

Review

Native Protein Template Assisted Synthesis of Non-Native Metal-Sulfur Clusters

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Abstract: Metalloenzymes are the most proficient nature catalysts that are responsible for diverse biochemical transformations introducing excellent selectivity and performing at high rates, using intricate mutual relationships between metal ions and proteins. Inspired by nature, chemists started using naturally occurring proteins as templates to harbor non-native metal catalysts for the sustainable synthesis of molecules for pharmaceutical, biotechnological and industrial purposes. Therefore, metalloenzymes are the relevant targets for the design of artificial biocatalysts. The search and development of new scaffolds capable of hosting metals with high levels of selectivity could significantly expand the scope of bio-catalysis. To meet this challenge, herein, three native scaffolds: [1Fe-4Cys] (rubredoxin), [3Fe-4S] (ferredoxin), and [S₂MoS₂CuS₂MoS₂]-ORP (orange protein) protein scaffolds are case studies describing templates for the synthesis of non-native monomeric to mixed metal-sulfur clusters, which mimic native Ni containing metalloenzymes including [Ni-Fe] Hydrogenase and [Ni-Fe] CO Dehydrogenase. The non-native metal-substituted metalloproteins are not only useful for catalysis but also as spectroscopic probes.

Keywords: designed metalloproteins; models of [Ni-Fe]-hydrogenase and [Ni-Fe]-CODH; orange-protein and spectroscopic probes



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1. Introduction

Nature has evolved in order to utilize metal ions and/or metal clusters within protein scaffolds to build up metalloproteins that accomplish diverse chemical reactions enabling to sustain of life [1–4]. The versatility of the metals and biological ligands available in proteins is amazing. The same metal (with a set of conserved amino acids as ligands) may show different electronic/physical properties, performing a wide range of biological roles in different metalloproteins [5–7]. Nature utilizes a range of different metals and recruits the correct metal into proper protein environments to execute selective functions [5–7]. The nuclearity of metal-cofactors varies from monomeric to multimeric. Monomeric metalloenzymes are well studied, such as cupredoxin [8], rubredoxin [9], cytochrome P450 [10,11], and molybdenum-enzymes [12], which are involved in a variety of biochemical transformations, and with relevant roles in electron transfer processes. Furthermore, many biochemical transformations occurred by a variety of complex metalloenzymes such as nitrogen-fixing nitrogenases [13,14], photosystem [15,16], hydrogenases [17,18], and carbon monoxide-dehydrogenase (CODH) [19,20].

However, many enzymes show intrinsic promiscuity [21,22] for various forms of chemical reactions, whereas other activities are obtained by only a small alteration of their active site or protein environment [23]. The diversity of promiscuous enzymatic activity can be expanded by the incorporation of a variety of metallic ions at the active sites of metalloproteins, catalyzing a wide range of chemical transformations [2,24–26]. Handling of the metal-binding site of metalloprotein is usually aimed for two main reasons: (i) to replicate the active site of other native metalloenzymes, and (ii) to design spectroscopic

probes for elucidating the structure and function of native metalloenzymes. Herein, three native protein scaffolds, [1Fe-4Cys] (rubredoxin) [9], [3Fe-4S] (ferredoxin) [27,28] and [S₂MoS₂CuS₂MoS₂]-ORP (orange protein) [29,30] polypeptides are considered as templates and have proven a handy platform for the synthesis of non-native metal–sulfur or mixed-metal–sulfur clusters with novel functions. The protein-assisted metal–sulfur cluster synthesis was started in the 1980s to investigate the structures and functions of metalloproteins [31–33] and then explored in many metalloproteins [34–36]. Indeed, Rubredoxin (Rd) is the simplest and smallest iron–sulfur protein possessing one Fe that is replaced by a series of non-native metal ions to study the biochemical properties of Rd [9] and to design the model compounds, such as Ni-Rd, that are considered as a model of [Ni-Fe]-Hydrogenase [37,38].

In the case of [3Fe-4S]-ferredoxin, the vacant Fe-site is filled up by a variety of metal ions to yield a wide range of hetero-metal–sulfur cubane clusters ([M,3Fe-4S]). Interestingly, designed [Ni,3Fe-4S]-ferredoxin mimics the native [Ni-Fe] carbon monoxide dehydrogenase [39]. The third protein, ORP, Ref. [29] is used as a template for the synthesis of hetero-metal-clusters as spectroscopic probes by replacing the diamagnetic Cu^I from [S₂MoS₂CuS₂MoS₂]^{3−} of ORP, enlarging the scope of the initial studies [40]. Therefore, this review focuses on non-native metal ions that are incorporated in active sites by chemical manipulation of protein template, aiming at the synthesis of derivatives that may be described as either model enzymes or spectroscopic probes.

2. Covalently vs. Non-Covalently Coordinated Metal-Cofactors

In metalloenzymes, metal cofactors are bound to certain amino acid residues, which are necessary to drive the many biochemical transformations. Naturally occurring metalloproteins possess a native metallocofactor that can be attached to protein templates through covalent or non-covalent interactions by a group of side chain amino acid residues. The covalently attached metallocofactors are commonly found in many metalloproteins such as rubredoxin [9], ferredoxin [27], molybdoenzymes [12], nitrogenase [13], and hydrogenase [17]. In contrast, non-covalently attached metallocofactors are observed in a very limited number of metalloproteins, such as orange protein (ORP) [29] and Mo-Fe protein [41]. However, covalent anchoring metallocofactors are more strongly embedded in protein scaffolds than non-covalent anchoring metallocofactors, but both types of metal-cofactors are directly tuned by protein scaffolds. Furthermore, unlike covalent assembly, non-covalent (supramolecular) assembly can allow the entry and exit of the guest molecule in the host cavity reversibly. The designed information of molecular structure is stored in the host-cavity (protein template) that builds the guest structure through non-covalent interactions, including hydrogen-bonding, ion-pair, and hydrophobic interactions [42,43]. Inspired by Nature, the non-native metal ions have been recruited into protein scaffolds by non-covalent or covalent chemical ways to design artificial metalloenzymes.

