Ni-Zn-[Fe₄-S₄] and Ni-Ni-[Fe₄-S₄] clusters in closed and open α subunits of acetyl-CoA synthase/carbon monoxide dehydrogenase

Claudine Darnault¹,², Anne Volbeda¹,², Eun Jin Kim³, Pierre Legrand¹, Xavier Vernède¹, Paul A. Lindahl³ and Juan C. Fontecilla-Camps¹

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The crystal structure of the tetrameric α₂β₂ acetyl-coenzyme A synthase/carbon monoxide dehydrogenase from Moorella thermoacetica has been solved at 1.9 Å resolution. Surprisingly, the two α subunits display different (open and closed) conformations. Furthermore, X-ray data collected from crystals near the absorption edges of several metal ions indicate that the closed form contains one Zn and one Ni at its active site metal cluster (A-cluster) in the α subunit, whereas the open form has two Ni ions at the corresponding positions. Alternative metal contents at the active site have been observed in a recent structure of the same protein in which A-clusters contained one Cu and one Ni, and in reconstitution studies of a recombinant apo form of a related acetyl-CoA synthase. On the basis of our observations along with previously reported data, we postulate that only the A-clusters containing two Ni ions are catalytically active.

Acetyl-coenzyme A (CoA) synthase/carbon monoxide dehydrogenase (ACS/CODH) is a bifunctional enzyme that catalyzes the reversible reduction of CO₂ to CO (CODH activity):

\[ 2H^+ + CO_2 + 2e^- \rightarrow CO + H_2O \] (1)

In addition, ACS/CODH catalyzes the synthesis of acetyl-CoA from CO, CoA and a metal group donated by the cobalt-containing corrinoid iron-sulfur protein (CoFeSP) (ACS activity). Such activities have been implicated in the 'Fe-S World' theory of the origin of life in which fixing CO₂ into activated acetyl groups on (Ni)Fe-S surfaces is thought to have produced autocatalytic metabolists¹. The ACS/CODH enzyme from the acetogenic bacterium Moorella thermoacetica (ACS/CODHMt) is a 310 kDa αβ₂ tetramer that plays a central role in synthesizing acetyl-CoA through the Wood/Ljungdahl pathway². The β subunits of ACS/CODHMt are homologous to homodimeric CODH enzymes found in carboxydotrophic bacteria, including Rhodospirillum rubrum (Rr) and Carboxythermus hydrogenoformans (Ch); these homodimeric CODH enzymes catalyze reaction (1) but not the synthesis of acetyl-CoA from CO³⁴. Each β subunit contains an [Fe₆S₆] B-cluster and a novel active site [Ni-Fe₄S₄] C-cluster. A single [Fe₄S₄] D-cluster bridges the two β subunits. Each α subunit of ACS/CODHMt contains a single metal center, the A-cluster, which is responsible for the ACS activity⁵. During catalysis, CO is generated by reducing CO₂ at the C-cluster migrates through a tunnel and binds to the A-cluster⁶⁷, which also gets methylated by CH₃-CoFeSP⁸⁹. Methyl migration/CO insertion forms a metal-bound acetyl group that is attacked by CoA to generate acetyl-CoA⁹¹⁰. A recently determined structure of ACS/CODHMt has suggested that the A-cluster consists of a Ni-Cu-[Fe₄S₄] center⁶. Here we report the crystal structure of CO-treated ACS/CODHMt solved at 1.9 Å resolution. Surprisingly, and contrary to the recently reported structure of the same enzyme⁸, the two α subunits display different conformations and have A-clusters with dissimilar coordination geometries and metal contents. On the basis of these observations, we discuss possible catalytic mechanisms for the synthesis of acetyl-CoA. In addition, we show that the hydrophobic tunnels connecting the C- and A-clusters extensively bind Xe, a heavy gas that mimics the affinity of CO for such tunnels.

Structure determination

The structure of ACS/CODHMt was initially solved by a combination of molecular replacement (MR) using the CODH Ch model and a MAD experiment performed at the Fe edge using native crystals. The model was progressively completed starting with the β subunits and followed by the N-terminal domain of the α subunits. Subsequently, the central and C-terminal domains of the α subunits could be built into an electron density map calculated with a combination of partially refined model and experimental phases. The current 1.9 Å resolution CO-treated ACS/CODHMt model, which includes all the protein atoms and >1,200 solvent molecules, has excellent refinement statistics and stereochemistry (see Methods).

