Review

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The extended reductive acetyl-CoA pathway: ATPases in metal cluster maturation and reductive activation

Abstract: The reductive acetyl-coenzyme A (acetyl-CoA) pathway, also known as the Wood-Ljungdahl pathway, allows reduction and condensation of two molecules of carbon dioxide (CO₂) to build the acetyl-group of acetyl-CoA. Productive utilization of CO₂ relies on a set of oxygen sensitive metalloenzymes exploiting the metal organic chemistry of nickel and cobalt to synthesize acetyl-CoA from activated one-carbon compounds. In addition to the central catalysts, CO dehydrogenase and acetyl-CoA synthase, ATPases are needed in the pathway. This allows the coupling of ATP binding and hydrolysis to electron transfer against a redox potential gradient and metal incorporation to (re)activate one of the central players of the pathway. This review gives an overview about our current knowledge on how these ATPases achieve their tasks of maturation and reductive activation.

Keywords: carbon dioxide; carbon monoxide; cobalt; energy coupling; nickel.

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Introduction

The reductive acetyl-coenzyme A (acetyl-CoA) pathway

The conversion of carbon dioxide (CO₂) into organic material drives the global carbon cycle and is a prerequisite for all life forms that evolved on earth. The biological conversion of CO₂ into reduced compounds needs the extraction of energy from the environment (Appel et al., 2013). Plants and many microbes use the reductive pentose phosphate cycle, also known as Calvin-Benson cycle, which is the predominating way of CO₂ fixation (Berg et al., 2010; Fuchs, 2011; Appel et al., 2013). Enzymes of the reductive pentose phosphate cycle are oxygen-tolerant, which is likely responsible for their wide distribution. The reductive acetyl-CoA pathway, also called Wood-Ljungdahl pathway, is a way along which CO₂ enters the microbial carbon cycle under anaerobic conditions (Ragsdale, 2007, 2008; Ragsdale and Pierce, 2008; Fuchs, 2011). Among the six different known pathways of autotrophic carbon fixation, the reductive acetyl-CoA pathway is most likely the most ancient and evolved long before the oxygenation of the atmosphere (Russell and Martin, 2004; Martin and Russell, 2007; Fuchs, 2011). This pathway was likely present in the last universal common ancestor (Russell and Martin, 2004; Martin and Russell, 2007; Fuchs, 2011).

In contrast to CO₂ fixation along the reductive pentose phosphate cycle, the reductive acetyl-CoA pathway is restricted to strictly anoxic conditions (Fuchs, 2011). It was likely because of the anoxic conditions found in the early history of life on earth that metalloenzymes with complex metal clusters could evolve to catalyse the respective reactions. The reliance on these oxygen-sensitive metalloenzymes is the likely reason why prokaryotes employing the reductive acetyl-CoA pathway are restricted to anoxic niches.

The reductive acetyl-CoA pathway is found in phylogenetically diverse microbes, including anaerobic acetogenic, sulfidogenic, methanogenic and hydrogenogenic bacteria, and archaea (Oelgeschläger and Rother, 2008; Ragsdale and Pierce, 2008). Accordingly, the pathway operates in diverse anaerobic habitats from the gastrointestinal tract of animals and insects to the exotic environment of hot springs. The reductive acetyl-CoA
pathway has a unique appeal to mechanistic biochemists and structural biologists, due to the unusual enzymes with unprecedented cofactors and unique reactions found in the pathway. Two of the central catalysts of the pathway contain Ni, Fe and S ions in a unique assembly that resembles Ni,Fe,S-containing minerals with related catalytic capabilities (Russell and Martin, 2004; Martin and Russell, 2007). When we study the organometallic mechanisms of present day enzymes, we find an unusually large number of different metal-carbon bonds in the reactions of the pathway through which we get a grasp of how the first metabolic reactions on earth may have looked like.

