Redox, haem and CO in enzymatic catalysis and regulation

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Abstract

The present paper describes general principles of redox catalysis and redox regulation in two diverse systems. The first is microbial metabolism of CO by the Wood–Ljungdahl pathway, which involves the conversion of CO or H_2/CO_2 into acetyl-CoA, which then serves as a source of ATP and cell carbon. The focus is on two enzymes that make and utilize CO, CODH (carbon monoxide dehydrogenase) and ACS (acetyl-CoA synthase). In this pathway, CODH converts CO_2 into CO and ACS generates acetyl-CoA in a reaction involving Ni·CO, methyl-Ni and acetyl-Ni as catalytic intermediates. A 70 Å (1 Å = 0.1 nm) channel guides CO, generated at the active site of CODH, to a CO 'cage' near the ACS active site to sequester this reactive species and assure its rapid availability to participate in a kinetically coupled reaction with an unstable Ni(I) state that was recently trapped by photolytic, rapid kinetic and spectroscopic studies. The present paper also describes studies of two haem-regulated systems that involve a principle of metabolic regulation interlinking redox, haem and CO. Recent studies with HO2 (haem oxygenase-2), a K^+ ion channel (the BK channel) and a nuclear receptor (Rev-Erb) demonstrate that this mode of regulation involves a thiol–disulfide redox switch that regulates haem binding and that gas signalling molecules (CO and NO) modulate the effect of haem.

Redox catalysis and regulation of microbial CO formation and utilization

The carbon cycle is one of the most important natural cycles in which various forms of carbon (e.g. CO_2, CO, methane and organic carbon) interconvert [1]. The fixation of CO_2 by autotrophic organisms replenishes the organic carbon lost through the respiratory and fermentative oxidation of sugars and fats to generate energy to fuel processes such as growth and movement in heterotrophs. The burning of organic carbon in the form of fossil fuels is leading to increased levels of CO_2 in the atmosphere, which is linked to climate change [2]. In fact, human activities are having detrimental impacts on all of the major natural cycles (carbon, nitrogen, oxygen, hydrogen and phosphorus); as the population increases and demands for resources intensify, we will increasingly experience problems related to global warming, and shortages of energy, food, water and fossil fuels. Because these are so important to our way of life, these

issues have significant social and geopolitical impacts. Our goals are to increase our understanding of these natural cycles and to contribute to improving the earth, the air and the water.

Figure 1 illustrates the anaerobic reduction of CO_2 to acetic acid by the Wood–Ljungdahl pathway [3], which is one of the six known CO_2 fixation pathways [4]. This pathway also leads to the fixation of CO through the action of CODH (carbon monoxide dehydrogenase), a nickel–iron–sulfur enzyme that interconverts CO and CO_2. There are two branches of this pathway. In the methyl branch, 1 mol of CO_2 undergoes six-electron reduction to a methyl group bound to tetrahydrofolate; then methyl group transfer to a CFeSP (corrinoid iron–sulfur protein) forms a methyl-cobalt intermediate in a reaction catalysed by a methyltransferase. Then, in the carbonyl branch, CODH catalyses the reduction of CO_2 to CO; then CO is transferred to ACS (acetyl-CoA synthase) to undergo condensation with the methyl group to yield an enzyme-bound acetyl intermediate that reacts with CoA to form acetyl-CoA. Acetyl-CoA serves as a source of cell carbon; alternatively, the high-energy C–S bond can undergo cleavage through the intermediacy of acetyl phosphate to generate energy in the form of ATP.

CODH and ACS are isolated as an $\alpha_2\beta_2$ complex, which consists of a central butterfly-shaped α_2 dimer of CODH

Figure 1 | The Wood–Ljungdahl pathway of anaerobic CO₂ and CO fixation

The pathway involves the six-electron reduction of CO_2 to methyl-tetrahydrofolate (CH_3-H_4folate), followed by the methyltransferase (MeTr)-catalysed transfer of the methyl group to CFeSP. CODH reduces CO_2 to CO, which is condensed with the methyl group and CoA to generate acetyl-CoA.