3. Iron–Sulfur Proteins

Iron–sulfur ([Fe-S]) clusters are the utmost ancient and ubiquitous biological inorganic cofactors that are involved in a wide range of biochemical processes, including electron transfer, gene regulation, and catalysis [44–46]. [Fe-S] clusters show a variety of frameworks in biology and the most common structural types are [1Fe-4Cys] in rubredoxin [9], [2Fe-2S] in plant ferredoxin [4,47], and [4Fe-4S] in bacterial ferredoxin [48]. In addition, [3Fe-4S] type clusters are found in biology, structurally related to [4Fe-4S] cores, namely in ferredoxins and several complex metalloproteins [27,28]. Apart from the classic [Fe-S]-clusters, the unique, bigger, and highly complex [Fe-S]-clusters are found in many proteins such as shiroheme-[4Fe-4S]-cluster in sulfite reductase [49–51], [Mo-7Fe-9S] (FeMo-cofactor) and [8Fe-7S] (P-cluster) clusters in nitrogenase [52], a unique H-cluster in [Fe-Fe] hydrogenases [53], a hetero-metal cluster in [Ni-Fe] hydrogenase [17] and [Ni,4Fe-5S] cluster in carbon monoxide dehydrogenase [54]. Furthermore, a complex [8Fe-9S] cluster in the ATP-dependent reductase from *Carboxydotherrmus hydrogenoformans* [55] and a non-cubane [4Fe-4S] cluster in the heterodisulfide reductase from methanogenic archaea are

observed [56]. Due to structural/functional variability, iron–sulfur clusters are attractive templates to be used for the design of artificial metalloproteins/enzymes, aiming at the elucidation of the structure and function of the intricate systems and possibly other performances such as catalysts. Therefore, in this review, we are focusing on two types of iron–sulfur proteins, mononuclear-rubredoxin, and trinuclear-[3Fe-4S]-ferredoxin.

3.1. Overview of Rubredoxin

Rubredoxins (Rds), a small (5.6 kDa) and simplest case among iron–sulfur proteins, are observed mainly in anaerobic bacteria and archaea [9,57] and one group of eukaryotes (photosynthetic algae and plants) [58,59]. The active site of Rd possesses one iron atom, tetrahedrally ligated by four cysteine residues from two $-CX_2C-X_n-CX_2C-$ segments in a polypeptide chain (Figure 1). A variation of this binding site contains two adjacent cysteine-binding modes ($-CX_2C-X_n-CC-$) in a protein, namely desulfoferredoxin [60]. Based on metal composition, Rds are mainly two types which are single Fe-centre systems (rubredoxins and flavo-rubredoxins) and complex Fe-centre systems, wherein the Rd center is coupled with other types of iron centers (rubrerythrins, nigerythrins, desulfoferredoxins, and desulfoferredoxins) [9,61]. The biological functions of Rds are still unclear, but it presumes to participate in the e^- transfer process cycling between ferrous and ferric forms, in many biochemical processes, which are fatty acid metabolism, detoxification of reactive oxygen species [62,63], and carbon fixation [64]. The redox potentials of all types of Rds fall in the range from -0.1 V to $+0.1$ V (vs. NHE) in spite of the same prosthetic group, FeS_4 [9,61]. It is well established that the redox potential of Rds is highly influenced by many parameters, which are Fe-S bonds, hydrogen bondings [65,66], variation in solvation, electrostatic interaction, and dipole moment. The crystal structures of reduced and oxidized Rds reveal that the average Fe-S bond distance in the oxidized Rd is smaller than the reduced Rd ($d_{Fe^{III}-S} = 2.25\text{--}2.30$ Å vs. $d_{Fe^{II}-S} = 2.30\text{--}2.40$ Å) (Figure 1) [67].

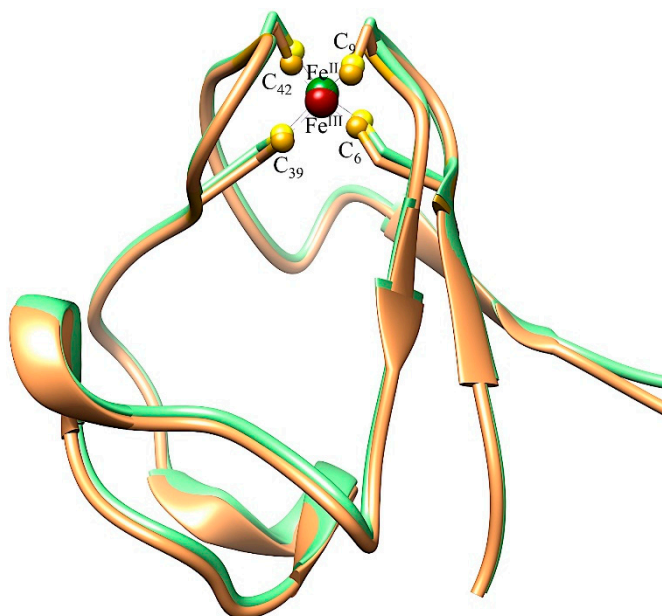


Figure 1. Superimposed of X-ray structures of native Rds from *Clostridium pasteurianum*; oxidized Fe^{III} -Rd (light brown ribbon with red Fe^{III} ball) (PDB: 1FHH) and reduced Fe^{II} -Rd states (light green ribbon with green Fe^{II} ball) (PDB: 1FHM).

