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Novel domain arrangement in the crystal structure of a truncated acetyl-CoA synthase from *Moorella thermoacetica*^{†,‡}

Anne Volbeda^{§,*}, Claudine Darnault[§], Xiangshi Tan^{||,#}, Paul A. Lindahl^{||}, and Juan C. Fontecilla-Camps^{§,*}

[§]Laboratoire de Cristallographie et Cristallogénèse des Protéines, Institut de Biologie Structurale Jean-Pierre Ebel, CEA, CNRS, Université Joseph Fourier, 41 Rue Jules Horowitz, F-38027 Grenoble, France

^{||}Departments of Chemistry and of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843.

Abstract

Ni-dependent Acetyl-CoA synthase (ACS) and CO dehydrogenase (CODH) constitute the central enzyme complex of the Wood-Ljungdahl pathway of acetyl-CoA formation. The crystal structure of a recombinant bacterial ACS lacking the N-terminal domain that interacts with CODH shows a large reorganization of the remaining two globular domains, producing a narrow cleft of suitable size, shape and nature to bind CoA. Sequence comparisons with homologous archaeal enzymes that naturally lack the N-terminal domain show that many amino acids lining this cleft are conserved. Besides the typical [4Fe-4S] center, the A-cluster contains only one proximal metal ion that, according to anomalous scattering data, is most likely Cu or Zn. Incorporation of a functional Ni₂Fe₄S₄ A-cluster would require only minor structural rearrangements. Using available structures, a plausible model of the interaction between CODH and the smaller ACS in archaeal multi-enzyme complexes is presented, along with a discussion of evolutionary relationships of the archaeal and bacterial enzymes.

Ni-dependent acetyl coenzyme A synthase (ACS) is a NiFeS-cluster enzyme that requires strict anoxic conditions to function. In the acetogenic bacterium *Moorella thermoacetica* (*Mot*), the ACS α -subunit forms an $\alpha_2\beta_2$ heterotetramer with the Ni-containing carbon monoxide dehydrogenase (CODH) β -subunit (1). This association constitutes the central enzyme complex of the anaerobic Wood-Ljungdahl pathway of acetyl-CoA synthesis from CO₂ (2,3). The structure of this complex is known in two crystal forms (4,5) and shows a long hydrophobic tunnel network that allows the CO produced at the CODH active site, the C-cluster, to be used at the ACS active site, the A-cluster, without being released to the medium. Our understanding of CO₂ reduction to CO by CODH has progressed significantly thanks to the recent crystal structures of key catalytic intermediates in the homodimeric β_2 enzyme from

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*To whom correspondence should be addressed. AV: Tel. +33438789606, Fax +33438785122, E-mail anne.volbeda@ibs.fr; JCFC: Tel. +33438785920, Fax +33438785122, E-mail juan.fontecilla@ibs.fr.

[#]Current address: Department of Chemistry, Institute of Biomedical Sciences, Fudan University, Shanghai, 200043, P.R. China

Supporting Information Available. Superposition statistics of bacterial CODH/ACS and archaeal CODH, sequence alignment of bacterial ACS with archaeal CODH and structure comparison of CODH and HCP. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Carboxydotherrnus hydrogenoformans (*Ch*) (6) and the heterotetrameric $\alpha_2\epsilon_2$ enzyme from the archaeon *Methanosarcina barkeri* (*Meb*) (7), see also (8). However, many mechanistic details of acetyl-CoA formation by ACS have not yet been elucidated. The active A-cluster has been characterized as a $\text{Ni}_2\text{Fe}_4\text{S}_4$ center in the crystal structures of both bi-functional CODH/ACS_{Mot} and mono-functional ACS_{Ch} (5,9), in agreement with many previous studies, as reviewed in (10,11). Catalysis involves three substrates, CoA, CO and the cobalt-containing methyl-carrying corrinoid iron-sulfur protein (CoFeSP):

At least 5 steps take place at the A-cluster during catalysis: (i) binding of CO, (ii) transfer of the methyl group from CoFeSP, (iii) formation of an acetyl intermediate, (iv) binding of CoA and (v) formation of acetyl-CoA. In addition, ACS requires reductive activation (12). This is so because the methyl group is provided by CoFeSP as a CH_3^+ cation (13) and two electrons are required to form a carbon-metal bond, most likely with the proximal Ni ion of the A-cluster. The two electrons are recovered upon reductive elimination of the acetyl intermediate to form acetyl-CoA. So far, crystal structures of well-defined catalytic intermediates of ACS have not been reported.

Here we report the crystallographic analysis of a truncated form of ACS_{Mot}, with a molecular mass of 49 kDa called α_{49} (14). Because it lacks the 310 residue-long N-terminal domain α_{49} resembles the homologous ACS β subunit of the acetyl-CoA decarbonylase/synthase (ACDS) multi-enzyme complex found in methanogenic archaea. To avoid confusion, we will use the nomenclature *A1*, *A2* and *A3* for ACS domains, and *C1*, *C2*, etc., for CODH domains, regardless their origin. In ACS_{Mot}, *A1* is involved in extensive inter-subunit interactions with CODH and contains a significant part of the hydrophobic tunnel that connects the C- and A-clusters (4,5). The isolated, recombinant archaeal ACS can be activated by adding NiCl_2 . According to metal analyses and spectroscopic studies, the reconstituted *Methanosarcina thermophila* (*Met*) ACS contains, like its bacterial counterparts, a classical $\text{Ni}_2\text{Fe}_4\text{S}_4$ A-cluster (15-17). The ACDS complex also contains $\gamma\delta$ CoFeSP heterodimers and $\alpha_2\epsilon_2$ hetero-tetramers, with α_2 corresponding to CODH (18). It has been shown that the hydrophobic tunnels of both the bacterial and the archaeal enzymes can bind xenon (5,7,19). The absence of the bacterial tunnel-containing *A1* domain in archaeal ACS raises the question how CO diffuses between the A- and C-clusters in the archaeal ACDS complex.

