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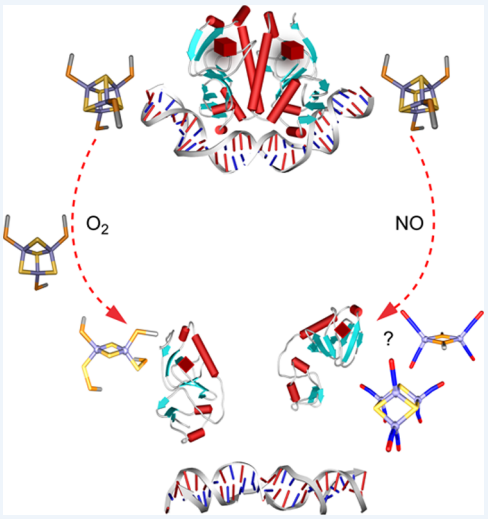
Iron–Sulfur Clusters as Biological Sensors: The Chemistry of Reactions with Molecular Oxygen and Nitric Oxide

Jason C. Crack,[‡] Jeffrey Green,[†] Andrew J. Thomson,[‡] and Nick E. Le Brun*[,‡]

[‡]Centre for Molecular and Structural Biochemistry, School of Chemistry, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, U.K.

[†]Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, U.K.

CONSPECTUS: Iron–sulfur cluster proteins exhibit a range of physicochemical properties that underpin their functional diversity in biology, which includes roles in electron transfer, catalysis, and gene regulation. Transcriptional regulators that utilize iron–sulfur clusters are a growing group that exploit the redox and coordination properties of the clusters to act as sensors of environmental conditions including O_2, oxidative and nitrosative stress, and metabolic nutritional status. To understand the mechanism by which a cluster detects such analytes and then generates modulation of DNA-binding affinity, we have undertaken a combined strategy of *in vivo* and *in vitro* studies of a range of regulators. *In vitro* studies of iron–sulfur cluster proteins are particularly challenging because of the inherent reactivity and fragility of the cluster, often necessitating strict anaerobic conditions for all manipulations. Nevertheless, and as discussed in this Account, significant progress has been made over the past decade in studies of O_2-sensing by the fumarate and nitrate reduction (FNR) regulator and, more recently, nitric oxide (NO)-sensing by WhiB-like (Wbl) and FNR proteins.



Escherichia coli FNR binds a [4Fe-4S] cluster under anaerobic conditions leading to a DNA-binding dimeric form. Exposure to O_2 converts the cluster to a [2Fe-2S] form, leading to protein monomerization and hence loss of DNA binding ability. Spectroscopic and kinetic studies have shown that the conversion proceeds via at least two steps and involves a $[3Fe\text{-}4S]^{1+}$ intermediate. The second step involves the release of two bridging sulfide ions from the cluster that, unusually, are not released into solution but rather undergo oxidation to sulfane (S^0) subsequently forming cysteine persulfides that then coordinate the [2Fe-2S] cluster. Studies of other [4Fe-4S] cluster proteins that undergo oxidative cluster conversion indicate that persulfide formation and coordination may be more common than previously recognized. This remarkable feature suggested that the original [4Fe-4S] cluster can be restored using persulfide as the source of sulfide ion. We have demonstrated that only iron and a source of electrons are required to promote efficient conversion back from the [2Fe-2S] to the [4Fe-4S] form. We propose this as a novel *in vivo* repair mechanism that does not require the intervention of an iron–sulfur cluster biogenesis pathway.

A number of iron–sulfur regulators have evolved to function as sensors of NO. Although it has long been known that the iron–sulfur clusters of many phylogenetically unrelated proteins are vulnerable to attack by NO, our recent studies of Wbl proteins and FNR have provided new insights into the mechanism of cluster nitrosylation, which overturn the commonly accepted view that the product is solely a mononuclear iron dinitrosyl complex (known as a DNIC). The major reaction is a rapid, multiphase process involving stepwise addition of up to eight NO molecules per [4Fe-4S] cluster. The major iron nitrosyl product is EPR silent and has optical characteristics similar to Roussin's red ester, $[Fe_2(NO)_4(RS)_2]$ (RRE), although a species similar to Roussin's black salt, $[Fe_4(NO)_7(S)_3]^-$ (RBS) cannot be ruled out. A major future challenge will be to clarify the nature of these species.

1. INTRODUCTION

Since iron–sulfur proteins were first identified in the 1960s from their unusual EPR signatures, they have been discovered in all forms of life.[1] They are thought to have originated in the seas of the ancient, anaerobic Earth where iron, as soluble Fe^{2+}, was available with sulfide ions (S^{2-}).[2] The protein chain coordinates the Fe–S cluster usually via the thiolate side chain of one or more cysteine residues. The diverse functions of iron–sulfur proteins can be grouped broadly into three classes, electron carriers, enzymatic catalysts, and gene regulators; their redox properties are crucial in all these roles.[3−5] The structural plasticity of iron–sulfur clusters in response to redox reactions with gaseous molecules allows the sensing of intracellular levels of molecular oxygen and nitric oxide (NO) and facilitates the regulation of gene expression through protein conformational

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changes that alter DNA binding affinities. Both gases have important biological roles: O_2 in aerobic respiration and as a precursor of reactive oxygen species; NO as a signaling molecule and as an antibacterial. This Account, drawing largely from work in the authors' laboratories, discusses the chemistry of the reactions with O_2 and NO of two iron−sulfur protein families involved in regulation, namely, the O_2-sensing fumarate and nitrate reduction (FNR) regulators and WhiB-like (Wbl) proteins that regulate cell development and react readily with NO. These studies have led to important insight into how bacteria sense and respond to these key environmental signals.[5,6]

2. PROPERTIES OF IRON−SULFUR CLUSTERS

The simplest iron−sulfur cluster is the [2Fe-2S] cluster, which consists of a [Fe_2-(μ_2-S)$_2$] rhomb, with each iron coordinated by two further ligands, normally amino acid residue side chains (Figure 1A). These are usually cysteine thiolates (RS^-), but

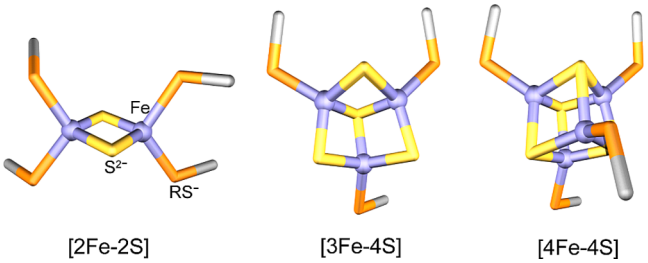

Figure 1. Commonly occurring iron−sulfur clusters. Structures of [2Fe-2S], [3Fe-4S], and [4Fe-4S] iron−sulfur clusters. Iron, sulfide, and cysteine residues are indicated.