**Individual reactions within the reductive acetyl-CoA pathway**

The reductive acetyl-CoA pathway can be divided into two branches along which two molecules of CO₂ enter the pathway and become reduced to finally condense to the acetyl-group of acetyl-CoA (Figure 1) (Ragsdale, 2007, 2008; Ragsdale and Pierce, 2008; Fuchs, 2011). In the methyl-branch, CO₂ is reduced to formate by formate dehydrogenase, which contains a pyranopterin cofactor bound to either Mo or W depending on the organism. Formate needs to be activated at the expense of ATP to undergo the condensation with tetrahydrofolate to 10-formyl-tetrahydrofolate, a reaction catalysed by formyl-tetrahydrofolate synthetase. In the following steps, 10-formyl-tetrahydrofolate is converted to 5,10-methyltetrahydrofolate, 5,10-methylene-tetrahydrofolate, and finally methyl-tetrahydrofolate. The methyl-branch and the carbonyl-branch are connected by the protein couple methyl-tetrahydrofolate:corrinoid iron-sulfur protein methyltransferase and the corrinoid iron-sulfur protein (Figure 1). Within the carbonyl-branch, CO₂ is also reduced in the first step by two electrons, however, CO is produced instead of formate. CO₂ reduction is catalysed by the Ni,Fe-containing carbon monoxide dehydrogenase (CODH). In the second step of the carbonyl branch, CO is condensed with coenzyme A and the methyl-group originating from the methyl-branch gives acetyl-CoA. The methyl-group is donated by the methylated corrinoid iron-sulfur protein (CoFeSP) and the condensation reaction is catalysed by the Ni,Fe-containing acetyl-CoA synthase (ACS) (Figure 1). The two Ni,Fe-containing enzymes, CODH and ACS, are typically found in a bifunctional complex, in which the two active sites are connected by an approximately 80 Å long gas channel allowing the controlled diffusion of CO formed in the active site of CODH to the active site of ACS (Doukov et al., 2002; Darnault et al., 2003). The pathways of bacteria and archaea differ in some of the aspects (Berg et al., 2010). Many of the genes encoding enzymes of the reductive acetyl-CoA pathway are arranged in a gene cluster (Figure 1).

**Figure 1** The extended reductive acetyl-CoA pathway.
Methyl transferase, corrinoid-iron/sulfur protein (CoFeSP), acetyl-CoA synthase, carbon monoxide dehydrogenase (CODH) and the ATPases, CoxC (CODH metal maturation) and RACo (reductive activator of CoFeSP), are indicated by coloured boxes. Genes encoding the corresponding proteins in *Carboxydothermus hydrogenoformans* are shown below. Enzymes responsible for generating methyltetrahydrofolate from carbon dioxide and tetrahydrofolate in the methyl branch were omitted.
The reductive acetyl-CoA pathway is used by acetogens to generate ATP and, for most bacteria, it is unclear how this may be achieved. The conversion of acetyl-CoA via acetyl-phosphate gives acetate and one ATP molecule, but one molecule of ATP is needed for formate activation, resulting in a net ATP formation of zero. Electron bifurcation schemes may be operative and could couple acetyl-CoA formation to generation of a proton motive force (Herrmann et al., 2008).

Several recent reviews describe the physiological function, structure and mechanism of the two main catalysts of the reductive acetyl-CoA pathway, CODH and ACS (Drennan et al., 2004; Ragsdale, 2004, 2006, 2007, 2008, 2009; Volbeda and Fontecilla-Camps, 2005, 2006; Fontecilla-Camps et al., 2009; Appel et al., 2013). The current review covers these enzymes only briefly. Emphasis is on ATPases from which we only recently learned more about their structures and functions. ATPases specific for the acetyl-CoA pathway couple the maturation of a metal cluster and the reductive activation of a metal ion to the hydrolysis of ATP. Compared to our knowledge about the structure and mechanism of the main catalysts of the reductive acetyl-CoA pathway, we are just beginning to understand what these ATPases do and how they achieve their tasks. Our current state of knowledge about the structure and function of these ATPases and their interaction partners will be described.

The extended acetyl-CoA pathway

Carbon monoxide dehydrogenases and the CooC-type ATPase

Carbon monoxide dehydrogenases

Carbon monoxide dehydrogenases (CODHs) are at the heart of the reductive acetyl-CoA pathway where they catalyse one of the two two-electron reductions of carbon dioxide (Figure 1).

The mechanism of CODHs has been discussed extensively in a recent review about the (bio)chemistry of CO and CO₂ (Appel et al., 2013). CODHs catalyse the reversible reduction of CO₂ to CO according to the following reaction:

\[ \text{CO} + \text{H}_2\text{O} \leftrightarrow \text{CO}_2 + 2\text{H}^+ + 2\text{e}^- \]

CODHs are not only found within the reductive acetyl-CoA pathway but are linked to different physiological functions. Monofunctional CODHs are found in aerobic and anaerobic prokaryotes using CO as source of electrons (Ragsdale, 2004; Robb et al., 2005). CODHs from aerobic bacteria contain a binuclear active site in which a Cu(I) ion is bridged to a pyranopterin bound Mo (Dobbek and Huber 2001; Dobbek et al., 2002). In contrast to the Ni-containing CODHs, which are bidirectional, Mo-containing CODHs catalyse only the oxidation of CO to CO₂ (Meyer et al., 2000). Anaerobic prokaryotes employ the Ni-containing CODHs (Ragsdale, 2004). Several microorganisms carry genes encoding isoforms of CODHs, an example being *Carboxydothermus hydrogenoformans* where five homologues of CODHs have been found in the genome (Wu et al., 2005). In *C. hydrogenoformans* the two electrons from CO oxidation can be transferred to a membrane bound hydrogenase, which couples proton reduction to the generation of a proton motive force (Svetlitchnyi et al., 2001, 2004). CODHs acting in the reductive acetyl-CoA pathway are typically associated with ACS in a stable complex, in which the generated CO binds to the Ni-Ni-[4Fe-4S] site of ACS (Ragsdale et al., 1985; Doukov et al., 2002; Darnault et al., 2003; Svetlitchnyi et al., 2004). Some archaea contain CODHs in a large complex, where it is associated with ACS and a corrinoid-iron/sulfur protein (Grahame, 1991; Gencic et al., 2010).