The crystal structure of ACS_{Mot} has been determined in closed (α_C) and open (α_O) conformations (5). The difference between the two structures is due to a large movement of *A1*, which in α_O blocks the connection between the A-cluster, bound to domain *A3*, and the tunnel network (5,20). Conversely, when the enzyme is in the α_C conformation, the A-cluster can bind the CO produced at the C-cluster, because the connection to the hydrophobic tunnel network is open. Only inactive $\text{NiCuFe}_4\text{S}_4$ and $\text{NiZnFe}_4\text{S}_4$ A-clusters have been found in crystal structures of this form (4,5). Functional $\text{Ni}_2\text{Fe}_4\text{S}_4$ A-clusters (10,11) have been observed in two α_O crystal structures, where the A-cluster is exposed to the medium (5,9). Therefore, the enzyme is likely to accept the methyl group from CoFeSP when it is in the α_O conformation. When the enzyme is treated with exogenous CO a Ni(I)-CO complex is formed. Furthermore, ^{57}Fe , ^{59}Ni and ^{13}C isotopic labeling experiments have shown that Ni, CO and the [4Fe-4S] cluster interact magnetically giving rise to an EPR spectrum known as the NiFeC signal (10,11 and references therein).

Purified recombinant α_{49} contains ~ 4 Fe and 0.5 Ni per A-cluster (14). Incubation with NiCl_2 followed by reduction with dithionite and exposure to CO yielded an EPR signal reminiscent of NiFeC in terms of g-values and relaxation/saturation properties. The ability to

generate this pseudo-NiFeC signal indicates that an A-cluster derivative can be reconstituted in the truncated subunit. The spin concentration of this signal was only $\sim 25\%$ of that normally observed for NiFeC, and the truncated subunit had no catalytic activity. In this respect, α_{49} resembles other constructs of the *Mot* enzyme, where metal heterogeneity at the active site and sub-stoichiometric amounts of nickel are a recurrent problem. The α_{49} structure presented here contains a novel metal-containing inactive form of the A-cluster, and a new arrangement of domains A2 and A3. We will call this form $\alpha_{49(T)}$. Below, we will discuss the relevance of these findings with respect to previous studies on the activity of bacterial and archaeal ACS's. We will also address the possible evolutionary implications of the striking similarities between ACS and CODH domains.

MATERIALS AND METHODS

Crystallization

α_{49} , a 49 kDa fragment of ACS_{Mot}, was purified after heterologous expression in *Escherichia coli*, as previously described (14). It contains residues 311–729 of the intact enzyme and a C-terminal His-tag. All crystallization experiments were performed at room temperature in an anaerobic glove box, using the vapor diffusion method. Crystals with maximal dimensions of $0.3 \times 0.3 \times 0.2 \text{ mm}^3$ were obtained after six to eight weeks. The best ones grew in hanging drops that were prepared by mixing 1 μl of a solution with a protein concentration of 20 mg/ml and 2 mM sodium dithionite in 50 mM Tris/HCl at pH 8.0, with 1 μl of a reservoir solution containing 1.4 M ammonium sulfate (AS), 2 mM sodium dithionite and 100 mM Tris/HCl at pH 7.1. After transfer to a stabilization solution containing 2.2 M AS and 30% glycerol, in addition to the other reservoir components, crystals were flash-cooled inside the glove box in liquid propane and subsequently stored in liquid nitrogen, as described (21).

X-ray data collection and processing

Diffraction data were collected to 3.0 Å resolution on a MAR CCD165 detector (MAR Research, Germany) by exposing the best crystal to monochromatic ($\lambda = 1.008 \text{ Å}$) X-rays under a cold ($\approx 100 \text{ K}$) nitrogen stream. A total of 450 images were collected with 0.4° oscillations, using the BM30A beamline of the European Synchrotron Radiation Facility in Grenoble, France. Diffraction spots were integrated, scaled, subjected to a zero-dose correction (22) and reduced to structure factor amplitudes with XDS (23). The first 333 images were selected to produce 99.5% complete data with good intensity statistics (Table 1). The crystal belonged to the trigonal space group P3₁21 with cell dimensions $a = b = 166.4 \text{ Å}$ and $c = 245.2 \text{ Å}$. A similar but less well-diffracting crystal with a halved crystallographic c-axis was used to collect data to 3.7 Å resolution at the high energy side of the Ni absorption edge ($\lambda = 1.4827 \text{ Å}$). These data were used to check the presence of Ni (statistics not shown).

