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REVIEW ARTICLE

Intermolecular disulfide bond to modulate protein function as a redox-sensing switch

N. Nagahara

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Abstract Recently, redox-regulated biological reactions have been elucidated. In the regulation of these reactions, redox-sensing molecular switches function as unique biological machineries that modulate the functional proteins present in enzymes, transcriptional factors, sensor proteins, and transcriptional factor modulators. The redox-sensing cysteine residues and the disulfide bond formed between these cysteine residues serve as redox-sensing molecular switches; these switches sense cellular oxidizing factors such as oxygen, reactive oxygen species, and cellular reducing factors such as thioredoxin (Trx), glutathione (GSH), and their family molecules. Depending on the redox status, the switch directly modulates the protein function via the "locking and unlocking" of the critically functional residue or indirectly modulates the protein function via "protein conformational changes," which affects the functioning of a distantly located critical residue in an allostery-like fashion or a topology change. Redox-sensing switches can be classified into two types—intramolecular (intrasubunit) and intermolecular (intersubunit) ones. Further, depending on the sensing specificity to reducing factors, the switch subtype is classified into Trx, GSH, or their family molecules-specific type. This review focused on the intermolecular redox-sensing switches found in various proteins.

Keywords Disulfide bond · Glutathione · Redox-sensitive cysteine · Redox-sensing switch · Thioredoxin

N. Nagahara (✉)
Department of Environmental Medicine, Nippon Medical School, 1-1-5 Sendagi Bunkyo-ku, Tokyo 113-8602, Japan
e-mail: noriyuki@nms.ac.jp

Introduction

Living organisms, which evolved during and after an increase in the levels and concentration of atmospheric oxygen on Earth, have used the abundant oxygen and molecules reacting with oxygen have evolved. In the cells, the overall reducing conditions are highly maintained (Giusn et al. 1999) because the reducing systems, including thioredoxin (Trx), glutathione (GSH), and their families, function to maintain redox equilibrium. However, these conditions are not uniform in the cell, and are locally inclined to oxidizing conditions due to newly produced oxidants. Oxygen is metabolized to produce active oxygen species in the cells, and further, the metabolism is facilitated in response to various stresses. Thus, the thiol status of proteins must be dynamic in the cells (Linke and Jakob 2003).

Two cysteines are oxidized to form a disulfide bond, depending on the topology, and the temperature- and pH-dependent redox potential of cysteine–cystine. In fact, oxidative stress easily leads to the formation of a large number of intra- and inter-protein disulfides between the two cysteines (Biswas et al. 2006; Brennan et al. 2004; Cumming et al. 2004). When a single cysteine residue is oxidized, sulfenate ($CysS\gamma O^-$), sulfinate ($CysS\gamma O_2^-$), and sulfonate ($CysS\gamma O_3^-$) are formed (Lugo-Mas et al. 2006; Nagahara and Katayama 2005; Poole et al. 1989), and sulfenate easily reacts with other thiols to form a disulfide. In the rare case of protein-tyrosine phosphatase IB (EC 3.1.3.48), the sulfenate reacts with the main chain nitrogen atom of an adjacent serine to form a sulfenyl amide (Salmeen et al. 2003; van Montfort et al. 2003).

While the two specific cysteines donate electrons to oxidizing factors, resulting in the formation of a disulfide bond, and the disulfide bond accepts electrons from

reducing reagents, these redox-sensitive cysteines and disulfide bond serve as redox-sensing switches to modulate the protein functions. The formation of the intermolecular disulfide bond induces a possible conformational change of the protein with an oligomeric transformation (oligomerization), which modulates the protein function. Then, reducing reagents cleave the disulfide bond to modulate the protein function via a possible conformational change. The investigation of the intermolecular switch has been progressed, because proteomics can be easily performed using an electron spray ionization, a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), or a reversed-phase liquid chromatography (LC) mass spectrometry for peptides (Bass et al. 2007) after two-dimensional polyacrylamide gel electrophoresis (PAGE), proteolysis with proteinases, and/or chemical modifications of cysteine residues (Nagahara et al. 2009a).

Classification of redox-sensing molecular switches

A redox-sensing switch consists of two redox-sensitive cysteine residues and the disulfide bond between them. Under oxidizing conditions, formation of an inter- and/or intra-molecular disulfide bond often leads to a change in the protein function, probably linked to a conformational change of the protein with or without oligomer transition. Then, cellular reducing factors (Trx, GSH, or their family molecules) cleave the disulfide bond with or without transformation of the oligomeric state, which regulates the protein function.

Redox-sensing switches can be classified into two types—intramolecular (intrasubunit) and intermolecular (intersubunit). Proteins, carrying intramolecular and/or intermolecular redox-sensing switches, include enzymes, transcriptional factors, transcriptional factor modulators, receptor proteins, and sensor proteins. Some reported proteins carrying intramolecular redox-sensing switches, are enzymes such as Ero1p (Sevier and Kaiser 2006), phosphatase Cdc25B (Sohn and Rudolph 2003), α-glucan, water dikinase (Mikkelsen et al. 2005), chloroplast fructose-1, 6-bisphosphatase (Clancy and Gilbert 1987), and protein disulphide-isomerase (Hawkins et al. 1991), and a transcriptional factor such as OxyR (Zheng et al. 1998).

Some proteins, carrying intermolecular redox-sensing switches, are listed in Table 1. The subtype of the switch classification depends on the sensing specificity to reducing factors (Trx, GSH, and/or their family molecules). Further, there are two regulation mechanisms, namely, the direct modulation of protein function via the "locking and unlocking" of a critical cysteine residue and indirect modulation via "a conformational change," which affects the function of a distantly located critical residue in an allostery-like fashion or a topology change.

Enzymes carrying thioredoxin-specific intermolecular redox-sensing switches

(3-)Mercaptopyruvate sulfurtransferase

General aspects

Rat mercaptopyruvate sulfurtransferase (EC 2.8.1.2) catalyzes the degradation of cysteine, detoxifies cyanide (Nagahara et al. 1999), and possibly serves as an antioxidant (Nagahara and Katayama 2005; Nagahara et al. 2007). The enzyme is widely distributed in prokaryotes and eukaryotes. Eukaryotic MST is localized in the cytosol and mitochondria (Nagahara et al. 1998; Nakamura et al. 2000). Rat enzyme is a 32.8-kDa simple protein (Nagahara and Nishino 1996). The promoter regions of the human and rat mercaptopyruvate sulfurtransferase genes are characterized as a house-keeping gene (Nagahara et al. 2004). The enzyme contains five cysteines and three of which are exposed cysteines: a catalytic site cysteine, Cys^{247} (Fig. 1), in the active site and Cys^{154} and Cys^{263} (Fig. 1) on the surface of the enzyme (Nagahara and Nishino 1996). Cys^{154} is a unique cysteine for the rat enzyme, and on other hand, the corresponding cysteine to Cys^{263} is conserved in mammalian mercaptopyruvate sulfurtransferases (Nagahara 2007).