Description of the ACS/CODHMt atomic model

The overall quaternary structure and organization of subunits is similar to that observed by Doukov et al.⁸: an αβ₂ tetrameric enzyme with dimensions of ~200 × 75 × 105 Å (Fig. 1a,b). The two β subunits contact each other extensively, yielding a structure similar to the reported structures of CODH Ch and CODHk⁵⁴. The subunits are attached to opposite faces of the β₂
The α subunits consist of three structural domains, designated as residues 1–312, 313–478 and 479–729. Only the N-terminal domain interacts with the β subunit. In contrast to the structure reported by Doukov et al., the α subunits in our structure have different conformations; packing analysis shows that they are not interchangeable in the crystal. As a consequence, the β subunits and most of the N-terminal domains of the α subunits satisfy the local two-fold symmetry axis of the αβ dimer, whereas the remainder of each α subunit does not (Fig. 1a,b). The two α subunits differ by a rotation of ~50° about a region found at the interface of the N-terminal and central domains. The central and C-terminal domains move as rigid bodies and are not significantly modified by the rotation. The α subunit N-terminal domain is also largely unaffected by the rotation, except for a helical segment discussed below.

The conformation of the α subunits in the ‘closed’ conformation (αc), seems to be similar to that reported by Doukov et al., whereas the other in the ‘open’ (αo) conformation, has a larger exposed surface and allows greater solvent accessibility to the A-cluster. We will also designate the corresponding clusters as Ao and Ao, and the tetramer as αββα.

### Hydrophobic tunnel network and Xe sites

The enzyme contains an extensive cavity network (Fig. 1c). A central S-shaped region runs from one N-cluster to the other, opening near to the Ni ions of these clusters. At the quarter positions of this S are long branches leading to each A-cluster. One branch opens near the Aα-cluster, but the other is blocked ~20 Å from the Aα-cluster by the movement of the α subunit N-terminal domain.

The distorted tetrahedral coordination of the Zn ion is completed by a cysteine thiolate to a proximal (relative to the cubane) Zn ion that, in turn, connects to a distal Ni ion (Niβ) through two additional bridging cysteines (Fig. 2a). The distorted tetrahedral coordination of the Zn ion is completed by a tightly bound and apparently heteroditaniolic exogenous ligand of unknown identity (Fig. 2c) that sits at the entrance of the hydrophobic tunnel mentioned above. The Niα ion has square planar coordination including, in addition to the two thiolates of Cys595 and Cys597, the two main chain N atoms from Gly596 and Cys597.

In the Aα-cluster, both the [Fe4-S4] cubane and the Niα ion with its S2N2 square planar coordination are preserved, whereas

The A-clusters of the α subunits

The most surprising observation concerning the two conformations displayed by the α subunit in our crystals is that the corresponding A-clusters are fundamentally different (Fig. 2). We have performed a series of anomalous scattering experiments to determine the nature of the metal centers present in the A-clusters by collecting X-ray data at the high energy side of the absorption edge for possible candidates, such as Fe, Co, Ni, Cu and Zn (Table 1). Double difference anomalous electron density maps indicate that the Aα-cluster is composed of a standard [Fe4-S4] cubane bridged by a cysteine thiolate to a proximal (relative to the cubane) Zn ion that, in turn, connects to a distal Ni ion (Niβ) through two additional bridging cysteines (Fig. 2a). The distorted tetrahedral coordination of the Zn ion is completed by a tightly bound and apparently heteroditaniolic exogenous ligand of unknown identity (Fig. 2c) that sits at the entrance of the hydrophobic tunnel mentioned above. The Niα ion has square planar coordination including, in addition to the two thiolates of Cys595 and Cys597, the two main chain N atoms from Gly596 and Cys597.

In the Aα-cluster, both the [Fe4-S4] cubane and the Niα ion with its S2N2 square planar coordination are preserved, whereas
the proximal site is occupied by a square planar Ni ion (Ni_p) coordinated by the three μ^2-bridging cysteine thiolates and an unidentified exogenous ligand (L) of unknown nature but probably unidentified exogenous ligand (Fig. 2a). The superposition of the A-cluster, a significant residual peak in the F_o – F_c electron density map suggests a partially occupied second position for the proximal metal ion that coincides with that of the Zn ion of the superimposed Ac-cluster (Fig. 3). Although we have not been able to use anomalous dispersion effects to identify the atom generating this peak, both Zn and Ni are plausible candidates, with occupancies of ~0.3 according to the crystallographic refinement.

Because Cu^1+ has been reported to be an essential component of the A-cluster, double difference anomalous maps were calculated from data collected at both low (λ = 1.482 Å) and high (λ = 1.376 Å) energy sides of the Cu absorption edge. However, no significant positive peak was observed in the [Δanom1.376 – Δanom1.482] double difference Fourier map, thereby ruling out the presence of any significant amounts of Cu in our A-clusters.

