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Purification and Characterization of the Oxygen-Sensitive Acetohydroxy Acid Synthase from the Archaebacterium *Methanococcus aeolicus*

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Acetohydroxy acid synthase (EC 4.1.3.18) of the archaebacterium *Methanococcus aeolicus* was purified 1,150-fold to homogeneity. The molecular weight of the purified enzyme was 125,000, and it contained only one type of subunit (M_r = 58,000). The amino-terminal sequence had 46 to 57% similarity to those of the large subunits of the eubacterial anabolic enzymes and 37 to 43% similarity to those of the yeast and plant enzymes. The methanococcal enzyme had a pH optimum of 7.6. The pI, estimated by chromatofocusing, was 5.6. Activity required Mg^{2+} or Mn^{2+} ions, thiamine pyrophosphate, and a flavin. Flavin adenine dinucleotide, flavin mononucleotide, and riboflavin plus 10 mM phosphate all supported activity. However, activity was strongly inhibited by these flavins at 0.3 mM. The Michaelis constants for pyruvate, $MgCl_2$, $MnCl_2$, thiamine pyrophosphate, flavin adenine dinucleotide, and flavin mononucleotide were 6.8 mM, 0.3 mM, 0.16 mM, 1.6 μM, 0.4 μM, and 1.3 μM, respectively. In cell extracts, the enzyme was sensitive to O_2 (half-life = 2.7 min with 5% O_2 in the headspace), but the purified enzyme was less sensitive to O_2 (half-life = 78.0 min with 20% O_2). Reconstitution of the enzyme with flavin adenine dinucleotide increased the sensitivity to O_2. Moreover, in the assay the homogeneous enzyme was rapidly inactivated by O_2, and the concentration required for 50% inhibition (I_{50}) was obtained with an atmosphere of 0.11% O_2. The methanococcal enzyme has similarities to the eubacterial and eucaryotic enzymes, consistent with the ancient origin of the archaebacterial enzyme.

Acetohydroxy acid synthase (AHAS) (EC 4.1.3.18) is a common anabolic enzyme in bacteria, yeasts, and plants. In these organisms, it catalyzes the first common step in the biosynthesis of the branched-chain amino acids, i.e., the formation of acetolactate from two molecules of pyruvate and the formation of 2-acetohydroxybutyrate from pyruvate and 2-ketobutyrate (29, 30). Some bacteria also contain a catabolic AHAS whose physiological function is acetolactate synthesis for the acetoin and 2,3-butanediol fermentations (28). Both the catabolic and anabolic enzymes contain thiamine pyrophosphate (TPP), and they have 20 to 30% amino acid sequence similarity (17). However, they differ in a number of important respects. While the pH optima of the anabolic enzymes are greater than 7, the pH optima of the catabolic enzymes are between 6 and 7 (13, 24, 26). The anabolic enzymes also contain flavin adenine dinucleotide (FAD), which is absent from the catabolic enzymes (27). Finally, the anabolic enzymes from bacteria contain a small subunit that is required for feedback inhibition by the branched-chain amino acids. When the catabolic enzymes from the same or closely related bacteria have been examined, this small subunit has not been found (17, 38).

The function of FAD in the anabolic AHAS is unclear. The enzyme does not catalyze an oxidation-reduction reaction, and FAD, but not other flavins, is essential for activity (21). In the plant enzyme, FAD plays a role in the subunit association (7). The presence of FAD has been proposed to be a vestige of AHAS evolution. Pyruvate oxidase from *Escherichia coli* has 29 to 30% sequence similarity to the large subunits of the enteric AHAS isozymes and a very similar coenzyme requirement (5, 12). In pyruvate oxidase, FAD functions as an electron carrier to quinones. Quinones (Q_0 and Q_1) are also inhibitors of AHAS (22). Presumably, if AHAS evolved from pyruvate oxidase, the FAD binding site may have been retained to maintain the enzyme structure (22).

Although the anabolic AHAS is generally a labile enzyme, it has been purified from overexpressing or recombinant strains of *E. coli*, *Salmonella typhimurium*, and *Saccharomyces cerevisiae* (3, 8, 18, 23). In addition, the anabolic enzyme has been purified from the wild-type strains of *Pseudomonas aeruginosa* and barley (1, 6). However, the enzyme from an archaebacterium or a strict anaerobe has never been examined.

The methanococci are strictly anaerobic, methane-producing archaebacteria (33). While the methanococcal enzymes in the branched-chain amino acid biosynthetic pathway have not been studied in detail, preliminary investigations with partially purified enzymes have demonstrated that the methanococcal AHAS and other enzymes of the pathway resemble the eubacterial and eucaryotic enzymes with regard to their catalytic properties and native molecular weights (35–37). This result suggests that this pathway is very ancient and that the AHASs from organisms of the three primary domains are homologous. To critically examine this hypothesis, the AHAS from *Methanococcus aeolicus* was purified and characterized.