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad \Delta G^{o'} = -95 \text{ kJ/mol}$$
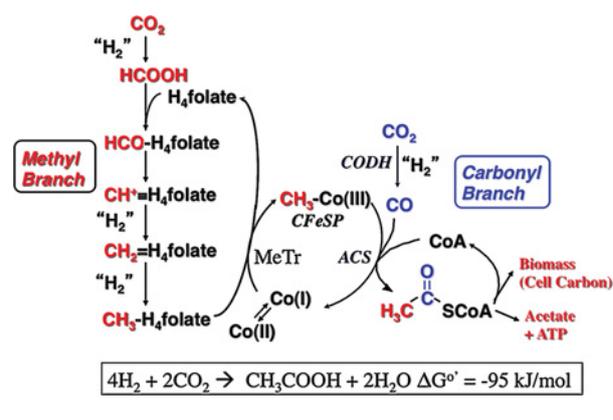
subunits that interface with a boot-shaped ACS β subunit on either side of the CODH core [5,6]. The active sites of both CODH and ACS are intriguing nickel–iron–sulfur metallocentres: an $NiFe_4S_4$ cluster in the case of the C-cluster and a dinickel centre bridged through the sulfur of cysteine to an Fe_4S_4 cluster for the A-cluster. The CODH–ACS complex acts as a machine in which the power source consists of reducing equivalents liberated from reactions catalysed by enzymes such as pyruvate ferredoxin oxidoreductase or hydrogenase, which produce low-potential electrons that are transferred to CODH through cellular redox mediators such as ferredoxin. In a two-electron redox reaction that occurs through a bimetallic reaction mechanism, oxidation of ferredoxin by CODH sends these electrons along a wire of Fe_4S_4 clusters (B- and D-clusters) within CODH to its C-cluster, thus driving the reduction of CO_2 to CO. The CO then travels through a tunnel to the A-cluster at the active site of ACS [7], which condenses the CO with a methyl group and an organic thiol (CoA) to generate acetyl-CoA. Despite having six redox-active transition metals, ACS catalyses a

transformation that is not a net redox reaction; however, it involves redox activation and internal redox reactions.

Co-ordinating the two active sites where CO is generated (the C-cluster of CODH) and utilized (the ACS A-cluster) is a tunnel [7]. By incubating crystals under xenon gas and performing anomalous scattering experiments, individual gas-binding sites could be visualized throughout the channel. At the end of the tunnel, a gas-binding cage is located ∼4 Å (1 Å = 0.1 nm) from the dinickel centre, which, in this structure, has a substitution-labile nickel ion closest to the cluster (thus termed the proximal nickel, Ni_p). The channel stretches over 138 Å from the A-cluster to the opposite A-cluster on the other end of the CODH–ACS molecule.

In order to understand the mechanism of ACS, ACS was cloned and heterologously expressed independently of CODH and a reconstitution protocol was developed that yields highly active protein [8]. Upon reaction with CO, this *Escherichia coli*-expressed protein exhibits IR and EPR signals (from a metal–CO adduct) that are characteristic of those elicited from the well-studied CO adduct with CODH–ACS. The yield of active enzyme is up to 80%, which enables a number of studies (e.g. X-ray absorption or Mössbauer spectroscopy).

As shown in Figure 2, ACS operates through a random-ordered mechanism [9], which allows the characterization of catalytically relevant binary complexes between enzyme and either CO (via Path A) or the methyl group (by Path B), both of which appear to be organometallic Ni_p–carbon species. Figure 3 shows the proposed mechanism of acetyl-CoA formation, depicting Path A in which CO binds before the methyl group. The first step is redox activation of the Ni_p^{2+} to Ni_p^{1+}, which binds CO. Then nucleophilic attack of Ni_p^{1+} on the methyl-Co^{3+} state of the CFeSP forms Co^{1+} and, presumably, an initial paramagnetic methyl-Ni_p^{3+}·CO intermediate [10–12]. However, the product is EPR-silent, whereas the Ni^{3+} state should be paramagnetic. One explanation is that the methyl-Ni_p^{3+} species is highly oxidizing, facilitating its one-electron reduction to generate methyl-Ni_p^{2+}, a step that is followed by C–C bond formation to make acetyl-Ni_p^{2+}·CO. Nucleophilic attack of CoASH liberates acetyl-CoA and two electrons, which bifurcate: one to reduce the methyl-Ni_p^{3+} to methyl-Ni_p^{2+} and the other to regenerate the Ni_p^{1+} for the next round of catalysis.

