The Smallest Active Carbamoyl Phosphate Synthetase Was Identified in the Human Gut Archaeon *Methanobrevibacter smithii*

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Key Words

Carbamoyl phosphate synthetase • Ancestral form • *Methanobrevibacter smithii* • Intestinal archaea • Evolution • Obesity

Abstract

The genome of the major intestinal archaeon *Methanobrevibacter smithii* contains a complex gene system coding for carbamoyl phosphate synthetase (CPSase) composed of both full-length and reduced-size synthetase subunits. These ammonia-metabolizing enzymes could play a key role in controlling ammonia assimilation in *M. smithii*, affecting the metabolism of gut bacterial microbiota, with an impact on host obesity. In this study, we isolated and characterized the small (41 kDa) CPSase homolog from *M. smithii*. The gene was cloned and overexpressed in *Escherichia coli*, and the recombinant enzyme was purified in one step. Chemical cross-linking and size exclusion chromatography indicated a homodimeric/tetrameric structure, in accordance with a dimer-based CPSase activity and reaction mechanism. This small enzyme, MS-s, synthesized carbamoyl phosphate from ATP, bicarbonate, and ammonia and catalyzed the same ATP-dependent partial reactions observed for full-length CPSases. Steady-state kinetics revealed a high apparent affinity for ATP and ammonia. Sequence comparisons, molecular modeling, and kinetic studies suggest that this enzyme corre-

sponds to one of the two synthetase domains of the full-length CPSase that catalyze the ATP-dependent phosphorylations involved in the three-step synthesis of carbamoyl phosphate. This protein represents the smallest naturally occurring active CPSase characterized thus far. The small *M. smithii* CPSase appears to be specialized for carbamoyl phosphate metabolism in methanogens.

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Introduction

In recent years, many studies [Dridi et al., 2011; Horz and Conrads, 2010; Nava et al., 2011; Plottel et al., 2011] have focused on the involvement of the human microbiome in human health and disease. While archaea colonize several different anaerobic niches within the human body [Belay et al., 1988, 1990], there is a single predominant species, *Methanobrevibacter smithii*, present in the human gut [Miller et al., 1982]. First isolated from feces, this methanogenic organism is a strict anaerobe that grows on a mixture of hydrogen and carbon dioxide [Balch et al., 1979].

Samuel and Gordon [2006] made the remarkable discovery that a synergic interaction in the colon between *M. smithii* and *Bacteroides thetaiotaomicron*, the major gut prokaryote, appears to have an important role in controlling host obesity [Samuel and Gordon, 2006]. Complex di-

etary polysaccharides that cannot be metabolized by the human host are digested by enzymes in the gut microbiome [Stams, 1994]. Bacterial fermentation of polysaccharides yields short-chain fatty acids that provide as much as 10% of the daily caloric intake of the host. Archaeal methanogenesis facilitates bacterial fermentation by preventing the accumulation of H₂ gas and other end products that would otherwise inhibit the process. Analysis of the cecal content of gnotobiotic mice colonized with *M. smithii* alone or together with *B. thetaiotaomicron* revealed that many genes that participate in the metabolism of these end products are upregulated when both organisms are present in the gut [Samuel et al., 2007]. Several lines of evidence suggest that obesity is associated with elevated levels of intestinal methanogenic archaea [Million et al., 2011]. Consequently, *M. smithii* is considered to be a potential therapeutic target in the treatment of obesity [Buck and Hansen, 2007]. The principle source of nitrogen in *M. smithii* is ammonia, resulting from the degradation of amino acids by the host or intestinal bacteria. *M. smithii* competes with *B. thetaiotaomicron* for the available ammonia, and when both organisms populate the gut, the transcription of several enzymes involved in ammonia transport and assimilation is upregulated in *M. smithii*. Although there have been numerous microbiological and physiological studies of human gut microbiota, little is known about the metabolism and key enzymes of the major archaeon in the gastrointestinal tract, *M. smithii*.

Carbamoyl phosphate synthetase (CPSase; EC 6.3.5.5) plays a major role in ammonia assimilation in all organisms. The enzyme catalyzes the synthesis of carbamoyl phosphate (CP), a common precursor of pyrimidine nucleotides, arginine, and, in terrestrial vertebrates, urea, where it represents the major means of eliminating ammonia. CPSases are multisubunit or multidomain proteins. *Escherichia coli* CPSase is composed of a 40-kDa glutaminase (CarA, GLN) subunit which hydrolyzes glutamine and transfers ammonia to a 120-kDa synthetase (CarB, SYN) subunit [Trotta et al., 1971]. The SYN subunit consists of two homologous domains having a nearly identical tertiary fold and active site residues that were thought to have evolved by ancestral duplication and fusion [Nyunoya and Lusty, 1983]. The amino half of the subunit, designated CPS.A, catalyzes the synthesis of carbamate from ATP, bicarbonate, and ammonia, while the carboxyl half, CPS.B, catalyzes CP synthesis from carbamate and a second ATP [Alonso and Rubio, 1995; Guy and Evans, 1996; Post et al., 1990]. *E. coli* CPSase GLN and SYN subunits associate to form heterodimers and tetramers [Anderson, 1986]. The partial reactions cata-

lyzed by CPSase, first demonstrated for the *E. coli* enzyme [Anderson and Meister, 1965; Meister, 1989], are thought to occur universally in nearly all organisms:

Each of the major CPSase domains is comprised of three subdomains designated A1, A2, A3 and B1, B2, B3 [Guillou et al., 1989; Guy and Evans, 1996; Post et al., 1990; Rubio et al., 1991]. The X-ray structure of *E. coli* CPSase [Thoden et al., 1997] showed that the A1-A2 and B1-B2 subdomains have nearly identical tertiary folds. A2 and B2 are catalytic subdomains, catalyzing reactions 1–2 and 3, respectively [Guy et al., 1997; Post et al., 1990; Thoden et al., 1997]. The A3 and B3 subdomains have distinctly different tertiary structures and functions. B3 is a regulatory subdomain that has been adapted to bind allosteric ligands [Rubio et al., 1991]. Remarkably, the active sites on the GLN, CPS.A and CPS.B domain are connected by a 96-Å-long intramolecular tunnel that sequesters the labile intermediates within the *E. coli* enzyme complex [Thoden et al., 1997].