other residues, such as histidine (-N=), serine (RO^-), and aspartate (RCO_2^-), are sometimes found. Because each iron is tetrahedrally coordinated, the four protein ligands are constrained to lie in a plane perpendicular to that of the rhomb. The [4Fe-4S] cluster can be thought of as comprising two Fe_2-(μ_2-S)$_2$ rhombs, one on top of the other and at right angles to each other. The [4Fe-4S] cluster may also be viewed as comprising two interpenetrating tetrahedra of iron and sulfide ions, generating a cube (Figure 1C). This geometry ensures that the coordinating amino acid residue side chains (again, usually cysteine) are positioned at the vertices of a tetrahedron. Removal of one iron from a [4Fe-4S] cluster generates a [3Fe-4S] cluster (Figure 1B). There are other, less common iron−sulfur clusters, including the recently discovered six cysteine-coordinated [4Fe-3S] cluster located close to the catalytic center of O_2-resistant NiFe hydrogenases.[7]

In iron−sulfur clusters, the oxidation state of iron is invariably +2 or +3 and, because of the tetrahedral coordination, is always high spin having either four or five unpaired electrons, respectively. The interactions between the electron spins on different iron ions, called exchange coupling, is most commonly antiferromagnetic, resulting in no or few unpaired electrons.[1] Ferromagnetic coupling can occur, leading to higher ground state spin systems. Clusters with unpaired electrons are paramagnetic and often have unique signatures that can be detected by electron paramagnetic resonance (EPR), magnetic circular dichroism (MCD), and Mössbauer spectroscopies.

Each cluster can in principle adopt several different overall charge states. In practice, however, protein bound clusters are normally confined to a pair of oxidation states within a range of reduction potentials, typically −100 to −600 mV versus SHE.[1] In [4Fe-4S] and [2Fe-2S] electron transfer proteins, the +2/+1 couple is most common, while the +1/0 couple is usually found in [3Fe-4S] proteins. Exceptions to this occur where the cluster coordination and, therefore, the environment are unusual. The HiPIPs (high potential iron proteins) are [4Fe-4S] proteins with reduction potentials in the range +50 to +450 mV. These proteins cycle through the +3/+2 couple, and because it becomes easier to add electrons to clusters in higher overall charge states, their reduction potentials are much higher. In some cases, particularly in enzymes associated with nucleic acid replication and repair that contain one or more functionally essential [4Fe-4S] clusters, the cluster probably plays a structural role but is not redox active within physiological range.[8]

Iron−sulfur clusters are susceptible to damage from redox reactions with reactive oxygen species, including molecular oxygen (O_2), superoxide ions, and hydrogen peroxide. This can lead to cluster interconversion or partial or complete loss of iron. Reactions with strongly coordinating species such as nitric oxide (NO) also lead to cluster damage/conversion. In the case of NO, coordination is coupled to redox processes (see below). These properties are utilized by several regulatory proteins that function as sensors of reactive oxygen and nitrogen species, enabling cells to respond to changing external conditions. Examples include the O_2-sensing fumarate and nitrate reduction (FNR) regulator, the NO-sensor NsrR, and the likely NO-sensing WhiB family of regulators.[5,6] The sensitivity of iron−sulfur regulatory proteins to O_2 poses major technical challenges, but to overcome these, our laboratories have developed a range of strategies, which were recently described.[9] Consequently, we are able to obtain stable homogeneous samples at high concentration for *in vitro* biophysical studies.

3. OXIDATION OF CLUSTERS BY O_2: THE FNR [4Fe-4S] TO [2Fe-2S] CLUSTER CONVERSION

A remarkable feature of bacteria is their ability to adapt to rapidly changing environments. In the absence of O_2, many can utilize alternative electron acceptors (e.g., nitrate), enabling them to remain competitive. Such bacteria must have mechanisms for monitoring the availability of O_2 so that they can respond by reprogramming gene expression, facilitating a switch between aerobic and anaerobic metabolism. This permits them, when O_2 becomes available again, to exploit the greater metabolic efficiency of aerobic respiration while avoiding the severe toxicity of O_2 toward anaerobic respiratory systems.

In many bacteria, an O_2-sensing DNA-binding protein called FNR, a homologue of the cAMP receptor protein (CRP), coordinates the switch between aerobic and anaerobic metabolism.[10] In *Escherichia coli* and some other bacteria, FNR is predicted to comprise two distinct domains that provide DNA-binding and sensory functions. The N-terminal sensory domain contains four essential cysteine residues that are capable of binding either a $[4Fe\text{-}4S]^{2+}$ or a $[2Fe\text{-}2S]^{2+}$ cluster,[11] while the C-terminal DNA-binding domain recognizes specific FNR-binding sequences within target promoters (Figure 2A). Under anaerobic conditions, FNR acquires one $[4Fe\text{-}4S]^{2+}$ cluster per protomer, resulting in dimerization and enhanced site specific DNA-binding to target promoters.[12] Thus, FNR either represses or activates genes associated with aerobic and anaerobic respiration, respectively. In *Bacillus*

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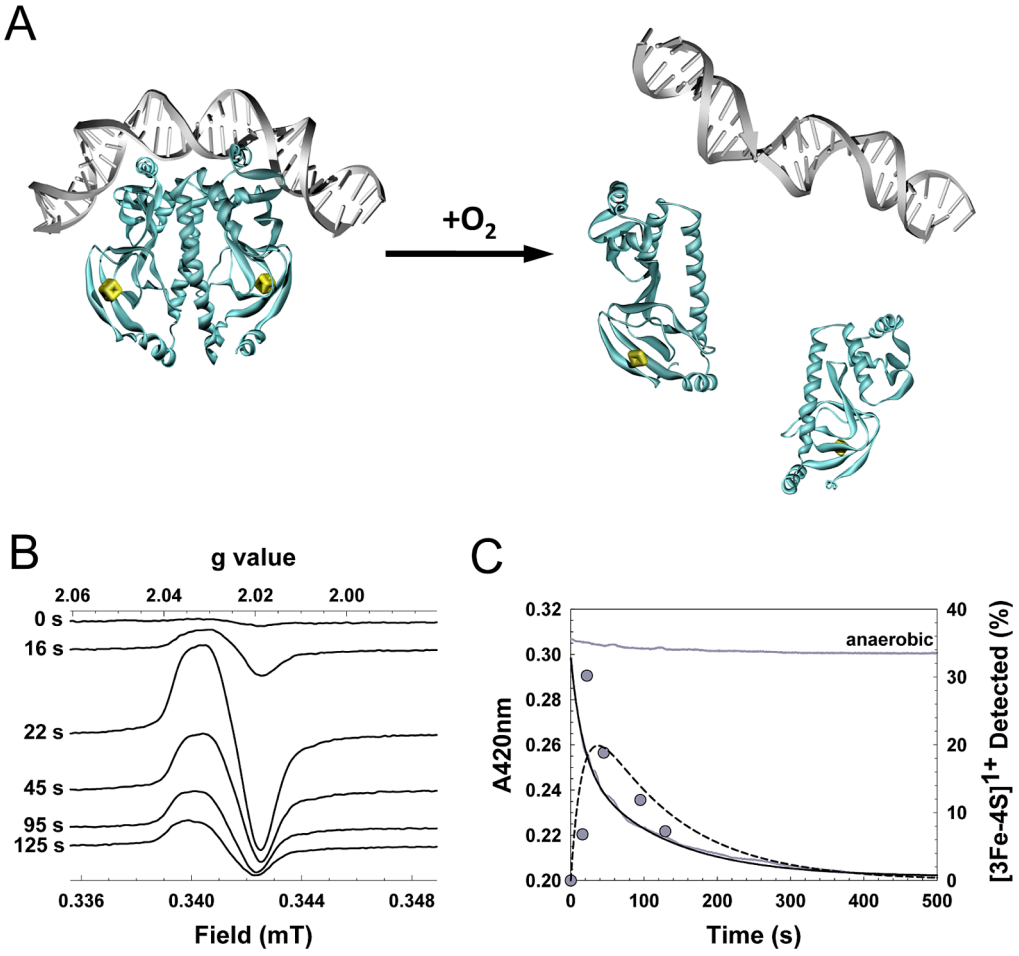