Figure 2 | Random mechanism of the ACS reaction

Shown are the random-order binding of CO and the methyl group to ACS, followed by C–C bond formation and thiolysis by CoA to generate acetyl-CoA.
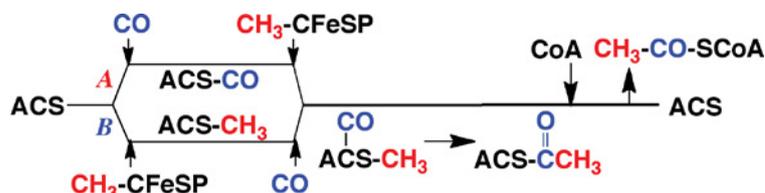
Figure 3 | The mechanism of acetyl-CoA formation by ACS
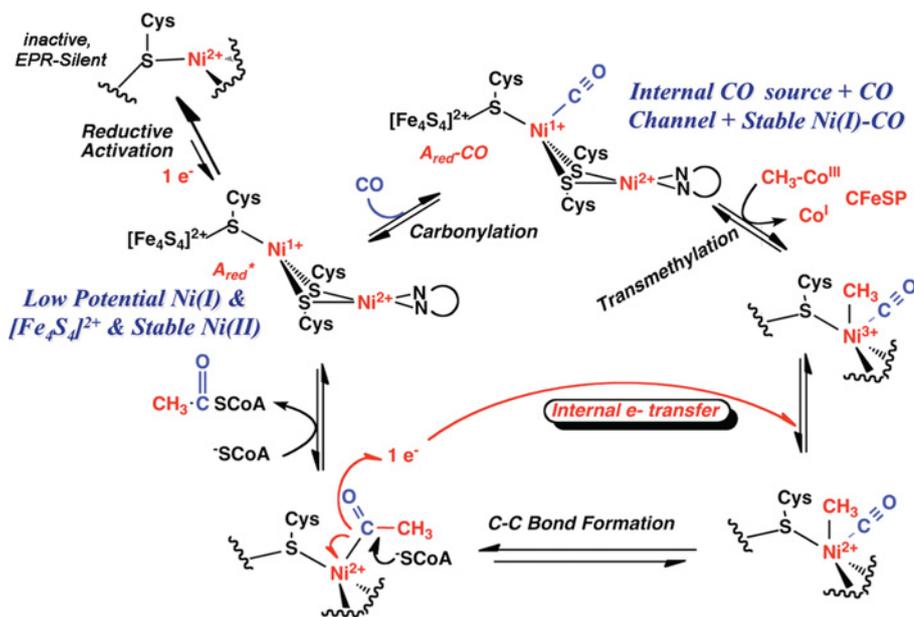
The active species in the mechanism described in Figure 3 is a nucleophilic Ni^{1+} species; however, this intermediate had not been observed in rapid kinetic studies. Thus it appeared that this must be a very unstable species and that binding of CO is tightly coupled to the one-electron reduction of the resting Ni^{2+} state of ACS. To test this hypothesis, the $Ni_p^{1+} \cdot CO$ intermediate was subjected to photolysis at low temperatures (<30 K), which led to loss of the IR band from the metal·CO and conversion of the characteristic $g = 2.074/2.028$ EPR signal into a new Ni(I) signal. [13]. Slight heating of the sample returned the enzyme to the ACS·CO state as measured by EPR and IR to have an extremely low activation energy for CO rebinding of ~1 kJ/mol [13]! We attribute this nearly activation-less reformation of the CO adduct to the existence of a cage for CO near the A-cluster of ACS that traps the CO upon photolysis. This cage is presumably the gas-binding site that is ~4 Å from Ni_p observed in the crystal structure of the xenon-incubated protein [7]. Thus formation of the highly labile Ni^{1+} intermediate is kinetically coupled to the carbonylation reaction. The use of unstable intermediates to catalyse reactions is rather common and has been illustrated in a homogeneous catalytic reaction. Reek and co-workers have shown that the most active catalysts are often the least stable catalysts [14]. In palladium-catalysed allylic alkylation, the rate-determining step consists of nucleophilic attack on the palladium–allyl complex. Raising the intermediate's energy decreases the activation energy, thus increasing the reaction rate.

Thus the enzyme seems masterfully 'designed' to keep levels of this very reactive Ni^{1+} state very low. In the absence of substrate, one only observes the unreactive Ni^{2+} form of the protein. Upon adding CO, the protein is converted into the paramagnetic $Ni^{1+} \cdot CO$ species through the intermediacy of the Ni^{1+} intermediate that has so far only been trapped by low-temperature photolysis. This CO cage also is important because it retains substrate near the active site to facilitate the kinetically coupled reaction. Then, the methylated CFeSP transfers its methyl group to form methyl-Ni, another reaction that involves a cryptic electron-transfer reaction, as shown in Figure 3. Finally, CoA releases the acetyl group as acetyl-CoA and ACS returns to its initial state.