All CODHs contain additional [4Fe-4S] clusters with short intercluster distances allowing rapid electron transfer (Figure 2A) (Dobbek et al., 2001; Drennan et al., 2001, 2004; Doukov et al., 2002; Darnault et al., 2003; Jeoung and Dobbek, 2007b; Gong et al., 2008). One [4Fe-4S] cluster termed cluster D is found in the dimer interface and is coordinated by two cysteine residues from each monomer. Cluster D is difficult to reduce and remains in the oxidized [4Fe-4S]⁺ state at redox potentials as low as -530 mV (Craft et al., 2002). CODHs belong to the Ni-containing enzymes and are the only known enzymes in which Ni is integrated into an iron/sulfur scaffold (Figure 2B–D), termed cluster C. Cluster C is a catalytic [Ni-Fe₄-S₄-OH] cluster in which a distorted Ni₃Fe₄S₄ cube with one open Ni-S edge sits next to a S-bridged Fe in exo with a water/hydroxo-ligand (Figure 2C). An additional sulfido ligand bridging Ni and Fe in a [Ni-Fe₄-S₅] cluster, previously assumed to be the active state of the cluster C (Dobbek et al., 2001, 2004; Ha et al., 2007), is connected to a more oxidized inactive state of the enzyme (Wang et al., 2013a,b). The central Ni ion of cluster C is the place where the substrates CO and CO₂ (Figure 2D) (Jeoung and Dobbek, 2007a), as well as inhibitors, such as cyanide, bind (Ha et al., 2007; Jeoung and Dobbek, 2009; Kung et al., 2009). The three Ni-ligands form a quasi-T-shaped arrangement leaving one coordination site at the Ni open to bind and activate the substrates.
The activity of CODHs is inhibited by several small molecules, many of which resemble CODH substrates to which they are isosteric and isoelectronic. Cyanide is a slow binding competitive inhibitor related to CO (Diekert and Thauer, 1978; Drake et al., 1980; Krzycki and Zeikus, 1984; Grahame and Stadtman, 1987) and binds to an open coordination site of the Ni ion (Ha et al., 2007; Seravalli and Ragsdale, 2008; Jeoung and Dobbek, 2009; Kung et al., 2009). Cyanate, acting as an analogue of CO$_2$, binds selectively to the reduced state of CODH where it competes with CO$_2$ for binding to the active site (Wang et al., 2013a,b). Along with these anionic inhibitors of CODH, sulfide also binds reversibly to the cluster C, likely acting as the bridging sulfido ligand (Dobbek et al., 2001), thus, inhibiting catalysis (Feng and Lindahl, 2004; Wang et al., 2013a).

**CooC and related maturases**

Within the last two decades, it became clear that the assembly of metalloenzymes in cells is a complex task depending on a large number of different accessory proteins. Within the Ni-containing enzymes (Kaluarachchi et al., 2010; Boer et al., 2014) the maturation of ureases and hydrogenases was especially well studied (Moncrief and Hausinger, 1997; Drapal and Bock, 1998; Watt and Ludden, 1999; Blokesch et al., 2002; Mulrooney and Hausinger, 2003; Forzi and Sawers, 2007; Leach and Zamble, 2007; Li and Zamble, 2009; Farrugia et al., 2013).

Comparatively less is known about the assembly of cluster C. The group of Paul Ludden, using *Rhodospirillum rubrum* as model organism, has reported most physiological studies on cluster C maturation. The structural gene of CODHRr (*cooS*) is part of the gene cluster *cooF-SCTJ*, whose transcription is controlled by the presence of CO (Roberts et al., 2004), acting as a switch via the CO-sensing hemeprotein CooA (Shelver et al., 1995; Roberts et al., 2005). The effect of deletions of *cooC, cooT* and *cooJ* are in agreement with their involvement in Ni uptake and CODH maturation in *R. rubrum*. Deletion of *cooT* and *cooJ* required approximately 50-fold higher Ni levels to sustain growth of *R. rubrum* on CO, while the deletion...
of cooC was only compensated at 1000-fold higher Ni levels (Kerby et al., 1997). All three genes are required for increased Ni\textsuperscript{2+} accumulation upon CO exposure (Watt and Ludden, 1999). The cooT gene encodes a 7.1 kDa protein, with weak similarity to the chaperone-type HypC protein, required for the maturation of hydrogenase in Escherichia coli. The cool gene encodes a 12.6 kDa protein with a histidine-rich Ni-binding domain, coordinating up to four Ni\textsuperscript{2+} ions per monomer (Watt and Ludden, 1998). Surprisingly, no homologues of CooT and CooJ are found in the acetogenic, hydrogenogenic, methanogenic and sulfido- genic bacteria and these two Coo proteins appear to be restricted to R. rubrum and close relatives. In contrast to CooT and Cool, ATPases with high similarity to CooC are found in all of the diverse microorganisms where CODHs are found.