Furthermore, the protein fold of Rd is remarkably stable under aerobic conditions, in a wide range of pH, solvents, and temperatures, and also stable toward mutagenesis [68]. The native iron in Rd can be replaced by a wide range of metal ions, including $^{57}Fe^{II}$, Co^{II} , Ni^{II} , Cu^{II} , Zn^{II} , Cd^{II} , Hg^{II} , Ga^{III} , In^{III} , and Mo^{VI} [9,38,69–72]. A series of non-native metal substituted Rd variants have been studied to explain the structural, electronic, and magnetic

properties of metal ions in active sites and are also useful as model systems. Indeed, The Cu-Rd derivative enlarges our knowledge of the redox chemistry interplay between Cu^{I} and Cu^{II} in a sulfur-rich protein environment [70,71]. Interestingly, cysteine-rich Cu^{II} -Rd is a remarkably stable compound under dark and argon, indicating thermodynamically unfavorable to “Cu-thiol-auto-reduction” [71]. In addition, molybdenum was also incorporated in apo-rubredoxin to afford the insertion of molybdenum in a cysteinyl coordination sphere, which is of interest as a model complex for resting or active state of molybdoenzymes, which hold a remarkable place in bioinorganic chemistry, because they perform a series of metabolic reactions in the carbon, nitrogen, and sulfur biocycles. However, the designed MoO_2 -Rd derivative promotes the oxygen atom transfer reactions, such as the oxidation of arsenite to arsenate [72]. Among these, designed Ni-Rd is mostly studied as a model of [Ni-Fe]-hydrogenase [37,38,73].

3.1.1. Ni-Substituted Rd: Model of [Ni-Fe]-Hydrogenase

Nature designed an efficient enzyme, Hydrogenases, that reversibly cleave hydrogen, a clean and alternative chemical feedstock to non-renewable energy sources. Hydrogenases have three distinct families, which are [Fe-Fe], [Ni-Fe], and Fe (only) hydrogenases [17,74]. Among them, the bimetallic active site in [Ni-Fe]-hydrogenases are covalently ligated by four cysteine residues. Two cysteines bridge the two metal centers (Ni and Fe), and the other two cysteines are terminally coordinated to the Ni-atom. In addition, unusual biological ligands, CO and CN^- are bonded to Fe-atom in the active site and make them fascinating examples of ‘organometallic’ cofactors [75–77] (Figure 2A). The Ni-atom is believed to be the center of activity for hydrogen evolution. The extensive study of Hydrogenases has a central focus on structure/function aspects for a future protein-based H_2 -evolving technology. Parallely, the bio-inspired small synthetic model systems have extensively progressed for hydrogen conversion catalysts but are not yet comparable to the naturally occurring enzymes [78–81]. It is known that the protein matrix plays a vital role in tuning the activity of the inorganic active site, but direct enzymological studies may be hampered by biological complications/complexities due to its large size with complex catalytic cofactor and toxicity toward the dioxygen molecule. Therefore, size scale intermediates between native enzymes and organometallic model compounds can provide many advantages for both regimes [82–84]. In this perspective, small stable proteins such as rubredoxin and its metal substituted derivatives are available for modeling the active site of bacterial [Ni-Fe]-Hydrogenases.

Ni-substituted Rd (Ni-Rd), an air-stable derivative (Figure 2B), is one such model showing identical structural features at the primary coordination sphere of Ni-fragment of native [Ni-Fe]-hydrogenase (Figure 2A) and native-enzyme-like activity including the evolution of hydrogen, deuterium-proton exchange, and inhibition of hydrogen-evolving activity by carbon monoxide [38]. The characterization of hydrogen-evolving Ni-Rd activity has been significantly expanded during the last decade [85–87]. The first study by Moura and Co-workers reported that recombinant Ni-Rd from *Desulfovibrio* exhibited lower hydrogen-evolving activity, but recently, Shafaat and Co-workers have reported that recombinant Ni-Rd from *Desulfovibrio desulfuricans* ATCC 27774 displays light initiated H_2 production with a high turnover frequency of about $20\text{--}100\text{ s}^{-1}$ at 4°C in solution upon electrochemical study. The electrocatalytic over-potential is about 550 mV, which is comparable to native [Ni-Fe]-hydrogenase at the same condition [85,87]. Furthermore, the covalent attachment of Ni-Rd to the graphite electrode, coupling through amide bond formation shows stable H_2 -evolving activity for many weeks with a higher turnover number of about 6700, but it shows lower turnover frequency due to the slower interfacial electron transfer (ET) rates that modulates the catalytic rate [88]. Ni-Rd is an ideal candidate for studying the molecular mechanism of the native [Ni-Fe]-hydrogenase. A combined experimental and theoretical data indicate that the proton-coupled electron transfer is a vital step for catalysis where Cys-thiolates act as the site for protonation, suggesting a similar mechanism as native [37,86]. The secondary coordination sphere of metalloprotein

plays a significant role in the catalytic cycle. In order to understand the effect of the protein environment on the H₂-activity of Ni-Rd, a group of mutated Rds is generated, which influences the H₂-evolving activity, suggesting the modulation of the H-bonding network at the vicinity of the active site. The result indicates that Cys35 is the primary site for protonation during catalysis [73]. Moreover, Ni-Rd is generated *in vivo*, and it is indistinguishable from chemically substituted Ni-Rd, presenting a structural and functional replica of native enzymes [89].