Structure solution

The phase problem was solved by molecular replacement with PHASER (24–26), using the 3 Å resolution data and domains A2 and A3 of ACS_{Mot} α_O as search models. For success, it was necessary to exclude the N-terminal region of domain 2 (residues 311–319) and a long inter-domain helix (residues 471–499). In addition, low peaks from the rotation function output had to be included in order to find well-contrasting solutions in the translation search. Checks with anomalous difference maps showed that good solutions gave strong peaks for the A-cluster. The asymmetric unit contains six molecules related by non-crystallographic symmetry (ncs) operations close to those of a regular 32 hexamer and a solvent content of about 63%. In the crystal with the smaller unit cell, which contains a trimer per asymmetric unit, the two-fold axes of the hexamer correspond to crystallographic symmetry operations.

Refinement

Because of the limited data resolution, refinement was carried out with tight ncs restraints, using *REFMAC* (27,28) and including TLS refinement (29) to model overall anisotropic displacements of the twelve domains present in the hexamer. Manual model corrections were performed with the *TURBO* computer graphics package (30). The quality of electron density maps was significantly improved by averaging, using the program *SUPERMAP* (31). A first six-fold averaged electron density map obtained with molecular replacement phases clearly showed the long helix between domains A2 and A3 that was left out of the starting model. Part of the main chain of the C-terminal His-tag also appeared in this map, at the dimer interfaces of the hexamer (Fig. 1), but the corresponding side chains were only partially resolved during refinement. The first six N-terminal residues of $\alpha_{49(T)}$ are disordered and therefore were not included in the model. Refinement statistics of the final model are given in Table 1.

Structure and sequence analyses

Figures 1-3, 5, 7-8 and S2 were prepared with the programs *MOLSCRIPT* (32) and *RASTER3D* (33), in addition to *CONSCRIPT* (34) that was used for the display of electron density maps (Fig. 3). The electrostatic potential surfaces of Figure 4 were determined at pH 7 with the APBS (35) plugin of PyMOL (<http://www.pymol.org>), after addition of hydrogen atoms with PDB2PQR (36) using the AMBER force field (37) and pK_a 's calculated with PROPKA (38), excluding the metal and inorganic sulfur atoms of the A-cluster from the protein. Structural superpositions were performed with the BIOMOL program *SUPPOS* (<http://www.xray.chem.rug.nl/Links/Biomol1.htm>). Tunnels and cavity maps in Figures 5, 7, 8 and S2 were calculated with the program *CAVsel* (A. Volbeda, unpublished). Amino acid sequences used in the manual structure-based alignments in Figures 6 and S1 were obtained from SWISSPROT (<http://www.ebi.ac.uk/swissprot/>).

RESULTS

Crystal structure determination

The structure of α_{49} in the novel $\alpha_{49(T)}$ conformation has been solved at 3.0 Å resolution by molecular replacement (Table 1). There is a regular trimer of dimers in the asymmetric unit (Fig. 1). A similar crystal form with an approximately halved c-axis contained a trimer in the asymmetric unit and diffracted to 3.7 Å resolution. After refinement of the 3.0 Å resolution structure, an average temperature factor of 57.3 Å² was obtained, suggesting small differences in subunit orientations between hexamers. The final model shows good refinement statistics (Table 1), as indicated by a satisfactory geometry and an R_{free} of 20.8% for all structure factor observations between 20 Å and 3.0 Å resolution. Although we were unable to obtain X-ray data to a better resolution, precluding a detailed study with $\alpha_{49(T)}$, the model is of sufficient quality to allow the analysis of both fold and major active site differences with previously obtained structures.

Changes with respect to previous ACS structures

In $\alpha_{49(T)}$, domains A2 and A3 have significantly moved to each other relative to their previously known conformations (Fig. 2). This movement corresponds to a rotation around a hinge region located close to the C-terminus of the long interdomain helix (residues 471–499, shown in orange in Fig. 1). The internal structure of the two domains in the new conformation does not change much: separate C_α -superpositions of domains A2 (residues 318 to 491) and A3 (residues 494 to 729) of $\alpha_{49(T)}$ to those of α_O give root-mean-square deviations (rmsd's) of about 0.5 Å. Taking A2 as a reference (Fig. 2), the structural change of $\alpha_{49(T)}$ with respect to α_O may be described as a rotation (κ) of domain A3 by 50° and a translation parallel to the rotation axis ($T_{||}$) of 4.6 Å, with a maximal shift of 34 Å in C_α positions. Although the domain conformation

of $\alpha_{49(T)}$ is somewhat closer to that of α_O in ACS_{Ch} (9), after superposition of their respective A2 domains there is still a substantial difference in the position of domain A3 ($\kappa = 35^\circ$, $T_{//} = 1.3\text{\AA}$). Our results indicate that there is much more flexibility in the relative positions of domains A2 and A3 than previously recognized (Fig. 2). In fact, the rearrangement of domain A3 in $\alpha_{49(T)}$ is as extensive as the one observed for domain A1 between α_C and α_O in ACS_{Mot} (5). When the corresponding A2 domains are superimposed, there are no bad clashes between α_O A1 and $\alpha_{49(T)}$ A3. However, the superposition of α_C to $\alpha_{49(T)}$ leads to the collision of these two domains (not shown). It follows that the $\alpha_{49(T)}$ conformation is compatible with the orientation of A1 in α_O , but not in α_C .