CODH maturation depends on ATP hydrolysis by CooC as a mutation of the canonical lysine residue of CooC\textsubscript{mo} to glutamine abolished ATP hydrolysis in vitro and the insertion of nickel in apoCODH\textsubscript{mo} in vivo (Jeon et al., 2001). In vitro nickel activation of apoCODH\textsubscript{mo} in cell extracts was enhanced by CooC and depended upon ATP hydrolysis (Jeon et al., 2001). The activation of isolated nickel-deficient CODH\textsubscript{mo} does not occur with CooC, ATP and Ni\textsuperscript{2+} alone, suggesting that so far unknown gene products contribute to the activation. An open reading frame within the acs-operon (acsF) is homologous to CooC and was suggested to act in the assembly of the Ni-Ni-[4Fe-4S] cluster of ACS (Loke and Lindahl, 2003), however experimental evidence for this claim is lacking.

Many organisms contain more than one gene encoding a homologue of CooC. The complete genome of C. hydrogenoformans harbours three paralogous CooC-type genes (Wu et al., 2005), whose physiological roles still have to be demonstrated (Techtmann et al., 2009, 2011). Genes encoding CooC proteins are typically found in direct vicinity of genes encoding CODH and ACS (Figure 1) (Wu et al., 2005).

CooC proteins contain a deviant Walker A motif (G\textsubscript{K}G\textsubscript{K}GhGK[ST]; where h denotes a hydrophobic amino acid), which is found in the SIMIBI class of NTPases (Leipe et al., 2002). Members of the SIMIBI class have diverse physiological roles such as regulation of cell division (MinD) (Lutkenhaus and Sundaramoorthy, 2003; Lutkenhaus, 2007), plasmid and chromosome segregation (Soj) (Leonard et al., 2005), pumping of anions (ArsA) (Zhou et al., 2000), the insertion of Mo-citrate into the scaffold protein NifEN (NifH, also called Fe protein), or one step of the complex maturation of the Mo,Fe-protein of nitrogenase (Schindelin et al., 1997; Chan et al., 1999; Hu et al., 2006, 2010). A common theme of these proteins, the coupling of conformational changes to nucleotide binding and hydrolysis, also affects their interaction with partner proteins.

Further in vitro studies have been conducted on CooC1, one of the three CooC proteins from C. hydrogenoformans. The oligomerization state of CooC1 depends on the presence of Ni and nucleotides. CooC1 is monomeric in the apo state, but a dimer is formed when Ni or ATP-Mg\textsuperscript{2+} is added (Jeoung et al., 2009). CooC1 is binding one equivalent of Ni\textsuperscript{2+} with a \(K_d\) of 0.41±0.05 \(\mu\)M. An exchange of any of the two conserved Cys residues of the GCXC motif (Cys112 and C114; CooC numbering) against alanine abolishes the capacity to bind Ni\textsuperscript{2+} (Jeoung et al., 2009).

Crystal structures of CooC1 have been determined in four different states: without nucleotides and metals, ADP-bound, Zn\textsuperscript{2+}/ADP-bound, and Zn\textsuperscript{2+}-bound (Jeoung et al., 2010). These structures reveal that CooC proteins consist of two separate functional modules: an ATPase module, which is similar to other SIMIBI-ATPases and a metal binding module in the dimer interface, which appears to be unique to CooC proteins (Figure 3A and B). The topology of the ATP-binding site classifies the CooC proteins as members of the MinD family of SIMIBI-NTPases. The overall topology of the ATP-binding site of CooC1 shows an \(\alpha\)-\(\beta\)-\(\alpha\) layout in which the central element is an eight stranded \(\beta\)-sheet. The crystal structure of CooC1 is most closely related to the structure of NifH (Schindelin et al., 1997; Jeoung et al., 2010) and to MinD (Cordell and Lowe, 2001; Hayashi et al., 2001; Sakai et al., 2001), a protein acting in the spatial regulation of cell division (Lutkenhaus and Sundaramoorthy, 2003; Lutkenhaus, 2007).