Figure 2. *E. coli* FNR and its reaction with O_2. (A) A model of FNR based on the structure of *E. coli* CRP (pdb file 1CGP). The [4Fe-4S] cluster is represented by a cube. Following the increase in O_2 concentration, the DNA-bound dimeric FNR protein undergoes reaction at its cluster, resulting in a [2Fe-2S] form (represented by a rhomb) that dissociates into monomers that no longer bind DNA. (B) Time-resolved EPR (left) and absorbance experiments (right) showing the formation and decay of a $[3Fe\text{-}4S]^{1+}$ intermediate. Simultaneous fits of both data sets are shown in the right-hand panel.[17]

Scheme 1. Reaction of *E. coli* [4Fe-4S] FNR with O_2[a]

Step 1

$$[4Fe\text{-}4S]^{2+} + O_2 \rightarrow [3Fe\text{-}4S]^{1+} + Fe^{2+} + O_2^{\cdot-}$$

Step 2

$$[3Fe\text{-}4S]^{1+} \rightarrow [2Fe\text{-}2S]^{2+} + Fe^{3+} + 2S^{2-}$$

Step 2 - one persulfide ligand reaction

$$[3Fe\text{-}4S](CysS)_3 + CysS^- + O_2 + 2H^+ \rightarrow [2Fe\text{-}2S](CysS)_3(CysSS) + Fe^{3+} + S^{2-} + H_2O_2$$

Step 2 - two persulfide ligand reaction

$$[3Fe\text{-}4S](CysS)_3 + CysS^- + O_2 + 4H^+ \rightarrow [2Fe\text{-}2S](CysS)_2(CysSS)_2 + Fe^{3+} + 2H_2O$$

Overall reaction

$$[4Fe\text{-}4S](CysS)_4 + nO_2 \rightarrow [2Fe\text{-}2S](CysS)_2(CysSS)_2 + Fe^{2+} + Fe^{3+} + \text{reduced } O_2 \text{ species}$$

[a]Note that the overall reaction is written in this way because it is currently unclear how many O_2 molecules are required and what the reduced oxygen products are, for example, whether superoxide generated in step 1 participates further in step 2.

subtilis and other Gram-positive bacteria, the [4Fe-4S] cluster is located at the C-terminal end, coordinated by three cysteines and one asparate. This type of FNR is a permanent dimer, with DNA-binding apparently controlled directly by the presence or absence of the cluster.[13] While reaction of the *B. subtilis* FNR with O_2 is not well-defined, in *E. coli* FNR, reaction with O_2 leads to the conversion of the $[4Fe\text{-}4S]^{2+}$ cluster into a $[2Fe\text{-}2S]^{2+}$ cluster, causing a conformational change in the protein leading to monomerization and loss of high affinity DNA binding[11,12] (Figure 2A). Over the past decade, we have

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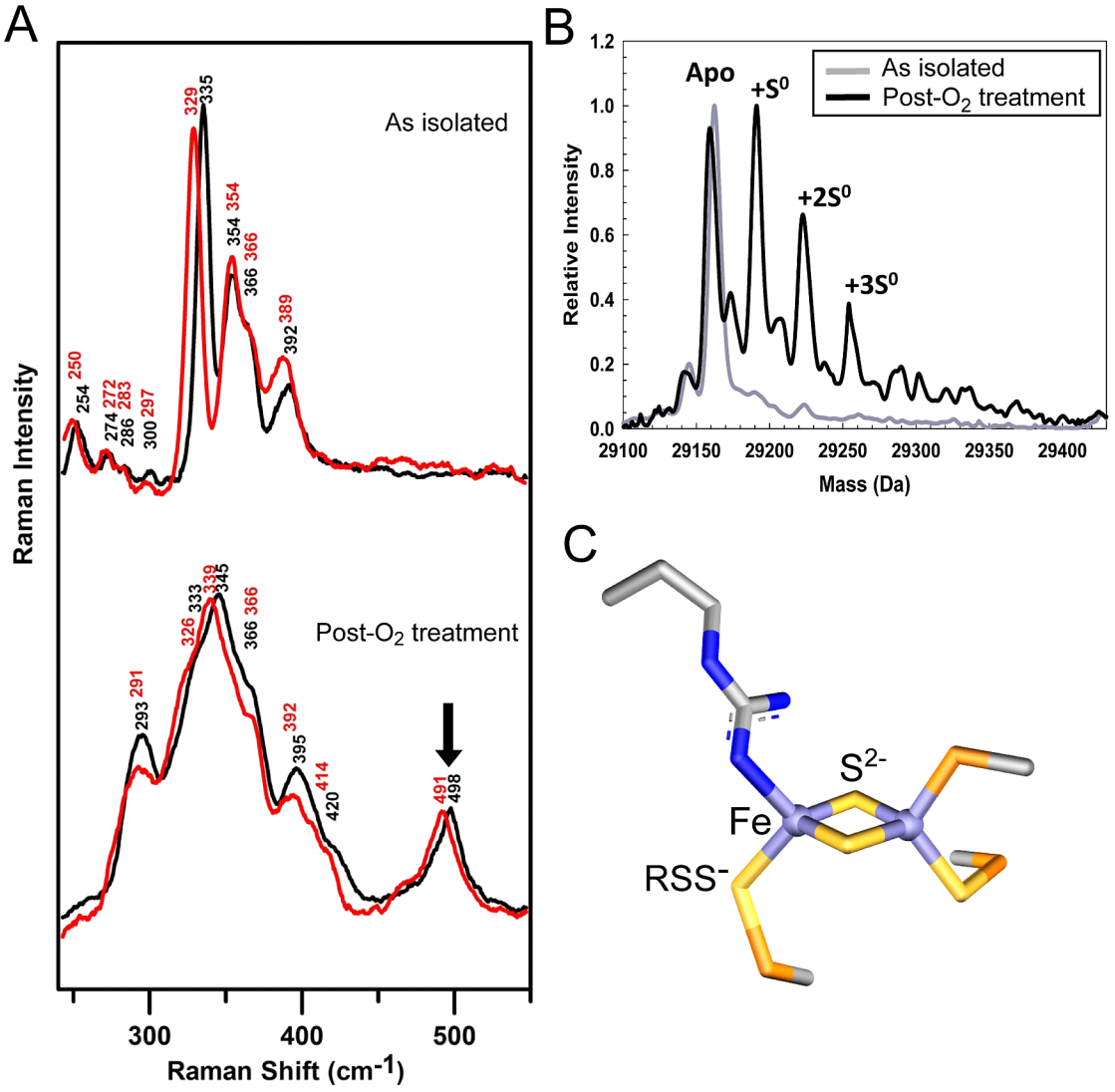