Remarkable aspects of the Wood–Ljungdahl pathway are its use of CO as a catalytic intermediate, the involvement of cryptic electron-transfer reactions that are coupled to one-carbon-transfer reactions, the use of substrate tunnels to sequester CO and direct this substrate to the active site and the inclusion of organometallic intermediates in the key stages of catalysis.

Redox, haem and CO in metabolic regulation in mammals

CO also plays an important role in mammalian metabolism. Although it is a potent toxin when present in the 500 p.p.m. range, it plays an important role in various signalling pathways, including mediating O_2-sensing and the hypoxic response, regulating the circadian modulation of haem biosynthesis, acting as a regulator of T-cell function in lymphocytes, serving as a regulator of caveolin-1 status in growth control and activating soluble guanylate cyclase and mitogen-activated protein kinases [15].

In higher animals, the only significant source of CO is HO (haem oxygenase) [15], which catalyses the degradation of haem to generate BV (biliverdin) and CO and release iron [16–19]. Overall, this reaction involves seven electrons donated

by NADPH and cytochrome P450 reductase and three molecules of O_2, leading to the cleavage of the tetrapyrrole haem ring and generation of BV. BV is converted into bilirubin in a reaction catalysed by BV reductase, which is a pleitrophic enzyme with various other functions in cell signalling, including haem transport into the nucleus and activity as a dual-specificity protein kinase [20].

Not only CO, but all of the substrates and products of the HO reaction have important physiological roles [17]. Haem is the prosthetic group of electron-transfer proteins and redox enzymes and regulates genes involved in oxygen utilization; however, it is toxic when haem levels rise above $\sim$100 μM [21]. BV undergoes conversion into bilirubin, which is a powerful antioxidant [22]. Finally, because iron is required for haem- and non-haem proteins, iron deficiency (anaemia) can lead to morbidity and death, yet iron overload also can lead to a variety of clinical syndromes [23]. Thus HO activity needs to be tightly regulated and closely linked to haem and iron homoeostasis and to cellular oxygen levels and redox poise.

Recent studies on HO2, the BK (large-conductance K^+) channel [24] and the nuclear receptor Rev-Erb [25] has led to a model for the regulation of various metabolic processes and links cellular haem levels to the cellular redox poise and CO concentrations [26]. In this model, haem activates or inhibits a target protein, while thiol-disulfide redox switches [HRMs (haem-responsive motifs), CXXCH motifs] regulate haem binding. CO can then reverse (or, in some cases, amplify) the effect of haem.

There are two HO isoforms in mammals: inducible HO1 and constitutively expressed HO2. HO1 and HO2 share similar physical and kinetic properties; however, they have different tissue distributions, with HO1 expressed in many tissues and HO2 highly expressed in the brain, testes and carotid body [27]. HO1 (33 kDa) and HO2 (36 kDa) share a high level of sequence and structural homology within their core catalytic domains, with distal and proximal helices sandwiching the haem and a histidine residue in the proximal helix, which donates a ligand (His[25] in HO1 and His[45] in HO2) to the iron [28–30] (Figure 4). These proteins share comparable enzymatic activities and a stretch of $\sim$20 hydrophobic residues at their C-termini that form a transmembrane anchor to the microsomal membrane [27]. In contrast with HO1, HO2 contains a $\sim$20-residue extension at its N-terminus and, whereas HO1 totally lacks cysteine residues, HO2 contains a region near the C-terminus (between residues 260 and 290, HO2 numbering), which includes two HRMs. These HRMs form a thiol–disulfide redox switch that is involved in regulating the affinity of HO2 for a single haem that binds to the catalytic domain [31]. It had initially been suggested that each of these HRMs in HO2 bind an additional haem (thus giving a total of three haems/mol of protein) [32]; however, the results of various experiments with the full-length and several truncated forms of HO2, including haem titrations and measurement of haem/protein stoichiometry, demonstrate that this protein, like HO1, binds only one haem per monomeric unit and that the C-terminal

Figure 4 | Domains of HO1 and HO2

HRMs are CP (Cys-Pro) motifs that in HO2 regulate binding of haem. TM helix is the C-terminal transmembrane helix that is found in both HO1 and HO2. See the text for details.
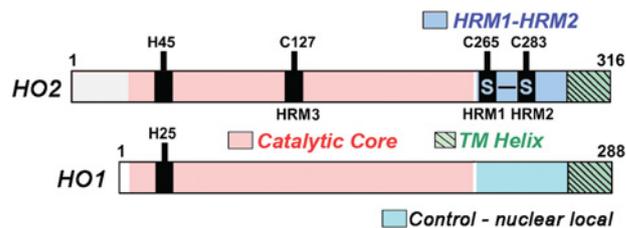
domain of HO2 acts as a regulator of haem binding to the catalytic domain [31,33].