Structures of CooC1 have been determined for monomeric and different dimeric assemblies. The surface area buried upon dimerization of CooC1 is small and predominately created around the metal binding site, while the two ATP binding sites have no contacts. As no crystals could be obtained of Ni-bound CooC1, the metal binding site was only indirectly characterized using the more stably bound Zn\textsuperscript{2+} ion (Figure 3C) (Jeoung et al., 2009, 2010). The small dimerization interface is in agreement with the observed equilibrium between monomeric and dimeric states in solution. The enforcement of a homodimeric structure by metal binding is similar to what has been observed for NifH and Get3, which show a covalent dimerization through coordination of a [4Fe4S] cluster and a Zn\textsuperscript{2+} ion, respectively (Georgiadis et al., 1992; Schindelin et al., 1997; Mateja et al., 2009). The Zn\textsuperscript{2+} ion in CooC1 is tetrahedrally coordinated by four thiolates originating from the two cysteine residues of the Gly-Cys-X-Cys motif.
conserved in CooC proteins (Figure 3C). Zinc had not been added to the crystallization medium and was likely captured by the protein during purification or crystallization from metal impurities in the medium.

As-isolated CooC1 does not contain Ni²⁺ and shows only the absorption bands typical for the contribution of its amino acids. When one equivalent of Ni²⁺ is added to a preparation of CooC1 under anoxic conditions a distinct peak with a maximum at 330 nm develops ($\varepsilon_{330} = 4.7 \times 10^3$ M⁻¹ cm⁻¹) (Jeoung et al., 2009). Intensity and position of the absorption maximum are in agreement with a ligand-to-metal charge-transfer (LMCT) between Cys-S- and Ni²⁺. Number and position of the maxima are changing when ATP or ADP are present in solution with absorption maxima at 286, 295 and 341 nm and a shoulder at 380 nm are being discernible, indicating a functional coupling between the nucleotide binding site and the metal binding site.

CooC proteins possess the P loop motif and ATPase activity is dependent on the presence of Mg²⁺ agreeing with a metal-assisted mechanism for ATP hydrolysis. The deviant Walker A motif with two essential lysine residues (GKGhGK[ST]) is part of the N-terminal portion of CooC. The ATPase activity of CooC is strictly dependent on both lysine residues, indicating that CooC acts as a dimer in which the N-terminal lysine of the motif stabilizes an ATP molecule bound in the opposed monomer by acting across the dimer interface, as found for other ATPases with the deviant Walker A motif. Dimer formation and the relative arrangement of the monomers of the dimer thus likely play a critical role in the mechanism of CooC proteins.

How CooC supports the assembly of the Ni,Fe-cluster of CODHs is unknown. The current preliminary data would be in agreement with it acting as an ATP-dependent nickel insertase. The flexible dimer arrangement could allow coupling of large-scale conformational changes to partner recognition and nickel release (Jeoung et al., 2010). However, the Fe,S-portion of cluster C needs to be assembled and we do not yet know if Ni is inserted into a preformed [Fe₄S₄OH] cluster or if the complete assembly of cluster C occurs on a scaffold protein, analogous to nitrogenase (Hu et al., 2006; Fay et al., 2010; Hu and Ribbe, 2011a,b, 2013a,b) and the Fe,Fe-hydrogenases (Kuchenreuther et al., 2012; Nicolet and Fontecilla-Camps, 2012).
Corrinoid-iron/sulfur protein and its reductive activator RACo

Corrinoid-iron/sulfur protein

The corrinoid-iron/sulfur protein (CoFeSP) connects the methyl-branch and the carbonyl-branch of the reductive acetyl-CoA pathway by engaging in two reversible methyltransfer reactions (Figure 1). It (I) accepts a methyl-group from CH₃-THF bound to methyltransferase and (II) transfers the methyl-group to the A cluster of acetyl-CoA synthase (Figure 1), according to the following reactions:

\[ \text{CH}_3\text{-THF} + \text{Co(I)FeSP} \rightleftharpoons \text{THF} + \text{CH}_3\text{-Co(III)FeSP} \]

\[ \text{CH}_3\text{-Co(III)FeSP} + \text{ACS} \rightleftharpoons \text{CH}_3\text{-ACS} + \text{Co(I)FeSP} \]

CoFeSP belongs to the large class of B₁₂-dependent methyltransferases, which participate in the amino acid biosynthesis of diverse organisms, as well as in the one carbon metabolism of bacteria and archaea. B₁₂-dependent methyltransferases typically catalyse the transfer of methyl groups between organic and inorganic acceptors and donors (Matthews, 2001; Matthews et al., 2008). The methyltransfer between CoFeSP and ACS is the only known example of a methyltransfer between two metals, the Co ion of CoFeSP and one of the two Ni ions of ACS in biology (Appel et al., 2013).