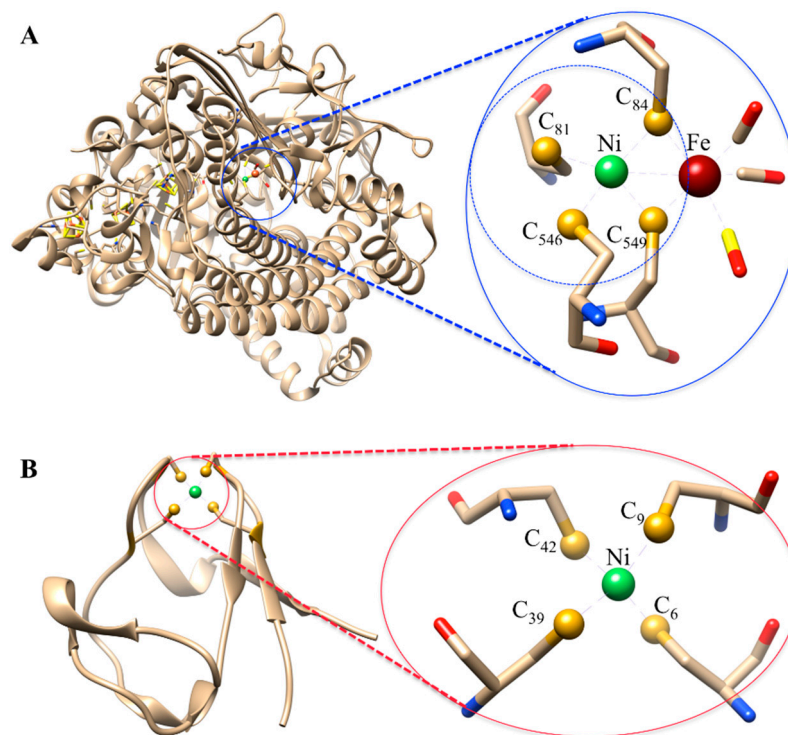


Figure 2. The Ni-Rd model shows identical structural features at the primary coordination sphere of Ni-fragment of native [Ni-Fe]-hydrogenase. **(A)** Crystal structures of [Ni-Fe]-Hydrogenase from *Desulfovibrio vulgaris Miyazaki F* (PDB:1H2R), highlighted the [Ni-Fe] active site with cysteine coordination and **(B)** Crystal structure of Ni-Rd from *Desulfovibrio gigas* (PDB:1R0J), highlighted the Ni-site with cysteine coordination.

Ni-Rd is also extended to design hybrid catalysts for light-driven H₂ evolution, namely “solar fuel”, which is an emerging research area [90–93]. In order to meet this, a ruthenium-based chromophore is covalently connected to mutated Ni-Rd through free Cys31 residue to build a hybrid enzyme, Ru,Ni-Rd, that is capable of photo-induced hydrogen production [87,94]. The hydrogen generation rate highly depends on the distance between Ru and Ni centers in Ru,Ni-Rd suggesting the intramolecular electron transfer in catalysis. A series of Rd variants are designed as probes where free cysteine residue is located at different positions 17, 31, 38, and 45 to understand the impact of chromophore attachment site on hydrogen evolving activity of Ru,Ni-Rd [87]. The hydrogen production efficiency is lowest when the Ru-chromophore is the longest distance from the Ni-active site. This approach is ideal for the manufacture of solar fuels [87]. The modification of metal-site in rubredoxin will be considered great progress and a breakthrough in the biosynthetic inorganic chemistry field. Therefore, the outstanding results and progress in this research would certainly have a great impact on the future as a potential alternative fuel source.

3.1.2. Spectroscopic Probes-M-Rd

A number of metal-substituted Rd derivatives are available and are also useful for the study of various spectroscopies due to their rich optical and magnetic nature in order to understand the structure and function of Rds [9]. Indeed, the non-native ⁵⁷Fe-Rd is

employed as a Mössbauer probe for the study of the oxidation state of the Fe center as well as the covalent nature of the Fe-S bond. Mössbauer spectroscopy study indicates larger Fe-S covalency nature in Desulfiredoxin over Rd [95,96]. The other spectroscopic probe is NMR, which gives the information about the cysteine coordination at Fe-site in Rd but paramagnetic Fe causes broadening of ^1H -NMR [97]. In order to overcome this paramagnetic NMR, the native Fe in Rd is replaced by a diamagnetic Zn^{II} [98,99]. The NMR study of Zn-Rd shows well-defined as well as well-resolved NMR peaks that indicate a similar structure to the native structure [100,101]. Likewise, Cd substituted Rd is more advantageous for ^{113}Cd -NMR study to gain more structural information [102,103]. The Cd-Rd is extensively used as a ^{113}Cd -NMR probe for studying the metal–cysteine ligation in various metalloproteins [104]. The potential redox value of Rds is highly affected by the six conserved H-bonding interactions which are observed between cysteine–sulfur and vicinal amide–protons [85]. The NMR study of ^{113}Cd -substituted Rd-Cp and WT-Rd-Cp shows a large ^1H chemical shift of amide proton in the vicinity of the active site (NH–S), suggesting the variation of the redox potential [105]. The small M-Rd derivative is an efficient NMR probe to study the Fe-S–HN interaction in other [M,Fe-S] proteins.