The source of the two different types of A-clusters in our crystals is not known. One possible explanation is that before crystalization, the A-clusters of the two α subunits of the heterodimer contained all combinations of metals: (Zn, Ni), (Zn, Ni) and (Ni, Ni). Either the crystal packing favored one of these forms directly (by selecting (Zn, Ni) with closed and open conformations, respectively) or, alternatively, the packing of the enzyme with one closed and one open form induced a rearrangement of metal ions at the metal centers in the crystal resulting in the observed distribution. A second alternative is that all the molecules in the crystallizing solution had the (Zn, Ni) configuration observed in our crystals, which may correspond to the natural active enzyme. The presence of an

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### Table 1 Metal assignments from anomalous diffraction experiments.

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<tr>
<th>Δanom1.376 (Zn edge)</th>
<th>F_o^c – F_c^c</th>
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<td>0.9</td>
<td>1.8</td>
<td>3.3</td>
<td>-0.5</td>
<td>0.9</td>
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</table>

1. λ is the X-ray wavelength (Å).
2. f'' is the imaginary component of the anomalous scattering factor.
3. p is the electron density, and s is the r.m.s. value of the map.
4. M_p and M_d correspond to the proximal (with respect to the [Fe_4-S_4] cluster) and distal metal center, respectively, with c and o designating closed and open forms of the α subunit.

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Fig. 2 Structure of the A-clusters in the α, and α, subunits depicted as ball-and-stick models. Atoms are colored as follows: Fe, red; Ni, green; Zn, black; S, yellow; O, orange; N, blue; and C, gray. a, The A-cluster. Double difference [Δanom1.376 – Δanom1.376] (blue) and [Δanom1.482 – Δanom1.482] (purple) anomalous electron density maps were solved at 2.7 Å resolution using data and 90°-shifted refined model phases from crystal CO

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Table 2. These maps, contoured at the 4 σ levels, show the presence of Zn and Ni at the proximal and distal sites, respectively (see also Table 1).

b, The A-cluster. [Δanom1.482 – Δanom1.482] anomalous electron density map shows the presence of two Ni ions. c, Same as (a), except that the maps shown are the Δanom1.482 difference map (red) calculated using refined 1.9 Å resolution model phases and the corresponding mF_o – F_c map that was contoured at the 4 σ (blue) and 10 σ (cyan) levels. The electron density shows the presence of an unidentified heteroatomic exogenous ligand (L) bound to Zn and modeled as SO. The Zn ion has distorted tetrahedral coordination. d, Same model as (b) depicting maps calculated in (c). The proximal Ni has square planar coordination and binds three cysteine thiolates and an exogenous ligand (L) of unknown nature but probably different from the one in (c). A residual electron density peak (P) that occupies a position equivalent to that of the Zn ion in (a) and (c) is also shown.
alternative position in the proximal metal of the A\(_\beta\)-cluster (Fig. 3) that could be occupied by either Zn or Ni compromises the interpretation.

**Metal clusters in the \(\beta\) subunit**

Distances between the B-, C- and D-clusters of the \(\beta\) subunits of ACS/CODHM\(_{1\alpha}\) are similar to those already reported for other CODHs\(^1\). The C-cluster consists of a Ni-Fe\(_4\)-S\(_4\) cage that can be viewed as a [Ni-Fe] subsite linked by three \(\mu_3\)-bridging sulfide ions emanating from one face of an [Fe\(_3\)-S\(_4\)] subsite. The Ni is four-coordinate with distorted tetrahedral geometry, including three endogenous ligands (the thiolate of Cys550 and two of the three \(\mu_3\)-bridging sulfide ions) and one unidentified exogenous axial ligand best modeled as CO with 0.5 occupancy (Fig. 4a). The unique Fe is coordinated by His283, Cys317 and the remaining \(\mu_3\)-bridging sulfide ion. Overall, our C-cluster is closer to the one reported by Drennan et al.\(^4\) in that it lacks the \(\mu_2\)-sulfide ion that bridges the Ni and unique Fe in CODH\(_{\alpha\beta}\). There are several residual electron density peaks in our final \(F_o - F_c\) difference Fourier electron density map indicating alternative conformations for some of the C-cluster atoms. These are generally difficult to model except for the peak between one sulfide of the [Fe\(_3\)-S\(_4\)] subsite and Cys316, suggesting a partially occupied persulfide and another peak that corresponds to an alternative position of the unique Fe (Fig. 4b). In our crystals, the C-cluster Ni ion is either disordered or only partially occupied because an electron density map containing no negative peak at the Ni site is obtained only when Ni occupancy is set to 0.5. Even the [Fe\(_3\)-S\(_4\)] subsite and the unique Fe are best modeled with 0.75 occupancies. A similar situation was observed in the CODH\(_{\alpha\beta}\) structure with a reported 0.80 occupancy for the C-cluster (PDB entry 1JYJ). A detailed discussion of the structure/function relationships of the C-cluster will be reported elsewhere.