CoFeSPₜₜ is a heterodimeric protein composed of a large subunit CfsA (48.4 kDa) and a small subunit CfsB (33.9 kDa) (Figure 1). As the name of the protein implies, CoFeSP has a [4Fe-4S] cluster and a cobalamin as cofactors (Figure 4). The [4Fe-4S] cluster is supposed to be involved in reductive activation of inactive Co(II), when electrons are supplied by CODH, pyruvate:ferredoxin oxidoreductase or reduced low potential ferredoxins (Menon and Ragsdale, 1998, 1999). The cobalamin cofactor is the principal reactant in the methyltransfer-reactions, while the protein matrix tunes the relative stabilities of the two active states Co(I) and CH₃-Co(III). Tuning in B₁₂-dependent enzymes is primarily provided by ligands to the Co ion, by either the base of the cobalamin cofactor (base-on) or a histidine side chain (his-on) from the protein. CoFeSP is unusual in that it has no additional ligand bound to the Co⁺ ion, being in a state designated as base-off/his-off (Figure 4). The corrin-ring is sandwiched between the large and the small subunit making the Co ion inaccessible to larger molecules. Therefore, conformational rearrangements appear mandatory for the interaction between CoFeSP and its partners ACS, methyltransferase and RACo.
(Svetlitchnaia et al., 2006; Goetzl et al., 2011; Ando et al., 2012; Kung et al., 2012).

Both cofactors of CoFeSP are bound to the large subunit, the \([4\text{Fe}-4\text{S}]\) cluster, and is coordinated by cysteines of the small N-terminal domain, which contains 58 residues and the cobalamin interacts primarily with the C-terminal domain consisting of 110 residues (Figure 4) (Goetzl et al., 2011). In between these domains is a \((\beta/\alpha)_8\) barrel domain to which the two cofactor carrying domains are fused by long linkers. The small subunit also contains a \((\beta/\alpha)_8\) barrel domain with obvious structural similarities to the \((\beta/\alpha)_8\) barrel domain of the large subunit. The two \((\beta/\alpha)_8\) barrel domains have a pseudotwofold symmetrical arrangement; \((\beta/\alpha)_8\) barrel domains are frequently observed in protein structures and do not necessarily indicate a common origin. However, a superimposition of the two \((\beta/\alpha)_8\) barrel domains of CoFeSP reveals that they are structurally more closely related to each other than to any other \((\beta/\alpha)_8\) barrel domain in the PDB (Svetlitchnaia et al., 2006; Goetzl et al., 2011). The resemblance is also reflected in a sequence identity of 21% calculated from the structure-based alignment, indicating a common origin of the two \((\beta/\alpha)_8\) barrel domains. Thus, both \((\beta/\alpha)_8\) barrel domains are likely derived from a homodimeric ancestral protein. Structure comparisons also revealed that the \((\beta/\alpha)_8\) barrel domains of CoFeSP are related to the \((\beta/\alpha)_8\) barrel domain of methyltransferase, which activates CH$_3$-tetrahydrofolate for the methyltransfer to CoFeSP. Methyltransferase is a homodimeric protein and from its similarity to CoFeSP we can infer that the proteins involved in methyl transfer reactions in the reductive acetyl-CoA pathway are likely derived by two successive duplications of a gene encoding an ancestral \((\beta/\alpha)_8\) barrel protein (Svetlitchnaia et al., 2006).

The crystal structure of a complex between CoFeSP and methyl transferase from Moorella thermoacetica has recently been elucidated (Ando et al., 2012; Kung et al., 2012), offering insights into the structural (re)arrangements required for protection and catalysis in a B$_{12}$-dependent methyltransferase system. The structure of the complex shows large rearrangements for the two cofactor-carrying domains. Surprisingly, these movements are not only found in the B$_{12}$-containing domain, but also in the \([4\text{Fe}-4\text{S}]\) cluster-coordinating domain (Ando et al., 2012; Kung et al., 2012). Several intermediate steps of the B$_{12}$-containing domain along a trajectory from its resting position to the interaction site between cobalamin and methyl-tetrahydrofolate were found in the crystals (Ando et al., 2012; Kung et al., 2012).

**RACo: the reductive activator of the corrinoid iron/sulfur protein**

\[
\text{ATP-Mg}^2++H_2O\rightarrow\text{ADP-Mg}^2++P_i
\]
\[
x\text{ATP-Mg}^2++\text{H}_2\text{O}+\text{Co(II)}-\text{CoFeSP}+\frac{1}{2}\text{DTT}^-\rightarrow x\text{ADP-Mg}^2++x\text{P}++\text{Co(II)}-\text{CoFeSP}+\frac{1}{2}\text{DTT}^-
\]

(x: The stoichiometry of ATP molecules hydrolysed per uphill electron transfer to Co(II)FeSP has not yet been determined. Based on the structure of RACo we expect x to be equal to 1.)

The physiological role of RACo appears to be the reductive activation of inactive Co(II)FeSP. As detailed above CoFeSP cycles between the active CH$_3$-Co(III) and the Co(I) states. The Co(I) state of CoFeSP is prone to oxidation and is frequently oxidized to the inactive Co(II) state. The Co(I)/Co(II) couple has a reduction potential of about -450 to -500 mV (Harder et al., 1989; Menon and Ragsdale, 1998) and under physiological conditions electrons needed to reduce Co(II) may be scarce. RACo couples the thermodynamically favourable hydrolysis of ATP to the unfavourable electron transfer from a site with a more positive reduction potential, its [2Fe2S]-cluster, to a site with a more negative reduction potential, the Co(II) ion of CoFeSP (Hennig et al., 2012).