Interestingly, the native Fe-Rd is also chosen as a paramagnetic NMR probe to be useful for the protein–protein interaction study [98]. Indeed, two proteins, Fe-Rd and cytochrome c3 (cyt c3), are considered binding partners. The paramagnetic probe Fe-Rd maps the interface of the target protein, cyt c3, by NMR study. The NMR study identifies the heme methyl groups in cyt c3 that participate in the binding surface interface of the Rd-cyt c3 complex [98]. This is a valuable approach to extending the study to other protein partners.

3.2. Overview of [3Fe-4S] Ferredoxin

Widespread distribution and multiple roles of iron–sulfur clusters with variable structure and oxidation states have been increasingly disclosed. [Fe-S]-proteins show diverse functions, which are electron transfer, nitrogen fixation, photosynthesis, enzymatic catalysis, signaling, respiration, gene regulation, and DNA repair and replication [106–110]. Ferredoxins (Fds) are an important class of [Fe-S] proteins and have different sub-classes according to their composition of iron–sulfur, including [2Fe-2S], which is known as plant-Fd [111,112], and [3Fe-4S] and [4Fe-4S] centers are known as bacteria ferredoxin [27,28]. Like Rd, the redox chemistry of [3Fe-4S] is also highly influenced by pH because it has an inherent tendency for protonation [7,113]. This protonation chemistry has been found in all [3Fe-4S]-ferredoxin such as [3Fe-4S] ferredoxins from *P. furiosus* [114], *D. gigas* (Fd II) [115], and beef heart aconitase [116], and [7Fe-8S] ([3Fe-4S] and [4Fe-4S]) ferredoxins from *A. Vinelandii* (Fd I) [117], and *D. africanus* (Fd III) [118]. The structure of the cuboidal [3Fe-4S] cluster is an open-faced crown-like structure (Figure 3) with three inorganic μ_2 -S atoms, which are active centers for protonation [113]. This chemistry is flourished for the synthesis of hetero-metal cluster synthesis by introducing the non-native metal into the open-faced site (below).

A common [4Fe-4S] cluster is covalently attached to a protein scaffold with a typical cysteine binding motif: –Cys- X_2 -Cys- X_2 -Cys- X_n -Cys– or Cys-X-X-Cys-X-X-Cys. The other type, the [3Fe-4S] cluster, differs in only one Fe-center missing at the corner site of the cubane [4Fe-4S] cluster [119] and is generally bound in a polypeptide chain with three cysteine residues. Interestingly, both trimeric cuboidal and tetrameric cubane clusters are reversibly interconverted. The first protein-bound [3Fe-4S] cluster was found in aconitase in 1984 [120]. The [3Fe-4S] is an inactive form of aconitase, and it is easily interconverted to form an active [4Fe-4S] cluster [120]. Indeed, an easy interconversion between [3Fe-4S] and [4Fe-4S] clusters is observed in *Pyrococcus furiosus* ferredoxin, where one cysteine out of four is replaced by aspartate residue (Figure 3) [121]. In addition, in *Desulfovibrio africanus* ferredoxin III, one of two [4Fe-4S] clusters coordinates with three Cys residues, and it readily converts to the [3Fe-4S] cluster [122]. The fourth iron is ligated by abiological water and hydroxide ligands [123] or protein-derived carboxylate ligands [124].

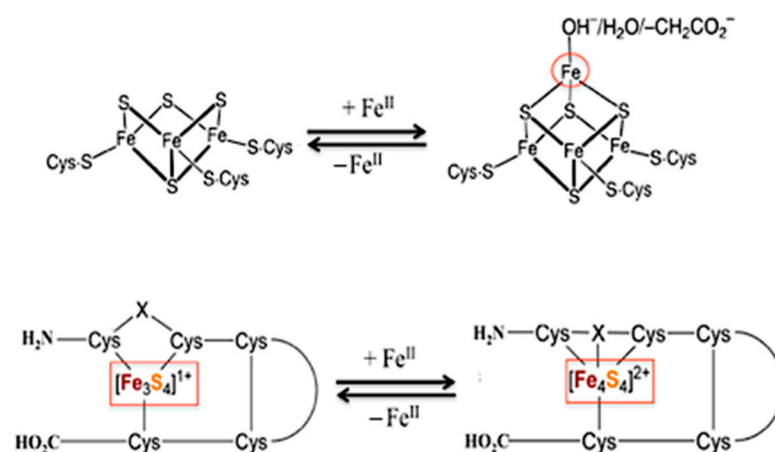


Figure 3. Inter-conversion between [3Fe-4S] and [4Fe-4S].

This conversion has suggested a possible route of recruiting other metal ions into the [3Fe-4S] core to yield a series of novel heteromeric [M,3Fe-4S] type clusters. The construction of [M,3Fe-4S] clusters is preceded by reactions with exogenous metal ions. Indeed, the first result was the formation of the [Co,3Fe-4S]²⁺ cluster that was recognized in 1986 [125]. Since that time, the [3Fe-4S] core has served as a precursor of the formation of [M,3Fe-4S]. Therefore, a series of metal ions are incorporated into the [3Fe-4S] core to yield various hetero-metal-sulfur clusters [M,3Fe-4S] within the protein environment [36,126–128].