**Properties of A-clusters**

Known properties of the A-cluster are instrumental in relating the structure to a plausible mechanism of catalysis. A-clusters can be stabilized in at least two redox states, called A\(_{ox}\) (S = 0) and A\(_{red}\)-CO (S = 1/2)\(^4\). The reduction of A\(_{ox}\) to A\(_{red}\)-CO is mediated by one electron that is most likely generated by the oxidation of CO at the C-cluster. The [Fe\(_3\)-S\(_4\)]\(^{1/2}\) cube is also reduced by one electron but the rate is too slow for reduction to occur with each catalytic cycle\(^1\). Both ACS/CODH\(_{1\alpha}\) and the isolated \(\alpha\) subunit can accept a methyl group from CH\(_3\)-Co\(^{3+}\)FeSP to form a stable methylated intermediate, but only after a reduction occurs at or near the A-cluster\(^10,11,16\). No EPR signal associated with this process has been observed\(^9\), suggesting a two-electron oxidation-reduction with S = 0 or integer in both states. The A\(_{red}\)-CO state shows the well-studied Ni-Fe-C EPR signal, which broadens by hyperfine interactions in samples of M. thermacetica grown on \(^{61}\)Ni (I = 3/2) or \(^{57}\)Fe (I = 1/2) or in samples exposed to \(^{13}\)CO (refs. 17,18). Mössbauer and UV-visible studies indicate that the cube is in the 2+ state, suggesting that Ni is 1+ (refs. 19,20). The EPR signal along with acetyl-CoA synthase activity are abolished after oxidized ACS/CODH\(_{1\alpha}\) is treated with 1,10-phenanthroline (phen) because this chelator removes Ni from the A-cluster\(^14,23,22\). The reaction is reversible, in that incubating phen-treated ACS/CODH\(_{1\alpha}\) with NiCl\(_2\) under reduc-
spin per removed/reinserted. This indicates that the purified ACS goes from the open to the closed form (closing the CO tunnel), and Niₚ goes from tetrahedral to distorted square pyramidal coordination, generating an exposed equatorial site that binds CH₃⁺ from CoFeSP while Niₚ goes from Ni(0) to Ni(II) to provide the required two electrons. Ni oxidation and the tetrahedral-to-CH₃-bound-square planar coordination change are probably concerted as Ni(0) would prefer the former and Ni(II) the latter. In the reaction of ACS to the open form, the Niₚ coordination reverts to trigonal pyramidal, and ACS adopts the closed conformation opening the CO tunnel as a new cycle starts. The [Fe₄S₄] cluster is depicted as Z₃Ni⁺ in all the steps of the reaction to indicate that, although we do not know its valence during catalysis, methylation can take place whether the cluster is reduced or not. The mechanism assumes that the α subunit stays open between methylation and CoA acetylation. There are at least two ways to picture CoA binding: either it binds to open α subunit productive site upon CoFeSP dissociation or, as suggested by the structure, the α subunit is forced to stay in an open configuration after methylation because of steric clash between the side chain of Ile146 and the methyl group bound to Niₚ that would result in the methylated A-cluster in the closed conformation. It is also possible to postulate methylation before CO binding, followed by partial closing of the α subunit and carboxylation of the A-cluster; however, the structure itself does not provide direct evidence concerning the order of events during catalysis. The reported A(ox) and A(red)-CO states are not catalytic intermediates in the model shown, and are not illustrated. b, Schematic depiction of the A-clusters in the closed and open conformations. L₁ and L₂ are unidentified exogenous ligands. From their corresponding electron densities, they seem to be different. The two A-cluster forms are the basis for the mechanism proposed in (a). If the reduced Niₚ site is tetrahedral (as in the closed Zn-containing form) and oxidized Niₚ is square planar (as in the open form), it is easy to rationalize why only the latter is extractable by phenanthrene based on their relative exposures to solvent.

**Catalytic role of the A-cluster metal ions**

Any proposed mechanism for acetyl-CoA synthesis by ACS is expected to provide a source for the two electrons (the previously postulated D-site) that are required to form the metal-methyl bond at the A-cluster (Fig. 5a). Because of the absence of any potential electron donors near the A-cluster, the reducing functionality is likely related to redox changes at the cluster itself. As mentioned above, the [Fe₄S₄]²⁺ cube is an unlikely candidate because it reduces at a rate 200-fold slower than methyl transfer. The presence of a Ni–Ni unit bridged by thiolasates raises the possibility of a reduced spin-coupled Niₚ₁⁺–Niₚ₂⁺ arrangement that could oxidize to Niₚ₁⁺–Niₚ₂⁺ upon methylation. However, based on its Niₚ₃ coordination sphere (with deprotonated N ligands from the protein main chain), Niₚ will not reduce to 1+ and, consequently, it likely remains as a diamagnetic square planar Ni³⁺ throughout catalysis. For Niₚ, we have direct evidence for coordination changes at the proximal site, as indicated by our open and closed α subunit conformations. If these are functionally relevant, they may be related to the redox state of Niₚ. Having ruled out [Fe₄S₄] and Niₚ as participating in the intramolecular redox events occurring with each catalytic cycle, we now propose that the Niₚ exists in at least two stable redox states: in the absence of reactants, it is in the 2+ state, but in the presence of CO in vitro, Niₚ⁻ can be reduced by one electron and bind CO, yielding Ni⁰-Co and the Aₕ-Coₕ state. Both in vivo and in vitro in the presence of CH₃-Co²⁺(FeSP), CoA