Genes encoding the proteins of the reductive acetyl-CoA pathway are typically clustered in the genomes. RACo and CoFeSP genes are typically found in close proximity in the firmicutes and δ-proteobacteria, but are either missing or are not near the homologous CoFeSP genes in the euryarchaeota (Hennig et al., 2012). Thus, it does not seem mandatory for all organisms having a CoFeSP homologue to also have a RACo homologue and another mechanism to reduce inactive Co(II)-CoFeSP, such as a flavin-based electron bifurcation mechanism (Herrmann et al., 2008; Li et al., 2008; Wang et al., 2010; Kaster et al., 2011) may exist in these organisms.

RACo belongs to the COG3894. Members of this group include the reductive activases of corrinoid enzymes (RACE), like the RACE protein from the veratrol O-demethylase system of Acetobacterium dehalogenans (Siebert et al., 2005; Schilhabel et al., 2009; Nguyen et al., 2013), as well as the activator of methylamine methyltransferase from the methanogenic archaean Methanosarcinaarkeri (Ferguson et al., 2009). The former RACE protein also contains a [2Fe2S] cluster domain like RACo and the latter has an additional C-terminal domain with two [4Fe4S] cluster-binding motifs but lacks the N-terminal [2Fe2S] cluster domain. The crystal structure of RACo (Figure 5) provided evidence that all these proteins belong to the...
ASKHA-ATPases, with prominent members of this ATPase-family being hexokinase, actin and Hsc70 (Hurley, 1996). Structure-based alignments revealed a sequence identity of 10–15% to other members of the ASKHA-family, a level usually deemed to be below the twilight zone of sequence identity. Residues conserved among the different ASKHA-ATPases cluster in the N-terminal region of the ASKHA domain where ADP and ATP are bound in the related ASKHA-ATPases (Flaherty et al., 1991; Kabsch and Holmes, 1995; Hurley, 1996).

The N-terminal domain of RACo is homologous to plant-type [2Fe-2S] ferredoxins and sequence identities between these proteins are in the range of 28–30%. The sequence motif CX₇CX₇CX₇C is typical for the [2Fe2S]cluster-binding domain of RACE proteins. The [2Fe2S] cluster itself is mildly oxygen sensitive, as a protein solution exposed to air loses its characteristic colour after 2 days.

RACo forms a homodimer with clearly distinguishable functional domains (Figure 5) (Hennig et al., 2012). The N-terminal [2Fe2S] cluster domain (residues 3–94) does not interact with the other domains in the crystal structure. Its mobility is also reflected in the lack of electron density for this region in most crystals measured for the RACo protein. The [2Fe-2S]-cluster domain has a β-grasp topology typical for plant-type [2Fe2S] ferredoxins with a β-sheet of five β-strands and one α-helix lying on top of the sheet. The [2Fe2S] cluster is near the surface of the domain (Figure 5A and C). The N-terminal domain is connected via a linker domain (residues 95–125) to the dimerization and ATPase domain. The dimerization domain (residues 126–206) consists of a short three-stranded antiparallel β-sheet and three α-helices (Figure 5A). Dimerization is mostly due to two of the α-helices interacting with the C-terminal domain of the other monomer. The large C-terminal domain (residues 207–630) shows the typical topology of the ASKHA-ATPase family with two subdomains of ββαβαβα topology. A cleft between the two domains harbours a phosphate-Mg²⁺ complex in the RACo structure and defines the ATP-binding site of RACo at the same position, as in other ASKHA-ATPases (Figure 5B).

The UV/Vis spectrum of as-isolated RACo shows the typical spectrum of [2Fe2S] cluster containing ferredoxins.
with maxima at 350, 410 and 460 nm and a shoulder at 550 nm. Addition of DTT bleaches the reddish-brown colour of RACo and decreases the absorption by about 80% at 460 nm. Reduction with DTT is complete within 10 min. Resonance Raman spectra of RACo with an excitation wavelength of 413 nm reveal bands at 288, 327, 344 and 390 cm⁻¹ and a shoulder at 320 cm⁻¹ (Meister et al., 2012).

The complete mechanism of ATP-driven electron transfer by RACo is not yet understood, but some of its aspects became clear during the last 2 years. Electron transfer between both proteins needs the close proximity between electron donor and electron acceptor. Complex formation between RACo and CoFeSP likely provides the necessary proximity. Formation of the complex depends on the oxidation state of the Co ion and occurs only with the inactive Co(II) state of CoFeSP (Hennig et al., 2012). The redox state of RACo seems to have no influence on complex formation. Analytical size exclusion chromatography, together with densiometric analysis, revealed a 2:2:2 stoichiometry of CfsA, CfsB and RACo, indicating a RACo:CoFeSP complex with a molecular mass of about 300 kDa. The rate of ATP induced electron transfer increases with ATP concentrations until about 2 mM ATP. The link between the ATP-binding site and electron transfer is also evident from studies of residues in the phosphate-Mg²⁺-binding pocket, whose exchange abolishes the ability of RACo to catalyse the ATP-dependent electron transfer to CoFeSP (Hennig et al., 2012).