Reduced Trx activated mercaptopyruvate sulfurtransferase which has been treated with DTT, but DTT did not activate the activity which has been treated with reduced Trx (Nagahara et al. 2007). MALDI-TOF mass spectrometric analysis (Nagahara and Katayama 2005) and protein chemical study using iodoacetate (Nagahara et al. 2007) revealed that the catalytic site Cys^{247} is a target of the oxidants. The enzymatic activity was inhibited by a stoichiometric concentration of hydrogen peroxide, and the activity was completely restored by DTT, reduced Trx or Trx with a reducing system containing thioredoxin reductase (TRD) and NADPH, but reduced GSH does not restore the activity (Nagahara and Katayama 2005). Further, Trx peroxidase activity was also detected in the reduction process. It was concluded that a sulfenate ($CysS\gamma O^{-}$) is formed at Cys^{247} and that the redox potential of the sulfenate is lower than that of reduced GSH. These findings suggested that reduced Trx reacted with cysteines other than Cys^{247}. Another target cysteine residue of Trx to activate the enzymatic activity with transformation of the dimer to the monomer was determined to be Cys^{154} or Cys^{263} on the surface of the enzyme (Nagahara et al. 2007).

Table 1 Intermolecular switch-carrying proteins

Subtype[a]	Protein type	Name	Reducing factors[b]	Reference[c]
Thioredoxin-specific	Enzyme	Mercaptopyruvate sulfurtransferase[d]	Trx ≫ DTT (No effect of GSH)	Nagahara et al. (2007)
Non-specific	Enzyme	Cyclic AMP-dependent protein kinase[d,f]	DTT > Trx > GSH	de Piña et al. (2008)
Undetermined	Enzyme	Chloroplast acetyl-CoA carboxylase[d,f]	DTT (No data for Trx and GSH)[e]	Sasaki et al. (2001)
		2Cys-peroxiredoxin[d,f]	Trx, DTT (No data for GSH)	Matsumura et al. (2008)
		Cytosolic malate dehydrogenase[d]	Trx, DTT (No data for GSH)	Hara et al. (2006)
		Group VIA phospholipase A_2[d,f]	DTT (No data for Trx and GSH)[e]	Song et al. (2006)
		Isocitrate dehydrogenase kinase/phosphatase[d]	DTT (No data for Trx and GSH)[e]	Oudot et al. (1999)
		Mitochondrial cyanide-resistant alternative oxidase[d,f,g]	DTT (No data for Trx and GSH)[e]	Rhoads et al. (1998)
	Receptor protein (Enzyme)	Protein-tyrosine phosphatase[d,f,g]	DTT (No data for Trx and GSH)[e]	van der Wijk et al. (2004)
	Sensor protein (Enzyme)	RegB (redox sensor histidine kinase)[d,g]	DTT (No data for Trx and GSH)[e]	Swem et al. (2003)
	Transcriptional factor	CprK[d,f]	DTT (No data for Trx and GSH)[e]	Gupta and Ragsdale (2008)
		OhrR[d,f]	DTT (No data for Trx and GSH)[e]	Panmanee et al. (2006)
		Yap1[d]	Trx (No effect of GSH) (No data for DTT)	Delaunay et al. (2002)
	Transcriptional factor modulator	NEMO[d]	DTT (No data for Trx and GSH)[e]	Herscovitch et al. (2008)
	Other protein	β actin[f]	Trx (No data for GSH and DTT)	Lassing et al. (2007)

[a] The switch subtypes depending on Trx specificity

[b] The symbol ">" or "≫"represents comparison of each reducing power

[c] The study determined the action of each reducing factor on the redox-sensing switch

[d] Details are described in the text

[e] There is no evidence about whether the reduction proceeds in vivo

[f] Lock and unlock type

[g] protein without a domain necessary for the membrane anchoring

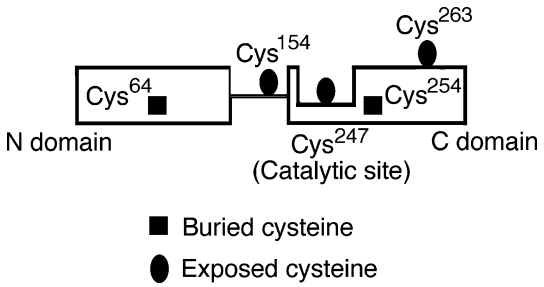

Fig. 1 Schema of rat mercaptopyruvate sulfurtransferase Rat MST contains 296 amino acid residues (Nagahara and Nishino 1996) and may consist of N- and C-domains

Under oxidizing conditions, a sulfur atom of each exposed cysteine (Cys^{247}, Cys^{154}, and Cys^{263}) donates an electron to the oxidants, and the cysteine is transiently converted to a sulfenate or is involved in the formation of a disulfide bond, resulting in the inhibition of the enzyme (Fig. 2). These findings imply that the enzyme partly contributes to maintain redox homeostasis.

Regulation mechanism of the enzymatic activity via the redox-sensing switch

Rat mercaptopyruvate sulfurtransferase has monomer–dimer equilibrium: overexpressed recombinant enzyme in *Escherichia coli* (*E. coli*) and authentic enzyme from rat liver exhibit the ratios of the monomer to the dimer as 92 to 8 and 2 to 1, respectively, in 0.2 M potassium phosphate buffer containing no reducing reagents under air-saturated conditions (Nagahara and Sawada 2006; Nagahara et al. 2007). The symmetrical dimer via an intersubunit disulfide bond between Cys^{154} and Cys^{154} and between Cys^{263} and Cys^{263}, or the asymmetrical dimer via an intersubunit disulfide bond between Cys^{154} and Cys^{263} may be formed under oxidizing conditions with H_2O_2. The high-performance liquid chromatography (HPLC) spectrum of double

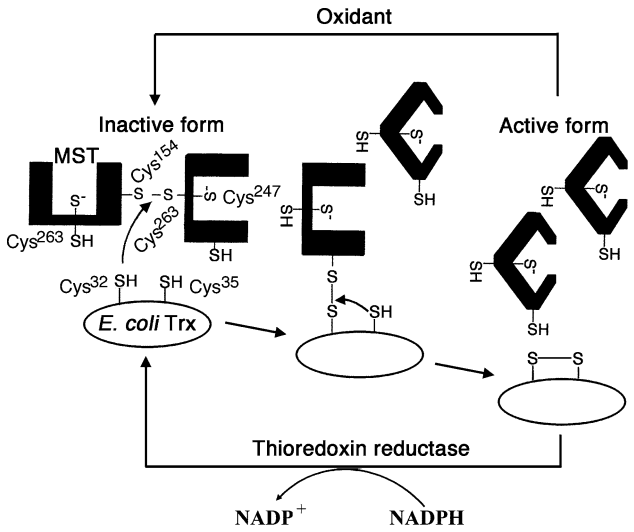

Fig. 2 Regulation of the enzymatic activity of mercaptopyruvate sulfurtransferase. Details are described in the text. MST, mercaptopyruvate sulfurtransferase

mutant enzyme, C154/263S, exhibited a single peak assigned to the monomer (Nagahara et al. 2007) (Fig. 2).