Although the structural organization of CPSases is very diverse, all are composed of homologous domains and subdomains and catalyze the same series of reactions. Generally, bacteria possess a single CPSase that catalyzes the formation of CP used for both arginine and pyrimidine nucleotide biosyntheses, while enteric and gram-positive bacteria contain two CPSases, each specific for one of these two metabolic pathways [Paulus and Switzer, 1979]. These enzymes are comprised of homologous SYN subunits of comparable size [Yang et al., 1997]. Most CPSases have a full-length SYN subunit consisting of fused CPS.A-CPS.B domains, but there are several organisms, including the thermophilic archaeon *Methanocaldococcus jannaschii* [Bult et al., 1996] and the hyperthermophilic eubacterium *Aquifex aeolicus* [Ahuja et al., 2001], that have a split *carB* gene encoding separate polypeptides corresponding to CPS.A and CPS.B domains. The only known exception is a 34-kDa carbamate kinase-like CPSase of an entirely different sequence and tertiary structure found in the hyperthermophilic archaeon *Pyrococcus furiosus* [Durbecq et al., 1997; Legrain et al., 1995; Marina et al., 1998; Uriarte et al., 2001], and in *Pyrococcus abyssi* [Purcarea et al., 1996, 2001].

The genome of *M. smithii* contains *carA* and *carB* genes coding for a 40-kDa GLN subunit and a 118-kDa SYN subunit (MS-I, SYN1), respectively, that are similar to the CPSase subunits found in most bacteria [Popa et

Fig. 1. MS-s sequence identity and domain structure. **a** Domain and subdomain organization of MS-s and the full-length SYN subunits. The full-length SYN domain consists of homologous CPS.A and CPS.B domains, each of which are comprised of subdomains designated A1, A2, A3 and B1, B2, B3, respectively, of the *E. coli* SYN subunit. **b** Protein sequence identity (%) was calculated by pair-wise alignment of CPSase synthetase subunits, as indicated in Experimental Procedures. The proteins analyzed in both panels are: *M. smithii* small (MS-s) and large (MS-l) SYN; *M. thermoautotrophicus* small (MT-s) and large (MT-l) SYN, *M. stadtmanae* small (MST-s) and large (MST-l) SYN; *E. coli* (EC) SYN, and *P. abyssi* (PA) CK-like CPSase.

al., 2009]. Surprisingly, the genome also contains two genes coding for much smaller proteins that are homologous to the CPSase synthetase domain. One of these proteins (SYN2) consists of 391 residues (45 kDa) with a sequence that resembles a truncated CPS.B domain, but it lacks active site residues that are known to participate in substrate binding and catalysis. Therefore, it is likely to be encoded by an inactive pseudogene. The second small CPSase homolog (MS-s, SYN3) is also homologous to a part of CPS.B and consists of 367 residues (41 kDa) including the residues implicated in catalysis [Popa et al., 2009]. Prior to this report it was not known whether this protein has catalytic activity or is an inactive remnant of an unproductive gene duplication event.

We have isolated and characterized the small *M. smithii* CPSase MS-s, showing that it is catalytically active, synthesizing CP from NH_3 , 2 ATP, and bicarbonate by the same mechanism utilized by the large CPSases. This is the smallest functional CPSase discovered thus far and may more closely resemble the putative ancestral kinase that gave rise to this diverse family of enzymes.

Results and Discussion

Sequence Analysis

The large SYN subunit of the CPSase from the methanogens *M. smithii* (MS-l), *Methanothermobacter thermoautotrophicus* (MT-l), and *Methanosphaera stadtmanae* (MST-l) are 70–73% identical to one another (fig. 1b). The alignment of the *E. coli* CPSase SYN subunit with the

large CPSases MS-l, MT-l, and MST-l gave sequence identities of 50–53%, indicating that the CPSases from *E. coli* and methanogens are closely related to each other and to the SYN subunit of most prokaryotic and eukaryotic organisms.

The small CPSase from *M. smithii*, MS-s, is homologous to the CPS.B subdomain of *E. coli* but is significantly smaller, i.e. 367 versus 513 residues, respectively (fig. 1a), representing about a third of the full-length subunit (1,073 residues). Based on the deduced amino acid sequence, the protein has a calculated molecular mass of 41,403 Da and an isoelectric point of 4.62. MS-s aligns with residues 560–947 of the *E. coli* SYN subunit corresponding to the B1 and B2 subdomains of CPS.B [Goillou et al., 1989] (fig. 1a) with a score of 20.1% sequence identity (fig. 1b) and therefore lacks the B3 subdomain at the carboxyl end of the polypeptide.

MS-s is also homologous to the *E. coli* CPS.A domain, as expected, since the CPS.A and CPS.B domains of the *E. coli* enzyme are quite similar to one another (19.3% identity, 34.5% similarity). However, the alignment score of MS-s is somewhat higher for CPS.B (20.1% identity, 40.1% similarity) than for CPS.A, and the modeling program selected CPS.B as the best structural template. By analogy to the function of B3 in other CPSases, which have binding sites for both allosteric activators and inhibitors [Fresquet et al., 2000; Goillou et al., 1989; Guy and Evans, 1996; Rubio et al., 1991], the lack of this domain in MS-s suggests that this enzyme is unregulated.