Figure 3. Formation of a persulfide coordinated [2Fe-2S] FNR form. (A) Resonance Raman spectra of FNR before and after the addition of O_2 showing vibrations characteristic of the [4Fe-4S] and [2Fe-2S] cluster frameworks, respectively. Note that the Raman shift represents the vibrational frequency. The vibration at 498 cm^{-1} that shifts to 491 cm^{-1} on isotopic substitution of S^{32} to S^{34} is an S−S stretching vibration (indicated by arrow). This confirms the presence of at least one persulfide ligand to the [2Fe-2S] cluster. (B) LC-MS spectrum of FNR showing the accumulation of S^0 by the protein after exposure to O_2. S^0 is bound either as a persulfide or, possibly, polysulfides of cysteine. (C) The first example of a structurally characterized [2Fe-2S] cluster coordinated by a persulfide ligand, found in *T. maritima* HydE (pdb file 4JXC). The resonance Raman spectrum of this center shows a band at 498 cm^{-1} characteristic of a coordinated persulfide, RS−S^-.[21]

contributed significantly to understanding of the chemistry of this cluster conversion reaction.

3.1. The [3Fe-4S] Intermediate

The seminal work of Kiley and colleagues established the basis of the O_2-mediated cluster conversion reaction,[14,15] but the underlying chemistry was not understood. Early reports indicated that a $[3Fe\text{-}4S]^{1+}$ cluster was formed during the conversion, but this was a relatively minor species[14] and its physiological relevance was questioned.[16] Using a combination of visible absorbance and EPR spectroscopies on identical samples, we demonstrated that the EPR active ($S = 1/2$) $[3Fe\text{-}4S]^{1+}$ species is a transient intermediate in the conversion process, which, therefore, can be described in two steps (Figure 2B).[17] Step 1 involves the release of Fe^{2+} to generate the $[3Fe\text{-}4S]^{1+}$ intermediate (Scheme 1). This is likely to occur following a one electron oxidation of the $[4Fe\text{-}4S]^{2+}$ cluster, yielding an unstable $[4Fe\text{-}4S]^{3+}$ cluster that releases a Fe^{2+} ion.[18] The rate of step 1 ($k \approx 250\ M^{-1}\ s^{-1}$) is linearly dependent on O_2, indicating that there is no rapid O_2 prebinding step and that the initial oxidation reaction occurs more slowly than the subsequent ejection of the Fe^{2+} ion.[17,19] Step 1 also results in the one electron reduction of O_2 to superoxide, $O_2^{-\bullet}$, which is likely to be rapidly recycled back to O_2 and H_2O by superoxide dismutase (SOD) and peroxidase, and FNR itself appears to have SOD activity.[17]

Step 2 is the O_2-independent conversion of the unstable $[3Fe\text{-}4S]^{1+}$ species to the $[2Fe\text{-}2S]^{2+}$ cluster ($k \approx 0.008\ s^{-1}$) with concomitant release of a further iron ion and two sulfide ions (Scheme 1).[17,19,20] The relative rates of steps 1 and 2 control the extent to which the $[3Fe\text{-}4S]^{1+}$ intermediate can be observed, and the rate of step 2 in particular is influenced by the reaction environment. For example, Fe^{3+}-chelators enhance the rate of step 2 such that step 1 becomes rate determining and the two steps are kinetically indistinguishable, with no intermediate observed.[19] Iron chelators also influence the

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oxidation state of the cluster-ejected iron. The Fe^{3+} lost from the cluster in step 2 is highly susceptible to reduction in the presence of Fe^{2+} chelators but is protected by Fe^{3+} chelators.[19] These observations have possible physiological relevance for cluster conversion as it occurs in the reducing environment of the cytoplasm.

3.2. Oxidation of Cluster Sulfide

Although two sulfide ions are released from the cluster during the $[4Fe\text{-}4S]^{2+}$ to $[2Fe\text{-}2S]^{2+}$ conversion, they are not ejected into solution.[20] Instead, they undergo two electron oxidation to form sulfane (S^0), reacting with cysteine side chains (RS^-) to give persulfide (RSS^-) that can coordinate the [2Fe-2S] cluster. Evidence was obtained by selective ^{34}S-labeling of the sulfide ions in the [4Fe-4S] form of FNR, followed by identification of resonantly enhanced $^{34}S-^{32}S$ stretching modes in the resonance Raman spectrum of [2Fe-2S] FNR after exposure to O_2 (Figure 3A).[21] Although a denaturing method that does not preserve iron−sulfur clusters, LC-MS can detect S^0 covalently attached to a protein. Multiple cysteine persulfides (at +32, +64, and +96 Da) upon O_2-exposure of [4Fe-4S] FNR were detected (Figure 3B),[21] consistent with early reports that only 70% of the sulfide in the original $[4Fe\text{-}4S]^{2+}$ cluster was detectable following O_2 exposure.[14]

Cluster conversion involving sulfide oxidation is apparently not unique to FNR but is more widespread. Recently, similar such conversions were observed in two different radical SAM enzymes: HydE from *Thermotoga maritima*[22] and biotin synthase (BioB) from *E. coli*.[21] We note that in BioB, the cluster responsible for reductive cleavage of SAM underwent conversion, while in HydE, it was the secondary [4Fe-4S] cluster. Hence step 2 is more complex than initially envisaged and may itself be multiphasic, depending on whether release of iron and sulfide from the cluster occurs simultaneously with sulfide oxidation. The electrons from sulfide oxidation (either two or four electrons for one and two sulfides, respectively) likely reduce, directly or indirectly, O_2 to either H_2O_2 or H_2O (Scheme 1).