At the X-ray wavelength of 1.008 Å used for data collection with the best $\alpha_{49(T)}$ crystal, a map calculated with Δ_{anom} coefficients displayed high peaks at the active site both for the [4Fe-4S] cluster and one metal ion (Fig. 3). This ion occupies the proximal site relative to the cluster, previously called M_p (5). An equivalent map was calculated with 3.7 Å resolution data collected at $\lambda = 1.4827$ Å from the related crystal with the smaller unit cell. In this map, the peak corresponding to the [4Fe-4S] cluster was still present but there was no peak at M_p (not shown). Thus, there is no evidence for the presence of Ni in the A-cluster in $\alpha_{49(T)}$. Because (i) the λ used is lower than the wavelength corresponding to the Ni absorption edge and (ii) there is a high electron density peak at M_p in the map calculated with $2F_o - F_c$ coefficients, it appears that a metal ion heavier than nickel occupies the proximal site. The most likely candidates are Cu and Zn, as these metals were also observed in previous crystal structures of ACS_{Mot} (4,5). A flat $F_o - F_c$ difference map for the ion at M_p was obtained with 75% occupied Zn²⁺. The metal ion is tetrahedrally coordinated by an external ligand, modeled as HS⁻ with the same occupancy, and by the thiolate groups of Cys509, Cys595 and Cys597 (Fig. 3).

The cleft formed between $\alpha_{49(T)}$ domains A2 and A3, is located opposite to where A1 would be in the enzyme. An analysis of electrostatic potential surfaces (Fig. 4) strongly suggests that this cleft may bind the highly negatively charged (39) CoA substrate and acetyl-CoA product: it contains the most extensive complementary positive charge distribution of the truncated structure, which is located mainly on the surface of A2. The same positive patch is visible in the structures of α_O (Fig. 4A) and α_C (Fig. 4B), but in these conformations it is approximately twice as far away from the A-cluster than in $\alpha_{49(T)}$. Only in the latter conformation (Fig. 4C) does the distance of about 15 Å between the patch and the A-cluster seem compatible with functional binding of the coenzyme.

Results from fluorescence quenching, chemical modification and protection experiments led Wood and co-workers to propose that CoA binding to ACS protects about five tryptophan residues and two arginines from chemical modification (40,41): i) the modification of these residues causes the loss of the ¹³CO/¹²CO exchange reaction in acetyl-CoA catalyzed by the A-cluster; ii) modification of the arginines modifies the fluorescence of the tryptophan(s) indicating that some are close to each other; iii) at least one of the modified tryptophans must be close to the A-cluster because it perturbs the NiFeC EPR signal; iv) both adenine and 3'-dephospho-CoA quench the tryptophan residue(s) fluorescence, suggesting that the 3'-phosphate group is not essential for CoA binding; v) the pyrophosphate bridge of CoA binds to arginine(s) as indicated by the protection that added pyrophosphate exerted against the chemical modification of these residues and vi) these modifications only affect the microenvironment around the targeted residues, as shown by CD. The cleft formed between A2 and A3 contains two tryptophans, 418 and 427, and four arginines, 334, 346, 429 and 616 (Fig. 5A). Trp418 is likely to be one of the modified residues because in $\alpha_{49(T)}$ it is the closest tryptophan to the A-cluster at 12.5 Å and, consequently, it could influence the NiFeC signal. Trp427 may be also a potential candidate for chemical modification being at 17.5 Å from the A-cluster. Consistent with the results described above (v), arginines 334, 346, 429 and 616 are close to the two tryptophans.

Coenzyme A could be stabilized through interactions of i) the adenine ring with Trp418 or Trp427 and ii) its pyrophosphate region with the guanidinium group of two of the arginines mentioned above (Fig. 5A). These interactions are also compatible with the approach of the cysteamine moiety of the coenzyme to the M_p site of the A-cluster through a narrow tunnel that does not exist in the other α -subunit conformations (Fig. 5B). Assisted by His419, His408 may abstract a proton from CoA-SH before its putative binding to the apical binding site of Ni_p (labeled with *). Next it may react with an acetyl group bound equatorially to Ni_p (at the site labeled with \$). A cavity above the S atom of the coenzyme shows that there is also enough space for the acetyl-CoA product. We conclude that, if $\alpha_{49(T)}$ has functional relevance, it may approximate the CoA binding conformation of ACS. The fact, as mentioned above, that the $\alpha_{49(T)}$ conformation is incompatible with α_C is not surprising because the acetylated ACS, that binds CoA, should not be able to adopt this conformation due to steric hindrance at the A-cluster.

Comparison to archaeal enzymes

In order to investigate whether the domain arrangement in $\alpha_{49(T)}$ could arise from crystal packing interactions, we compared the amino acid sequences of the two bacterial enzymes of known structure with two methanogenic (*Met* and *Meb*) counterparts. Out of the 403 aligned residues, 147, or 36.4%, are invariant (Fig. 6) suggesting that the bacterial and archaeal ACS's have very similar structures. Out of the 30 residues involved in interactions ($d < 3.8 \text{ \AA}$) between domains A2 and A3 in $\alpha_{49(T)}$, 18, or 60%, are invariant. By comparison, only 13 residues are involved in A2/A3 contacts in both α_O and α_C . Seven of these residues, or 54%, are invariant and 6 of them are also involved in A2/A3 interactions in $\alpha_{49(T)}$. If the conformation of the latter were a crystallization artifact, the fraction of invariant residues involved in its domain interactions would not be expected to be higher than the overall 36.4% identity among the four aligned sequences. Therefore, the high degree of residue conservation at the A2/A3 interface suggests a functional role for the $\alpha_{49(T)}$ conformation. The invariant residues in Figure 6 are also highly conserved in an alignment of 7 bacterial and 13 archaeal ACS amino acid sequences (10).