MATERIALS AND METHODS

Bacterium and growth conditions. *M. aeolicus* was grown in a 400-liter fermentor containing mineral (McN) medium with 0.5% sodium formate at 37°C under an N_2 atmosphere. During growth, the pH of the culture was maintained at 7.0 by the frequent addition of formic acid (37). After being harvested, the cells were stored frozen at −70°C as a cell paste for up to 1 year.

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AHAS assay. AHAS was measured under anaerobic conditions as described previously (35). The standard assay mixture contained 0.1 M Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine} (pH 7.5), 50 mM pyruvate, 10.0 mM $MgCl_2$, 0.1 mM TPP, and 0.1 mM FAD. Results reported are averages of duplicate assays. In some assays, the decrease in A_{333} of pyruvate was monitored (23). Anaerobic assay solution (1 ml) was prewarmed in a sealed cuvette at 37°C for 20 min. The reaction was initiated by the addition of 1.6 μg of AHAS. One unit of activity was the amount of enzyme required to form 1 μmol of acetolactate per min. To determine the pH optimum, Bis-Tris [bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane] chloride, Tris chloride, and sodium CHES [2-(*N*-cyclohexylamino)ethanesulfonate] buffers were used for pH values from 5 to 6.5, 7 to 8, and 8.5 to 10, respectively, at a concentration of 0.1 M.

Purification of AHAS. All of the purification steps were performed anaerobically at 3°C (36). The basic buffer contained 20 mM KH_2PO_4 (pH 7.5), 20% (vol/vol) glycerol, 5 mM $MgCl_2$, 0.05 mM TPP, 0.005 mM FAD, 0.5 mM dithiothreitol, and 0.5 mM Na_2EDTA.

(i) Preparation of cell extract. Cell extract was prepared as described previously except that 20% (vol/vol) glycerol was added to the PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonate)] buffer, pH 7.5 (35).

(ii) Ultracentrifugation. Cell extract (640 ml from about 400 g of cell paste) was centrifuged twice with a VTi50 rotor at 4°C and 242,000 × *g* for 5 h. After the first centrifugation, the pellet was collected and resuspended in one-half volume of basic buffer prior to the second centrifugation. After an additional centrifugation for 30 min, about 300 ml of supernatant was collected.

(iii) Phenyl Sepharose chromatography. The supernatant was loaded onto a phenyl Sepharose CL-4B column (3.0 by 28.0 cm), which had been equilibrated with 1.0 M KH_2PO_4 (pH 7.5) in the basic buffer. The column was washed with 200 ml of 0.6 M KH_2PO_4 (pH 7.5) in basic buffer at a flow rate of 50 ml/h followed by a linearly decreasing gradient generated from 500 ml of 0.6 M KH_2PO_4 in basic buffer to 500 ml of basic buffer containing 40% (vol/vol) ethylene glycol. An additional 200 ml of basic buffer containing 40% (vol/vol) ethylene glycol was then applied. Enzyme activity eluted as a single peak at about 0.3 M KH_2PO_4. Fractions containing activity were pooled (260 ml) and dialyzed overnight against 2 liters of 20 mM Tris-Cl buffer (pH 9.2) containing the all components present in the basic buffer except phosphate. Less that 10% of the activity was lost during dialysis.

(iv) PBE-94 chromatography. A polybuffer exchanger 94 (PBE-94) column (3.0 by 28 cm) was equilibrated with the dialysis buffer (pH 9.2) prepared without FAD. The dialyzed sample was loaded at a flow rate of 50 ml/h. The column was washed with 200 ml of dialysis buffer followed by a linearly increasing gradient produced from 500 ml of basic buffer without FAD and 500 ml of 1.2 M KCl in basic buffer without FAD. An additional 200 ml of 2 M KCl in basic buffer without FAD was then applied. Enzyme activity eluted as a single peak at about 0.3 M KCl, and fractions with activity were pooled (210 ml) and diluted to 0.3 M KCl with basic buffer that contained FAD.

(v) Red Agarose chromatography. After dilution, the material was applied onto a Red Agarose column (2.8 by 19 cm) that had been equilibrated with basic buffer at a flow rate at 40 ml/h. The column was washed with 100 ml of 0.3 M KH_2PO_4 (pH 7.5) in basic buffer, a linearly increasing gradient generated from 300 ml of this buffer and 300 ml of 1.5 M KCl in basic buffer, and 100 ml of 2 M KCl in basic buffer. The enzyme eluted as a single peak at about 0.7 M KCl, and the fractions which contained activity (220 ml) were pooled.

(vi) Phenyl Sepharose chromatography. A small phenyl Sepharose column (2.2 by 8.0 cm) was equilibrated with 0.6 M KH_2PO_4 (pH 7.5) in basic buffer. The sample from the Red Agarose column was loaded at a flow rate of 6 ml/h and washed with 50 ml of the equilibration buffer followed by a linearly decreasing gradient produced from 80 ml of equilibration buffer and 80 ml of basic buffer containing 20% (vol/vol) ethylene glycol. Finally, the column was washed with 30 ml of basic buffer containing 40% (vol/vol) ethylene glycol. The enzyme activity eluted as a single peak at about 0.4 M KH_2PO_4. Fractions containing activity were pooled (35 ml) and diluted to 0.1 M KH_2PO_4 with basic buffer.