3.3. Modulating the O_2 Reactivity of $[4Fe\text{-}4S]^{2+}$ Clusters

The inherent reactivity of iron−sulfur clusters to O_2 means that protein-bound clusters can be susceptible to O_2-mediated "damage". However, the extent of this inherent reactivity is highly variable. Hence, as discussed above, the FNR proteins react rapidly with O_2, while Wbl proteins (see below), which contain an iron−sulfur cluster, are relatively stable under aerobic conditions (their reaction with O_2 is ∼270 times slower than that of FNR with O_2[23]). In the case of FNR, O_2 accessibility is a key requirement for its functional role as an O_2-sensor. However, the reduction potential of the 3+/2+ couple of the [4Fe-4S] cluster, which is under the control of the protein environment, is also important for controlling the ease with which the resting $[4Fe\text{-}4S]^{2+}$ becomes oxidized.[18] Substitutions of residues near to the cluster in FNR significantly affect O_2 reactivity, for example, a decrease in O_2 sensitivity was observed in the case of the substitutions L28H FNR[24] and S24F FNR,[25] both of which involve a residue lying adjacent to a cluster-coordinating cysteine. However, the chemical factors modulating O_2 reactivity are not clear. For L28H, disruption of hydrogen bonding that influences cluster stability and hence redox potential was proposed to be important,[24] while for S24F FNR, lower accessibility to the cluster caused by the larger side chain of phenylalanine was proposed to be the major influence.[25] Although further investigations are needed to elucidate all the factors influencing cluster reactivity, the available data imply that substitution of neighboring residues can tune the O_2-sensitivity of FNR proteins to elicit responses at physiologically relevant O_2 concentrations that could range from ≤3 nM to ∼250 μM.[25,26]

4. A COMMON MECHANISM OF NITROSYLATION OF REGULATORY PROTEIN [4Fe-4S] CLUSTERS

The gaseous diatomic molecule NO is a reactive, membrane-permeable radical. In eukaryotes, it is generated by nitric oxide synthase(s) and functions (at nanomolar concentrations) as a signaling molecule via its reversible coordination to the heme group of guanylate cyclase, activating it to produce cGMP and resulting in vasodilation. NO is also a defense molecule deployed by mammalian macrophages (at micromolar concentrations) in response to infection by bacteria. Host generated NO reacts readily with several bacterial targets, leading to thiol S-nitrosation, N-nitrosation of certain amino acids (e.g., tryptophan), and nitrosative DNA damage. Metal cofactors are major targets for NO, with iron−sulfur proteins being especially susceptible to NO-induced damage. As well as exposure to exogenous NO, some bacteria generate endogenous NO via bacterial nitric oxide synthase and by the reduction of nitrite to NO.[27] Thus, bacteria must adapt to counteract the deleterious effects of NO (at high concentration) and respond to lower NO concentrations that signal environmental stress conditions. For example, because NO rapidly reacts in the presence of O_2, buildup of detectable concentrations of NO is associated with hypoxia.[28] As a result, many bacteria contain proteins that function as NO sensors, to control detoxification, repair, and long-term survival systems as part of a stress response mechanism.

4.1. A Multistep Reaction Involving up to Eight NO Molecules per Cluster

WhiB-like (Wbl) proteins ($M_r \approx 10-15$ kDa) contain a highly conserved cysteine residue motif, $C(X_n)C(X_2)C(X_5)C$, which can bind a [4Fe-4S] cluster.[23] The Wbl family is restricted to the Actinobacteria, a phylum that includes *Streptomyces*, the most abundant source of clinically relevant antibiotics, and important pathogens, such as *Mycobacterium tuberculosis*. Actinomycetes invariably contain multiple Wbl proteins and determining their individual functions is a challenge. In *S. coelicolor*, several Wbl proteins, including WblA, WhiB, and WhiD, are required at various stages of sporulation.[29] In *M. tuberculosis*, Wbl proteins are implicated in the ability to persist within the human host for decades, as well as resistance to a wide range of antibiotics. *M. tuberculosis* WhiB3 regulates lipid and polyketide biosynthesis, including triacylglycerol accumulation in response to activated macrophages.[30] [4Fe-4S] WhiB3 is O_2-sensitive and reacts with NO,[31] suggesting that WhiB3 acts as a sensor of O_2 and NO to control expression of genes involved in intermediary metabolism. This may explain the well documented accumulation of triacylglycerol in *M. tuberculosis* in response to hypoxia and NO exposure.[32] The *M. tuberculosis whiB1* gene is essential, and whereas [4Fe-4S] WhiB1 did not bind to the *whiB1* promoter DNA, following cluster nitrosylation, binding was observed.[33] These observations strongly suggest that the reaction of NO with Wbl proteins may be an important signaling process in Actinomycetes by controlling DNA-binding.

Our *in vitro* studies of the reaction of the Wbl proteins WhiD from *S. coelicolor* and WhiB1 from *M. tuberculosis* with NO have

