The Archetype γ-Class Carbonic Anhydrase (Cam) Contains Iron When Synthesized in Vivo†

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Received December 10, 2008; Revised Manuscript Received January 7, 2009

ABSTRACT: A recombinant protein overproduction system was developed in *Methanosarcina acetivorans* to facilitate biochemical characterization of oxygen-sensitive metalloenzymes from strictly anaerobic species in the *Archaea* domain. The system was used to overproduce the archetype of the independently evolved γ-class carbonic anhydrase. The overproduced enzyme was oxygen sensitive and had full incorporation of iron instead of zinc observed when overproduced in *Escherichia coli*. This, the first report of in vivo iron incorporation for any carbonic anhydrase, supports the need to reevaluate the role of iron in all classes of carbonic anhydrases derived from anaerobic environments.

Methane-producing species from the *Archaea* domain of life (methanoarchaea) are terminal organisms of anaerobic microbial food chains that convert complex organic matter to methane, a component of the global carbon cycle. The pathways producing methane utilize several specialized metalloenzymes unique to the methanoarchaea that contain a diversity of metals in the active site such as cobalt, iron, nickel, molybdenum, and zinc (*1*). There are no published examples of metalloenzymes overproduced in the methanoarchaea, and only a few have been heterologously produced in eukaryotic or bacterial systems, impeding a biochemical understanding. There are also problems associated with recombinant production of archaeal proteins in eukaryotic or bacterial systems, including poor secretion of product, protein misfolding, differences in codon bias, requirement for the specialized amino acid pyrrolysine (*2*), and insertion of active metal centers (*3, 4*). Of particular importance is the need for overproduction of metalloenzymes that play key roles in diverse metabolisms of the *Archaea* domain, including methane-producing pathways. A prime example is Cam, the archetype of the independently evolved γ-class CA[1] first isolated from the methanoarchaeon *Methanosarcina thermophila* (*5*). Cam's from *Methanosarcina* species function in the pathway for conversion of acetate to methane (*1*) which accounts for two-thirds of the nearly 1 billion metric tons of methane produced each year in the Earth's biosphere (*6*). CA's from the γ-class have been identified in a diversity of species from all three domains of life, although the archetype (Cam) from *M. thermophila* is the only one biochemically characterized.

The active-site metal in Cam is in question. Although no other class of CA is reported to contain iron, in vitro evidence suggests the possibility that Cam from *M. thermophila* contains Fe^{2+}. Cam overproduced in *Escherichia coli* and purified in the presence of air (*7*) contains zinc, the physiologically relevant active-site metal reported for all CA's except the Cd-containing ζ-class CA from the marine diatom *Thalassiosira weissflogii* (*8*). When zinc is exchanged in vitro for either Co^{2+} or Fe^{2+}, Cam activity increases relative to that of the zinc enzyme, consistent with a physiological role in vivo for any of these three metals (*9*). Thus, the physiologically relevant active-site metal in Cam produced in vivo is unknown. The exceptionally small amounts of Cam produced in vivo in *M. thermophila* (*5*), and the difficulties in obtaining large quantities of cell material, have deterred isolation aimed at addressing the in vivo metal content and biochemically characterizing the enzyme.

A system was developed for overproduction of recombinant metalloproteins in the methanoarchaeon *Methanosarcina acetivorans* to circumvent problems associated with overproduction of archaeal proteins in eukaryotic or bacterial systems. Cam was chosen as a model metalloprotein since it is synthesized at very low levels in vivo (*5*) and it is difficult to obtain enough material to determine the metal composition of the enzyme synthesized in vivo in the native host *M. thermophila*. The system is based on a plasmid shuttle vector with selectable markers for both *M. acetivorans* and *E. coli* (Figure 1). The promoter for the *cdh* operon encoding CODH/ACS was ligated upstream of a multiple cloning site. In vivo studies using translational fusions with LacZ showed this promoter to have 40−60-fold greater expression than basal levels when *M. acetivorans* is grown on acetate. The expression vector has the same plasmid

† This work was supported in part by grants from the Department of Energy, Energy Biosciences Program (DE-FG02-95ER20198 to J.G.F. and DE-FG02-93ER20106 to K.R.S.), the National Science Foundation, Division of Cellular and Bioscience (MCB0110762 to J.G.F. and K.R.S.), and the Thomas H. Maren Foundation to (J.G.F.).