3.2.1. Ni-Incorporated in [3Fe-4S]-Fd: Model of Ni-Containing CODH

CO dehydrogenases (CODHs) catalyze the reversible and selective oxidation of CO to CO₂ [129,130]. Nature evolves two types of CODHs: (1) Mo-Cu containing CODHs found in aerobic organisms [131,132], and (2) [Ni-Fe] containing CODHs found in anaerobic organisms [133,134]. Anaerobic bacteria such as *Carboxydotherrhus hydrogenoformans* (CODH_{Ch}), *Moorella thermoacetica* (CODH_{Mt}), and *Rhodospirillum rubrum* (CODH_{Rr}) use CO as a carbon source [135]. The crystal structures of the active sites of [Ni-Fe]-CODH have been reported and harbor a [Ni,4Fe-4S-OHx], [Ni,4Fe-5S] (μ_2 -sulfide covalently bridges the Ni-atom and the distal Fe-atom) and [Ni,4Fe-4S] cluster (Figure 4) [135,136].

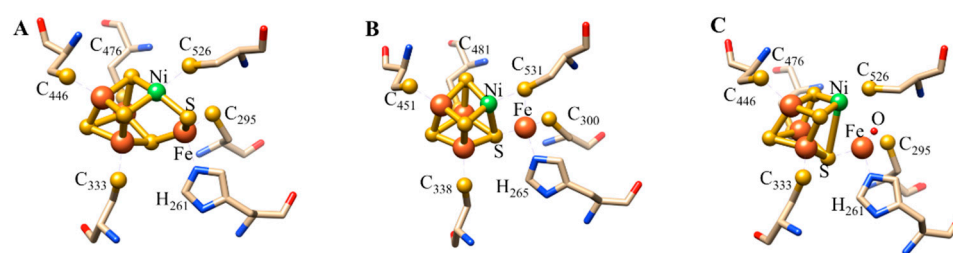


Figure 4. Structures of [Ni-Fe]-CODH from different sources, [Ni,3Fe-5S] (A) from CODH_{Ch} (PDB file 1SU6), [Ni,3Fe-4S] (B) from CODH_{Rr} (PDB file 1JQK) and [Ni,3Fe-4S-O] (C) from CODH-II_{Ch} (PDB file 3B53).

A wide range of metal substituted [3Fe-4S]-ferredoxin derivatives are reported. Among them, [Ni,3Fe-4S] ferredoxin can be considered as a simplified protein-based model of the native [Ni-Fe]-CODH [39]. Indeed, *Pyrococcus furiosus* ferredoxin (*Pf*-Fd), a small e[−] transfer protein contains a cubane [4Fe-4S] cluster that facile interconverts to [3Fe-4S] cluster under suitable experimental conditions due to the presence of non-cysteinylligation (mainly aspartate) at the specific one Fe site [121,137–139]. The apo-*pf*-Fd protein has been recruited with a [Ni,3Fe-4S] cluster that mimics the active site core of the native CODH. Recently, Lewis et al. reported that the [Ni,3Fe-4S] cluster is reconstituted in *pf*-Fd to generate a protein-based simplified model for [Ni-Fe]-CODH that shows reversible e[−] transfer process and binding of both CO (as substrate) and CN[−] (as inhibitor) of CODH [39].

3.2.2. Spectroscopic Probes-[M,3Fe-4S] Ferredoxin

A wide range of non-native metals such as Cu [140], Mn [127], Ni [36,115], Co [115,125,127], Zn [118,141,142], Cd [115,118] and Ag [143] are incorporated into the vacant space in the cuboidal [3Fe-4S] core for the studies of the optical, electronic, magnetic, and redox properties of mixed metal cluster, [M,3Fe-4S]. Indeed, [Cd,3Fe-4S] cluster shows the ground state spin value of $S = 2$ whereas [Cu,3Fe-4S] shows $S = 1/2$ [128]. Furthermore, the redox potentials of [Cd,3Fe-4S]^{2+/+} and [Cu,3Fe-4S]^{2+/+} couples are -470 mV and +190 mV (vs. NHE) respectively [128], suggesting that the ground spin state and redox potential are functions of the incorporation of metal ions in the [3Fe-4S] fragment. The [3Fe-4S]⁰ core has two sites, including a delocalized Fe^{II}•Fe^{III} pair and a localized Fe^{III} site. Upon addition of Zn^{II} ion in [3Fe-4S] fragment, the formation of [Zn,3Fe-4S]⁺ shows unusual hyperfine interaction with spin state of 5/2 using Mossbauer spectra. In contrast, Mossbauer spectra of three Fe^{II} sites in [Ni,3Fe-4S]⁺ show the same quadrupole splitting with the same isomer shifts suggesting that three Fe^{II} sites are delocalized rather than localized Fe^{II} sites [141]. Therefore, small [M,3Fe-4S] derivatives are efficient spectroscopic probes that can be applied to other cubane iron-sulfur proteins.