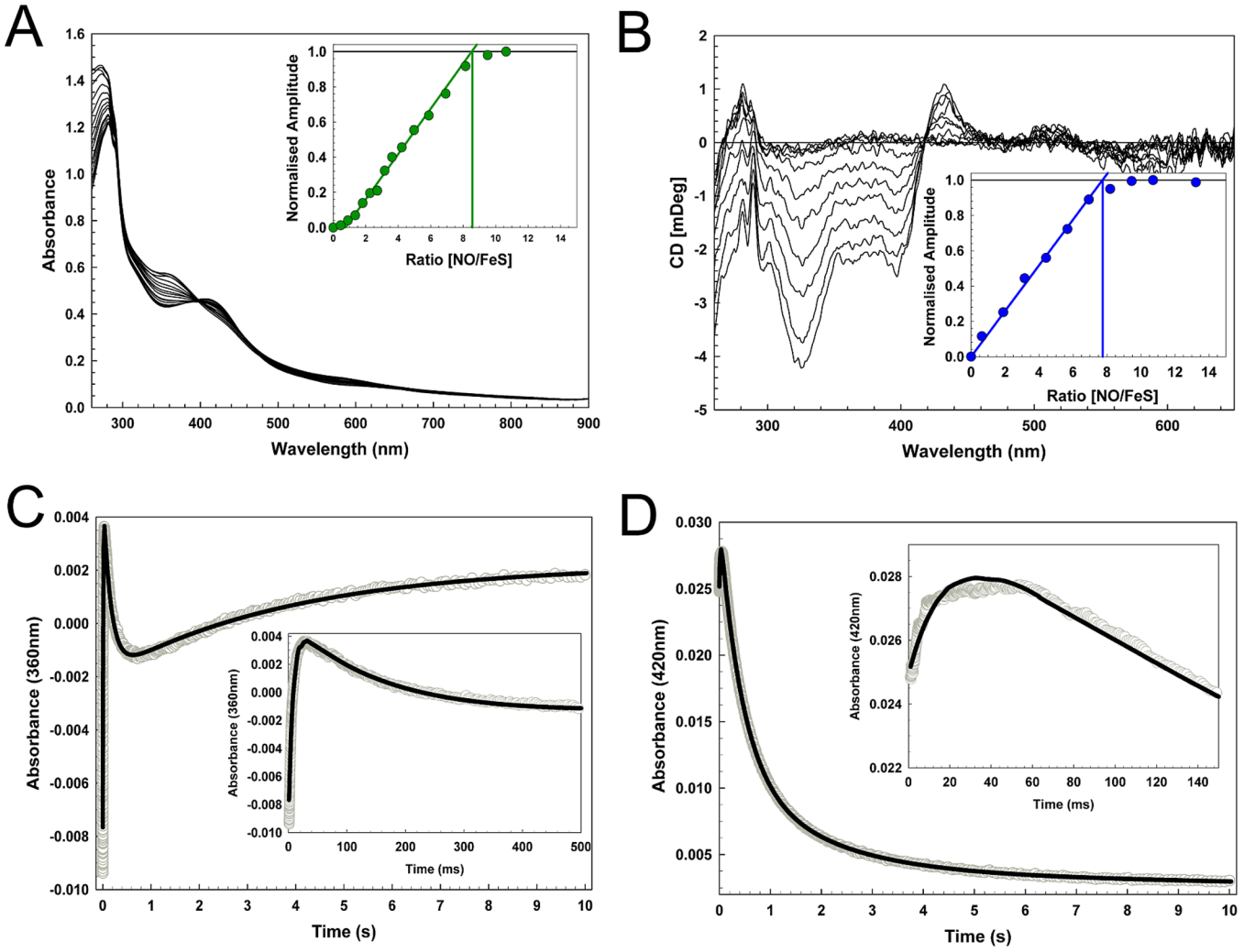

Figure 4. Nitrosylation of *S. coelicolor* [4Fe-4S] WhiD. (A) UV−visible absorbance and (B) CD spectra of the [4Fe-4S] cluster in WhiD as a function of added NO. The insets show plots of the spectroscopic change as a function of NO concentration expressed per cluster revealing, in both cases, saturation of NO binding at a ratio of eight NO per [4Fe-4S] cluster. Panels C and D show the time dependence of absorbance at 360 and 420 nm following addition of an excess of NO to WhiD [4Fe-4S]. The reaction is complete within ∼10 s but consists of at least four phases as shown by the changes at shorter times between 0 and 500 ms, giving evidence for at least three intermediates.

revealed several quite unexpected aspects of cluster nitrosylation.[34] Titration of these proteins with NO resulted in changes in their absorbance and CD spectra that were not complete until approximately eight NO molecules were added per cluster (Figure 4A,B). The form of the titrations suggested that the reaction was complex, and this was confirmed by kinetic studies that revealed a multiphase reaction. A remarkable aspect was the rapidity of the reaction with NO, which with a $t_{1/2}$ of ca. 1 s was ∼10^4-fold faster than the reaction of these clusters with O_2 and much more rapid than previously reported [4Fe-4S] cluster nitrosylation reactions (Figure 4C,D). Four distinct kinetic phases were observed, that is, A → B → C → D → E. Plots of the observed pseudo-first-order rate constants (k_{obs}) against NO concentration were linear for each step, indicating a first order dependence on NO for each step, consistent with the stepwise addition of NO.

Although FNR functions primarily as an O_2 sensor, it also responds to physiological concentrations of NO.[35−37] *In vitro* studies of additions of NO to [4Fe-4S] FNR revealed a rapid reaction similar to that observed for Wbl proteins, with multiple NO-dependent steps and absorbance spectra indicating a similar final product.[37] Interestingly, the rate constant for the slowest step of the NO reaction with FNR is at least ∼3 orders of magnitude greater than that for the slowest step with O_2, indicating that FNR is much more sensitive to NO than to O_2. However, *in vivo* studies showed that the extent of the FNR-regulated transcriptional response was ∼4-fold lower for NO compared with O_2, leading to the proposal that FNR is partially protected in the cell by stress responses initiated by the dedicated NO sensors NorR and NsrR.[37] Thus, NO reacts via a common mechanism with [4Fe-4S] clusters in phylogenetically unrelated regulatory proteins (Wbl and FNR). This implies that, unlike the reaction with O_2, reactivity with NO can be relatively insensitive to cluster environment.

4.2. Cluster Nitrosylation Intermediates and Products

Identification of the intermediates and final product(s) of cluster nitrosylation is challenging because distinguishing between different iron-nitrosyl species is not straightforward, particularly if they are formed only transiently. EPR measurements following addition of substoichiometric and excess NO to [4Fe-4S] regulators revealed the presence of low concentrations (<10% iron) of a dinitrosyl iron complex,

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$[Fe(NO)_2(RS)_2]^-$ (DNIC, Figure 5, where RS^- is $CysS^-$), which has a distinctive $S = 1/2$ EPR signal at $g = 2.03$[34,37] and is

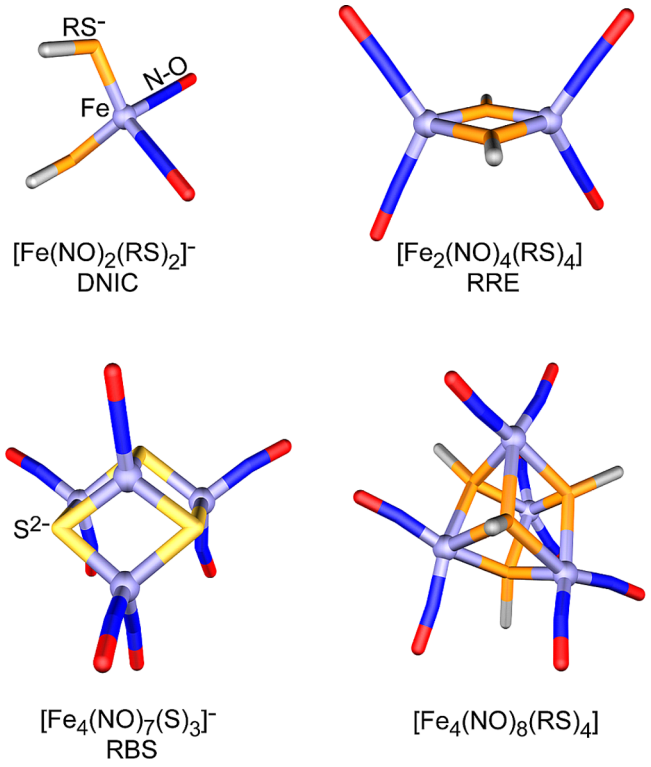

Figure 5. Structures of iron-nitrosyl species. Structures of (mononuclear iron) dinitrosyl iron complex (DNIC), Roussin's red ester (RRE), Roussin's black salt (RBS), and a putative tetranuclear iron octanitrosyl cluster species. Iron, sulfide, thiol, and NO are indicated.

often represented using the Enemark–Feltham notation as $\{Fe(NO)_2\}^9$.[38] Contrary to many reports of reactions of NO with protein-bound iron–sulfur clusters, DNIC is not a major product of Wbl cluster nitrosylation. The absorbance spectra of the final products were similar to those of a dinuclear iron dinitrosyl species known as Roussin's red ester, $[Fe_2(NO)_4(RS)_2]$ (RRE, Figure 5), an EPR-silent complex composed of two $\{Fe(NO)_2\}^9$ units. RRE species have been obtained following the reaction of NO with model iron–sulfur clusters[39] and with a Rieske-type protein.[40] For WhiD, it was proposed that two RRE-like species might be formed, each with two bridging cysteine residues, within the iron–sulfur cluster binding pocket. The proximity of two such species imposed by the cysteine coordination suggests the possibility that a dimer of RRE-like species might be formed, composed of four iron ions bridged by cysteine residues with each iron bound by two NO ligands ($[Fe_4(NO)_8(Cys)_4]$ (Figure 5). DFT calculations based on model complexes indicated that such a species may be stable.[34] Another well characterized small molecule iron-nitrosyl species, Roussin's black salt, $[Fe_4(NO)_7(S)_3]^-$ (RBS, Figure 5), in which a tetranuclear array of irons is bridged by sulfides,[41] has been reported after nitrosylation of *Pyrococcus furiosus* [4Fe-4S] ferredoxin.[42] Further work is underway to identify not only the products of the reaction of FNR/Wbl iron–sulfur clusters with NO but also the intermediates.