The archaeal A-cluster-containing subunit resembles α_{49} in that it lacks the N-terminal A1 domain. Therefore, it is remarkable that 16 of the 20 residues from A2 and A3 that interact with A1 in α_C are conserved in the ACS subunit of the ACDS complex from methanogens (Fig. 6). Doukov et al. noted structural similarities between a large fraction of domains C2 and C4 of CODH and the A1 domain of ACS in CODH/ACS_{Mot} (4). The recently reported structure of the archaeal *Meb* CODH was found to be very similar to its bacterial counterparts, except for its N- and C-terminal regions and the presence of an extra FeS-cluster binding domain (7). Intrigued by these similarities, we performed a detailed comparison of the available structures (Table S1). We found that A1_{Mot} resembles more the archaeal CODH than the bacterial one. This is indicated by (i) a larger number of superimposed residues (285 vs. 217), (ii) a corresponding lower root-mean-square deviation (rmsd) of C_α -positions (2.23 $\text{\AA}$ vs. 2.54 $\text{\AA}$) and (iii) a higher percentage of sequence identities (16% vs. 12%). When the superposition is carried out separately for two sub-domains of A1, the statistics are further improved (Table S1). Furthermore, a DALI search (42) with A1_{Mot} gives a Z-score of 34.1 for the archaeal CODH subunit (ACDS_{Meb- α}), compared to a value of 20.8 for the bacterial one (CODH_{Mot- β}).

The bacterial ACS/CODH_{Mot} A1 domain has a tunnel for CO diffusion between the C- and A-clusters and an equivalent tunnel is present in the superimposed archaeal CODH (Fig. 7). However, like the tunnel in α_O , it does not reach the surface, being blocked by the helix that in ACS_{Mot} acts as a tunnel gate, allowing a connection between the A- and C-clusters only in α_C (5,20). If we assume that the archaeal CODH domains that correspond to C2 and C4 of their

bacterial counterparts have the same tunnel function as ACS_{Mot} domain A1, the A-cluster in the superimposed ACDS-complex is positioned like in α_C . Accordingly, the distance between the catalytic Ni ions in CODH and ACS would change from 66 Å in the bacterial (α_C) enzyme complex to only about 34 Å in its archaeal counterpart.

We have taken advantage of the high structural (Table S1, Fig. 7) and amino acid sequence similarities (Fig. 6) among domains of the bacterial and archaeal enzymes to model the relative position of CODH and ACS components in the ACDS complex (Fig. 8). Our model predicts that the smaller archaeal ACS interacts with the CODH C2/C4 domains as the bacterial A2/A3 domains interact with ACS_{Mot}A1 (see also Fig. 4). The C α -atom superposition of α_C domain A1 to ACDS_{Meb}- α generates only one potential collision between the rest of the bacterial ACS and the archaeal CODH. It involves Gly327, which aligns with Ser11 of the ACS subunit of ACDS (Fig. 6). However, only a small readjustment of the N-terminal region of the archaeal ACS would be required to remove this too short contact. Domain 1 of the archaeal ACS, which corresponds to A2 in ACS_{Mot}, would also interact with the C-terminal region of one of the CODH subunits (Fig. 8). Seven of the 16 residues at the *Mot* A1/A3 interface are invariant in the superimposed ACDS_{Meb} CODH structure, where they are exposed to solvent (see also Fig. S1). This 44% sequence identity is much higher than the overall value of 16% found for the 276 aligned residues (Table S1). Even more strikingly, these conserved residues establish 31 of the 41 contacts ($d < 3.8$ Å) between A1 and A3 in α_C . We conclude that the CODH-ACS portion of the ACDS complex shown in Fig. 8 is a plausible model for the “closed” form in which there is a continuous tunnel between the A- and C-clusters. Many short (< 2 Å) contacts leading to van der Waals collisions are generated when similar superpositions are performed with either α_O or $\alpha_{49(T)}$. Thus, modeling of other putatively functional conformations of the CODH-ACS component of the ACDS complex is not possible with the currently available structures.

DISCUSSION

The structure of $\alpha_{49(T)}$ reported here (Fig. 1) shows major differences with previous ACS structures, both in the metal composition of the A-cluster and in the arrangement of domains A2 and A3 (Fig. 2). Indeed, this is the first structure where the distal site of the A-cluster (M_d) is not occupied by a Ni ion and is empty (Fig. 3). This does not prevent the tetrahedral binding of a metal ion heavier than Ni at the proximal labile Ni site (M_p). In this respect $\alpha_{49(T)}$ resembles the CODH/ACS_{Mot} “closed” structures, which show either tetrahedral Zn or Cu bound at the labile Ni M_p site. Since no metal ions were added during the crystallization experiments, the bound Zn or Cu ion must come from either the reagents or the cell extracts used for purification. In fact, the binding of Zn or Cu to the α_{49} subunit in cell extracts would explain why it cannot be activated by the addition of NiCl₂ (14). Metal heterogeneity greatly complicates the interpretation of spectroscopic studies. In addition to the observed presence of Ni, Cu and Zn at M_p (4,5), the results reported here show that A-cluster heterogeneity might also arise from fractions with an empty M_d site. Attempts to generate a Ni₂Fe₄S₄ A-cluster by soaking crystals in NiCl₂ solutions resulted in crystal cracking and/or loss of diffracting power. Nevertheless, manual model building suggests that only minor changes in the $\alpha_{49(T)}$ structure would be necessary to incorporate Ni at the M_p and M_d sites. For the latter, a small rearrangement of the Cys595-Gly596-Cys597 loop would be required (Fig. 5B).