Fig. 2. Phylogenetic tree of CPS SYN subunits. The phylogenetic tree was constructed as indicated in Experimental Procedures, using the amino acid sequences of CPS SYN subunits or domains from the methanogens *M. smithii* small (MS-s) and large (MS-l), *M. thermoautotrophicus* small (MT-s) and large (MT-l), *M. stadtmanae* small (MST-s) and large (MST-l), *M. jannaschii* (MJ), *A. aeolicus* (AA), *E. coli* (EC), *Helicobacter pylori* (HP), the archaea *Picrophilus torridus* (PT), *Sulfolobus acidocaldarius* (SA), *P. furiosus* (PF), *P. abyssi* (PA) CK-like CPSase, *Saccharomyces cerevisiae* (SC) pyrimidine-specific enzyme, and the hamster pyrimidine biosynthetic multifunctional protein (CAD).

Open reading frames encoding large and small putative CPSases found in *M. smithii* are also present in the genomes of the thermophilic archaeon *M. thermoautotrophicus* and in the human intestinal archaeon *M. stadtmanae*; therefore, this unusual complement of CPSase genes may represent a common feature of methanogens. The overall sequences of the small CPSases from these three methanogens are well conserved (46.9–48.1% identity) and are much less similar to the synthetase domain of *E. coli* CPSase (20.1–25.3% identity).

Moreover, the deduced amino acid sequence of the small *M. smithii* CPSase has a limited overall identity score (19.1–24.2%) with the corresponding SYN subunits of full-length CPSases from *E. coli* and methanogens, but an appreciably higher identity score (47.6–48.1%) with the small CPSases from methanogens (fig. 1b). The large SYN subunit of the CPSase from the methanogens *M. smithii* (MS-l), *M. thermoautotrophicus* (MT-l), and *M. stadtmanae* (MST-l) are 70–73% identical to one another (fig. 1b). The alignment of the *E. coli* CPSase SYN subunit with the large CPSases MS-l, MT-l, and MST-l gave sequence identities of 50–53%, indicating that the full-length CPSases from *E. coli* and methanogens are closely related to each other and to the SYN subunit of most prokaryotic and eukaryotic organisms.

Since both MS-s and the CK-like CPSases are small CP-synthesizing enzymes having a molecular mass of 41 kDa and 33 kDa, respectively, we investigated the possibility that MS-s may be more closely related to the CK-like CPSases than to the larger forms of the SYN CPSase subunits found in most bacteria. However, while the percent identity between MS-s and *E. coli* CPSase is relative-

ly low (20.1%), the percent identity between the MS-s and *P. abyssi* CK-CPSase is only 11.5%. This difference is highly significant at these low levels of sequence similarity. Four randomized versions of the *P. abyssi* CK-CPSase sequence were generated of identical in size and amino acid composition but completely unrelated amino acid sequences. Sequence alignments yielded a percent identity between MS-s and the four randomized *P. abyssi* proteins of 7.4, 13.0, 14.1, and 15.1%. This analysis suggests that MS-s and *P. abyssi* CPSase matches are coincidental and that the proteins are not homologs. This interpretation was reinforced by the conservation of *E. coli* CPSase active site residues and the kinetic studies described below.

The calculated phylogenetic tree (fig. 2) illustrates the relationship between the CPSase SYN subunit from methanogens, *E. coli* and other organisms belonging to all three domains of organisms, Bacteria, Archaea, and Eukarya. The small CPSases from the methanogens were the first to diverge, forming a separate branch. These proteins are more distantly related to the other bacterial, archaeal, and eukaryotic CPSases. Distinct branches cluster the eukaryotic CPSases from yeast (SC) and mammals (CAD), the archaeal large CPSases, and the bacterial full-length CPSases. One unanticipated result is that CPSase from *A. aeolicus* (AA), an organism of ancient lineage which has a split gene encoding separate CPS-A and CPS-B polypeptides [Ahuja et al., 2001], is a close relative of *E. coli* CPSase and other bacterial CPSases. Interestingly, the large methanogenic CPSases cluster together forming a separate lineage distinct from other bacterial and archaeal enzymes. The carbamate kinase-like CPSase from

Fig. 3. Active site conservation of MS-s. The alignment of MS-s with *E. coli* CPS.A and CPS.B domains was carried out as indicated in Experimental Procedures. The residues that interact with the bound substrates AMPPNP and Mg²⁺ and those that are part of the loop closing the active site identified in the X-ray structure of *E. coli* CPSase [Thoden et al., 1999] are indicated in both the

alignment and the table. The interacting residues in each *E. coli* subdomain are shaded grey and their position (residue number) is indicated. Residues in MS-s that are not conserved are underlined in the alignment and indicated by an asterisk in the table. The corresponding active site residues that are not interacting in CPS.A or CPS.B are enclosed in parentheses.

P. abyssi (PA) [Purcarea et al., 2001] is located on a distant branch of the phylogenetic tree, consistent with its distinctive sequence and structure.

The residues involved in substrate binding and catalysis in MS-s were identified based on X-ray structural analysis of *E. coli* CPSase with a nonhydrolyzable ATP analog [Thoden et al., 1999]. The alignment of *M. smithii* MS-s with the *E. coli* CPS.B and CPS.A domains (fig. 3) showed that all of the putative active site residues from one or the other domain have been conserved. The only exception is Arg848 in *E. coli* CPS.B that has been replaced in the archaeal enzyme by Gly274.