One of the major challenges of an ATP driven electron transfer is rendering the electron transfer unidirectional (Rees and Howard, 1999). This is important because the intended flow of electrons is energetically uphill, thus, the reverse direction, which must be diminished, is thermodynamically favoured. To diminish a thermodynamically favourable reaction, its kinetics must be slow. The kinetics of electron transfer is strongly dependent on the distance between donor and acceptor (Marcus and Sutin, 1985; Page et al., 1999). To reach physiologically meaningful electron transfer rates the donor-acceptor couples are typically in a distance of shorter than 15 Å (Page et al., 1999). As RACo only forms a complex with Co(II)FeSP, a RACo:CoFeSP complex will dissociate after the ATP-induced electron transfer. The lack of a complex between Co(II)FeSP and RACo keeps the donor and acceptor couple apart and therefore decreases the rate of electron transfer between Co(I) and the oxidized [2Fe-2S] cluster.

A direct interaction between the Co(II) ion and RACo appears mandatory to enable redox-selective complex formation, which is supported by EPR- and resonance Raman spectroscopy (Meister et al., 2012). Using an excitation wavelength of 413 nm, three bands in the RR spectrum are observed for the corrin ligand of CoFeSP in the region 1400–1700 cm⁻¹. While in oxidized Co(II)FeSP bands are observed at 1604, 1543 and 1498 cm⁻¹, they are shifted to 1607, 1545 and 1496 cm⁻¹ upon complex formation with RACo (Meister et al., 2012). Complex formation does not induce changes in the RR bands of the [4Fe4S] cluster of Co(II)FeSP and the [2Fe2S] cluster of RACo. Changes upon complex formation are observed in a comparison of the EPR signals of Co(II), which become sharper and better resolved indicating a tightening of the ligand – Co(II) interaction in the complex (Meister et al., 2012).

The main questions remaining about the mechanism of ATPase coupled electron transfer by RACo are (I) how ATP-binding or its hydrolysis can trigger and ultimately drive electron transfer and (II) how redox-selective complex formation between the two proteins are achieved. Further structural and other biophysical investigations are currently underway to answer these remaining questions.

Concluding remarks and future directions

The principal catalysts of the reductive acetyl-CoA pathway were discovered more than 30 years ago (Ragsdale and Pierce, 2008). Spectroscopic investigations already suggested the presence of unusual metal sites in some of the proteins and the crystal structures, whose determination was additionally motivated by the spectroscopic investigations, confirmed these assumptions and revealed unique metal clusters, in which Ni, Fe and S have unprecedented arrangements. We only now begin to slowly understand how these metal clusters achieve substrate activation.

Much less is known about the maturation of the Ni, Fe-clusters of ACS and CODH. We are only starting to understand which proteins are involved in the maturation of CODH and ACS. The slow start is partially due to the uninformative genetic context, which, in contrast to hydrogenases and ureases, does not hint to a series of accessory genes clustered around the genes encoding ACS/CODH, but only to one protein, namely CooC, which is involved in the maturation of CODH, and possibly, ACS in a yet not understood fashion. Given the rising interest in CODH and ACS in biotechnological applications mimicking the water-gas shift reaction (Appel et al., 2013) and acetic acid formation by the Monsanto/Cativa process (Appel et al., 2013), it is clear that we have to better understand how these enzymes are matured before we can produce them efficiently in recombinant hosts.
That the reductive acetyl-CoA pathway is still good for surprises is also evident from the recent discovery of RACo (Hennig et al., 2012) and the RACE-ATPases (Siebert et al., 2005; Ferguson et al., 2009; Schilhabel et al., 2009). RACo, long known as Orf7 and predicted to be an Fe/S-protein (Loke and Lindahl, 2003), was unpredicted in its function until the landmark publications of the groups of Gabriele Diekert and Joseph Kryzky, who suggested it to be a reductive activator of CoFeSP, a prediction we could recently show to be correct (Hennig et al., 2012).

Many open questions about the function of the individual proteins, as well as the mechanism and maturation of the principal catalysts ACS and CODH, remain to be resolved. The common origins for proteins such as methyltransferase and CoFeSP, and again a common origin of the two CoFeSP subunits, revealed first glimpses of how this old pathway may have evolved. Together with biochemical and structural studies, phylogenetic studies to elucidate the molecular evolution of the reductive acetyl-CoA pathway and its individual proteins may bring the historical dimension into our focus. Comparing the enzymes from bacteria and archaea can additionally inform us about possible evolutionary scenarios for the development of this ancient pathway.

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