Although the structures of the catalytic domains of HO1 and HO2 (residues 30–240, HO2 numbering) closely overlay (Figure 5) with average root mean square deviation for $C\alpha$ atoms of 0.8–0.9 Å [30], their C-terminal domains (preceding the 20 hydrophobic residues that form the transmembrane helices) significantly diverge in sequence and function. The C-terminus of HO1 appears to be involved in regulating whether HO1 localizes to the nucleus or to the ER (endoplasmic reticulum) in the cytoplasm, with proteolytic truncation of the C-terminus linked to nuclear import [34]. On the other hand, the C-terminus of HO2 acts as a redox switch [31]. When the C-terminal HRMs (from Cys[265] and Cys[283]) are in the oxidized disulfide state, the affinity of HO2 for haem increases $\sim$10-fold [31] relative to that of the reduced ditholate state. The K_d (310 nM) for the haem–HO2 complex, when the thiols are in the reduced state, is above the normal concentration of free (or exchangeable) haem in the cytoplasm of most cells, thus the activity of HO2 is dependent on the redox state of the cell. To confirm that this redox switch is operative *in vivo*, an isotope-coded affinity tag approach (called OxICAT [35], developed by U. Jakob), which traps the redox state of thiols within the cell, confirmed that the HRMs undergo oxidation to the disulfide state when cells are treated with various oxidants, convert into the reduced forms when *N*-acetylcysteine is added to the growth medium and exhibit an approximately 50:50 mixture of oxidized/reduced forms when they are grown under normoxic conditions [33]. These results coincide with the midpoint potential of the disulfide–thiol switch (-200 mV) [33] and with the ambient intracellular redox potential (~-200 mV) under normal aerobic growth conditions [36].

We have proposed that other important proteins [including eIF2α (eukaryotic initiation factor 2α) kinase, 5-aminolaevulinate synthase and Bach1 transcription factor] that contain HRMs may exhibit redox-controlled ligand binding as described above for HO2 [24]. We recently confirmed this for the HRM in the Rev-Erb transcription factor [25]. Thiol–disulfide switches can also be found in other motifs, such as CXXC; however, this pattern is also found in proteins, such as thioredoxin and disulfide oxidoreductases, that undergo thiol–disulfide interchange [37] and proteins

Figure 5 | Overlay of HO1 and HO2

The structures of these proteins are known for the core catalytic domains. The region beyond Glu[245] in HO2 appears to be unstructured, and the electron density for this region of the protein was not present in the diffraction data.
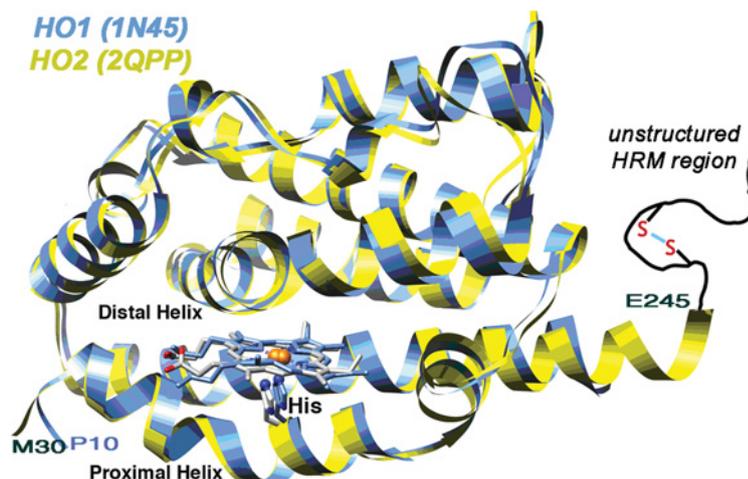
that are associated with metal–thiolate co-ordination, as in zinc-finger proteins [38].