All of the active site residues identified in the X-ray structure of *E. coli* CPSase with a nonhydrolyzable ATP analog bond [Thoden et al., 1999] are located between residues 129–306 of the A2 subdomain and 677–848 of the B2 subdomain (fig. 3). Sequence alignment showed that of the 12 active site residues in CPS.A, which catalyze carbamate formation (reactions 1 and 2), all are conserved in the corresponding positions in CPS.B, which catalyzes CP synthesis (reaction 3). This high degree of conservation is consistent with the similarity of the reactions catalyzed by the two domains: ATP-dependent phosphorylations of bicarbonate and carbamate. The

Fig. 4. Molecular modeling of MS-s. Superposition of the modeled three-dimensional structure of MS-s (red) with the *E. coli* CPSase 1BXR [Thoden et al., 1999] X-ray structure (grey). The position of the ATP analog AMPPNP [Thoden et al., 1999] is shown (blue) bound to both CPS.A and CPS.B domains of the *E. coli* enzyme; the box indicates the region of the carbamate phosphorylation domain in *E. coli* CPSase, corresponding to B1-B2 subdomains.

substrates are isosteric; the only difference is that one of the oxygens in bicarbonate is replaced by an N-H in carbamate. When MS-s was included in the sequence alignment, 10 of these same 12 residues were found to be identical or highly conservative substitutions (K/R or L/I) with residues in CPS.A and/or CPS.B. Two MS-s residues, Asp253 and Gly274, are not conserved (fig. 3), but these are interactions with the backbone amide, not the side chain. As will be subsequently discussed, the alignments are consistent with the observation that MS-s can catalyze both carbamate and CP formation (reactions 1–3). It is significant that the putative active site residues are nearly perfectly conserved while the overall sequence identity to the *E. coli* subdomains is only 20%.

Molecular Modeling

Modeling of the three-dimensional structure of MS-s was attempted using Modeller 9v9. The three-dimensional model was computed using the X-ray structure of the *E. coli* CPSase SYN subunit (pdb: 1BXR) as a template. The program fit (fig. 4) MS-s to the B1-B2 subdomains of the carbamate phosphorylation domain (CPS.B). The Ra-

machandran plot of the model structure indicated that 95.6% of the residues are in most favored or allowed regions, 2.2% in generously allowed regions, and 2.2% in disallowed regions (data not shown). Due to the low sequence identity between MS-s and *E. coli* B1-B2 (20%), the model statistics were poor [ERRAT2 score of 74 and Z score (ProSa) of -5.78], precluding an accurate positioning of MS-s active site residues. However, the superposition of the model with *E. coli* CPSase gives a root mean square deviation of $1.66 \text{ \AA}$ for 144 α -carbons and $1.66 \text{ \AA}$ for backbone atoms, suggesting that the overall tertiary fold is likely to be approximately correct.

Cloning, Expression, and Purification of Recombinant MS-s

M. smithii ATCC 35061 (DSM 861) cells were grown anaerobically at 37°C using an $\text{H}_2\text{:CO}_2$ gas mixture, as described in Experimental Procedures. Under these conditions, the culture reached the stationary phase after 200 h (data not shown). Chromosomal DNA was isolated in the presence of mutanolysin, as described in Experimental Procedures, yielding 86.5 mg/ml archaeal DNA. The MS-s

carB gene (YP_001273061) coding for the *M. smithii* 367-residue CPSase was amplified by PCR, and the resulting DNA fragment was inserted into the pRSETC expression vector, using *Bam*HI-*Nco*I restriction sites. This vector allows the expression of the recombinant CPSase with a 3-kDa His-tag fused to the amino end of the protein. The resulting plasmid (pMS-s) was analyzed by double digestion with the cloning restriction enzymes *Bam*HI-*Nco*I, and both strands were sequenced. The construct pMS-s encoding MS-s was expressed in *E. coli* BL21(DE3) by isopropyl- β -D-thiogalactopyranoside (IPTG) induction at 20°C, and the recombinant MS-s was purified from the soluble fraction in a single step, by Ni²⁺ affinity chromatography (fig. 5a), as indicated in Experimental Procedures. The yield of the purified recombinant MS-s obtained was 14 mg/l of culture. SDS-PAGE analysis of the purified protein gave the expected molecular mass of 44 kDa, taking into consideration the fused 3-kDa His-tag polypeptide.

Oligomeric Structure

The oligomeric structure of MS-s was assessed by chemical cross-linking with dimethyl suberimidate [Davies et al., 1970]. The time course of the cross-linking reaction (fig. 5b) shows that monomeric MS-s gradually disappeared as the reaction proceeded, and was converted into two discrete high molecular species, possibly a tetramer and a higher oligomeric species. The formation of sharp bands in the gels argues against nonspecific association of the monomers which would tend to give a broad, highly heterogeneous species forming a smear.

E. coli CPSase monomers associate to form dimers and tetramers [Kim and Raushel, 2001]. Since MS-s is homologous to the truncated CPS.B domain, the dimer would correspond to a full-length CPS.A-CPS.B synthetase monomer without the A3 and B3 subdomains. The MS-s tetramer would be equivalent to a dimer of the *E. coli* synthetase domain again without A3 and B3. The expected molecular mass of the MS-s dimer and tetramer would be 88 and 176 kDa, respectively, taking into account the appended 3-kDa His-tag polypeptide. Failure to observe the dimeric species is not unusual during chemical cross-linking and can occur if the residues involved in forming the cross-links are not optimally positioned so that the rate of cross-linking of the tetramer and higher oligomers occurs much more rapidly than the dimer.

The oligomeric structure of MS-s was further investigated by size exclusion chromatography on a calibrated Hiload 16/60 superdex 200 column (fig. 5c). Two protein peaks were observed by measuring the absorbance at 280 nm and SDS-PAGE analysis. Calibration of the column

with known proteins gave a molecular mass of 91 kDa and 185 kDa for these two peaks, in good agreement with the expected size of the MS-s dimer (88 kDa) and tetramer (176 kDa). No monomeric MS-s was present, indicating that the protein associates to form homodimers and dimers of dimers.

Catalytic Activity of MS-s

The ammonia-dependent CPSase activity of the recombinant *M. smithii* CPSase MS-s was measured in the coupled reaction with ATCase, as indicated in Experimental Procedures. The enzyme catalyzes CP synthesis from ATP, ammonium chloride, and bicarbonate, with a specific activity at 37°C of 77 ± 5.9 nmol/min/mg. Temperature had a very limited effect on the activity of MS-s, as the specific activity only decreased to 58.3 ± 4.2 nmol/min/mg when the assays were conducted at 22°C.