The high percentage of conserved residues between bacterial and archaeal enzymes at the A2/A3 domain interface (Fig. 6) suggests that the novel $\alpha_{49(T)}$ conformation, or a closely related one, may be functionally relevant. In terms of A-cluster accessibility, the new structure is intermediate between α_C and α_O . In the former, the A-cluster is connected to the CO-producing C-cluster through a long hydrophobic tunnel. An accessibility analysis (not shown) indicates that in this conformation the active site cannot react with bulky substrates such as CoFeSP or

CoA. We have postulated that α_O , in which the connection between the A- and C-clusters is blocked, may be the conformation that accepts a methyl group from CoFeSP (5,20). Conversely, the novel $\alpha_{49(T)}$ could correspond to the conformation that binds CoA. Indeed, this idea is supported by i) the presence of tryptophan and arginine residues that could be protected from chemical modification by CoA in the cleft formed between domains A2 and A3 (Fig. 4); ii) the electrostatic complementarity between this region and CoA (Fig. 4C); and iii) the corresponding distribution of the adenine, pyrophosphate and cysteamine moieties of CoA relative to tryptophanes, arginines and the M_p site of the A-cluster, respectively (Fig. 5A). Furthermore, in $\alpha_{49(T)}$ His408 is ideally positioned to abstract a proton from CoA-SH before its S^- atom binds to Ni_p (Fig. 5B). This is consistent with the prediction (43) that a base with a $pK_a \sim 6$ deprotonates CoA.

The structures of α_C and α_O contain another extensive positive patch on the surface of domain 1 (not shown), not far from the A-cluster, involving several arginine residues located around Trp151. According to the results of Shanmugasundaram et al. (40,41), this region could also bind CoA. However, in α_C the A-cluster is buried in the protein and therefore not accessible for CoA, whereas in α_O access of the cysteamine moiety of the coenzyme to the active site would be blocked by domain A3, assuming binding of its adenine and pyrophosphate moieties to Trp151 and surrounding arginines. The binding mode of CoA is likely to be evolutionary conserved. Sequence comparisons (Fig. S1 and 10) show that this is not the case for the Trp151 surface region. On the other hand, several crucial residues of the A2/A3 cleft are invariant: His408, proposed above to be involved in the deprotonation of CoA-SH, His419, to which His408 is hydrogen-bound (Fig. 5B), Trp427 and the nearby Arg334 and Arg429 (Fig. 5A). These could be the two arginines that are protected from chemical modification by CoA (41). In conclusion, the combination of a large number of observations strongly suggests the involvement of the A2/A3 cleft in CoA binding.

Substantial ACS domain movements during catalysis would explain the reported relatively low rates of acetyl-CoA synthesis ($<10 \text{ s}^{-1}$). According to kinetic data, CoA binds to the A-cluster after CH_3^+ and CO (44,45). This makes sense in a scheme where these two substrates bind to the two available Ni_p coordination sites prior to the migration/insertion reaction that results in acetyl synthesis and that liberates one coordination site for subsequent CoA binding. CoA does not bind to CO-treated ACS (43), suggesting that these two substrates might compete for the same binding site and/or that the enzyme conformation is not suitable for coenzyme binding. Similar chemistry involving the sequential binding of three substrates (methyl, CO and thiol) to a single nickel ion to produce a thioester has been reported for synthetic Ni(II) complexes (46). If our interpretation is correct CH_3^+ , CO and CoA binding would require the enzyme to adopt conformations similar to α_O , α_C and $\alpha_{49(T)}$, respectively. In $\alpha_{49(T)}$, two A2 conserved residues, Arg405 and His408, could respond to changes at the active site and trigger domain movements towards other enzyme conformations (Fig. 3,5D and 5E). The order of CH_3^+ and CO binding during catalysis is still controversial (44,45). If CH_3^+ were to bind Ni_p before CO, a more open α_C conformation would be needed to prevent the collision of the bound methyl group with the enzyme (20). Structures of enzyme-substrate complexes and reaction intermediates will be required to determine whether additional conformational changes take place during catalysis. It appears that protein domain movements finely modulate substrate accessibility to the A-cluster by generating suitable interacting surfaces (Fig. 4).