With regard to cleavage of the disulfide bond, the ratio in the most effective reducing system was 1:1:0.05:12.5 for [rat MST]:[rat Trx]:[rat TRD]:[NADPH], and 1:5:0.02:12.5 for [rat MST]:[*E. coli* Trx]:[*E. coli* TRD]:[NADPH] (Nagahara et al. 2007). In the *E. coli* Trx-TRD-NADPH system, MST activity increased to 4.5-fold that of the control. On other hand, in the rat Trx-TRD-NADPH system, MST activity increased to threefold that of the control (Nagahara et al. 2007). These findings imply that the *E. coli* reducing system is more effective than the rat system. Thus, theses cysteines and the disulfide bond serve as redox-sensing switches.

Escherichia coli reduced Trx cleaved the intersubunit disulfide bond to increase the activity to 2.3- and 4.9-fold that of DTT-treated and DTT-untreated mercaptopyruvate sulfurtransferase, respectively. *E. coli* Trx has only two exposed cysteine residues (Cys^{32} and Cys^{35}) and they are redox-active (NP_418228) (Musso et al. 1977). Rat Trx also activated mercaptopyruvate sulfurtransferase, but less effectively than *E. coli* reduced Trx. Rat Trx has three exposed cysteine residues: redox-active Cys^{31} and Cys^{34}, and Cys^{72} (NP_446252) (Wang et al. 2002). Cys^{72} is easily oxidized to form an intermolecular disulfide bond (Powis and Montfort 2001) and to undergo glutathionylation (Casagrande et al. 2002). Trx function changes with these modifications. Thus, *E. coli* Trx has been used to probe a Trx-dependent modification of mammalian protein function (Lundstrom and Holmgren 1990; Holmgren 1979; Park and Thomas 1989).

Escherichia coli C35S (Cys^{35} was replaced with Ser) Trx activated MST after treatment with DTT, and further, revised-phase HPLC analysis revealed some adducts with the enzyme (Nagahara et al. 2007). Thus, Cys^{32}, another redox-active cysteine, reacts with a sulfenyl Cys^{247} of mercaptopyruvate sulfurtransferase to form an intermolecular disulfide bond. Then, Cys^{35} of Trx cleaves the disulfide bond between Trx and the enzyme, resulting in activation of the enzymatic activity (Fig. 2).

Thioredoxin-specific reduction

It is noteworthy that DTT and reduced GSH much less activated the activity (cleave the disulfide bond) than reduced Trx (Nagahara et al. 2007). These findings imply that cleavage of the disulfide bond is Trx-specific.

As a similar case, the enzymatic activation of plant α-glucan water dikinase is also Trx-specific; oxidation inhibited via formation of a disulfide bond between Cys^{1004} and Cys^{1008}, and Trx_f more effectively restored the enzyme activity via reduction of the intrasubunit disulfide bond than DTT (Mikkelsen et al. 2005). The specificity of Trx_f for this enzyme is determined by a sequence "CFATC" contained in the active site (Mikkelsen et al. 2005), which is similar to a consensus sequence essential for Trx binding (a CXXXXC motif) (Sandalova et al. 2001; Waksman et al. 1994). On other hand, MST does not contain a sequence "CFATC" and the CXXXXC motif although sequences around Cys^{154} and Cys^{263} of rat MST are related or identical to parts of rat and *E. coli* TRD sequences (Nagahara et al. 2007). The reason for the affinity of thioredoxin for the enzyme has not been clarified.

Molecular evolution of the redox-sensing switch

Mercaptopyruvate sulfurtransferase and thiosulfate sulfurtransferase (rhodanese) are evolutionarily related enzymes (Nagahara and Nishino 1996; Nagahara et al. 1995). The redox-active cysteine corresponding to rat Cys^{263} is conserved in the C-terminal catalytically active domain of mammalian sulfurtransferases (Horowitz and Criscimagna 1988; Nagahara and Katayama 2005).

Eukaryota emerged after Prokaryota in the ancient atmosphere of the Earth around 2.2 billion years ago. Then, cyanobacteria emerged 2.7 billion years ago and produced oxygen via photosynthesis, resulting in an increase of the oxygen concentration (Canfield 2005; Kasting and Siefert 2002). A certain codon [CTG] coding a leucine residue in the C-terminal catalytically active domain of prokaryotic enzymes was replaced with [TGT] or [TGC] coding a cysteine residue in that of eukaryotic enzymes (a transition of the first base, a transversion of the second base, and a transversion of the third base; Fig. 3). The cysteine residue in the Earth's atmosphere under oxidizing conditions serves as a redox-sensing switch, which regulates cellular

redox homeostasis. Plants and fungi began to diverge from one another 1 billion years ago, and then animals diverged from fungi (Brenner et al. 1993). As plants evolved under low oxygen concentration, it is reasonable that the leucine residue would be conserved. Fungi emerged while the oxygen concentration in atmosphere was increasing; the [CTG] coding a leucine was replaced with [TAC] coding a tyrosine (a transition of the second base) and [TGT] coding a cysteine in aspergillus and candida enzymes, respectively (Fig. 3).

The oxygen concentration increased to ca. 35% about 290 million years ago, but decreased to ca. 12% about 250 million years ago. The oxygen concentration increased to ca. 21% again, and then stabilized around 170 million years ago. Although fish emerged in the water around 570 million years ago, the [CTC] coding a leucine (a transversion of the third base) was conserved in the medaka enzyme in about 1/30 oxygen concentration of the air (Fig. 3). In amphibians (evolved around 370 million years ago), the frog enzyme contained the evolved cysteine [TGC], and its rhodanese conserved leucine [CTT] (Fig. 3). In insects (evolved around 360 million years ago), the fruit fly enzyme also had the evolved cysteine [TGT], and its rhodanese does not conserve the leucine but did have a glutamine [GCT] (inexplicable replacements) (Fig. 3). In birds (evolved around 200 million years ago), and mammals (evolved around 250 million years ago), both the enzymes contained the evolved cysteine ([TGT] or [TGC]) (Fig. 3).

Enzymes carrying non-specific intermolecular redox-sensing switches

Cyclic AMP-dependent protein kinase

General aspects

Rat cyclic AMP-dependent protein kinase (commonly abbreviated as PKA; EC 2.7.11.11) is distributed as a holoenzyme in eukaryotes and critically contributes to glucose, glycogen, and lipid metabolisms. It has two isoforms—type I and type II enzymes—and both are heterotetramer, consisting of two catalytic and two regulatory subunits. The regulatory subunits of the type I and type II enzymes are referred to as RI and RII, respectively, and, further, each regulatory subunit is classified into α and β subtypes (Taylor et al. 2005). The enzyme is usually activated with increasing amounts of cAMP by adrenalin (Taylor et al. 2004). Type I enzyme contains redox-active cysteine residues, Cys^{17} and Cys^{38}, in regulatory subunit, and H_2O_2 oxidizes the enzyme to form intermolecular disulfide bonds between these cysteine residues of different subunits, which changes the structure to increase the kinase activity (phosphorylation) independently of β-adrenergic stimulation and cAMP elevations (Brennan et al. 2006).