This small *M. smithii* CPSase also catalyzed the ATP-dependent partial reactions in accordance with the three-step mechanism demonstrated for *E. coli* CPSase [Kothe et al., 2005; Meister, 1989] (table 1). The bicarbonate-dependent ATPase reaction in the presence (reactions 1 and 2) and absence (reaction 1 only) of ammonia measured at 22°C showed a ratio of 1.7:1, indicating that 2 ATP molecules are consumed in the CP synthesis when all of the substrates are present. Moreover, the CP-dependent ATP synthetase reverse reaction rate is very low (4.52 nmol/min/mg) relative to the ATP hydrolysis, representing 8% of the forward ATPase reaction in the presence of ammonia, as in the case of *A. aeolicus* CPSase [Ahuja et al., 2001].

Enterococcus faecium carbamate kinase [Marina et al., 1998] does not catalyze the first partial reaction, i.e. the bicarbonate-dependent ATP hydrolysis in the absence of ammonia, whereas the second partial reaction measured in the reverse direction as a CP-dependent ATP synthesis occurs at a very high rate (table 1). In contrast, for MS-s, like *E. coli* CPSase [Rubio et al., 1987], the rate of the bicarbonate-dependent ATP hydrolysis is high relative to the CP-dependent ATP synthesis activity. The kinetic studies provided additional strong evidence that MS-s is a canonical CPSase, not a carbamate kinase.

Steady-State Kinetic Parameters

Substrate saturation curves for ATP, NH₄Cl, and NaHCO₃ of the recombinant MS-s were measured at 37°C (data not shown), and the kinetic parameters were calculated from the least squares fit to either the Michaelis-Menten or the Hill equation. While both NH₄Cl and NaHCO₃ saturation curves follow Michaelis-Menten kinetics, the ATP saturation curve is slightly sigmoidal, with

Fig. 5. MS-s purification and oligomeric structure determination. **a** Purification of the recombinant MS-s was carried out by affinity chromatography on Ni²⁺-Probond resin as described in Experimental Procedures, and the elution fractions were analyzed by 12% SDS-PAGE. The lanes correspond to the molecular weight marker (Std), fractions 1–3 eluted with TN buffer (50 mM TrisHCl, pH 8, 200 mM NaCl), fractions 4–6 eluted with TN buffer containing 50 mM imidazole, and fractions 7–9 eluted with TN buffer containing 100 mM imidazole. **b** Chemical cross-linking

was carried out at 22°C for 0, 5, 10, and 15 min (lanes 1–4) using 20 µg recombinant MS-s in 100 mM TEA buffer, pH 8.5, 100 mM NaCl, and 10 mM dimethylsuberimidate. The reaction mixture was quenched with 100 mM TrisHCl, pH 8, and analyzed by 10% SDS-PAGE. **c** Size exclusion chromatography was carried out as indicated in Experimental Procedures. The elution profile was obtained by measuring the absorbance at 280 nm. The peak fractions (fractions 15–18 and 35–41) were analyzed by 10% SDS-PAGE and found to contain MS-s.

a Hill coefficient of $n_H = 1.4 \pm 0.1$. The steady-state kinetic parameters (table 2) indicated a relatively high apparent affinity for ATP ($K_m = 0.61$ mM), 2- and 12-fold higher than the K_m (ATP) of the *E. coli* SYN subunit (1.3 mM) [Rubio et al., 1987] and *A. aeolicus* CPS.A-CPS.B complex

(7.43 mM) [Ahuja et al., 2001], respectively. The k_{cat} value for this substrate (0.043 s⁻¹) is 170-fold lower than the corresponding value (7.3 s⁻¹) for the *E. coli* SYN subunit [Rubio et al., 1987]. The apparent second-order rate constant k_{cat}/K_m for ATP (70.5 M⁻¹s⁻¹) is 21–23-fold higher than that

for the other two substrates NH_4Cl and NaHCO_3 , indicating a high efficiency for ATP reaction. Moreover, this second-order rate constant is 79-fold lower than that of the *E. coli* SYN subunit ($5,600 \text{ M}^{-1}\text{s}^{-1}$). Surprisingly, the $K_m(\text{NH}_4\text{Cl})$ value of MS-s (15.6 mM) is 13-fold lower than that for the *E. coli* enzyme (211 mM) [Huang and Raushel, 2000], indicating a high affinity for ammonia of this enzyme, while the value of $K_m(\text{NaHCO}_3)$ is comparable to that of the *E. coli* SYN subunit (10.8 mM). Furthermore, this small *M. smithii* CPSase shows similar k_{cat} and k_{cat}/K_m values for both NaHCO_3 and NH_4Cl substrates. While MS-s clearly catalyzed CP synthesis, the k_{cat} values were surprisingly low compared to other CPSases. Whether the kinetic parameters accurately reflect the intrinsic activity or are the result of instability of the protein or other factors involved in the assay is under investigation.

Mechanism of CP Synthesis

Besides the canonical CPSases, the only other enzymes that have been found to synthesize CP are the archaeal carbamate kinases from the hyperthermophilic organisms, *P. furiosus* and *P. abyssi*. These enzymes use ATP and carbamate formed chemically from ammonia and bicarbonate to synthesize CP. Several lines of evidence suggest that MS-s is much more closely related to the longer CPSases found in all mesophilic organisms: (1) MS-s is significantly larger (41 kDa) than either carbamate kinase or the hyperthermophilic enzymes (33 kDa); (2) MS-s exhibits no statistically significant sequence similarity to carbamate kinases; (3) the residues implicated in catalysis in *E. coli* CPSase are highly conserved in MS-s, whereas the enzyme lacks the carbamate kinase active site residues; (4) carbamate kinases do not catalyze the first partial reaction, the bicarbonate-dependent ATPase activity, but rapidly catalyze the CP-dependent ATP synthesis, while the reverse is observed for MS-s; (5) the stoichiometry of the reaction catalyzed by carbamate kinases is 1 mol of ATP consumed in CP synthesis, whereas 2 ATPs are required for the CP synthesis catalyzed by MS-s, and (6) the levels of ammonia are likely to be too low in the nitrogen-limited environment of the gut to provide sufficient carbamate to sustain significant CP synthesis. Determination of the three-dimensional structure of MS-s, now underway, should provide definitive evidence that the protein is more closely related to the mesophilic CPSases than carbamate kinase.