4.3. Oxidation of Cluster Sulfide

Chemical analysis of clusters in Wbl and FNR before and after the addition of NO revealed significant oxidation of S^{2-} to S^0 during the reaction and the retention of the majority of this S^0 with the protein throughout gel filtration.[34,37] Application of LC-MS to pre- and post-NO treated samples of [4Fe-4S] FNR also indicated a significant increase in persulfide bound protein, with additional peaks at +32, +64, +96, and +128 Da, corresponding to apo-FNR containing between 1 and 4 S^0 adducts per protein. Consistent with this, whole cell studies of the effects of NO revealed the formation of persulfide coordinate iron-nitrosyl species that were, it was proposed, associated with FNR.[43]

Thus, generation of cysteine persulfides appears to be a common feature of the cluster reactions with NO and O_2 in both FNR and Wbl (section 3.2) and may, indeed, be much more widespread than previously suspected. However, the mechanism of sulfide oxidation must be different for the NO reaction compared with the O_2 reaction (Scheme 1). NO is a strong π-acceptor ligand that complexes with iron typically in the +1 state. Hence, six electrons are required to reduce all of the iron in a $[4Fe\text{-}4S]^{2+}$ cluster to the Fe^{1+} state. Three sulfide ions could thus act as the source of electrons yielding up to three S^0.

5. CLUSTER REPAIR FOLLOWING REACTION WITH O_2 OR NO

The [2Fe-2S] form of FNR decays ($k \approx 0.0025\ s^{-1}$) in the presence of O_2 to form cluster-free apoprotein.[44] The apoprotein can incorporate a "new" cluster following oxidative disassembly of the original one, as demonstrated by *in vitro* reconstitution experiments and, more importantly, *in vivo* through reactivation of FNR in cells where protein synthesis was inhibited.[45] However, the relatively slow formation of apo-FNR from the [2Fe-2S] form suggests that the reverse conversion of [2Fe-2S] to [4Fe-4S] could take place if O_2 levels become sufficiently low to stabilize the [4Fe-4S] form.[14,21] Storage of sulfide ions as persulfide ligands to the [2Fe-2S] cluster implies that only Fe^{2+} and a supply of electrons is needed to drive the reverse conversion. *In vitro* experiments show that this is indeed possible: $[4Fe\text{-}4S]^{2+}$ FNR could be regenerated by anaerobic incubation of the cysteine persulfide-coordinated $[2Fe\text{-}2S]^{2+}$ cluster with the reductant dithiothreitol (DTT) and Fe^{2+}, in the absence of exogenous sulfide (Figure 6A). Approximately 75% of the original $[4Fe\text{-}4S]^{2+}$ clusters was restored within 20 min of addition of excess Fe^{2+} to [2Fe-2S] FNR (Figure 6B–D).[21] In contrast, no significant $[2Fe\text{-}2S]^{2+}$ to $[4Fe\text{-}4S]^{2+}$ conversion occurred using glutathione in place of DTT. An overview of FNR cluster conversion and repair is provided in Figure 7. *In vivo* demonstration of the importance of this novel mode of cluster repair, which does not require the intervention of the iron–sulfur cluster biosynthetic machinery, is now needed.

It is also interesting to consider the fate of nitrosylated clusters. Because they also appear able to store sulfur as cysteine persulfide, they may also have the potential for cluster repair. *E. coli* cells lacking the *ytfE* gene showed enhanced susceptibility to nitrosative stress and a deficiency of several iron–sulfur cluster proteins. This led to the proposal that YtfE, a di-iron protein, is required for the repair of iron–sulfur cluster proteins following nitrosative stress.[46] Recently, it was shown that YtfE (renamed RIC for repair of iron–sulfur clusters) can donate iron for iron–sulfur cluster biosynthesis.[47]

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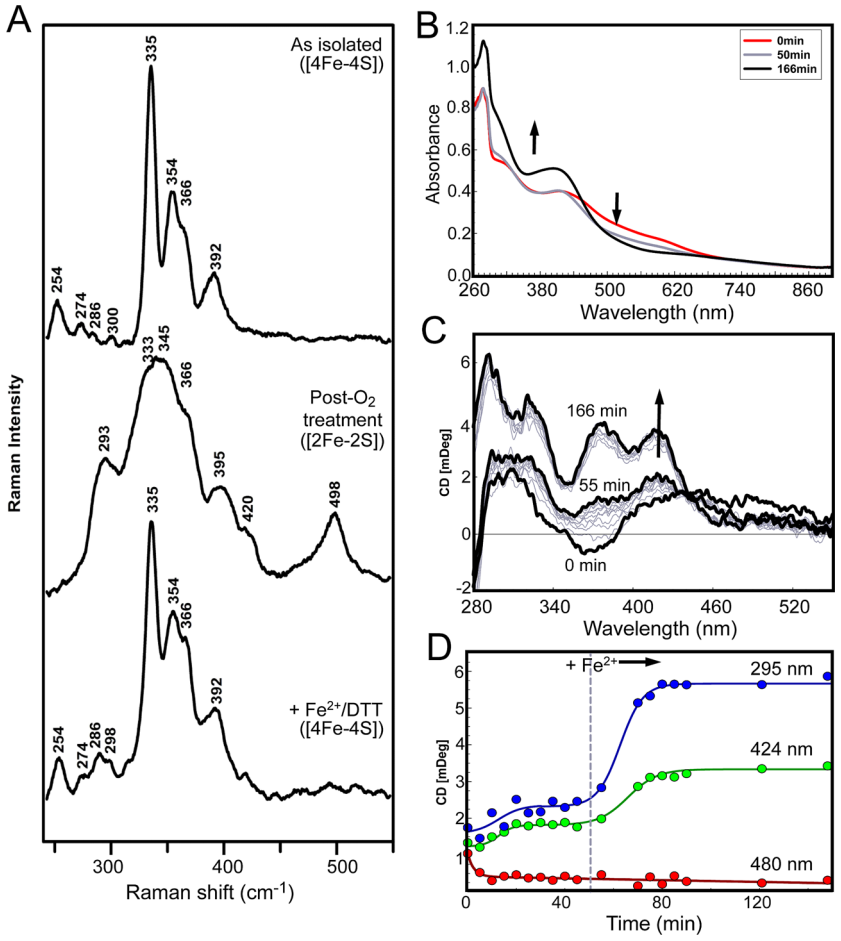

