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Clostridium pasteurianum F_1F_o ATP Synthase: Operon, Composition, and Some Properties

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The *atp* operon encoding F_1F_o ATP synthase in the fermentative obligate anaerobic bacterium *Clostridium pasteurianum* was sequenced. It consisted of nine genes arranged in the order *atpI*(i), *atpB*(a), *atpE*(c), *atpF*(b), *atpH*(δ), *atpA*(α), *atpG*(γ), *atpD*(β), and *atpC*(ε), which was identical to that found in many bacteria. Reverse transcription-PCR confirmed the presence of the transcripts of all nine genes. The amount of ATPase activity in the membranes of *C. pasteurianum* was low compared to what has been found in many other bacteria. The F_1F_o complexes solubilized from membranes of *C. pasteurianum* and *Escherichia coli* had similar masses, suggesting similar compositions for the F_1F_o complexes from the two bacteria. Western blotting experiments with antibodies raised against the purified subunits of F_1F_o detected the presence of eight subunits, α, β, γ, δ, ε, a, b, and c, in the F_1F_o complex from *C. pasteurianum*. The F_1F_o complex from *C. pasteurianum* was activated by thiocyanate, cyanate, or sulfhydryl compounds; inhibited by sulfite, bisulfite, or bicarbonate; and had tolerance to inhibition by dicyclohexylcarbodiimide. The target of thiol activation of the F_1F_o complex from *C. pasteurianum* was F_1. Thiocyanate and sulfite were noncompetitive with respect to substrate Mg ATP but competitive with respect to each other. The F_1 and F_o parts of the F_1F_o complexes from *C. pasteurianum* and *E. coli* bound to each other, but the hybrid F_1F_o complexes were not functionally active.

The F_1F_o ATP synthase is a multisubunit enzyme complex found exclusively in cytoplasmic membranes of bacteria, inner membranes of mitochondria, and thylakoid membranes of chloroplasts (4, 14, 54, 59). It synthesizes ATP from ADP and inorganic phosphate (P_i), utilizing the transmembrane chemiosmotic energy of a proton or sodium gradient (4, 14, 18, 59). Reversibly, it hydrolyses ATP to ADP and P_i which, when coupled to proton extrusion, generates chemiosmotic energy (4, 59). Structurally, the enzyme consists of two parts, a membrane-intrinsic F_o and a membrane-extrinsic F_1. When detached from the membrane, F_1 functions exclusively as ATP hydrolase. The most investigated bacterial F_1F_o complex is that from *Escherichia coli* (F_1F_{oEc}) (18, 19, 21). It consists of eight subunits, with a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10-12}$ (21). The α, β, γ, δ, and ε subunits constitute the F_1 moiety, and the a, b, and c subunits constitute the F_o moiety. All eight subunits are essential for the function of the enzyme complex (14, 58).

The *atp* operon of the *E. coli* ATP synthase consists of nine genes arranged in the order *atpI*(i), *atpB*(a), *atpE*(c), *atpF*(b), *atpH*(δ), *atpA*(α), *atpG*(γ), *atpD*(β), and *atpC*(ε) (64). A similar composition of the *atp* operon has been reported for the gram-positive, obligate anaerobic bacteria *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*) (11) and *Clostridium acetobutylicum* (GenBank accession no AAD16419). Some anaerobic bacteria have *atp* operons consisting of only eight genes. They lack the first gene, *atpI*, encoding subunit i. Examples are *Enterococcus hirae* (*Streptococcus faecalis*) (GenBank accession no M90060), *Lactobacillus lactis* (GenBank accession no AF059739), and *Lactobacillus acidophilus* (GenBank accession no AF098522). The *atp* operon of the anaerobic acetogenic bacterium *Acetobacterium woodii* consists of 11 genes, including those normally found in bacterial *atp* operons, plus two additional copies of *atpE*, encoding the c subunit (55). The *A. woodii* ATP synthase is sodium rather than proton dependent. It should be noted that the product of the first gene of the *atp* operon, *atpI*, has not been found in any purified F_1F_o complex (14).

Early work indicated that the F_1F_o complex purified from the gram-positive, obligate anaerobic bacterium *Clostridium pasteurianum* (F_1F_{oCp}) could function with only four polypeptides with apparent molecular masses of 65,000, 57,500, 43,000, and 15,000 Da (8, 9). The last polypeptide was not found in the purified F_1 ATPase, and it was identified as the dicyclohexylcarbodiimide (DCCD)-binding proteolipid or the c subunit (8, 9). Due to this simple composition, F_1F_{oCp} was postulated to be an ancient version of ATP synthase (25, 45). In this study, we show that the composition of the F_1F_{oCp} complex, at both the genetic and the protein levels, is very similar to those of the F_1F_o complexes from other bacteria (14, 23, 27, 41, 64). Inactivation by sulfite and activation by thiocyanate and sulfhydryl compounds of F_1F_{oCp} are also reported.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. pasteurianum* strain DSM 525 was obtained from G. Gottschalk, University of Göttingen, Göttingen, Germany. It was grown in a semidefined liquid medium containing 1.0% glucose or glycerol as a carbon source in 3.5-liter batch cultures in 4-liter flasks at 37°C under 100% N_2 or CO_2 (34). Cells were harvested at the late log phase (after 16 to 18 h of growth; optical density at 600 nm, 1.2 to 1.5) by centrifugation at 6,000 × *g*, washed in TMG buffer (100 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 10% glycerol), and stored at −80°C until used. *Moorella thermoacetica* (ATCC 39073) was grown in a semidefined liquid medium with 1% glucose as a carbon source at

