMed: 3053703]
- (21). Morgan CA; Hurley TD Development of a high-throughput in vitro assay to identify selective inhibitors for human ALDH1A1. *Chem. Biol. Interact* 2015, 234, 29–37. [PubMed: 25450233]
- (22). Grabowska D; Chelstowska A The ALD6 gene product is indispensable for providing NADPH in yeast cells lacking glucose-6-phosphate dehydrogenase activity. *J. Biol. Chem* 2003, 278, 13984–13988. [PubMed: 12584194]
- (23). Longtine MS; McKenzie A 3rd; Demarini DJ; Shah NG; Wach A; Brachet A; Philippsen P; Pringle JR Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 1998, 14, 953–961. [PubMed: 9717241]
- (24). Funakoshi M; Hochstrasser M Small epitope-linker modules for PCR-based C-terminal tagging in *Saccharomyces cerevisiae*. *Yeast* 2009, 26, 185–192. [PubMed: 19243080]
- (25). Kilgore HR; Raines RT π - π^* Interactions Modulate the Properties of Cysteine Residues and Disulfide Bonds in Proteins. *J. Am. Chem. Soc* 2018, 140, 17606–17611. [PubMed: 30403347]
- (26). Ginestier C; Hur MH; Charafe-Jauffret E; Monville F; Dutcher J; Brown M; Jacquemier J; Viens P; Kleer CG; Liu S; Schott A; Hayes D; Birnbaum D; Wicha MS; Dontu G ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007, 1, 555–567. [PubMed: 18371393]
- (27). Richardson JS; Videau LL; Williams CJ; Richardson DC Broad Analysis of Vicinal Disulfides: Occurrences, Conformations with Cis or with Trans Peptides, and Functional Roles Including Sugar Binding. *J. Mol. Biol* 2017, 429, 1321–1335. [PubMed: 28336403]
- (28). Sugawara K; Suzuki NN; Fujioka Y; Mizushima N; Ohsumi Y; Inagaki F Structural basis for the specificity and catalysis of human Atg4B responsible for mammalian autophagy. *J. Biol. Chem* 2005, 280, 40058–40065. [PubMed: 16183633]

- (29). Kursula P; Sikkila H; Fukao T; Kondo N; Wierenga RK High resolution crystal structures of human cytosolic thiolase (CT): a comparison of the active sites of human CT, bacterial thiolase, and bacterial KAS I. *J. Mol. Biol* 2005, 347, 189–201. [PubMed: 15733928]

Figure 1. Activation of Ald4 by reducing reagent.

Rate of Ald4/Ald6 was shown as increase in NADH/NADPH absorption at 340 nm. (A) The rates of Ald4 and Ald6 with and without reductants (DTT or TCEP, 1 mM). (B) The rates of Ald4 with 1 mM glutathione (GSH) or 1 mM CoA or no reductant. (C) Identification of C324-C325 disulfide bond in Ald4 by tandem MS. Disulfide formation was indicated by the black line on the peptide. The peptide mass is (D) DTT treatment reduced the disulfide bond formed between C324 and C325 and carbamidomethylation of C324 and C325 was observed.

Figure 2. The response to glutathione by yeast Ald4, Ald4 C325S, and human Aldh1a1.
A) The rates of Ald4 WT and C325S mutant were plotted against glutathione concentration.
B) The rates of Aldh1a1 was plotted against glutathione concentration.

Figure 3. Ald4 WT with adjacent cysteine can be inactivated by H_2O_2 and activated by DTT reversibly.

The rates after H_2O_2 treatment or H_2O_2 followed by DTT treatment were normalized to that before H_2O_2 treatment. Significance was calculated with two-tailed t-test (*** $P < 0.001$).

Figure 4. Ald4 WT yeast survives better in oxidative stress than Ald4 C325S mutant.

(A) Yeast growth assay. Ald4 WT or Ald4 C325S were expressed in *ald4* strain and the cells were cultured on SC agar plates with 2% ethanol as the carbon source with or without 1 mM H₂O₂ treatment. (B) Yeast survival assay. The survival of *ald4* strain re-expressing either Ald4 WT or Ald4 C325S were tested by plating the cultures on YPD agar plates. The survival rate after H₂O₂ treatment was calculated as the colony number after H₂O₂ incubation divided by that without H₂O₂ incubation. Significance was calculated with two-tailed t-test (**P<0.01). (C) NADPH measurement in *ald4* knock-in with Ald4 and Ald4 C325S with or without 2 mM H₂O₂ treatment. NADPH concentration in the yeast was normalized to yeast cell number. Significance was calculated with two-tailed t test. (*P<0.05)