(vii) Hydroxyapatite chromatography. A hydroxyapatite column (2.2 by 5 cm) was equilibrated with basic buffer. After application of the diluted enzyme from the phenyl Sepharose chromatography step, the column was washed with 20 ml of basic buffer and a linearly increasing gradient generated from 40 ml of basic buffer and 40 ml of 0.5 M KH_2PO_4 (pH 7.5) in basic buffer at a flow rate at 4 ml/h. Enzyme activity eluted as a single peak at about 0.2 M KH_2PO_4. Fractions containing activity were pooled (17 ml) and dialyzed overnight against 2 liters of 0.1 M Tris-Cl buffer (pH 7.5) containing 20% (vol/vol) glycerol, 0.5 mM dithiotreitol, and 0.5 mM disodium EDTA. Purified enzyme was stored at −20°C.

Protein and pI determinations. The protein concentration was determined with the bicinchoninic acid reagents (Pierce Co., Rockford, Ill). The pI value of AHAS was measured by PBE-74 chromatography under anaerobic conditions. The PBE-74 column (0.9 by 15.0 cm) was equilibrated with 50 ml of buffer containing 25 mM Tris-Cl (pH 7.5), 20% glycerol, 5 mM $MgCl_2$, 0.1 mM TPP, and 0.5 mM cysteine hydrochloride. Purified enzyme (1.0 ml) was loaded onto the column at a constant flow rate of 8 ml/h. The column was washed with 25 ml of the equilibration buffer followed by 110 ml of polybuffer 74, which was diluted eightfold and contained 20% (vol/vol) glycerol, 5 mM $MgCl_2$, 0.1 mM TPP, and 0.5 mM cysteine hydrochloride. Fractions of 4 ml were collected. The pI value was obtained by the pH measurement of each fraction with high activity.

Molecular weight determination. The molecular weight of the native enzyme was determined anaerobically with a Sephadex G-200 column (1.3 by 75 cm) (37). The subunit molecular weight was measured by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS). Purified enzyme (1.0 ml) containing 12 μg of protein was dialyzed in a membrane with a molecular mass exclusion limit of 3.5 kDa (Spectrum Medical Industries, Inc., Los Angeles, Calif.) against 50 mM ammonium acetate buffer (pH 7.0) overnight at 4°C. Dialyzed enzyme was concentrated for electrophoresis by a speed vacuum concentrator (Savant Instrument Inc. model RH 40-II). For routine analyses, slab gels (16 cm by 1.5 mm) containing 12% polyacrylamide were prepared (14). After electrophoresis, the protein was visualized by Coomassie blue staining. The standard proteins (molecular weights) were myosin (200,000), β-galactosidase (116,000), phosphorylase *b* (97,000), bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), and trypsin inhibitor (20,000).

Protein sequencing. Purified enzyme (1.4 ml; 16 μg of protein), was dialyzed against 2 liters of 3 mM sodium acetate buffer (pH 9.5) overnight at 4°C and concentrated on a speed vacuum concentrator to 0.1 ml. The amino-terminal amino acid sequence was determined by using the 477A protein

TABLE 1. Purification of AHAS of *M. aeolicus*

Step	Total U	Total protein (mg)	Sp act (U/mg)	Yield (%)	Purification (fold)
Cell extract	786	22,900	0.03	100.0	1
Ultracentrifugation	561	5,490	0.10	71.3	3
Phenyl Sepharose	575	470	1.23	73.2	36
PBE-94	203	96	2.11	25.8	62
Red Agarose	81	10	8.36	10.3	244
Phenyl Sepharose	20	0.5	38.27	2.6	1,116
Hydroxyapatite	8	0.2	39.30	1.0	1,147

sequencer (Applied Biosystems Inc., Foster City, Calif.) at the University of Georgia.

Materials. PBE-94, PBE-74, polybuffer 74, and Sephadex G-200 were obtained from Pharmacia Fine Chemicals Inc. (Piscataway, N.J.). Hydroxyapatite (Bio-Gel HT) and the molecular weight standards for polyacrylamide gel electrophoresis were purchased from Bio-Rad Laboratories (Rockville Center, N.Y.). Polyacrylamide (30%) and methylene bisacrylamide (2%) solutions were purchased from National Diagnostics (Manville, N.J.). Other chromatography materials, protein markers for gel filtration, and biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Purification. In a representative purification, the AHAS of *M. aeolicus* was purified 1,150-fold under anaerobic conditions (Table 1). Protection from oxygen was necessary because the activity in extracts was rapidly inactivated by air (35). The synthase was further stabilized by the inclusion of glycerol, $MgCl_2$, TPP, FAD, dithiothreitol, and EDTA in a phosphate buffer during most of the purification. It was possible to pellet the synthase by ultracentrifugation because it has a high molecular weight in cell extracts (36). This step enriched the enzyme threefold and reduced the volume of cell extract. Subsequent chromatography on phenyl Sepharose achieved high enrichments due to the hydrophobicity of the enzyme. After this step, chromatographic purification of the enzyme was achieved only with high losses in yield. Although the chromatography on PBE-94 provided less than a twofold enrichment of the AHAS and yielded a low recovery, this step was essential to obtain homogeneous enzyme. Various dye columns were tested, but only Red and Green Agarose bound the enzyme. Of these two, Red Agarose had the highest capacity and provided the greatest enrichment of the synthase. After chromatography on Red Agarose, a small phenyl Sepharose column was utilized to lower the phosphate concentration and obtain additional purification prior to the hydroxylapatite column step. The final specific activity of the synthase was 39 U/mg. In two other purifications, specific activities of 31 U/mg, representing an 870-fold purification, and 34 U/mg, representing a 3,620-fold purification, were obtained. The low yield was typical of purifications of AHASs from other organisms (6, 18).