Figure 6. Cycling FNR clusters between [2Fe-2S] and [4Fe-4S] forms. (A) Resonance Raman spectra of FNR in the [4Fe-4S] form (upper), after the addition of O_2 showing the [2Fe-2S] persulfide ligated form (middle), and following incubation of the [2Fe-2S] form with Fe^{2+} and DTT to regenerate the [4Fe-4S] state (lower). Panels B, C, and D show the time course of the conversion process from [2Fe-2S] to [4Fe-4S]. (B, C) Absorption and CD spectra of the [2Fe-2S] form after the addition of DTT at 0 min. At 50 min, excess Fe^{2+} was added under anaerobic conditions. Arrows indicate the direction of changes of intensity over time. (D) Plots of the CD intensity at 295, 424, and 480 nm as a function of time show that [2Fe-2S] to [4Fe-4S] conversion requires the addition only of Fe^{2+} to yield ~75% of the [4Fe-4S] form. Since no sulfur was added, persulfide ion must be the source of sulfur required to rebuild the [4Fe-4S] cluster.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

The inherent sensitivity of iron−sulfur cluster proteins to a range of reactive oxygen and nitrogen species has been exploited in nature through the evolution of a range of regulatory proteins, in which a cluster acts as the sensory unit that modulates the DNA-binding activity of the regulator. The chemistry described in this Account has established the remarkable complexity of iron−sulfur cluster reactions with both O_2 and NO, both of which are multistep reactions. Some key general features have been discovered. The O_2-mediated cluster conversion of [4Fe-4S] FNR to a persulfide-coordinated [2Fe-2S] form, in which cluster-released sulfur is stored on the protein, is also observed in radical SAM enzymes,[21,22] and as in FNR, these conversions are likely to proceed via a [3Fe-4S] intermediate. In the case of NO, reaction with the [4Fe-4S] clusters of regulatory proteins occurs extremely rapidly (even compared with the high rate of the O_2 reaction of FNR) and involves stepwise addition of NO molecules leading to the formation of iron-nitrosyl species. While details of the molecular products have yet to be conclusively demonstrated, our work has shown unequivocally that they are multinuclear iron nitrosyl species rather than the long-known mononuclear iron DNICs.[34,37]

A major challenge now is to determine the factors that control cluster reaction specificity and modulate the relative reactivities of a cluster toward O_2 and NO. These new results suggest that clusters are inherently more reactive toward NO than they are to O_2. Thus, it is not immediately obvious why the primary function of a regulator such as FNR is O_2 sensing, rather than NO sensing. It is likely that this is not determined by the cluster chemistry *per se* but rather through the control of NO accessibility to FNR in the cell.[37] Although not yet demonstrated, the *E. coli* NO sensors NorR and NsrR most likely have a greater reactivity toward NO than does FNR, such that the latter is protected from NO when these regulators are present. This suggests a hierarchy of protein reactivities toward NO.[37] To enable NO sensing regulators to function in aerobically growing cells (such as *S. coelicolor* and *M. tuberculosis*), it is likely that their O_2 reactivities have evolved to be as low as possible (for example, Wbl proteins react very slowly with O_2[23]).

Structural characterization of iron−sulfur cluster regulators has thus far proved to be extremely difficult; SoxR is the only example of a high resolution structure of a cluster bound form.[48] This undoubtedly reflects the difficulties of working with oxygen sensitive proteins. More structural information is

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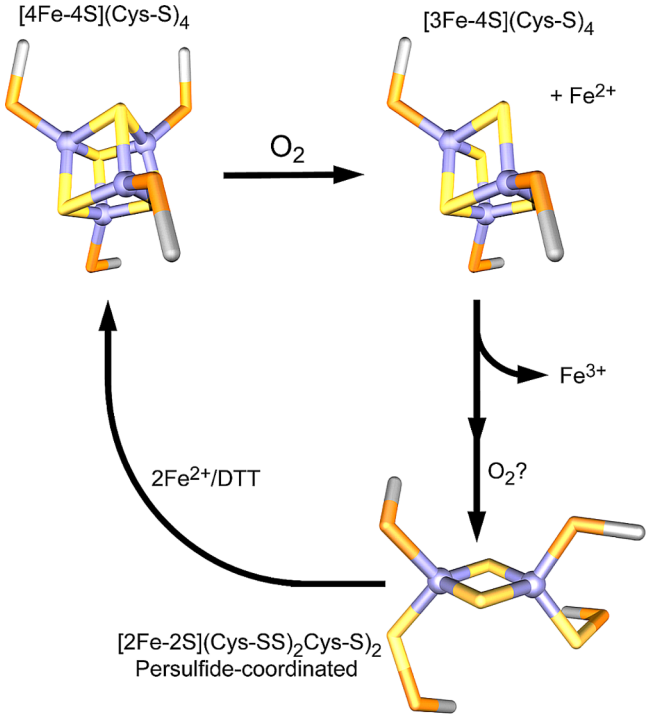

Figure 7. Overall summary of cluster conversion reactions in FNR.

urgently needed to complement the growing mechanistic understanding of how these regulators function.

Finally, cluster repair is an important theme to emerge from our recent work. The demonstration that the persulfide-coordinated [2Fe-2S] cluster of FNR can convert back to the [4Fe-4S] form with only the addition of iron, and a source of electrons has potentially important consequences for understanding iron–sulfur cluster metabolism *in vivo*.[21] *In vivo* evidence for the repair of nitrosylated iron–sulfur clusters has been available for some time.[46] Recent advances in understanding NO-sensing by iron–sulfur cluster regulatory proteins may now provide a useful system to uncover the chemistry of the repair process.

AUTHOR INFORMATION

Corresponding Author

*Nick E. Le Brun. Tel: +44 1603 592699. Fax: +44 1603 592003. E-mail: n.le-brun@uea.ac.uk.

Notes

The authors declare no competing financial interest.

Biographies

Jason Crack received his B.Sc. in biochemistry from Royal Holloway, University of London, in 1999. His Ph.D. (2004) was in iron–sulfur protein chemistry under Andrew Thomson, FRS, at the University of East Anglia (UEA). He has held postdoctoral positions at York and UEA and is currently a senior postdoctoral associate with Nick Le Brun.

Jeffrey Green was awarded B.Sc. (1980) and Ph.D. (1983) degrees in biochemistry by the University of Hull. After postdoctoral positions with Howard Dalton, FRS (Warwick), and John Guest, FRS (Sheffield), he took up a BBSRC Advanced Fellowship at the University of Sheffield, where he is currently professor of microbiology.

Andrew Thomson, FRS, received the D.Phil. degree from Oxford in 1965. During postdoctoral work at Michigan State University with Barnet Rosenberg, he established the biological role of cisplatin. Subsequently, with the late Colin Greenwood, he founded at UEA a multidisciplinary research center where he pioneered the application of MCD spectroscopy to metalloproteins.

Nick Le Brun obtained his B.Sc. (1990) and Ph.D. (1993) in chemistry from UEA. After a Wellcome Trust Fellowship with Andrew Thomson, FRS, and Geoff Moore at UEA and an EMBO Fellowship with Lars Hederstedt in Lund, Sweden, he joined the faculty at UEA where he is currently professor of biological chemistry and Director of the Centre for Molecular and Structural Biochemistry (CMSB).

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