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[1] Abbreviations: CA, carbonic anhydrase; CODH/ACS, CO dehydrogenase/acetyl-CoA synthase.

10.1021/bi802246s CCC: $40.75
Published on Web 01/20/2009

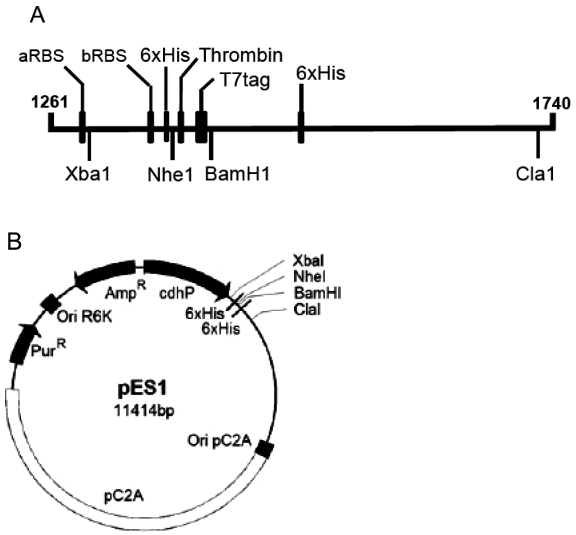

FIGURE 1: Expression vector pES1. Panel A shows the details of the cloning sites, and panel B shows the entire plasmid. The vector contains genes for selection by ampicillin resistance in *E. coli* and puromycin resistance in *M. acetivorans*. The origins of replication are OriR6K and Ori pC2A for *E. coli* and *M. acetivorans*, respectively. Downstream of the *cdh* promoter is a multiple cloning site flanked by sequence for six-His tags and the T7 tag for optional protein fusions to aid in protein purification. An N-terminal six-His fusion may be cleaved by digestion with thrombin using the thrombin digestion site. After the *cdh* promoter are shown an archaeal ribosomal binding site (aRBS) and a bacterial ribosomal binding site (bRBS).

backbone as pEA103, which is present at low copy numbers of approximately 15 copies per cell during growth of *M. acetivorans* on acetate and is stable for at least 22 generations without selection, negating the requirement for puromycin during high-volume scale-up (*10*). This construct includes a multiple cloning site flanked by sequence for six-His translational fusions to allow for optional C- and N-terminal fusion proteins. The effectiveness of the six flanking His residues was tested by cloning *Methanocaldococcus jannaschii* prolyl tRNA synthetase (MJ1238, MjProRS) into the *Nhe*I and *Bam*HI restriction sites and purifying the gene product from cells grown in acetate with Ni^{2+} affinity resin (His-Link resin, Promega, Inc.). The activity observed by active-site titration (*11*) followed by aminoacylation of the Mj $tRNA^{Pro}$ (UGG) transcript with proline (*12*) confirmed that the recombinant enzyme had K_m (0.52 ± 0.15) and k_{cat} (0.32 ± 0.12) values similar to those for recombinant tRNAPro expressed in *E. coli* (0.30 ± 0.09 and 0.27 ± 0.00, respectively).

Recombinant *M. thermophila* Cam, without the putative 34-amino acid leader signal peptide, was overproduced using the *M. acetivorans* system to determine the physiological active-site metal and to provide enough cell material for future biochemical and biophysical investigations. Cam was produced without the six-His tag to prevent contamination with Ni^{2+}. Scale-up of the growth of *M. acetivorans* with acetate in a 14 L volume using the pH auxostat method (*13*) yielded 2.2 g wet weight per liter which provided enough material to produce up to 10 g of purified recombinant Cam. The soluble enzyme was purified anaerobically to homogeneity as judged by SDS−PAGE with a final yield of 0.25 g per g wet weight of cell material. Metal analysis showed iron was present with approximately one atom per monomer (Table 1). Kinetic analyses of the purified enzyme revealed

Table 1: Michaelis−Menten Steady-State Kinetic Parameters for Cam

overproduction system	K_m (mM)	k_{cat} ($\times 10^{-4}$ s^{-1})	k_{cat}/K_m ($\times 10^{-6}$ M^{-1} s^{-1})	metal/ monomer
E. coli[a]	44.8 ± 7.4	23.5 ± 2.3	5.4 ± 1.6	0.97 Fe
M. acetivorans[b]	58.9 ± 8.4	23.1 ± 2.2	3.9 ± 0.9	1.17 Fe
E. coli[c]	21.8 ± 2.2	6.1 ± 4.0	2.8 ± 0.5	0.92 Zn