Type II enzyme also contains reactive cysteine residues. H_2O_2 oxidizes the enzyme to form a disulfide bond

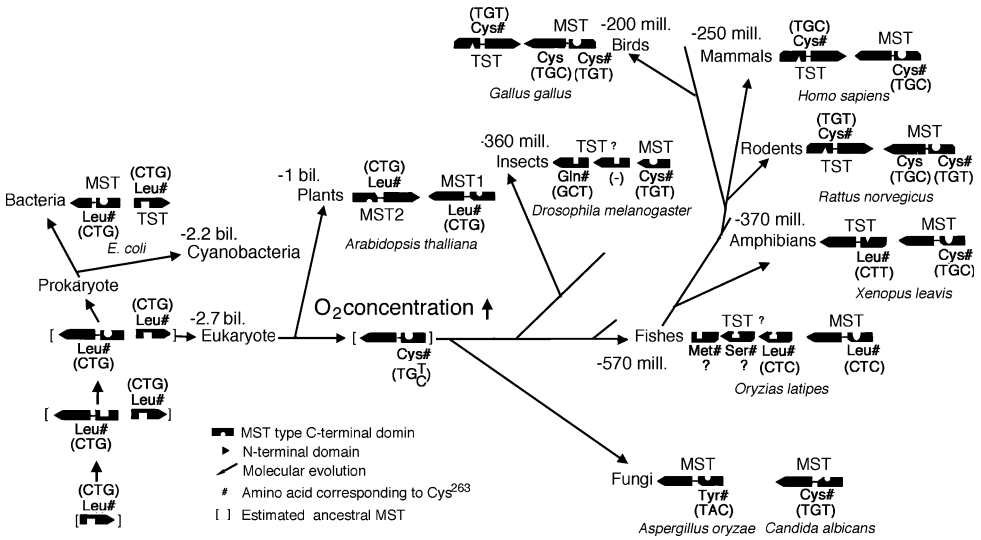

Fig. 3 Molecular evolution of the redox-sensing switch of mercaptopyruvate sulfurtransferase. In this phylogram of a rhodanese family, data for codons and deduced amino acid residues were based on cDNA or incomplete genomic DNA data: *Aspergillus oryzae* (AP007175 for MST); *Candida albicans* (XM_709437); *Drosophila melanogaster* (BLAST data form FlyBase; National Center for Biotechnology Information; and Berkeley Drosophila Genome Project); *Gallus gallus* (D50564 or XM_001231690 for MST, P25324 or XP_416284 for TST); *Oryzias latipes* (BLAST data form Medaka Expressed Sequence Tags data, the National Bio Resource Project Medaka Genome Project, and National Institute of Genetics, DNA sequencing center); *Xenopus laevis* (BC08421 for MST and BC084422 for TST). Sequence identity was analyzed using GENTYX. *Hash* symbol indicates the amino acid corresponding to Cys^{263} of rat mercaptopyruvate sulfurtransferase as a redox-sensing switch. Details are described in the text

between Cys^{199} of catalytic α subunits and Cys^{97} of regulatory IIα subunits (First et al. 1988; Humphries et al. 2002, 2005; Nelson and Taylor 1983), resulting in the inhibition of the cAMP activation of the enzyme (Raynaud et al. 1997) (Fig. 4). The kinase activity also decreased with a decreasing amount of cAMP (cAMP hydrolysis) via an insulin-induced activation of cAMP phosphodiesterase. In this review, I focus on the redox-regulated type IIα cyclic AMP-dependent protein kinase.

Regulation mechanism of the enzymatic activity via the redox-sensing switch

It is noteworthy that, in the molecular evolution of cyclic AMP-dependent protein kinase, Cys^{97} is conserved only in the type II and not type I regulatory subunits (de Piña et al. 2008). H_2O_2 oxidizes the type II enzyme to form a disulfide bond between Cys^{97} of regulatory IIα or IIβ subunit and Cys^{199} of the catalytic α subunits (de Piña et al. 2008) (Fig. 4). H_2O_2 inhibited cAMP activation of type II enzyme and the cAMP activation was restored by GSH, DTT, or Trx with TRD (de Piña et al. 2008). It is an interesting finding that the catalytic subunit of C199A (Cys^{199} is replaced with Ala) is insensitive to H_2O_2 (Takio et al. 1982). When the wild-type catalytic subunits in type II holoenzyme were substituted with mutant catalytic α subunits or the enzyme was preincubated with cAMP, H_2O_2 is ineffective (de Piña et al. 2008). These findings suggest that Cys^{97} in regulatory IIα or IIβ subunit and Cys^{199} in the catalytic α subunit are critical for both cAMP activation and redox-regulation of type II enzyme activity.

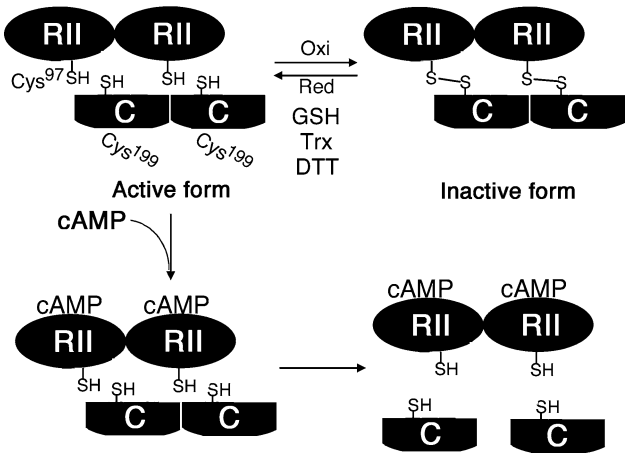

Fig. 4 Regulation mechanism of cyclic AMP-dependent protein kinase activity via the redox-sensing switch. When RII is modified with cAMP, the heterotetramer is separated to homodimeric regulatory subunits and two monomeric catalytic subunits. *C* catalytic subunit α, *Oxi* oxidation, *RII* Type II regulatory subunit, *Red* reduction. Details are described in the text

It is concluded that the disulfide bond serves as intermolecular redox-sensing switch to regulate type II enzyme activity.