Because the homogeneous methanococcal enzyme had a high specific activity, the purification did not appear to yield partially inactive enzyme. The synthase was homogeneous on SDS-polyacrylamide gel electrophoresis (Fig. 1), and the amino-terminal sequence also revealed only a single N-terminal peptide (see below). Freezing and thawing of the homogeneous enzyme resulted in loss of more than 50% of the activity. When stored in the absence of cofactors, the enzyme retained full activity for several months at −20°C. In the absence of cofactors, it was also stable at 37°C for 120 min (data not shown).

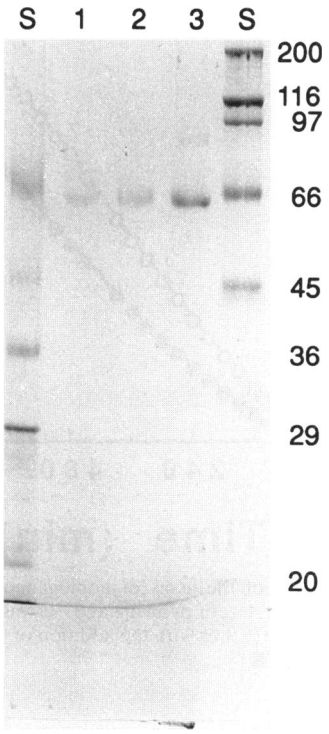

FIG. 1. SDS-polyacrylamide gel electrophoresis of purified AHAS. Lanes 1, 2, and 3 contained 0.5, 1.0 and 2.0 μg of methanococcal AHAS, respectively. Lanes S contained the molecular weight markers. Numbers are molecular weights in thousands.

Physical properties of AHAS. The molecular weight of the AHAS was determined with extracts, partially purified preparations, and the homogeneous enzyme by gel filtration. In extracts or after ultracentrifugation, the AHAS had a high M_r of 530,000 to 550,000 (36). During the purification, lower-molecular-weight forms became abundant. Following one or two chromatography steps (depending on the purification), high- and medium-molecular-weight forms (M_r of 530,000 to 340,000) predominated, and only small amounts of a lower-molecular-weight enzyme (M_r of about 100,000) were present. At the end of the purification, the molecular weight of the homogeneous enzyme was 125,000, and only one peak of activity was detected. On SDS-polyacrylamide gel electrophoresis, only one type of subunit was found, and the small subunit, which is common in the bacterial synthases, was not detected (Fig. 1). The M_r of the large subunit (mean ± standard deviation) was 58,000 ± 2,000 (six determinations). Thus, the homogeneous methanococcal synthase was a dimer with an M_r of about 120,000.

The conclusion that only one type of subunit was present in the homogeneous methanococcal AHAS was supported by the amino-terminal sequence analysis. After Edman degradation, the sequence of the first 35 amino acids was Met-Asn-Gly-Ala-Glu-Ala-Met-Ile-Lys-Ala-Leu-Glu-Ala-Glu-Lys-Val-Val-Ile-Leu-Phe-Gly-Tyr-Pro-Gly-Gly-Ala-Leu-Leu-Pro-Phe-Tyr-Asp-Ala-Leu-His. There was no evidence for a second polypeptide, which would be expected if a small type of subunit was present. This sequence was very similar to portions of the amino-terminal sequences of the anabolic enteric AHASs (46 to 57%) and the AHASs from cyanobacteria (43 to 51%), *S.*

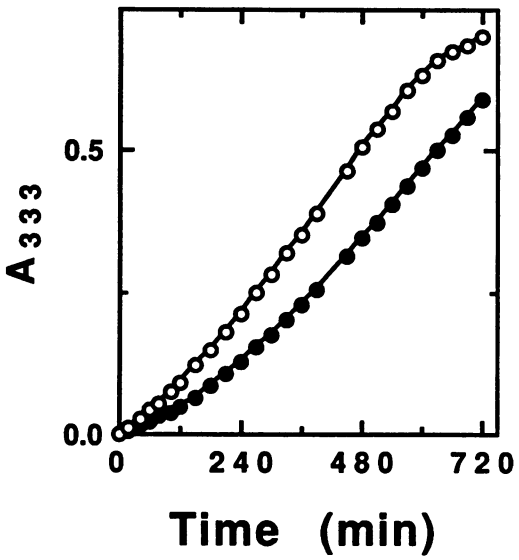

FIG. 2. Time course of the assay of homogeneous AHAS of *M. aeolicus*. The decrease in A_{333} of pyruvate at 37°C was measured in the standard anaerobic assay (○) or with the addition of 0.016% O_2 to the headspace of the assay (●).