Archaeal ACS shows extensive amino acid sequence similarities with A2 and A3 from the bacterial enzyme (Fig. 6). However, the absence of A1 in the former implies that the tunnel network between the C- and A-clusters of archaeal ACDS must be different from the one found in bacterial CODH/ACS. Suitable candidates for replacing the tunnel section of bacterial A1 are two tunnel-containing domains in the CODH portion of a recent ACDS_{Meb- $\alpha_2\epsilon_2$} crystal structure (7). These domains can be superimposed to more than 90% of sub-domains A1_a and

Al_b (Fig. 7, Table S1). Drennan *et al.* have reported a similar fold for the bacterial CODH_{Mot} domains *C2* and *C4* (4), but their resemblance to *Al* is less extensive (Table S1) and they do not contain a tunnel. Although a tunnel is present in the same domains in mono-functional CODHs of CO-oxidizing bacteria (47,48), their similarity to *Al*, reflected by DALI Z-scores (42) of about 20, are comparable to the value found for the *Mot* enzyme. This Z-score is much lower than the value of 34.1, obtained when *Al* and the archaeal *Meb* CODH are compared. Moreover, more than 75% of the residues involved in contacts between *Al* and *A3* in *Mot* α_C are conserved in the aligned sequences of the archaeal domains (Fig. 6 and S1). All these similarities suggest a plausible model for the CODH/ACS interaction in archaeal ACDS that includes a continuous tunnel for CO diffusion between the two active sites, obtained by superposition of *Al* from *Mot* α_C to *C2* and *C4* of CODH_{Meb} (Fig. 7).

In the obtained partial model of the ACDS complex (Fig. 8), all the [4Fe-4S] centers of the archaeal CODH are more than 45 Å away from the A-cluster, indicating that there is no direct electron transfer between the two enzymes. Consequently, the reductive activation of the A-cluster should require, like in the bacterial CODH-ACS complex, an external electron source (see also 49). Ferredoxin (Fd) may be the redox partner that activates both bacterial and archaeal enzyme complexes by two successive one-electron reductions (11,50). It is noteworthy that an *Al* region of CODH/ACS_{Mot} that lies close to the A-cluster and that can be cross-linked with Fd (51) is also accessible in the corresponding *C4* region in our partial model of ACDS.

Ni-containing CODH and ACS are considered to be ancient enzymes originally involved in primordial anaerobic carbon fixation pathways (52). Their occurrence in both bacteria and archaea may imply that they were already present in the Last Universal Common Ancestor (LUCA), but horizontal gene transfer is also a possibility. Domains *C2* and *C4* (bacterial *Mot* enzyme nomenclature) are found in bacterial and archaeal CODH's and hybrid cluster proteins (HCP's) (53,54) and in *Al* from bacterial ACS (Fig. 8). The *C2* domain is involved in extensive contacts with its counterpart in all CODH homo-dimers, and it has similar contacts with a homologous domain in monomeric HCP's (Fig. S2). In CODH's and HCP's there is an additional domain *C3* that is related to *C4* by pseudo two-fold symmetry (Fig. 8, Fig. S2). A metal-containing active site is bound at the interface of these two domains: the C-cluster in CODH and the hybrid cluster in HCP. Domains *C2* and *C4* may have originated from a common monomeric ancestral protein of unknown function, possibly binding a simpler metal site. The bacterial ACS domain *Al* lacks *C3* and, consequently, it is not suited to coordinate a metal cluster. However, it is remarkable that its structural similarity to the archaeal CODH *C2* and *C4* domains is comparable to that of the latter to the corresponding bacterial CODH domains (Table S1). Another common feature of CODH, HCP and bacterial ACS is the presence of a tunnel segment at the *C2/C4* interface (*Al_a/Al_b* in ACS, see Fig. 8 and S2). In all these enzymes, except CODH_{Mot}, the tunnel continues for an equivalent distance in *C4*. In CODH_{Mot} the tunnel extends inside *Al* instead.

Taken together, our results suggest that bacterial ACS originated from the duplication of the DNA region coding for archaeal CODH *C2* and *C4* domains (generating *Al*) and its fusion to the archaeal ACS gene, consisting of domains *A2* and *A3*. This modification would have allowed for the release of ACS activity from the multi-enzymatic ACDS methanogenic complex. In this scenario the archaeal enzymes existed before the bacterial ones (see also 55). The *Al*-containing ACS in the CO-tolerant anaerobic thermophilic bacterium *C. hydrogenoformans* is a monomer under high CO concentrations, but associates with CODH-III when CO levels are low. This ACS/CODH-III complex, very similar to its counterpart in the acetogenic *Mot*, is postulated to be involved in the Wood-Ljungdahl pathway of CO₂ fixation (56). Upon evolution of the acetogenic CODH/ACS complex, most of the tunnel function of the CODH *C4* domain disappeared, presumably to prevent escape of CO. This is

different from the situation in the archaeal ACDS multi-enzyme complex where some CO leakage has been observed (57).

CONCLUSIONS

The domain rearrangement found in truncated ACS adds important new information on how protein movements may control the complex catalytic mechanism of acetyl-CoA synthesis. Previously determined closed and open ACS structures appear well suited to bind CO and CH_3^+ , respectively. Several lines of evidence suggest that the truncated ACS structure reported here is closely related to the conformation that binds the third substrate, CoA. Further studies, including mutagenesis of selected residues, will be required to establish whether this is indeed the case. The truncated ACS structure has also been used to put forward a scenario for the evolutionary origin of the bacterial ACS from the archaeal ACDS complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ACS	acetyl-CoA synthase
ACDS	acetyl-CoA decarbonylase/synthase
<i>Ch</i>	<i>Carboxydotherrmus hydrogenofomans</i>
CoA	coenzyme A
CoFeSP	corrinoide iron-sulfur protein
CODH	carbon monoxide dehydrogenase
HCP	hybrid cluster protein
<i>Meb</i>	<i>Methanosarcina barkeri</i>
<i>Met</i>	<i>Methanosarcina thermophila</i>
<i>Mot</i>	<i>Moorella thermoacetica</i>

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Figure 1. Polypeptide fold of α_{49} in the new $\alpha_{49(T)}$ conformation. **(A)** Hexamer. **(B)** Monomer. Domain A2 is shown in red, domain A3 in lime green, the connecting α -helix in orange and the C-terminal His-tag in black. A-cluster atoms are depicted as brown (Fe), yellow (S) and black (Zn or Cu) spheres. The bottom trimer in **(A)** is shown as a grey C_α -trace.