Enzymes carrying intermolecular redox-sensing switches (undetermined reducing factors)

Chloroplast acetyl-CoA carboxylase

General aspects and regulation mechanism of the enzymatic activity via the redox-sensing switch

Pea chloroplast acetyl-CoA carboxylase (EC 6.4.1.2), a multi-enzyme complex, which consists of four subunits, two biotin carboxylases, and a carboxyltransferase (α and β subunits), catalyzes the first committed step of fatty acid synthesis (Ohlrogge and Browse 1995; Sasaki et al. 1995). The redox-regulated activation of carboxyltransferase may correlate with facilitation of chloroplast fatty acid synthesis in response to light (Sasaki et al. 2001). Carboxyltransferase contains 11 cysteines, including 2 redox-sensitive cysteines, Cys^{267} in α subunit and Cys^{442} in β subunit, which are required for redox-regulated enzymatic activity (Sasaki et al. 2001). The formation of a disulfide bond between Cys^{267} in α subunit and Cys^{442} in β subunit activates carboxyltransferase, and DTT cleaves the disulfide bond.

2-Cys peroxiredoxin

General aspects and regulation mechanism of the enzymatic activity via the redox-sensing switch

2-Cys peroxiredoxin (EC 1.11.1.15) is an example of a protein carrying an intermolecular redox-sensing switch, which directly modulates the protein function via the "locking and unlocking of the critical site." Hofmann et al. (2002) proposed a catalytic mechanism of functionally dimeric 2-Cys peroxiredoxin. A resent crystallographic study on the protein by Matsumura et al. (2008) revealed that the redox-active Cys^{52} is oxidized to form sulfenate. When the cysteine sulfenate reacts with Cys^{52} of the other subunit, an intermolecular disulfide bond is formed and this oxidized form does not possess peroxidase activity (an inactive form). Then, the reduced Trx or DTT cleaves the disulfide bond to convert the inactive form to the active form. On other hand, when the cysteine sulfenate reacts with excess H_2O_2, the peroxidation reaction proceeds to form sulfinate and/or sulfonate, resulting in inactivation (Matsumura et al. 2008; Nagahara et al. 2009b). These cysteines and the disulfide bond serve as redox-sensing switches to modulate the peroxidase activity.

Cytosolic malate dehydrogenase

General aspects

Malate dehydrogenase catalyzes the reaction of oxaloacetate to malate/oxaloacetate to malate utilizing the NAD^+/NADH or $NADP^+$/NADPH and is distributed in eukaryotes and most prokaryotes. NAD-dependent malate dehydrogenase (EC 1.1.1.37) exists as a homodimer of a 32–37 kDa subunit (Hock and Gietl 1982; Walk and Hock 1978), and NADP-dependent malate dehydrogenase (EC 1.1.1.82) consists of a larger subunit of 42 kDa (Crétin et al. 1990; Scheibe et al. 1991). There are five isoforms of malate dehydrogenase in plants (Gietl 1992)—cytosolic NAD-dependent malate dehydrogenase, mitochondrial NAD-dependent malate dehydrogenase, glyoxisomal and peroxisomal NAD-dependent malate dehydrogenases, chloroplast NADP-dependent malate dehydrogenase, and chloroplast NAD-dependent malate dehydrogenase. Chloroplast NADP-dependent malate dehydrogenase is a thiol enzyme, which has been intensively studied. It is noteworthy that the enzyme has N- and C-terminal extensions containing a redox-sensitive cysteine pair (Scheibe et al. 1991).

In this review, I focus on the cytosolic NAD-dependent malate dehydrogenase. Yamazaki et al. found that *Arabidopsis thaliana* enzyme was a target protein of *A. thaliana* cytosolic Trx-*h* using a Trx affinity chromatography (Yamazaki et al. 2004). The enzyme exhibits monomer–dimer equilibrium; oxidizing reagents dimerize the enzyme via a disulfide bond (between Cys^{330} and Cys^{330}) to decrease the enzymatic activity and then reducing reagents monomerize the dimeric enzyme to restore the enzymatic activity (Hara et al. 2006) (Fig. 5). The disulfide bond also serves as a redox-sensing switch to regulate the enzymatic activity.

Regulation mechanism of the enzymatic activity via the redox-sensing switch

Native PAGE and ultracentrifuge analyses revealed that recombinant cytosolic NAD-dependent malate dehydrogenase was oxidized by $CuCl_2$ to decrease the enzymatic activity via conversion of the monomeric state to the dimeric state. The disulfide bond is formed between Cys^{330} of each subunit (Yamazaki et al. 2004) (Fig. 5). Cys^{330} is an exposed residue on the surface of the enzyme, and further, is not the catalytic site. Then, the oxidized (dimeric) enzyme was reduced to restore the activity with conversion of the dimeric state to the monomeric state by DTT (~400 μM) or Trx-*h* (~3 μM) with or without DTT (10 μM) (Yamazaki et al. 2004) (Fig. 5). In the experimental system with Trx-*h* and DTT, DTT may reduce oxidized Trx-*h*. It has not been clarified whether reduced GSH or reduced Trx-*h* reduces the disulfide bond.

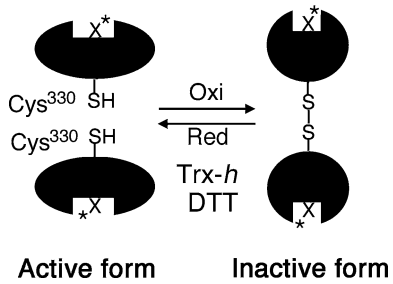

Fig. 5 Regulation mechanism of cytosolic malate dehydrogenase activity via the redox-sensing switch. *Oxi* oxidation, *Red* reduction, *X** catalytic sites, His and Asp. Details are described in the text

Group VIA phospholipase A_2

General aspects and regulation mechanism of the enzymatic activity via the redox-sensing switch

Human group VIA phospholipase A_2 ($iPLA_{2b}$) [classical Ca^{2+}-independent phospholipase A_2 (EC 3.1.1.4)] catalyzes a hydrolysis of phospholipids to a free fatty acid and is essential in several biological processes such as transcriptional regulation and cell proliferation in human and rat (Winstead et al. 2000). H_2O_2 inhibits the enzyme via formation of an intermolecular disulfide bond between two enzymes at Cys^{651} (not the catalytic site) and/or sulfenic acid at Cys^{651} (redox-active cysteine) with a decrease in the activity. On other hand, the activity is restored by DTT (Song et al. 2006). However, excess H_2O_2 inactivates the enzyme due to the formation of sulfenic acid or sulfonic acid at Cys^{651}. Song et al. (2006) proposed that modification such as sulfenylation or oxidation of Cys^{651} may regulate the enzymatic activity.

Isocitrate dehydrogenase kinase/phosphatase

General aspects and regulation mechanism of the enzymatic activity via the redox-sensing switch

Escherichia coli isocitrate dehydrogenase kinase/phosphatase (EC 2.7.11.5) is a homodimeric enzyme and is essential for the rapid adaptation to the carbon source supply as the oxidative metabolism. The enzyme is a homodimeric enzyme (Rittinger et al. 1996) and its activity is redox-regulated (Oudot et al. 1999). Under oxidative conditions (by treatment with cupric 1, 10 phenanthrolinate), an intermolecular disulfide bond is formed between the 2 Cys^{67} of each subunit (not the catalytic site), leading to the covalent dimerization of the enzyme with an increase in the enzymatic activity (Oudot et al. 1999). On other hand, DTT converts the functional dimer to the monomer (Oudot et al. 1999).