cerevisiae (43%), and plants (37 to 40%) (percent identical amino acids identical in parentheses) (10, 11, 15, 16, 20, 25, 32). Particularly striking was the conservation of 7 of the first 15 amino acids between the methanococcal synthase and the enteric isozyme II and the sequence Val-X-X-Leu-Phe-Gly-Tyr-Pro-Gly-Gly-Ala in nearly all of the 12 anabolic AHAS sequences examined. In contrast, the similarity to the *Klebsiella* catabolic AHAS was only 34%, and only five of the nine amino acids in this highly conserved amino-terminal portion remained the same (17).

The apparent pI of the homogeneous methanococcal enzyme, determined from its elution from PBE-74, was 5.6.

Catalytic properties. Under the standard assay conditions, full activity was obtained after a lag of about 1 h (Fig. 2). After that time, the activity was about 80% higher than the initial activity and was constant until the substrate was depleted. This lag was not affected by the omission of cofactors from the assay, but it was abolished by preincubation in the presence or absence of cofactors (see footnotes to Table 2). Because of the complexity of the effects of preincubation (see below) and the relatively small effect of the lag on the reaction velocity, the lag was not studied further.

The methanococcal AHAS required FAD, TPP, and $MgCl_2$ for full activity (Table 2). In contrast to case for the other anabolic enzymes described, flavin mononucleotide (FMN) and riboflavin were also stimulatory. For instance, FMN stimulated activity two- to threefold more than FAD, and the further addition of AMP had no effect (Table 2). While riboflavin alone had a modest effect, i.e., less than 70% stimulation in most experiments or slight inhibition, the stimulation by riboflavin plus 10 mM phosphate was similar to that by FMN (Table 2). Following a preincubation, the highest activity was obtained with Mg^{2+} and TPP in the absence of FAD (Table 2). One interpretation of these results was that the enzyme contained bound flavin after purification and that preincubations in the presence of flavins were inhibitory. While insufficient enzyme was available to analyze the flavin content, tightly bound FAD has been identified in other bacterial synthases (1, 23).

TABLE 2. Cofactor requirements of the AHAS of *M. aeolicus*[a]

Cofactor(s) omitted[b]	Sp act	
	No preincubation[c]	Preincubation[d]
$MgCl_2$ + TPP + FAD	1.1	3.9
FAD	6.5	30.9
TPP	5.3	3.8
$MgCl_2$	6.2	10.0
None	17.0	11.4
FAD (+ FMN)	29.9	ND[e]
FAD (+ FMN + AMP)	30.6	ND
FAD (+ riboflavin)	3.0	ND
FAD (+ riboflavin + P_i)	40.6	ND

[a] Homogeneous AHAS was dialyzed overnight against 0.1 M Tris-Cl, pH 7.5, containing 20% (vol/vol) glycerol.
[b] Cofactors omitted from the assay and preincubation. The concentrations of cofactors were 10 mM $MgCl_2$, 0.1 mM TPP, 0.1 mM FAD, 0.1 mM FMN, and 0.1 mM riboflavin. KH_2PO_4 was at 10 mM.
[c] Specific activities were obtained at 120 min of assay, after the initial lag.
[d] AHAS was preincubated without pyruvate and the indicated cofactor(s) at 37°C for 30 min prior to the assay. Assays were linear with time.
[e] ND, not done.

To confirm that flavins were inhibitory at high concentrations, the methanococcal enzyme was preincubated with either 0.1 mM FAD or FMN (Table 3). Following the preincubation with FAD, only 66 to 69% of the activity remained regardless of whether the enzyme was assayed with FAD or FMN. Following the preincubation with FMN, 83 to 93% of the activity remained. Thus, FAD was more inhibitory than FMN in the preincubation, but the two flavins gave identical responses in the assay. Following the preincubation of the enzyme with different concentrations of flavins, FMN showed the highest stimulation at 0.1 mM, and only 40% of this activity remained at 0.3 mM (data not shown). Riboflavin plus phosphate promoted the highest activity at 0.05 mM, and 60% of this activity remained at 0.1 mM. By contrast, FAD inactivated the enzyme even at the lowest concentration tested, 0.02 mM. Therefore, the higher apparent stimulation of FMN and riboflavin plus phosphate probably resulted from the lower inhibition by these compounds.