The question then arises of how a protein one third the size of the synthetase domain of *E. coli* CPSase can catalyze the complex series of reactions (reactions 1–3) involved in CP synthesis. A model is proposed (fig. 6) that

Table 1. ATP-dependent partial reactions

Enzyme	HCO ₃ ⁻ -dependent ATPase, nmol/min/mg		CP-dependent ATP synthetase nmol/min/mg
	+NH ₃	–NH ₃	
<i>M. smithii</i> MS-s	58 ± 4	35 ± 2	4 ± 1
<i>E. faecium</i> CKase*	427	8	4,533
<i>E. coli</i> CPSase**	2,000 ± 400	400 ± 50	390 ± 50

ADP and ATP formation catalyzed by *M. smithii* small CPSase (MS-s) was measured at 22°C as indicated in the Experimental Procedures. The specific activity and standard deviations were calculated from three experiments. * Results from [Marina et al, 1998]. ** Results from [Miran et al., 1991]

Table 2. Steady state kinetic parameters of MS-s

Substrate	K_m mM	V_{max} , nmol/min/mg	k_{cat} s ⁻¹	k_{cat}/K_m M ⁻¹ ·s ⁻¹
ATP*	0.61 ± 0.01	62 ± 1	0.043 ± 0.003	70.5
NH ₄ Cl	15.6 ± 2.6	75 ± 3	0.052 ± 0.003	3.3
Bicarbonate	14.5 ± 3.6	64 ± 7	0.044 ± 0.006	3.0

The steady state kinetic parameters were determined at 37°C, from the ATP, ammonium chloride and sodium bicarbonate saturation curves of MS-s (10 mg) in the coupled reaction with *A. aeolicus* ATCase as indicated in the Experimental Procedures.

* The sigmoidal ATP saturation curve was fit to the Hill equation, so the K_m was replaced with $[S]_{0.5}$. The steady state kinetic parameters and standard deviations were obtained by a least squares fit of eight data points in each saturation curve.

takes into consideration the overall structural and functional characteristics of MS-s, the smallest naturally occurring active CPSase identified so far. As discussed above, MS-s can catalyze both partial reactions, the formation of carbamate from ATP, ammonia, and bicarbonate and the ATP-dependent synthesis of CP from carbamate. In principle, the carbamate synthesized once released from the complex could react with a second ATP to form CP (fig. 6a), but this process would be expected to be inefficient because carbamate is hydrolyzed in the aqueous milieu to ammonia and bicarbonate, setting up in effect a futile cycle resulting in wasteful ATP hydrolysis. We propose instead that the functional unit is the MS-s dimer (fig. 6b). In this species, the two MS-s monomers would be related to each other by a two-fold axis of symmetry exactly analogous to the fused CPS.A and CPS.B domain in the *E. coli* enzyme. The model also pos-

Fig. 6. Functional model of MS-s. Schematic representation of CP synthesis by MS-s. **a** Carbamate is synthesized from bicarbonate, ammonia, and ATP, released from the enzyme, and then binds once again where it is phosphorylated by a second ATP molecule forming CP. This process would be expected to be inefficient since carbamate once released from the complex would be degraded to bicarbonate and ammonia. **b** The synthesis of carbamate is catalyzed by one of the monomers in the MS-s dimer (a) which binds bicarbonate, ammonia, and ATP; the carbamate is sequestered and transferred to the second monomer (b) which catalyzes the phosphorylation of carbamate to form CP. In a subsequent catalytic cycle, the roles of subunits a and b may be reversed.

tulates that, as in *E. coli* CPSase, the two active sites are connected by an intramolecular tunnel. When ATP, ammonia, and bicarbonate bind to monomer (a in fig. 6b) it becomes the site of synthesis of carbamate. Carbamate is then transferred to monomer b which now becomes the CP synthesis domain by default. In a subsequent catalytic cycle the roles may be reversed such that subunit b catalyzes carbamate synthesis and subunit a converts carbamate to CP. In this scheme, carbamate remains sequestered within the complex and protected from hydrolysis.

While further structural and biochemical studies are required to validate this model, there is precedence for this mechanism in mammalian CPSase. It was shown that both CPS.A and CPS.B domains of mammalian CAD separately cloned and expressed in *E. coli* could each dimerize and catalyze both ATP-dependent reactions and the overall synthesis of CP [Guy et al., 1997]. Moreover, the dissociation of CPS.A or CPS.B homodimers by high hydrostatic pressure abolished CP synthe-

sis, while the dissociated subunits could still catalyze the CPSase partial reactions [Guy et al., 1998].

Putative Physiological Function of MS-s

In addition to MS-s, *M. smithii* possesses an active, full-length glutamine-dependent CPSase, MS-l (unpubl. data), so the question arises of what selective advantage the unusual CPSase MS-s provides for the organism. Ammonia is the major source of nitrogen in *M. smithii* for the biosynthesis of nucleotides, amino acids, and many other metabolites. The organism has a specific ammonia transporter and two mechanisms for assimilation of ammonia, the glutamine synthetase-glutamate synthase and the glutamate dehydrogenase pathways [Samuel et al., 2007]. However, the availability of ammonia is strictly limited in the intestine and there is a competition for this scarce resource between *M. smithii* and *B. thetaiotaomicron*. This conclusion is supported by several lines of evidence. For example, analytical studies [Samuel et al., 2007] demonstrated that, in *M. smithii*, the ratio of glutamine to 2-oxoglutarate, a good indication of the nitrogen status of the organism, was reduced 32-fold when *M. smithii* was co-colonized with *B. thetaiotaomicron*. Moreover, when both organisms are present in the intestine, there is an appreciable upregulation of the expression of enzymes involved in nitrogen assimilation in *M. smithii* [Samuel et al., 2007].