Figure 2.

Stereo image of ACS C α -backbone superposition of *Mot* $\alpha_{49(T)}$ (blue), α_O (green) and α_C (red) and of *Ch* α_O (grey) to domain A2, excluding domain A1.

Figure 3. Stereo image of the active site in $\alpha_{49}(T)$. The blue grid depicts a six-fold averaged F_o-F_c electron density map with the metal site next to the [4Fe-4S] cluster and its protein ligands (Cys509C β and S γ , Cys595C α to Cys597S γ) omitted from the phase and structure factor calculations. The red grid corresponds to a six-fold averaged 3.0 Å resolution anomalous difference map.

Figure 4. Relative domain movements and stereo images of electrostatic potential surfaces of three ACS_{Mot} structures after superposition of their respective A2 domains. (A) α_0 ; (B) α_c ; (C) $\alpha_{49(T)}$. Domains are shown as thin ribbons and they are labeled A1, A2, A3a and A3b, with dashed ellipsoids showing their positions in α_0 . In (C) the hypothetical position of A1 is depicted in light blue, with the A-cluster position in α_0 shown in semi-transparent mode. Color code of the electrostatic potential surfaces: red negative, blue positive.

Figure 5.

Possible interactions of CoA with the narrow cleft formed in $\alpha_{49(T)}$. **(A)** Arrows indicate the overall complementarity between moieties of CoA (structure taken from pdb deposition 1II2) and putatively protected amino acid residues and reacting metals in the cleft: they connect the 3'-phosphate with K335, adenine with W418/W427, pyrophosphate with R334/R346/R429 and the thiol with Ni_p of the A-cluster. **(B)** Tentative docking of the cysteamine tail of CoA close to the A-cluster in $\alpha_{49(T)}$ inside a cavity map calculated for an accessible probe radius of 1.4 Å. Apical and equatorial substrate binding sites of the A-cluster Ni_p ion (taken from the superimposed A3 structure of α_o) are labeled with * and \$, respectively.

Figure 6.

Sequence alignment of bacterial and archaeal ACS's. The consensus_23 line highlights residues that are involved in close interactions ($< 3.8 \text{ \AA}$) between *Mot* domains A2 and A3; colors: grey in α_C and/or α_O , blue in $\alpha_{49(T)}$, red in all three structures. The consensus_1 line does the same for interactions with *Mot* domain A1: grey in α_C , blue in α_O , red in both. Color codes in sequences: β -strands green, α -helices pink, 3_{10} -helices violet.

Figure 7.

Superposition of the C_{α} -backbone of *Al* of ACS_{Mot} α_C (red) and α_O (green) with domains *C2* and *C4* (using the bacterial CODH domain numbering) of ACDS_{Meb} (blue). Corresponding tunnels are shown in yellow, green and light-blue, respectively. Ni_p indicates the location of the catalytic Ni ion of the A-cluster in α_C , the * highlights the nearby flexible helix. Ni-C shows the Ni position in the CODH C-cluster.

Bacteria

Archaea

Figure 8.

Proposed evolutionary relationship of ACS in bacteria and archaea, based on structural comparisons. A tentative model of the complex of the CODH and ACS in the archaeal ACDS_{Meb} is shown with domains numbered as in the bacterial complex. Two of the subunits are shown as C_α-traces, using different colors for the various domains. The latter are depicted as ellipses in the other, two-fold-symmetry-related subunits. Cluster locations in the top subunits are shown, excluding the D-cluster, which is located on the two-fold axis (indicated by a closed diamond) that relates subunits; hydrophobic tunnels are depicted in green; asterisks indicate potential ferredoxin binding sites; the C-terminus of one CODH subunit of ACDS is labeled with “C”; the open diamond indicates a pseudo two-fold symmetry between C3 and C4, perpendicular to the two-fold axis relating subunits.

Table 1

Crystallographic statistics for the truncated ACS crystal structure.

Data		Refinement	
Space group	P3 ₁ 21	Resolution (Å)	20.0–3.0
Cell dimensions:		Reflections in work set	74440
a, b (Å)	166.4	R _{work} (%)	17.1
c (Å)	245.2	Reflections in test set	3902
Molecules/asymmetric unit	6	R _{free} (%)	20.8
Resolution (Å) *	20–3 (3.1–3.0)	Total number of atoms	20121
R _{sym} (%) *	5.3 (24.3)	Water molecules	161
<I>/<σ(I)> *	19.6 (6.3)	Sulfate molecules	24
Number of observations *	588420 (55207)	Glycerol molecules	6
Unique reflections *	78314 (7323)	σ _{bond} (Å)	0.015
Completeness (%) *	99.5 (99.7)	σ _{angle} (°)	1.59
		average B factor (Å ²)	57.3

* numbers in parentheses refer to the highest resolution shell