In the presence of FAD, 0.1 mM ATP, ADP, AMP, NAD^+, NADH, $NADP^+$, and NADPH had no effect on activity. Metal salts showed various effects. $MnCl_2$ replaced the requirement for $MgCl_2$ in the assay. In the presence of 10 mM $MgCl_2$, a 10 mM concentration of other metal salts had little effect or was inhibitory. (Percent activities remaining are as follows: $FeCl_2$, 88%; $FeSO_4$, 97%; $ZnCl_2$, 74%; $NiCl_2$, 45%; $CuCl_2$, 20%; and

TABLE 3. Effect of flavins on O_2 sensitivity during the preincubation

Preincubation[a] with:		Sp act[b] with:	
20% O_2	FAD or FMN	FAD	FMN
−	−	20.8	19.0
+	−	19.6	17.2
−	FAD	13.8	13.1
+	FAD	2.8	2.9
−	FMN	17.3	17.7
+	FMN	1.5	1.2

[a] Homogeneous AHAS was incubated at 37°C for 20 min prior to the preincubation with 0.1 mM flavins and O_2 for 60 min. −, not added.
[b] Assays were performed under the standard conditions with 0.1 mM FAD or FMN. Following preincubation with O_2, the O_2 carried over into the assay was no more than 0.01% of the headspace.

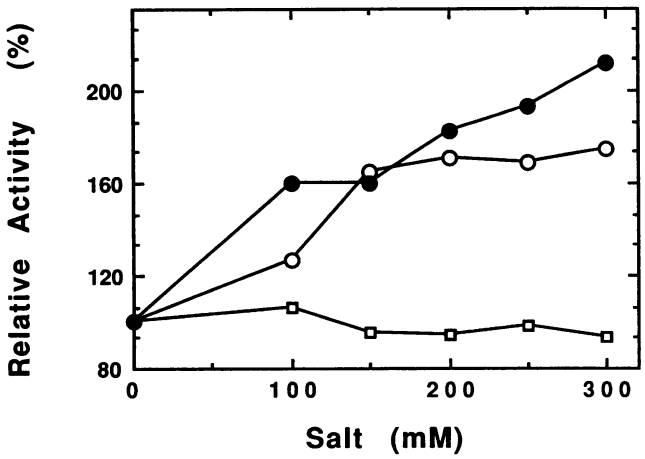

FIG. 3. Stimulation of the methanococcal AHAS by NH_4^+ ions. Salts were added to the standard assay, which also contained 10 mM $MgCl_2$. A relative activity of 100% was 9.7 U mg of protein^{-1}. The salts used were ammonium acetate (●), $(NH_4)_2SO_4$ (○), and $MgSO_4$ (□).

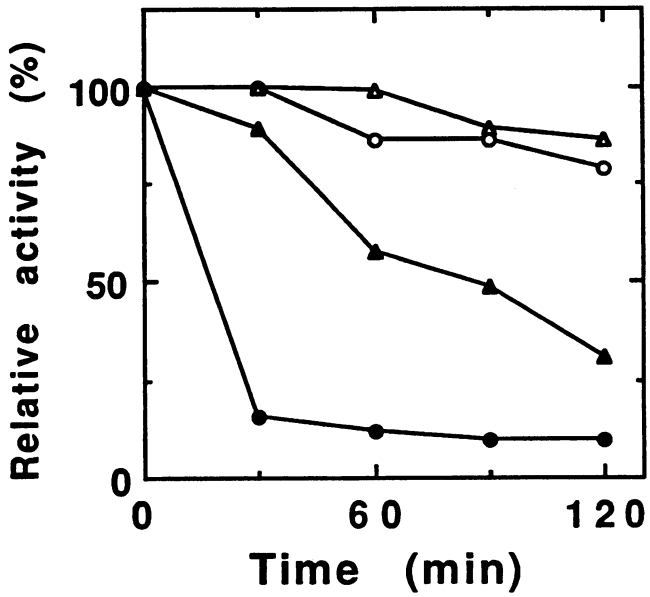

FIG. 4. Effect of cofactors and O_2 on the activity of methanococcal AHAS. The enzyme was dialyzed two times overnight in 0.1 M Tris-Cl, pH 7.5, with 20% (vol/vol) glycerol. AHAS was preincubated for the indicated times with 10 mM $MgCl_2$, 0.1 mM TPP, and 0.1 mM FAD at 37°C in the presence of 20% O_2 (●) or in the absence of O_2 (○) and assayed under the standard conditions. The amount of O_2 carried over into the assay was no more than 0.003%. A relative activity of 100% represented a specific activity of 17.4 U/mg. AHAS was incubated without cofactors in the presence of 20% O_2 (▲) or in the absence of O_2 (△). The assay was performed in the absence of exogenous cofactors, and 100% represents a specific activity of 1.6 U/mg.

$CoCl_2$, 16%.) In the presence of 0.5 mM $AgNO_3$, only 6% of the activity remained.