4. Overview of Orange Proteins

Orange protein (ORP), a monomeric small (~12 kDa) protein possesses a novel hetero-metallic cofactor of the type [S₂MoS₂CuS₂MoS₂]³⁻ that was first isolated from sulfate-reducing bacteria, *Desulfovibrio gigas* in 2000, and still, the function is unknown [29,30]. Now, the ORP encoded gene is found in many anaerobic bacteria [47,144]. The mixed metal-sulfur cluster is non-covalently attached to the protein matrix through hydrophobic and electrostatic interactions [29]. The ORP-Cu cluster is stable in solution for a long time, but the cofactor can be released from the host cavity by disrupting the non-covalent interaction in an irreversible process due to the self-rearrangement to yield a larger cluster that cannot be fit into the protein cavity [145]. Unfortunately, the holo-ORP is crystalized as apo-ORP without a metalocofactor, and the crystal structure of apo-ORP is shown in Figure 5 [146]. Furthermore, NMR data of apo-ORP [147,148] reveal a metal cluster-binding region in D21–A27, H53–N58, and L72–F81 amino acid residues. However, the exact metal cofactor binding region of ORP remains uncharacterized yet. This unusual mixed metal-sulfide cofactor in ORP is the result of the Mo/Cu antagonism that was first found in ruminants [149] and is now being utilized in Wilson's diseases and various states of cancer for the applications of anticopper therapies [149,150].

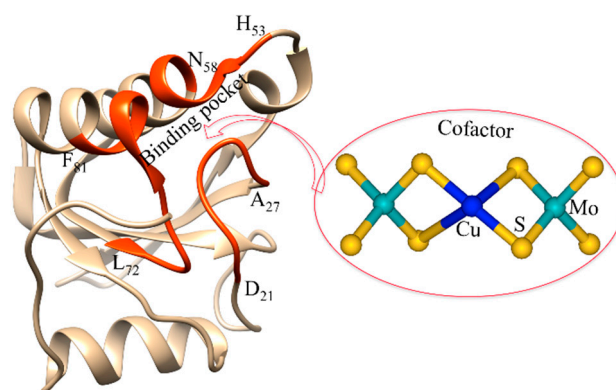


Figure 5. Crystal structure of apo-ATCUN-ORP (PDB: 2WFB) where metal-cofactor [S₂MoS₂CuS₂MoS₂]³⁻ (EXAFS structure) binding region amino acid residues are highlighted with red color in ribbon.

Protein-assisted syntheses of metal-cluster methodologies are quite inspired by the large experience of the Mour's group acquired in the past on self-assembly of iron-sulfur centers and hetero-metal sites in iron-sulfur proteins (described above) [125,142]. During the reconstitution procedure, firstly CuCl₂ was added, followed by TTM (Tetrathiomolyb-

date; MoS_4^{2-}), and the $[\text{Cu}(\text{TTM})_2]^{3-}$ -ORP was obtained with the exact composition of Mo:Cu stoichiometric ratio of 2:1, but in reverse additions, different mixed metal–sulfur composition were obtained. This result can be concluded since TTM has a tendency to absorb protein by either non-covalently interaction or charge interaction with positive charge amino acid; consequently, TTM does not go smoothly towards the protein cavity site. Therefore, the addition of TTM, followed by the addition of CuCl_2 , makes TTM sulfur ligands bind more copper ions resulting in the formation of different types of non-native Mo/Cu composition that is not allowed into the apo-ORP cavity. We can also propose that, in the first step of metal-cofactor formation, copper goes into the cavity region of apo-ORP and promotes the entry of TTM due to the nature of Mo-Cu antagonism, suggesting a driving force for this process. In an alternative way, we can propose that the apo-ORP protein does not promote a specific cavity, but upon the addition of CuCl_2 and TTM into apo-ORP, the protein makes a cavity for the accommodation of the discrete a native mixed metal–sulfur cluster. The simple inorganic cofactor, $[\text{S}_2\text{MoS}_2\text{CuS}_2\text{MoS}_2]^{3-}$ [151], in the solution is no longer stable and itself rearranges to afford a bigger cluster, but in the presence of a protein matrix, the cluster is stable for long days. Apart from vitro reconstitution, for understanding the cluster assembly in vivo in ORP, the ATCUN tag (Amino terminus Cu and Ni binding motif) [152] is inserted at N-terminus in the ORP (Ala1Ser2His3-native amino acid residues). The NMR spectrum of that event indicates that metal-cofactor formation has occurred through inter-molecular protein–protein interaction [30].

A series of metal derivatives replacing the native copper with iron, cobalt, nickel, and cadmium ions were reported. All derivatives, $[\text{S}_2\text{MoS}_2\text{FeS}_2\text{MoS}_2]^{3-}$ (ORP-Fe), $[\text{S}_2\text{MoS}_2\text{CoS}_2\text{MoS}_2]^{3-}$ (ORP-Co), $[\text{S}_2\text{MoS}_2\text{NiS}_2\text{MoS}_2]^{3-}$ (ORP-Ni) and $[\text{S}_2\text{MoS}_2\text{CdS}_2\text{MoS}_2]^{2-}$ (ORP-Cd), were synthesized using the apo-ORP as template [40]. These substitutions of the diamagnetic Cu^{I} ion ($[\text{S}_2\text{MoS}_2\text{CuS}_2\text{MoS}_2]^{3-}$) gave origin to either paramagnetic or NMR active derivatives. Therefore, the ORP-Fe cofactor is also interested as a minimal model of Fe-Mo-co in nitrogenase [13] and other derivatives such as $[\text{S}_2\text{MoS}_2\text{FeS}_2\text{MoS}_2]^{3-}$ (ORP-Fe), $[\text{S}_2\text{MoS}_2\text{CoS}_2\text{MoS}_2]^{3-}$ (ORP-Co), and $[\text{S}_2\text{MoS}_2\text{NiS}_2\text{MoS}_2]^{3-}$ (ORP-Ni) are EPR probe whereas $[\text{S}_2\text{MoS}_2\text{CdS}_2\text{MoS}_2]^{2-}$ (ORP-Cd) is NMR probe. This work represents the power of the protein matrix to determine the final shape and structure of metal-cofactor in situ formation. Moreover, host molecules encapsulate a variety of guests but the specific size and shape into the plastic cavity through non-covalent interactions.