Mitochondrial cyanide-resistant alternative oxidase

General aspects

Arabidopsis thaliana mitochondrial cyanide-resistant alternative oxidase catalyzes the oxidation of NAD(P)H and transfers electrons from reduced ubiquinone to oxygen, which is a bypass of cytochrome *c* oxidase. A cyanide-resistant, alternative respiratory pathway exists in the mitochondria in all higher plants, many fungi and certain protozoa. The enzyme is a homodimeric and transmembrane protein and is anchored in the mitochondrial inner membrane. The alternative oxidases of higher plants have two conserved cysteine residues in the NH_2-terminal domain (Vanlerberghe and McIntosh 1997). Two redox-active cysteines, Cys^{78} and Cys^{128}, are found in the *Arabidopsis* enzyme. Siedow and Umbach (1995) summarized the structural properties of the enzyme. The enzymatic activity is regulated by an intersubunit disulfide bond between the two enzymes (Umbach and Siedow 1993; Umbach et al. 1994) and by pyruvate (α-keto acid) (Millar et al. 1993) (Fig. 6). Pyruvate (α-keto acid) activates the enzyme via a thiohemiacetal formation at Cys^{128} under reducing conditions but not under oxidizing conditions (Rhoads et al. 1998; Ribas-Carbo et al. 1997; Umbach and Siedow 1996; Umbach et al. 1994; Vanlerberghe et al. 1995) (Fig. 6).

Regulation mechanism of the enzymatic activity via the redox-sensing switch (without a domain necessary for the membrane anchoring)

Rhoads et al. (1998) clarified the roles of two redox-active cysteines, Cys^{78} and Cys^{128}, and the intermolecular disulfide bond (a redox-sensing switch) in the redox-dependent regulation of *A. thaliana* mitochondrial cyanide-resistant alternative oxidase by studying site-directed mutants of each cysteine residue to alanine and chemical cross-linking of two adjacent enzymes with ethylene glycol bis (succinimidyl succinate) (a crosslinker of proteins).

Diamide oxidized two enzymes to form an intermolecular disulfide bond between Cys^{78} of each enzyme, resulting in dimerization (Fig. 6). Pyruvate (α-keto acids) activated the C128A (Cys^{128} is replaced with Ala), while the C78A exhibited low activity but pyruvate activation was not observed. C78E showed higher activity than the wild-type enzyme, but pyruvate (α-keto acid) activation was not observed. The side chain of Glu^{78} mimics a thiohemiacetal. This result of C78E was consistent with that pyruvate (α-keto acid) activation that occurred through a thiohemiacetal formation at Cys^{78} (Rhoads et al. 1998).

It is concluded that Cys^{78} serves as both the redox-sensing switch and the site of activation by pyruvate (α-keto acid) and, on other hand, Cys^{128} serves as the redox-sensing switch.

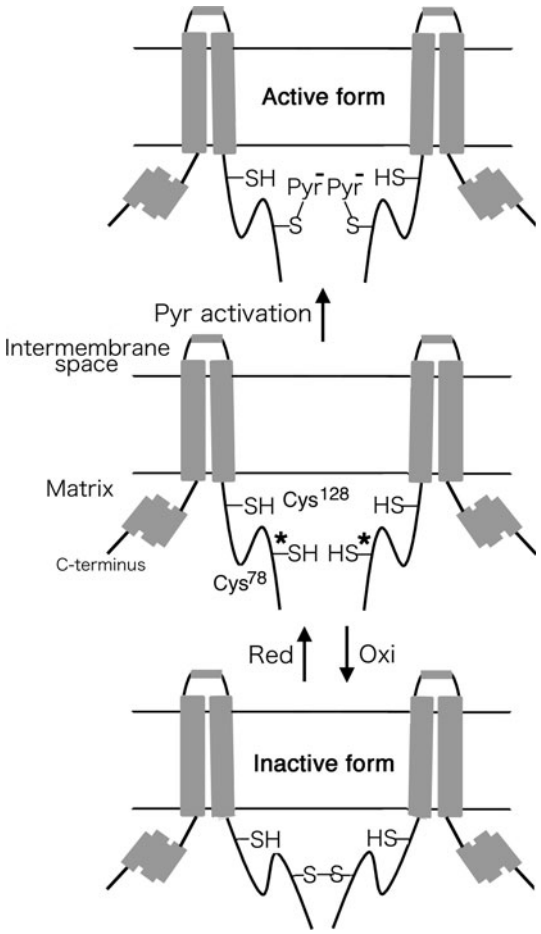

Fig. 6 Regulation mechanism of mitochondrial cyanide-resistant alternative oxidase activity via the redox-sensing switch. *Oxi*, oxidation; *Pyr activation*, Pyruvate activates the enzymatic activity via a covalent bond formation with Cys^{78}*; *Red*, reduction. Details are described in the text

However, there is no information regarding the reducing factors, such as GSH and Trx, for the dimeric enzyme.

Receptor proteins carrying intermolecular redox-sensing switches (undetermined reducing factors)

Protein-tyrosine phosphatases

General aspects and regulation mechanism of the enzymatic activity via the redox-sensing switch (without a domain necessary for the membrane anchoring)

Receptor protein-tyrosine phosphatase (EC 3.1.3.48) catalyzes the dephosphorylation of tyrosine residues in proteins. The enzyme consists of a single transmembrane domain and 1 or 2 catalytic domains, including a catalytic cysteine (Alonso et al. 2004). Cys^{723} in this domain forms a thiol-phosphate intermediate in the dephosphorylation

reaction and is essential for enzyme activity (Barford et al. 1995). As a response to changes in the cellular redox state, the intermolecular disulfide bond is formed between the catalytic Cys^{723} in the C-terminal catalytic domain (van der Wijk et al. 2004). H_2O_2 oxidized the 2 Cys^{723} to form a disulfide bond and to inhibit the enzyme activity (van der Wijk et al. 2004). Then, DTT abolished the intermolecular disulfide bonds and the activity was restored, but not the stable dimer formation (van der Wijk et al. 2004).