The Michaelis constants for the methanococcal AHAS were determined under the standard assay conditions for the nonvaried substrates and coenzymes from initial velocities obtained without preincubation of the enzyme. Because of the nonlinearity of the assay and other effects, these constants probably do not provide a precise estimate of the kinetic properties of this complex enzyme. However, they do provide a useful description of the observed properties for comparison with AHASs from other sources. The Michaelis constants (K_m, V_{max} in units milligram^{-1}) were as follows: pyruvate, 6.8 mM, 55; $MgCl_2$, 0.3 mM, 15; $MnCl_2$, 0.16 mM, 16; TPP, 1.6 μM, 8.8; FAD, 0.4 μM, 5.5; and FMN, 1.3 μM, 7.6. The end products of the branched-chain amino acid biosynthetic pathway, valine and isoleucine but not leucine, were competitive inhibitors with respect to pyruvate. The K_is for valine and isoleucine, which were determined at amino acid concentrations of 0.5 to 1.0 mM, were 0.3 and 0.4 mM, respectively. The methanococcal synthase also had a pH optimum of 7.6.

Ammonium sulfate stimulated AHAS activity up to 1.6-fold at a concentration of 200 mM (Fig. 3). Because ammonium acetate was stimulatory and $MgSO_4$ was not, this effect was due to the cation. In other experiments, 100 mM sodium acetate, potassium acetate, KH_2PO_4, or NaH_2PO_4 was not stimulatory (data not shown). Therefore, this effect was specific for NH_4^+ ions. The stimulation occurred following preincubation with NH_4^+ prior to dilution in the assay or upon NH_4^+ addition to the assay. Following a preincubation with 200 mM $(NH_4)_2SO_4$ for 30 min at 37°C, activity was increased by 38%. In the same experiment, the addition of $(NH_4)_2SO_4$ to the assay stimulated activity by 55%. Inclusion of $(NH_4)_2SO_4$ in both the preincubation and assay stimulated activity by 259%. While preincubation with cofactors was slightly stimulatory in these experiments, it had no effect on the relative stimulation by $(NH_4)_2SO_4$ during the preincubation or assay (data not shown). These results were consistent with a slow activation of the enzyme by NH_4^+ that was independent of the effects of the cofactors.

Oxygen lability. Incubation of the methanococcal AHAS with O_2 in the absence of substrate and coenzymes inactivated the enzyme. However, during the purification, the sensitivity to O_2 under this condition decreased. In cell extracts, the enzyme had a half-life of 2.7 min with 5% O_2 at 37°C in buffer without coenzymes (35). After 100-fold purification to a specific activity of 2.7 to 2.8 U mg of protein^{-1}, the half-life was extended to 35 min. The homogeneous AHAS was relatively insensitive to 5% oxygen, with a half-life of ≥200 min. The half-life with 20% oxygen was 78 min under the same conditions. The O_2 sensitivity of homogeneous AHAS was somewhat restored by the addition of cofactors (Fig. 4). After dialysis, the enzyme retained only a small amount of activity without coenzymes. This enzyme was slowly inactivated by O_2. If the enzyme was preincubated with Mg^{2+}, TPP, and FAD, activity was largely restored, and it was much more sensitive to O_2, with a half-life of <13 min with 20% O_2 at 37°C (Fig. 4). Since preincubation with FAD or FMN alone was sufficient to restore the O_2 sensitivity (Table 3), the O_2 sensitivity was apparently mediated by flavins. A similar inhibitory pattern was also obtained both in the light and in the dark, suggesting that the flavin-mediated O_2 inhibition was not affected by light (data not shown). For the partially purified enzyme, inhibition was not reversible by 4,000 U of catalase or removal of O_2 by flushing with N_2 (data not shown). Moreover, O_2 inhibition was not relieved by additional dialysis in anaerobic buffer containing cofactors, EDTA, and dithiothreitol (data not shown).

In contrast to its preincubation condition, homogeneous methanococcal AHAS was especially O_2 labile during the assay. When 0.6% O_2 was added to the headspace of the assay, enzyme activity was inhibited completely. Because the rate of inactivation was rapid, it was not possible to determine a half-time. Smaller amounts of O_2 inhibited both the initial and

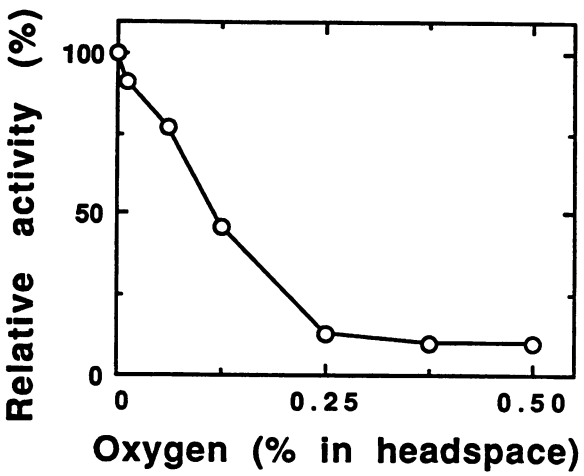

FIG. 5. Oxygen inhibition of AHAS during the assay. O_2 was added to the assay solution prior to the addition of enzyme. The assay was performed under standard conditions at 37°C for 40 min.

final AHAS activities as well as increased the apparent lag in the assay (Fig. 2). With 0.016% O_2, the initial and final velocities of the assay were 60 and 80% of those of the control assay without O_2, respectively. The lag also increased from 1 to 2 h. The concentration of O_2 required for 50% inhibition (I_{50}) under this condition was obtained with an atmosphere of 0.11% O_2 (Fig. 5). This inhibition was not reversible by the addition of 1,000 U of catalase or by removal of O_2 by flushing with N_2 (data not shown).