**Fig. 5** Proposed catalytic mechanism and schematic representation of the A-clusters. a. A simple depiction of a plausible catalytic mechanism based on the closed and open structures of ACS and on available data concerning the possible redox properties of the metal centers of the A-cluster. In reaction 1 to 2, CO produced at the C-cluster of the β subunit comes out of the tunnel and binds to trigonal pyramidal Niₚ(0). In steps 2 to 3, ACS goes from the closed to the open form (closing the CO tunnel), and Niₚ goes from tetrahedral to distorted square pyramidal coordination, generating an exposed equatorial site that binds CH₃⁺ from CoFeSP while Niₚ goes from Ni(0) to Ni(II) to provide the required two electrons. Ni oxidation and the tetrahedral-to-CH₃-bound-square planar coordination change are probably concerted as Ni(0) would prefer the former and Ni(II) the latter. In the reaction of ACS to the open form, the Niₚ coordination reverts to trigonal pyramidal, and ACS adopts the closed conformation opening the CO tunnel as a new cycle starts. The [Fe₄S₄] cluster is depicted as Z₃Ni⁺ in all the steps of the reaction to indicate that, although we do not know its valence during catalysis, methylation can take place whether the cluster is reduced or not. The mechanism assumes that the α subunit stays open between methylation and CoA acetylation. There are at least two ways to picture CoA binding: either it binds to open α subunit productive site upon CoFeSP dissociation or, as suggested by the structure, the α subunit is forced to stay in an open configuration after methylation because of steric clash between the side chain of Ile146 and the methyl group bound to Niₚ that would result in the methylated A-cluster in the closed conformation. It is also possible to postulate methylation before CO binding, followed by partial closing of the α subunit and carboxylation of the A-cluster; however, the structure itself does not provide direct evidence concerning the order of events during catalysis. The reported A(ox) and A(red)-CO states are not catalytic intermediates in the model shown, and are not illustrated. b, Schematic depiction of the A-clusters in the closed and open conformations. L₁ and L₂ are unidentified exogenous ligands. From their corresponding electron densities, they seem to be different. The two A-cluster forms are the basis for the mechanism proposed in (a). If the reduced Niₚ site is tetrahedral (as in the closed Zn-containing form) and oxidized Niₚ is square planar (as in the open form), it is easy to rationalize why only the latter is extractable by phenanthrene based on their relative exposures to solvent.
articles

Table 2 Data and refinement statistics

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1Except for the native and COα data sets (collected at 0.9801 and 0.9340 Å, respectively) statistics were calculated with Friedel mates treated independently.
2MAD data were collected at either side of the Fe absorption edge. Native data were used as the remote wavelength set.
3Data sets at the high energy (low wavelength) side of Zn (1.282 Å), Cu (1.376 Å), Ni (1.482 Å) and Co (1.602 Å) were collected from this CO-treated crystal.
4The numbers given within parentheses refer to the highest resolution shell.
5For COα collected at wavelength 0.9340 Å. This is the highest resolution data set.
6After conversion of TLS parameters and their inclusion in individual B-factors by tlsanl56.
7Values are for the opened and closed forms, respectively.

and either CO or CO2/reductant, Ni6+ will be reduced by two electrons, forming Ni0 and the Ared2 state (Ni d2+-Ni0 [Fe4-S4]2+/1+). The redox potential of the Ni0/Nip2+ pair should be ~530 mV versus Normal Hydrogen Electrode (NHE), the value reported for the D site 10. The redox state of the [Fe4-S4] cube appears irrelevant in the ability of the enzyme to accept a methyl group15, and we have indicated this by designating its core oxidation as ‘2+ or 1+’ in the Ared2 state.