Sensor proteins carrying intermolecular redox-sensing switches (undetermined reducing factors)

RegB (redox sensor histidine kinase)

General aspects and regulation mechanism of the sensor protein activity via the redox-sensing switch

Rhodobacter capsulatus RegB (EC 2.7.13.3) is one of the two-component signal-transduction systems, which consists of a membrane-spanning histidine-sensor kinase (RegB) and a DNA-binding response regulator (RegA). The photosynthetic bacterium uses the RegB–RegA signal-transduction cascade (phosphotransfer reaction from RegB to RegA) (Swem et al. 2007). RegB is oxidized to form an intermolecular disulfide bond between two RegB proteins containing the highly conserved, redox-reactive Cys^{265}. This dimerization is a metal-dependent reaction. In vitro study using RegB without domain necessary for the membrane anchoring revealed that the formation of a disulfide bond converts a kinase-active dimer to a kinase-inactive tetramer state (Swem et al. 2003). The "redox control" means that the histidine-sensor kinase senses oxidative stress to form a metal-dependent, intermolecular disulfide bond, which acts as a molecular switch for controlling kinase activity in vitro (Swem et al. 2003). DTT restores the kinase activity with conversion of the inactive oligomeric state to the active state. Under anaerobic conditions, RegB autophosphorylates at His^{225} and subsequently phosphate is transferred from RegB to RegA as a signal-transduction.

Transcriptional factors carrying intermolecular redox-sensing switches (undetermined reducing factors)

CprK

General aspects and regulation mechanism of the transcriptional factor activity via the redox-sensing switch

Desulfitobacterium dehalogenans CprK [a chlorophenol reductase gene (*cpr*) product] is a transcriptional regulator of anaerobic dehalorespiration for energy yielding (Smidt and de Vos 2004; Utkin et al. 1994). CprA and CprK are expressed from the *cpr* gene cluster. CprA catalyzes the reductive dehalogenation of chlorinated aromatic compounds. CprK is constitutively expressed at low levels and acts as a transcriptional regulator for the *cpr* gene cluster (Pop et al. 2004). Reduction of CprK increases the transcriptional activity. Further, the activity is also increased by effectors such as 3-chloro-4-hydroxyphenylacetate and chlorinated aromatic compounds (polychlorinated biphenyls), and electron acceptors (Wiegel and Wu 2000; Wiegel et al. 1999).

CprK has a helix-turn-helix DNA-binding motif in the C-terminal region (Aravind et al. 2005) and an effector-binding motif in the N-terminal region (Joyce et al. 2006). Of five cysteines (Cys^{11}, Cys^{105}, Cys^{111}, Cys^{161}, and Cys^{200}), only Cys^{161} is not involved in redox-regulation of CprK (Fig. 7). Under oxidizing conditions, an intermolecular disulfide (a redox-sensing switch) between the two redox-active cysteine residues (Cys^{11} and Cys^{200}) and/or an intramolecular disulfide bond between Cys^{105} and Cys^{111} is formed, which loses the DNA-binding activity (Pop et al. 2004) (Fig. 7). On other hand, DTT restores the DNA-binding activity. Further, the fluorescence spectroscopic study and the electrophoretic mobility shift assay revealed that the effector-binding affinity remained intact in the C11S (Cys^{11} is replaced with Ser) and C11D, but loses DNA-binding activity and resists redox inactivation with a change in the tertiary structure of the protein (Gupta and Ragsdale 2008). Thus, Cys^{11} plays a dual role as a redox switch and in maintaining the correct tertiary structure that promotes DNA-binding (Gupta and Ragsdale 2008)

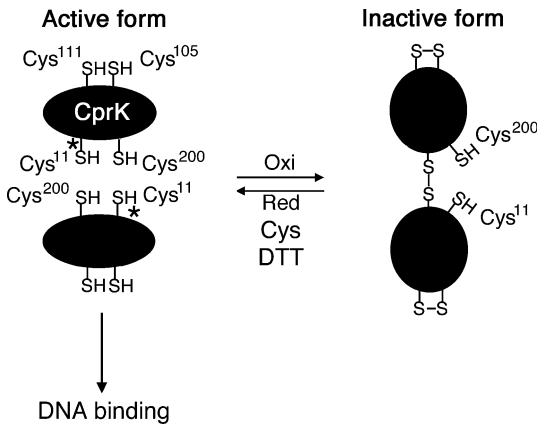

Fig. 7 Regulation mechanism of CprK activity via the redox-sensing switch. Cys^{11}* is a critical residue for the binding with DNA. The functions of Cys^{105} and Cys^{111} have not been determined. *Oxi*, oxidation; *Red*, reduction. Details are described in the text

(Fig. 7). However, there is no information on reducing process by GSH or Trx.

OhrR

General aspects and regulation mechanism of the transcriptional factor activity via the redox-sensing switch

The organic hydroperoxide resistance regulator (OhrR) is a peroxide-sensing transcription factor that regulates the expression of thiol-dependent peroxidases (Cussiol et al. 2003; Sukchawalit et al. 2001). There are two types of OhrR: 1-Cys (*Bacillus subtilis* OhrR) and 2-Cys (*Xanthomonas campestris* OhrR). In the N-terminal region, a cysteine residue is conserved in both groups of OhrRs. Cys^{15} of 1-Cys OhrR is required for sensing peroxides (Fuangthong and Helmann 2002). The redox-active Cys^{15} is initially oxidized by cumene hydroperoxide to form sulfenic acid (Fuangthong and Helmann 2002), which is not sufficient to inactivate OhrR (Lee et al. 2007). It is considered that the presence of low-molecular-weight thiols or excess concentration of oxidants inactivates OhrR (Lee et al. 2007). In the presence of low-molecular-weight thiols, a new disulfide is formed at Cys^{15}. Excess concentration of oxidants reacts with Cys^{15} to form a sulfinate and/or sulfonate. On other hand, in the inactivation of the 2-Cys OhrR, Cys^{22} is oxidized to form cysteine sulfenate, which consequently reacts with Cys^{127} of the other subunit to form an intermolecular disulfide bond between Cys^{22} and Cys^{127}, resulting in the inactivation of OhrR (Fig. 8). On other hand, DTT can restore the OhrR activity with monomerization (Panmanee et al. 2006) (Fig. 8). Thus, Cys^{22} serves as a H_2O_2 sensor. However, there is no information on whether GSH or Trx restores the activity.