[a] Aerobically purified and containing zinc that was reconstituted with Fe^{2+} in vitro. [b] Anaerobically purified and no metal replacement. [c] Aerobically purified and containing zinc that was reconstituted with zinc in vitro (*9*).

similar Michaelis−Menten steady-state parameters when compared to those of Cam produced in *E. coli* and reconstituted with Fe^{2+} in vitro. Cam produced in vivo in *M. acetivorans* and purified anaerobically (Table 1) had k_{cat} values nearly 4-fold greater than those previously reported for the zinc-containing enzyme produced in *E. coli* and purified aerobically (*9*). The enzyme produced in vivo was sensitive to oxygen, losing 90% of activity in 5 min after exposure to air, a result identical to that reported for Cam produced in *E. coli* and reconstituted with Fe^{2+} in vitro (*9*). The results show that *M. acetivorans* preferentially incorporates iron into Cam and demonstrates that the enzyme is synthesized in vivo with iron in the active site.

The initial biochemical, kinetic, and structural characterizations of Cam concluded that the active-site metal was zinc (*7, 14*−*16*), consistent with zinc reported for all CA's characterized from the extensive α- and β-classes. The conclusion that Cam contains zinc was based on the enzyme overproduced in *E. coli* cultured under aerobic conditions and purified aerobically. However, the methanoarchaea such as *M. thermophila* are strict anaerobes that live in ferrous-rich oxygen-free environments and synthesize an abundance of oxygen-sensitive enzymes. Indeed, it was shown previously that Cam reconstituted with Fe^{2+} in vitro is exquisitely sensitive to oxygen with rapid loss of activity on exposure to air (*9*). The loss of activity results from oxidation of Fe^{2+} to Fe^{3+} and loss of iron from the active site that is subject to replacement with zinc during aerobic purification. As discussed previously (*9*), the Irving−Williams series predicts that the stability of complexed Zn^{2+} is at least 1 order of magnitude greater than that for Fe^{2+} ligated with the nitrogen atoms of the His residues coordinating the active-site metal in Cam. Thus, aerobic purification leads to loss of Fe^{3+} and incorporation of Zn^{2+} that is a common contaminant of untreated buffers.

The results reported here establish that Cam, the archetype of the independently evolved γ-class CA, synthesized in vivo contains iron in the active site. This result, and the observation that iron-containing Cam has several-fold greater activity than the zinc-containing enzyme, indicates that iron is the physiologically relevant metal for Cam from *M. thermophila* in contrast to that previously reported for Cam aerobically purified from *E. coli* (*7, 14*−*16*). This conclusion is consistent with the Fe^{2+}-rich anaerobic environments of methane-producing species that synthesize an abundance of diverse iron-containing enzymes (*1*). Although this is the first report of any CA containing iron, it is interesting to note that a role for iron has been proposed in duck erythrocyte CA which was shown to have higher activity in the presence of iron when compared to that induced by similar concentrations of zinc (*17*).

Orthologs of *M. thermophila* Cam are predicted for the annotated genomes of the acetate-utilizing methane-producing species *M. acetivorans* (MA2536), *Methanosarcina barkeri* (MbarA 3020), and *Methanosarcina mazei* (MM3080) with deduced amino acid sequence identities of 86, 77, and 76%, respectively (*18*). This high level of sequence identity extends to the active-site residues of Cam, consistent with Cam orthologs from *Methanosarcina* species containing iron in the active site. Genomic sequencing (*18*) has identified putative Cam orthologs in anaerobes from the *Bacteria* domain such as *Clostridium* species that are suspected of containing iron in the active site. Clearly, the overproduction system described here is paramount to understanding the active-site metal in CA's and other metalloenzymes isolated from the methanoarchaea and other species in the *Archaea* and *Bacteria* domains of life. In particular, the results raise a caution for enzymes from anaerobic species overproduced in heterologous systems that are reported to contain zinc.

In summary, a system was developed for overproduction of oxygen-sensitive metalloproteins in *M. acetivorans*. Overproduction of the archetype γ-class CA in preparative yields provided sufficient material to establish iron as the physiological metal in the active site of this metalloenzyme, the first for any CA establishing a novel role for iron. The results support the need to reevaluate the role of iron in CA's and other enzymes originating from anaerobic environments that are overproduced and purified under aerobic conditions in bacterial or eukaryotic systems.

SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI802246S