The Nip0 proposal is reasonable only if µ2-bridging ‘metallo-cysteinate’ ligands to Ni0 show bonding properties that mimic neutral ligands with π-accepting capabilities (for example, trialkylphosphines or CO). Darensbourg et al.25 have found that the negative charge of thiolates is effectively neutralized by bridging to two metal centers (so called ‘metalated’ thiolates). The effect of such ligands on CO stretching frequencies of Fe carboxyl complexes is similar to that of phosphines26. Metalation increases Ni2+–E0 values, rendering such compounds more easily reduced. Along the same lines, Reedijk et al.27 synthesized a dinuclear Fe-S complex containing a thiolate ligand µ2-bridged to Ni0(CO)3. Stretching CO frequencies in this complex are similar to those of (t-butyl)3P–Ni0(CO)3 (ref. 28), indicating similar electronic properties for the phosphine and metallothiolate.

Accordingly, the mechanistic roles of Ni d2+ and the [Fe4-S4]2+ cube could be restricted to metalate the cysteine ligands of Nip to facilitate reduction to the Nip0 state.

If validated by experimental and theoretical studies, our proposal for Nip0 would be appealing because there is a rich history of Ni0 organometallic phosphine complexes that are catalysts for related reactions, such as the copolymerization of CO with ethylene29,30. Hsiao et al.31 have shown that CH3I reacts with mononuclear NiP2S2 (P = phosphine and S = thioether) complexes to yield Ni2+-CH3 adducts into which CO inserts rapidly to form...
Ni$^{2+}$-acetyl. The parent Ni$^{2+}$ compounds can be reduced to the Ni$^{0}$ state that binds CO. Corresponding Ni$^{2+}$($\text{N}_{3}S_{2}$) (S = thiolate) complexes neither reduce to Ni$^{1+}$ or Ni$^{0}$ states nor bind CO. Taken together, the reactivity of these compounds supports the possible role of Ni$_{d}^{0}$ in catalysis and suggests, as indicated above, that Ni$_{d}$ does not participate directly in acetyl synthesis.

The occurrence of Ni$_{a}$ in the active site of this enzyme would be unprecedented in biology. Thus, subsequent studies are required to assess the feasibility of this hypothesis. The Ni$^{0}$ assignment is formal, as the electron density will be significantly delocalized over the $\pi$-accepting ligands$^{22,23}$. The important point is that Ni$_{a}$ and its ligands can donate two electrons to form the CH$_{3}$Ni$_{2}^{+}$ species. A formal Ni$^{0}$ assignment has also been proposed for the reduced Ni-R form of NiFe hydorgenases; in this case, the Ni ion is thought to be protonated and, consequently, isoelectronic with Ni$^{2+}$-H$^{+}$ (ref. 33).

**Acetyl-CoA synthesis by ACS/CODH$_{\text{int}}$**

As stated above, one important point is that the enzyme is active for catalysis when Ni$_{d}$ is in the Ni$^{2+}$ redox state and thus capable of binding CO and CH$_{3}$Co$^{3+}$FeSP. This state is derived from reduction of a species containing Ni$^{2+}$ at the proximal site, as suggested by the results of phen treatment$^{14}$. Ni$_{d}^{0}$ with or without bound CO would be a powerful nucleophile that would attack the methyl group of CH$_{3}$Co$^{3+}$FeSP directly, forming Co$^{1+}$FeSP and either Ni$_{d}^{2-}$-CH$_{3}$ or cis C(O)-Ni$_{d}^{2-}$-CH$_{3}$. With both CO and methyl bound to Ni$_{d}^{2-}$, insertion of the former or migration of the latter would form the acetyl intermediate Ni$_{d}^{2-}$C(O)CH$_{3}$. Finally, CoA-SH would bind, deprotonate, and attack this intermediate, yielding acetyl-CoA and the Ni$_{d}^{0}$ state by reductive elimination (Fig. 5a).

A second important point is the presence of two $\alpha$ subunit conformations. This raises the possibility that conformational changes that occur during catalysis dictate the sequence in which substrates and products enter, react and leave the active site. Also, $\alpha_{c}$ appears to have a CoA-binding pocket that is much less obvious in $\alpha_{o}$ (data not shown). Because the tunnel entrance at the A$_{c}$-cluster is open while the counterpart at the A$_{o}$-cluster site is blocked, delivery of CO may be causally connected to the conformation of the $\alpha$ subunit. The protein coordination of the proximal metal site also differs in $\alpha_{c}$ and $\alpha_{o}$ subunits, with trigonal pyramidal coordination in $\alpha_{c}$ and mostly distorted trigonal planar in $\alpha_{o}$, suggesting that the geometry of the proximal site is causally related to the conformation of the $\alpha$ subunit. From the structure, the suggestion follows that CO binds to a trigonal pyramidal Ni$_{d}^{0}$ in the $\alpha_{c}$ conformation and that CH$_{3}$Co$^{3+}$FeSP binds subsequently. The process of methyl group transfer converts $\alpha_{c}$ to $\alpha_{o}$, and the Ni$_{d}$ conformation becomes square-planar (with CO bound axially and methyl bound in the plane). Subsequent insertion and CoA attack yield acetyl-CoA, Ni$_{d}^{0}$ and the $\alpha_{c}$ conformation (Fig. 5a). One should bear in mind, however, that it is possible to methylate the A-cluster in the absence of CO and then generate acetyl-CoA after subsequent incubation in CO and CoA$^{15}$; therefore, the actual sequence of events cannot be deduced from the structure alone.