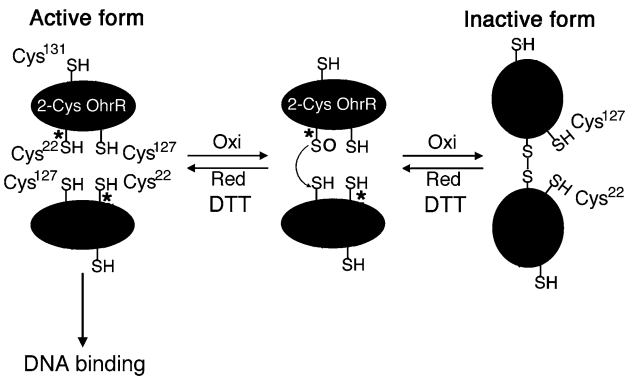

Fig. 8 Regulation mechanism of 2-Cys OhrR activity via the redox-sensing switch. Oxi, oxidation; Red, reduction. Details are described in the text

Yap1

General aspects and regulation mechanism of the transcriptional factor activity via the redox-sensing switch

When the hydroperoxide levels increase, the 20-KDa Yes associated protein 1 (Yap1) transcription factor is not directly oxidized; it instead facilitates to regulate hydroperoxide homeostasis in *S. cerevisiae* (Delaunay et al. 2002). To activate Yap1 activity, interaction with GSH peroxidase (GPx)-like enzyme, Gpx3 is essential. Yap1 contains 6 cysteines—Cys^{303}, Cys^{310}, Cys^{315}, Cys^{598}, Cys^{620}, and Cys^{629} (P19880-1). On other hand, Gpx3 contains three cysteines—Cys^{36}, Cys^{64} and Cys^{82}. H_2O_2 oxidizes two different proteins to form an intermolecular disulfide bond between Cys^{36} of Gpx3 and Cys^{598} of Yap1 (Fig. 9). Then, in Yap1, a disulfide exchange occurs from the intermolecular disulfide bond between Cys^{36} of Gpx3 and Cys^{598} of Yap1 to an intramolecular disulfide bond between Cys^{303} and Cys^{598} (Fig. 9). On transcriptional activation of Yap1 by H_2O_2, formation of the intramolecular disulfide bond causes a conformational change probably due to masking of the Yap1 nuclear export signal (Delaunay et al. 2000). In this reaction, Gpx3 serves as a H_2O_2 sensor and a regulator of transcriptional activity of Yap1 via an intermolecular redox-sensing switch. Trx cleaves the disulfide bond, but GSH cannot cleave the

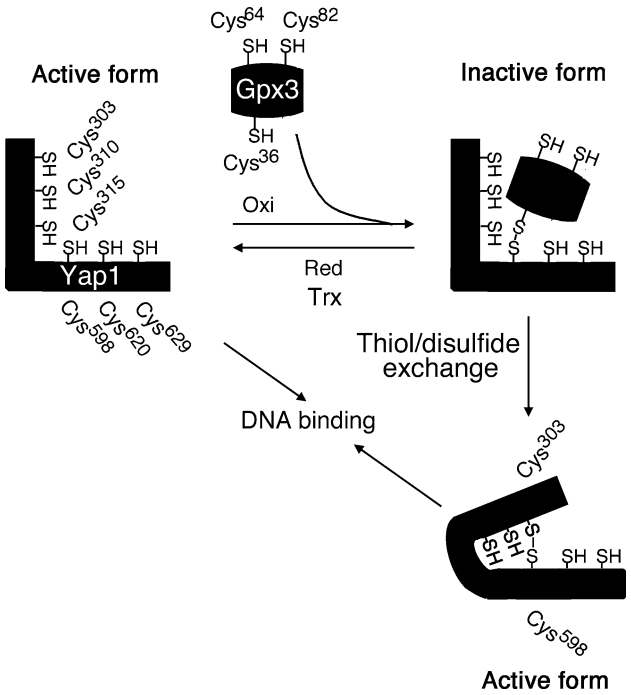

Fig. 9 Regulation mechanism of Yap1 activity via the redox-sensing switch. *Gpx3*, GSH reductase; *Oxi*, oxidation; *Red*, reduction. Details are described in the text

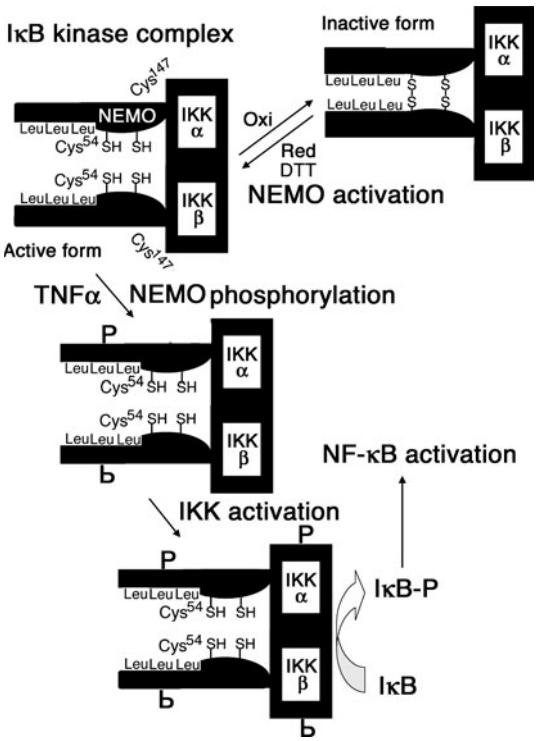

Fig. 10 Regulation mechanism of NEMO activity via the redox-sensing switch. *Oxi*, oxidation; *P*, phosphorylation; *Red*, reduction. Details are described in the text

disulfide bond (Fig. 9). However, there is no information on the effect of DTT on the disulfide bond.

Transcriptional factor modulator carrying intermolecular redox-sensing switches (undetermined reducing factors)

NEMO

General aspects and regulation mechanism of the transcriptional factor activity via the redox-sensing switch

NF-κB essential modulator (NEMO) is a regulatory component of the IκB kinase (IKK) complex, which controls activation of the NF-κB signaling pathway. The IKK complex consists of two catalytic subunits (IKKα and IKKβ) and the regulatory subunit NEMO. The IKK is activated to phosphorylate the IκB (NF-κB inhibitor), resulting in facilitation to translocate NF-κB to the nucleus and bind to DNA. NEMO carries multiple protein domains for integration of signals and bind to kinases (IKKα/β) (Yamamoto et al. 2001). There are two different regulation-systems for the NEMO activity. First, NEMO activity is modified by ubiquitination, phosphorylation, and SUMOylation (Sebban et al. 2006). Second, H_2O_2 induces formation of a NEMO dimer via disulfide bonds between Cys^{54} and Cys^{54}, and Cys^{347} and Cys^{347} to decrease TNFα-induced IKK activity (Fig. 10). On other hand, DTT or β-mercaptoethanol converted the dimeric state to monomeric state with restoration of the activity (Fig. 10). When wild-type NEMO is replaced with mutant NEMO (C54/347A), activation system of TNFα-induced IKK activity to facilitate the NF-κB DNA-binding is diminished because of loss of NEMO activation system. These findings imply that NEMO is a redox-regulated molecule to control the NF-κB signaling pathway (Herscovitch et al. 2008). However, there is no information on reducing process of NEMO by GSH or Trx.

Conclusions

1. Intermolecular and intramolecular disulfide bonds and cysteine residues serve as redox-sensing switches to regulate critical biological functions via modulation of protein functions.
2. Proteins carrying these switches are found in enzymes, transcriptional factors, sensor proteins, and transcriptional factor modulators.
3. The action mode of intermolecular redox-sensing switches is classified to direct modulation of protein function via the "locking and unlocking" of a critical residue or indirect one via "a conformational change."
4. Mercaptopyruvate sulfurtransferase carries thioredoxin-specific intermolecular redox-sensing switch.

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