DISCUSSION

AHAS from *M. aeolicus* was purified to homogeneity. The low recovery and complex purification result from the extreme lability of the enzyme. This instability is accompanied by changes in molecular weight and sensitivity to oxygen. At least some of these changes may have been due to loss of tightly bound cofactors, because the addition of flavins restored the O_2 sensitivity. In addition, FAD promotes the formation of high-molecular-weight forms of the barley AHAS (7). Possibly, the removal of flavin during the purification of the methanococcal enzyme explains the decrease in native molecular weight. Because the homogeneous AHAS retained high specific activity, at least some of its physiologically important properties remained after the purification.

The eubacterial anabolic AHASs are composed of two large (~60,000-M_r) and two small (~9,000- to 17,000-M_r) subunits in an $\alpha_2\beta_2$ structure (2, 3, 8, 23). In contrast, the homogeneous methanococcal AHAS contains only large subunits, like the yeast and plant anabolic AHASs and the eubacterial catabolic AHASs (6, 18). However, it is possible that a small subunit was removed during the purification of the methanococcal enzyme. While examination of the gene(s) may be necessary for a definitive resolution of this question, this possibility is unlikely. For the eubacterial enzymes, the small subunits are regulatory (4, 19), and the AHASs formed in strains containing a deletion of the gene for the small subunit have low specific activities and are relatively insensitive to feedback inhibition (9, 31). The homogeneous methanococcal AHAS remains sensitive to feedback inhibition in the apparent absence of small subunits and has a high specific activity. *M. aeolicus* is also a lithotroph that grows with H_2 plus CO_2 or formate as its only energy and carbon source and has little capacity to utilize exogenous amino acids (34, 35). Thus, the regulation of its AHAS should differ from those of the enzymes from the heterotrophic bacteria previously studied. Therefore, a small subunit probably is not a property of the methanococcal enzyme.

Methanococcal AHAS, like the AHASs of bacteria, yeasts, and plants, requires $MgCl_2$, TPP, and FAD for full activity. Partial activity is also obtained from the purified enzyme in the absence of exogeneous cofactors, suggesting that these cofactors are tightly bound. TPP and $MgCl_2$ are known to take part in the catalytic reaction of condensing pyruvate to acetolactate, while the function of FAD is not clear. The catabolic AHAS from *Aerobacter aerogenes* does not contain FAD (27), and in the anabolic AHAS the role of FAD may be structural (21). The interactions of the methanococcal AHAS with flavins are complex. In contrast to the eubacterial enzymes examined so far, the flavin requirement is nonspecific, and FAD, FMN, and riboflavin plus phosphate all stimulate activity. However, these flavins also inactivate the methanococcal AHAS during preincubations in the absence of pyruvate and potentiate the sensitivity to O_2. Therefore, it is not possible in the absence of additional information to assign a specific function at this time.

The O_2 inactivation of the methanococcal AHAS is remarkable. Because the properties of the inactivation during the preincubation and the assay are different, more than one mechanism may be involved. In the preincubation without pyruvate, the sensitivity to O_2 decreases during the purification, high levels of O_2 are required for inactivation, and the inactivation is relatively slow. When the enzyme is preincubated with flavins, the sensitivity to O_2 increases dramatically. Therefore, it is possible that the inhibition by O_2 is due to the oxidation of a tightly bound flavin on the enzyme or to a flavin-mediated oxidation. Consistent with this hypothesis, the partially purified enzyme does not require flavins for activity, suggesting that this coenzyme is already bound, and is much more sensitive to O_2. However, because of the small amounts of enzyme available, this hypothesis could not be tested directly. During the assay, the methanococcal enzyme is sensitive to much lower concentrations of O_2, and the inactivation is very rapid. O_2 also slowly inactivates the enteric AHAS II during catalysis (23). In this case, inactivation is reversed by dialysis, and formation of the thiazolone derivative of TPP is the suspected mechanism of inactivation. For the methanococcal synthase, inhibition is not reversed by dialysis, suggesting that another mechanism may be involved.

In general, the methanococcal AHAS closely resembles those from bacteria, yeasts, and plants. These enzymes also show high similarity in their amino acid sequences, subunit molecular weights, cofactor requirements, feedback inhibitions, and sensitivities to sulfonylurea herbicides. Differences may indicate specific adaptations of the methanococcal AHAS or inherited properties which have been lost in other lineages. For example, the small subunit is not present in the methanococcal and eucaryotic AHASs and may represent a relatively modern elaboration of the eubacterial lineage. Likewise, the extreme oxygen sensitivity of the methanococcal AHAS may represent an ancestral trait conserved in this strictly anaerobic bacterium. If so, the AHASs from other anaerobic bacteria may prove to be O_2 sensitive. In conclusion, AHAS appears to be very ancient and to have acquired substantial diversity during its biological evolution.

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