Spectroscopic Probes ORP

The metal cofactor of ORP contains Cu^{I} and Mo^{VI} , which are EPR inactive metal species and have no satisfactory NMR reporters that can help to investigate the location of metal-cofactor in the protein cavity. The 3D structure obtained by NMR spectroscopy displays that the mixed metal cluster is located inside the ORP protein cavity by non-covalent interactions, including H-bondings, electrostatic and hydrophobic interactions with an array of amino acid residues [146,148]. However, the exact binding locations of the metal cofactor in apo-ORP remain elusive. The amino acids are crucial for stabilizing the mixed metal–sulfur center within the protein cavity, and their identification is crucial for understanding the structure and the function of the metalloproteins. Therefore, the biophysical characterization of the binding pocket within the protein is important and different types of spectroscopic tools are now available for a diagnostic. Therefore, the metal cofactor is manipulated by substituting the diamagnetic Cu^{I} atom with paramagnetic or interesting NMR active metal reporters or coordinating the metal core with a small organic thiol ligand (^1H or ^{19}F NMR). In order to achieve our aims, we have reported a few synthetic inorganic semi-model compounds, where Mo-Cu cluster coordinated nonbiological thiols and fluorinated thiols are characterized by ^1H -NMR and ^{19}F -NMR respectively [153,154]. In addition, reconstitution of the NMR active cadmium atom into $[\text{S}_2\text{MoS}_2\text{CuS}_2\text{MoS}_2]^{3-}$ is originated a ^{113}Cd -NMR active compound, $[\text{S}_2\text{MoS}_2\text{CdS}_2\text{MoS}_2]^{2-}$ [153]. The ^{19}F -NMR of Mo/Cu-thiol complexes shows a higher chemical shift, which is advantageous for the NMR probe as an alternative to ^1H -NMR, circumventing the narrow window of observation of

protons and also the large number of protein-bearing protons that mask the protons in synthesized compounds. Thus, our synthetic strategy of synthesis was directed toward ^{19}F -NMR, because ^{19}F -NMR has no background signal, a broad window of chemical shifts (+400 to -400 ppm), and high sensitivity compared to ^1H -NMR. Moreover, because the chemical shift of ^{19}F -NMR is highly influenced by the local environment at the active site, the ^{19}F -NMR may be tuned by the vicinity of amino acids in proteins.

Transition metal ions (such as iron, cobalt, and nickel) have been used extensively as spin probes for the study of metalloproteins by means of NMR and EPR spectroscopy techniques. In this respect, the diamagnetic Cu^{I} ion in $[\text{S}_2\text{MoS}_2\text{CuS}_2\text{MoS}_2]^{3-}$ is replaced by a variety of paramagnetic metal ions ($\text{M} = \text{Fe}^{\text{I/II}}$, $\text{Co}^{\text{I/II}}$ and $\text{Ni}^{\text{I/II}}$) to obtain paramagnetic probes of ORP [40].

5. Conclusions

Enzymes are complex molecules that may or not contain metals at the catalytic site, where chemical transformations occur with amazing selectivity and at high rates. Of the known enzymes, one-third contain metals coordinated by the side chains of amino acids of the polypeptide chain and/or cofactors. In this case, the substrate is activated at the metal site.

Due to the chemical complexity of the system (large molecular mass, multiple subunit composition, and intricate architectural structures involving metals), the study of model compounds, retaining functional, structural (or both) characteristics has the advantage of working with a smaller size problem, more suitable for biophysical studies enabling to an inorganic chemistry approach for revealing the metal active site properties. Metalloenzymes use a wide range of metals in a variety of structural arrangements and geometries, most in parallel with inorganic compounds, but others are still a challenge for synthetic chemistry. Iron contained in iron–sulfur centers and in hemes are the most ubiquitous, but several other transition metals have specific roles, such as Ni, Mo, Cu, Zn, and others. Modeling efforts also represent an opportunity for further exploring new applications and functionalities.

The chemical design of models for metalloprotein active sites can be based on small inorganic compounds and now extend to peptides, protein-based synthetic analogs, and simple proteins that are used as templates (or scaffolds). As explained in this short review, we use as case studies three native scaffolds that provide very rich sulfur environments, used as templates for the synthesis of non-native metal clusters that can be mono, multi, and mixed metal–sulfur clusters, which mimic native metalloenzymes involved in key biological steps. Another advantage is the design of metal sites that can be quite useful as spectroscopic probes.

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Abbreviations

Rd	Rubredoxin
ORP	Orange Protein
Fd	Ferredoxin
CODH	Carbon monoxide dehydrogenase
cyt-c3	cytochrome c3
Pf	<i>Pyrococcus furiosus</i>
Dg	<i>Desulfovibrio gigas</i>
Dd	<i>Desulfovibrio desulfuricans</i>
Ch	<i>Carboxydotherrmus hydrogenoformans</i>
Rr	<i>Rhodospirillum rubrum</i>
Mt	<i>Moorella thermoacetica</i>
TTM	Tetrathiomolybdate
EXAFS	Extended X-ray absorption fine structure
PDB	Protein data bank
ET	Electron transfer
NHE	Normal hydrogen electrode
NMR	Nuclear magnetic resonance

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