**Comparison to a Cu-based mechanism.**

In the crystal structure reported by Doudouk et al.$^{8}$ the $\alpha$ subunits from each heterotetramer are identical, related by a noncrystallographic two-fold symmetry axis and, as far as we can tell, equivalent to our closed conformation. Their A-clusters contain the same Ni$_{d}$ site and [Fe$_{2}$S$_{3}$] cluster as we observe, but a Cu$^{2+}$ ion replaces the Zn$_{d}^{2+}$ of our A$_{d}$-cluster and, most important, the Ni$_{d}$ of our A$_{d}$-cluster. During the first step of acetyl-CoA synthesis, the authors propose that CO binds Cu$^{2+}$ as it emerges from the hydrophobic tunnel. In their $\alpha_{c}$ conformation,$^{8}$ as in our $\alpha_{c}$, the proximal (Cu) and Ni$_{d}$ ions are buried below the molecular surface, requiring a significant conformational change to get these metal ions more exposed to the protein surface to accept a methyl group from CH$_{3}$Co$^{3+}$FeSP and subsequently donate an acetyl group to CoA. Although not mentioned explicitly, the cubane appears to have been assigned, at least partially, as the required reducing functionality, so that the methyl group transfers to Ni$_{d}^{2-}$ in concert with oxidation of the all-ferrous cube (although Ni$_{d}$ redox could also be implicitly involved). Methyl migration from CH$_{3}$Ni$_{d}^{2+}$ to Cu$^{2+}$ then follows this step, yielding a Cu-acetyl intermediate. Finally, CoA binds, deprotonates and attacks the acetyl group, yielding acetyl-CoA.

The presence of Cu (and possibly Zn)$^{8}$ in the A-clusters and the presence of Zn and Ni in the A-clusters reported here indicate that the proximal site of the A-cluster can bind, and possibly exchange, at least three different metal ions. We believe, however, that once either Cu or Zn binds to the proximal site, the $\alpha$ subunit stays in an inactive conformation because, out of the three ions, only Ni$_{d}^{0}$ would be able to undergo the postulated two-electron redox process required for methylation. Although their chemical properties are certainly different, Zn$^{2+}$, Cu$^{1+}$ and Ni$^{0}$ are all $d^{10}$ metal ions with identically occupied d orbitals and geometry preferences. It is difficult at this point to assert whether metal substitutions occurred within the cell or during or after purification of ACS/CODH.

We are skeptical about the proposed role for Cu in the catalytic mechanism$^{8}$. The reported correlation between Cu content and catalytic activity is not present, as may be shown by normalization of the metal contents reported in Table 2 of Doudouk et al.$^{8}$. As discussed above, the electron spin of the A-cluster in the state that yields the Ni-Fe-C signal is substantially delocalized among the Ni$_{d}$, [Fe$_{2}$S$_{3}$] and CO$^{17,18}$. With CO bound to Cu (I = 3/2) as proposed, it seems unusual that a [Ni$^{2+}$-Cu$^{1+}$-CO-[Fe$_{2}$S$_{3}$]] spin-coupled cluster would show strong hyperfine splitting at the Ni, Fe and CO portions but not at the Cu located at the center of the system. The Cu-based structure neither explains the heterogeneity that characterizes this enzyme in terms of nonlabile and labile Ni because only a single type of Ni is observed. Another point concerns the migratory insertion reaction. In model compounds, alkyl-to-acyl insertions typically involves a single metal center with CO and methyl groups bound cis to each other$^{15}$ and not two different metal centers (CO bound to Cu and methyl bound to Ni), as proposed.

**Additional evidence for a Ni-Ni-[Fe$_{2}$S$_{3}$] active site**

During the preparation of this paper, an additional mechanism has been proposed by Gencic and Grahame$^{88}$ based on metal reconstitution experiments using a recombinant apo ACS subunit from the methanogen *Methanosarcina thermophila*. The Gencic-Grahame mechanism is similar to our own in three respects: (i) it considers only the Ni-Ni-Fe--containing A-clusters as catalytically active, ruling out any Cu-based catalysis, (ii) it does not assign any catalytic properties to Ni$_{d}$ and (iii) it does not assign a catalytic role to the one-electron reduced species giving rise to the Ni-Fe-C EPR signal because methylation is a two electron process. Instead of proposing a Ni$^{0}$/Ni$^{2+}$ pair as the two-electron donor required to form the methyl-metal bond, Gencic and Grahame postulate a Ni$^{0}$/Ni$^{2+}$ pair. In subsequent steps, their mechanism requires one-electron redox chemistry by the [Fe$_{2}$S$_{3}$] cluster. This is a major drawback of this model.
because, as mentioned above, data from one of our laboratories rules out a catalytically relevant redox active [Fe₄S₄] center at the A-cluster. The authors also claim that no external reductant is required to generate the Ni-Fe-C species. However, in this scheme, the unpaired electron originates from the [Fe₄S₄] cluster, implying that the starting material was already at least partially reduced by an external reductant.