A CXXCH motif forms the thiol–disulfide redox switch in the HBD (haem-binding domain) of the BK channel [24] that is involved in oxygen sensing in the carotid body, which is the chemosensor for oxygen levels in the bloodstream. Various studies indicate that the histidine residue within this motif serves as a haem ligand. Replacement of the histidine residue by arginine abolishes sensitivity of the BK channel to haem and CO [39]; furthermore, haem was shown to bind to a synthetic 23-residue peptide containing the CXXCH motif [40]. Finally, the binding of haem and redox regulation of haem affinity was characterized in studies with the independently expressed HBD [24].

It was first proposed by Kemp and co-workers that haem and CO regulate the activity of this channel [41]. According to this hypothesis, under normoxic conditions, there is sufficient oxygen to promote the HO-dependent degradation of haem to generate CO, which activates the channel to increase the extracellular levels of K^+ (leading to membrane hyperpolarization). Alternatively, under hypoxic conditions, low levels of oxygen result in low HO2 activity and increased haem, conditions that lead to inhibition of the channel and membrane depolarization. We proposed that intracellular redox poise is linked to regulation of channel activity by CO and haem because, on the basis of work with the HBD of the BK channel, the two cysteine residues in the CXXC motif can reversibly form a disulfide bond and this oxidized state of the protein has a markedly lower affinity for haem than the reduced state, with the two cysteine residues in the dithiolate form [24].

Interestingly, the HBD of the BK channel forms a linker between two domains called RCK1 and RCK2, which interact to form a Ca^{2+}-binding site, thus conferring Ca^{2+}-sensitivity [42–44]. Recent studies of the independently expressed 134-residue linker region indicate that this domain does indeed bind haem tightly and that haem binding is robustly regulated by the redox state of the haem, with the reduced state binding haem ~15-fold more tightly than the oxidized state (with a K_d of ~2.7 μM, which is well above the concentration of free/exchangeable haem in the cell) [24]. Furthermore, the HBD was shown to interact with HO2 [24], congruent with immunoprecipitation studies indicating that HO2 binds to the full-length channel [41]. Thus it appears that redox, CO, haem and protein–protein interactions between HO2 and the HBD in the BK channel converge to help cells regulate the chemoreflex response to hypoxia.

To summarize the recent studies interlinking redox, haem and CO to modulation of the BK channel, cysteine residues in the CXXC**H** motif of the HBD (the linker region between RCK1 and RCK2) form a thiol–disulfide redox switch with a midpoint potential (− 184 mV) that is within the cellular redox poise. The reduced dithiolate state of the HBD binds haem tightly and with 1:1 stoichiometry, with histidine as the haem ligand, while oxidation to the disulfide state leads to haem release.

Recent studies on a nuclear receptor protein called Rev-Erbβ also illustrate a similar mode of haem, thiol–disulfide and gas molecule regulation [25]. Rev-Erb represses a broad spectrum of target genes involved in regulating metabolism, the circadian cycle and pro-inflammatory responses. A thiol–disulfide redox switch controls the interaction between haem and the ligand-binding domain of Rev-Erbβ. The reduced dithiol state of Rev-Erbβ binds haem (K_d ~20 nM) 5-fold more tightly than the oxidized disulfide state. This low dissociation constant is in the range of the intracellular free haem concentration. In addition, the regulatory Fe^{2+}-haem of Rev-Erbβ has high affinity for CO (K_d = 60 nM), which replaces one of the internal ligands when bound. On the basis of recent results, it was proposed that the thiol–disulfide redox switch is one mechanism by which oxidative stress is linked

to circadian and/or metabolic imbalance and that oxidative stress leads to oxidation of cysteine(s), thus releasing haem from Rev-Erbβ and altering its transcriptional activity [25].

In a more general context, the studies described in the present paper relate to what appears to be an emerging theme of redox, haem and gas signalling molecules (CO and NO, and perhaps H_2S) regulating metabolic processes at three levels. At one level, haem activates or inhibits a target protein, as shown in studies described above on HO2, the BK channel and Rev-Erb β nuclear receptor. This concept is related to the recent work on haem–thiolate-sensor proteins [45]. At another level, thiol–disulfide redox switches (e.g. HRMs, CXXCH motifs) regulate haem binding, thus allowing haem binding to be linked to the cellular redox poise. Finally, CO can modulate the activating/inhibiting function of the haem through conformational changes at the haem-binding site. We suggest that further studies of haem-binding proteins that contain thiol–disulfide switches will expand the list of processes regulated similarly to the systems described here. Further studies of these systems will also allow deeper understanding of the substantial conversation between sensors/switches on proteins and cellular haem levels, redox poise and CO concentrations as these parameters change according to the metabolic state of the cell.

Funding

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