MS-s is a streamlined CPSase that is ideally suited to the task of capturing ammonia and converting it to pyrimidines and arginine. The kinetic studies showed that it has an appreciably higher affinity for ammonia than other CPSases. Moreover, the enzyme is also probably unregulated since the B3 or allosteric subdomain, the locus of regulation of all known CPSases, is not present in MS-s. While eukaryotic mitochondrial CPSase I, the enzyme that initiates the urea cycle, can be allosterically activated, there are no known allosteric inhibitors [Rubio and Cervera, 1995] presumably because it is essential in ureolytic animals to continuously and rapidly dispose of any ammonia formed via urea biosynthesis. Similarly, MS-s would be expected to be constitutively active if one of its major functions is to sequester ammonia, so that allosteric regulation would confer no advantage.

Conclusions

MS-s is a unique enzyme, the smallest functional CPSase discovered thus far and one that is only distantly related to other bacterial and archaeal CPSases. It consists of a catalytic domain but lacks the module involved in al-

losteric regulation. The catalytically active species is an MS-s homodimer or larger species and the mechanism of CP synthesis appears to occur in three steps by the same mechanism utilized by the full-length prokaryotic and eukaryotic CPSases. CPSase are postulated to be derived from an ancestral kinase resembling MS-s. Thus, the characterization of this enzyme may provide a glimpse of the evolution of CPSases, which are thought to have arisen by gene duplication, fusion, and the recruitment of domains that confer regulatory functions. In addition to supplementing the function of the full-length CPSase MS-I by bolstering the supply of pyrimidine nucleotides and arginine, MS-s may provide an additional mechanism for harvesting ammonia, a scarce nutrient in the intestine. These minimal CPSases may be a characteristic of archaeal methanogens, since they are also present in *M. thermoautotrophicus* and *M. stadtmanae*. Considering that there is apparently no mammalian counterpart of this unusual protein, MS-s may be an attractive drug target in the treatment of human obesity.

Experimental Procedures

Materials

Pfu DNA polymerase, nucleotides, restriction enzymes, *Bam*HI and *Nco*I, thermosensitive alkaline phosphatase (FastAP), and T4 DNA ligase were from Fermentas Life Sciences; Ni²⁺-ProBond resin was purchased from Invitrogen; aspartate, carbamoyl aspartate, antipyrine, diacetyl monoxime, triethanolamine (TEA), phosphoenol pyruvate, dimethyl suberimidate, and the enzymes pyruvate kinase, lactate dehydrogenase, hexokinase, and glucose 6-phosphate dehydrogenase were from Sigma-Aldrich; *A. aeolicus* aspartate transcarbamoylase (ATCase) recombinant enzyme was expressed in *E. coli* and purified as previously described [Purcarea et al., 2003].

Strains and Plasmids

The *M. smithii* ATCC35061 strain (DSM 861) was from the DSMZ Bacteria Collection [Samuel et al., 2007]. The *E. coli* DH5 α and BL21(DE3) strains and the pRSETC expression vector were from Invitrogen.

M. smithii Growth

The methanogen *M. smithii* ATCC35061 was cultivated anaerobically at 37°C in DSMZ 119 medium containing fatty acids and supplemented with DSMZ 320 salt solutions, in the presence of an 80% H₂:20% CO₂ gas mixture [Samuel et al., 2007]. Cultures of 20 ml were inoculated with 1-ml cultures under an N₂ atmosphere. The flasks were flushed every 24 h with fresh H₂:CO₂ mixture, and cell viability was analyzed by microscopy.

Cloning and Expression

M. smithii chromosomal DNA was isolated from a 20-ml culture using a DNeasy Blood and Tissue Kit for genomic DNA extraction (Qiagen), with an additional cell lysis step using 50 units

of mutanolysin (Sigma). The *carB1* gene (YP_001272934) from *M. smithii* was amplified by PCR using 2.5 units of *Pfu* DNA polymerase, 170 ng of *M. smithii* chromosomal DNA as a template, and 100 pmol of 5MS-s (5'-GGAGATTGGATCCAAATTTTATTTAT-CGGTTCAAG-3') and 3MS-s (5'-GTGTTTAAAGCCATGG TTA-AATCAAATCTTCAACATATC-3') primers defining the 5' and 3' ends of the *M. smithii carB1* gene, respectively. The DNA fragment was amplified after 30 cycles of 95°C for 45 s, 52°C for 1 min, and 72°C for 3 min each cycle, and purified with a DNA Clean & Concentrator Kit (Zymo Research). After digestion with *Bam*HI/*Nco*I, the gene (750 ng) was inserted into the pRSETC expression vector (130 ng) digested with the same restriction enzymes, and dephosphorylated with thermosensitive alkaline phosphatase. Both strands of the resulting construct were sequenced using a Genetic Analyzer 3500 (Applied Biosystems). The *M. smithii* CPS gene was expressed in *E. coli* BL21(DE3) after a 5-hour induction at 20°C with 1 mM IPTG.

Purification

The recombinant *M. smithii* enzyme expressed in *E. coli* as an N-terminal fused protein with a 3-kDa His-tag polypeptide was purified in one step at 4°C by affinity chromatography. Cells from a 100-ml culture were resuspended in 3 ml of 50 mM TrisHCl, pH 8, containing 2 mM 2-mercaptoethanol and 1:100 protease inhibitor cocktail (Sigma), and disrupted by sonication 6 times for 30 s using a Sonifier Cell disruptor W-350 (Bronson Sonic Power). The cell extract was centrifuged at 20,000 g for 30 min, and the supernatant was applied to a 1-ml Ni²⁺-ProBond column equilibrated with 50 mM TrisHCl, pH 8, and 200 mM NaCl (TN buffer). After 20-ml washes with the same buffer, the enzyme was eluted with 1-ml fractions of TN buffer containing increasing (50, 100, 200, and 500 mM) imidazole concentrations. The elution fractions were analyzed by electrophoresis on 12% SDS-PAGE gels. Fractions containing pure recombinant CPSase were collected and the buffer was changed to 50 mM TrisHCl, pH 8, and 5 mM 2-mercaptoethanol, using PD-10 desalting columns (GE Healthcare), to eliminate imidazole and NaCl.