In light of the binuclear Ni-based A₃ structure reported here, the known requirement of ACS for two Ni equivalents to support catalytic activity, the inorganic chemistry and spectroscopy favoring Ni, the promiscuity and lability of the proximal site and the varying levels of Cu observed in active enzyme solutions, we do not favor a Cu-based mechanism for ACS catalysis. Instead, we believe that active A-clusters are composed of a [Fe₄S₄]Ni₃-Ni₃ unit where only Ni₃ is active, possibly as a Ni²⁺/Ni⁴⁺ redox pair.

Methods

Purification and crystallization. M. thermoacetica cells were grown as described. ACS/CODHM₄ was purified as reported with only minor modifications. A monoclinic, space group C2 crystal form with a = 245 Å, b = 82 Å, c = 167 Å, β = 95° and one αβ dimer per asymmetric unit was obtained in 2–4 μl hanging drops in an anaerobic glove box at 20 °C. Hanging drops were prepared by mixing an equal volume of a ACS/CODHM₄ solution at 10 mg ml⁻¹ in 50 mM Tris-HCl, pH 8.0, 2 mM sodium dithionite and 10 mM dithiothreitol with the crystallization solution. The latter contained 1.95–2.10 M ammonium sulfate, 100 mM HEPES buffer, pH 7.0–7.3, 3–5% (w/v) PEG 400, 2 mM sodium dithionite and 100–200 mM dimethylammonium propane sulfonate (the NDSB195 reagent). Crystals were detected after one year after setting the crystallization plates. CO- and Xe gas–binding experiments and anaerobic flash-cooling of ACS/CODHM₄ crystals were carried out inside the glove box as described.

Data collection. All the diffraction data were collected at the European Synchrotron Radiation Facility (ESRF), Grenoble, France (Table 2). A 2.2 Å resolution native data set and Fe-edge MAD data were collected at the BM-30A beamline. To identify the nature of the other transition metals present in the protein, four data sets were collected using a CO-treated crystal at the high-energy side of the K-edges of Zn, Cu, Ni and Co at the ID29 beamline of the ESRF. All data were processed with the July 2002 version of XDS.

Structure determination and refinement. The structure was solved by combining MR and MAD phases. MR was performed with AMoRe using the known CODHM₂ structure (PDB entry 1J1V). A contrasted solution was found for the two β subunits of the dimer using reflections in the 15–3 Å resolution range (correlation coefficient of 0.35 and R-factor of 0.48.) The positions of seven metal clusters were found both in a native anomalous difference map calculated with MR phases and from Patterson maps calculated with MAD data set using SOLVE. These sites were subsequently treated as low-resolution super-atoms for refinement and phase calculations. Phases were combined with SIGMAA and subsequently significantly improved and extended to 2.2 Å resolution by density modification, including two-fold electron density averaging, using RESOLVE.

After applying the correct amino acid sequence to the ACS/CODHM₄ β subunit placed using MR, the protein model for the α subunit was progressively built with the initial help of automatic tracing using warpNtrace and RESOLVE and the graphics program TURBO-FRODO. Initial density-averaged electron density maps showed well-defined electron density at the N-terminal domain of the α subunit but ill-defined density elsewhere. This was subsequently explained by the different orientations of the central and C-terminal domains in the two β subunits, which did not allow for automatic noncrystallographic two-fold symmetry electron density averaging to be effectively applied. Phases from the partially refined model containing the β, dimer and the α-N-terminal domains of ACS/CODHM₄, combined with the experimental MAD phases, produced increasingly well-defined electron density maps. Several cycles of model building and model and MAD phase combination resulted in the complete structure. The same unique set of reflections (5% of the total) was excluded for the refinement of all crystal structures. Crystallographic refinement was performed with REFMACS (ref. 46) using eight TLS groups, one for each of the three domains of each α subunit and one for each β monomer. When the CO₄ data set became available, the model was further refined to a resolution of 1.9 Å. Refinement statistics are given in Table 2.

Coordinates. Atomic coordinates and structure factors corresponding to the 1.9 Å resolution model of the CO-treated crystal have been deposited with the Protein Data Bank (accession code 1OAO).

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Competing interests statement

The authors declare that they have no competing financial interests.

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