Chemical Cross-Linking

Fractions containing 20 μ g *M. smithii* CPSase in 100 mM triethanolamine, pH 8.5, and 100 mM NaCl were incubated with 10 mM dimethyl suberimidate [Davies et al., 1970] for 0, 5, 10, and 15 min at room temperature in a total volume of 45 μ l. The reaction was stopped by addition of 5 μ l of 1 M TrisHCl, pH 8, and the cross-linked proteins were visualized by SDS-PAGE (10% gels).

Size Exclusion Chromatography

Size exclusion chromatography was carried out at 4°C using a Hiload 16/60 Superdex 200 prep grade column equilibrated with 50 mM TrisHCl, pH 8, containing 5 mM 2-mercaptoethanol. The column was loaded with 500- μ l (5 mg) of the purified MS-s, and the protein was eluted at 1 ml/min with the equilibration buffer, monitoring the absorbance at 280 nm. The fractions containing the protein peaks were analyzed by SDS-PAGE.

CPSase Assay

Ammonia-dependent CPSase activity was measured using the radioactive assay in which the CPSase reaction is coupled with *A. aeolicus* aspartate transcarbamoylase (ATCase) [Ahuja et al., 2001]. Expression and purification of the *A. aeolicus* ATCase were

carried out as previously described [Purcarea et al., 2003]. The reaction mixture contained 100 mM sodium [^{14}C] bicarbonate (100 dpm/ μmol), 20 mM ATP, 22 mM MgCl_2 and 200 mM ammonium chloride, 50 mM TrisHCl, pH 8.0, 100 mM KCl, in the presence of 6 mM aspartate, pH 7, and 1 μg of purified *A. aeolicus* ATCase and various concentrations of recombinant MS-s CPSase, in a total volume of 0.3 ml. After a 20-min incubation at 37°C, the reaction was stopped by addition of 0.7 ml 10% trichloroacetic acid. After evaporation of the liquid phase at 95°C to decompose the unreacted bicarbonate, the radioactivity incorporated in the stable reaction product carbamoyl aspartate was measured after addition of 8 ml Aquasol scintillation liquid. Alternatively, the carbamoyl aspartate formed in the coupled reaction was measured by a colorimetric assay previously described [Purcarea et al., 2001].

Partial Reactions

The bicarbonate-dependent ATPase reaction was measured using a pyruvate kinase/lactate dehydrogenase coupled assay [Post et al., 1990] by monitoring the rate of ADP synthesis in the presence and absence of ammonium chloride. The reaction was performed in a final volume of 0.5 ml containing 100 mM sodium bicarbonate, 20 mM ATP, 22 mM MgCl_2 , 50 mM TrisHCl, pH 8.0, 100 mM KCl, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 10 units of pyruvate kinase, and 20 units of lactate dehydrogenase, and, optionally, 200 mM ammonium chloride. The reaction rate was measured spectrophotometrically at 340 nm, and the concentration was calculated using $\epsilon_{\text{NADH}} = 6.22 \text{ M}^{-1}\cdot\text{cm}^{-1}$. The CP-dependent ATP synthetase activity was assayed by measuring the rate of ATP formation from ADP and CP in the coupled reaction with hexokinase/glucose-6-phosphate dehydrogenase, following a similar procedure [Miran et al., 1991]. The reaction mixture contained 50 mM TrisHCl, pH 8.0, 100 mM KCl, 22 mM MgCl_2 , 1 mM NAD, 20 mM ADP, 2 mM CP, 5 mM glucose, 20 units of hexokinase, and 10 units of glucose-6-phosphate dehydrogenase in a final volume of 0.5 ml. Both partial reactions were assayed at 22°C and the initial rate was calculated from the time course up to a 5-min time course.

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Structure Analysis and Molecular Modeling

The genes encoding the CPSase subunits in the *M. smithii* ATCC35061 genome sequence were identified by BLAST-NCBI genome database screening. Sequence analysis was performed using the genomic and proteomic software package ExPASy SIB Bioinformatics Resource Portal. Protein pair alignment was carried out with EMBOSS needle alignment (EMBL-EBI) and multiple alignment was performed using ClustalW. Amino acid sequence randomization was performed using random protein sequence generator RandSeq software (ExPASy). Modeling of the three-dimensional structure was carried out using Modeller 9v9 [Sali and Blundell, 1993], based on the 1BXR X-ray structure of *E. coli* CPSase in complex with the ATP analog AMPPNP [Thoden et al., 1999]. Fifty models were generated and the best candidate was chosen based on DOPE (discrete optimized protein energy), zDOPE (normalized discrete optimized protein energy), and GA341 scores. The energy minimization was performed with Gromos 96 implemented in Swiss PDB Viewer. The model was further refined by loop optimization using ModLoop [Fiser and Sali, 2003] followed by energy minimization. In every round of optimization, at least 10 models were generated, followed by energy minimization. The quality of the model was assessed by the Ramachandran plot obtained with PROCHECK [Laskowski et al., 1993], ERRAT2 [Colovos and Yeates, 1993], and ProSa [Sippl, 1993] programs. Superposition of the MS-s model with 1BXR and calculation of the root mean square deviation were obtained using Swiss PDB Viewer [Guex and Peitsch, 1997]. The structures were visualized using the PyMol Molecular Graphics System 0.99rc6 (Schrodinger, LLC).

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