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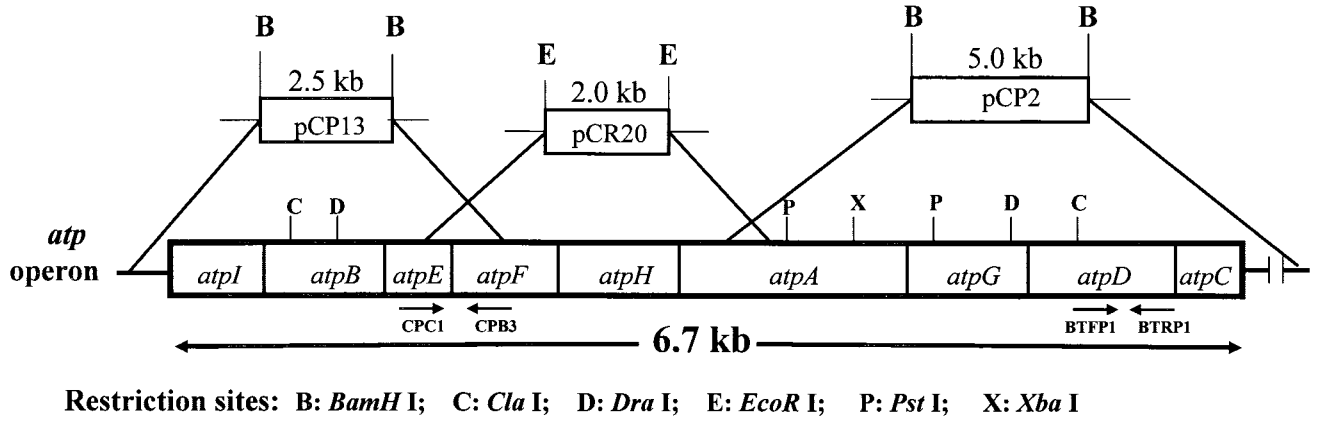

FIG. 1. Cloning and sequencing strategy. The *atp* operon of *C. pasteurianum* was sequenced from three plasmids, pCp2, pCp13, and pCR20. Plasmids pCp2 and pCp13 were obtained from the genomic library after screening with DIG-labeled PCR products, and pCR20 was obtained by cloning into pCR2.1 of a 2.0-kb PCR product containing *atp* genes amplified from *C. pasteurianum* genomic DNA. The primers used for PCR are indicated below the target genes.

58°C under 100% CO_2 (12). The following *E. coli* strains were used: strain DH5α was used as a reference for ATP synthase, strain INVαF′ (Invitrogen, Carlsbad, Calif.) was used as a host for plasmids carrying PCR clones, and ATP synthase-negative (Δ*unc*) mutant DK8 (35) (obtained from M. Futai, Osaka University, Osaka, Japan) was used as a host to screen the genomic library of *C. pasteurianum* in plasmid pBR322. All *E. coli* strains were grown and maintained in either Luria-Bertani broth or M9ZB salt medium with glucose (20 mM) as a carbon source (13).

Antibodies used. All antibodies used in this study were polyclonal. Antibodies against purified *M. thermoacetica* F_1 ATPase (F_{1Mt}) (12) and against a 20-mer synthetic peptide designed from the NH_2-terminal end of the δ subunit of F_{1Mt} (11) were raised in rabbits at the Animal Facility of the University of Georgia. Antibodies against purified subunits γ and ε of F_1 ATPase of *E. coli* (F_{1Ec}) were obtained from R. Aggeler, University of Oregon. Antibodies against purified subunits a, b, and c of F_{oEc} were obtained from G. Hebestreit-Deckers, University of Osnabrück, Osnabrück, Germany.

DNA source and *C. pasteurianum* genomic library. Genomic DNA of *C. pasteurianum* was isolated by the method of Marmur (46). The *C. pasteurianum* genomic library was a gift from G. Sawers, John Innes Centre, Norwich, United Kingdom. It was constructed in pBR322 by ligating partial *Sau*3AI digests of *C. pasteurianum* genomic DNA into the *Bam*HI site of the plasmid (65).

ATP synthase probes used to screen the *C. pasteurianum* genomic library. The cloning and sequencing strategy is outlined in Fig. 1. The *atp* operon of *C. pasteurianum* was sequenced from three plasmids, pCP2, pCP13, and pCR20. Plasmids pCP2 and pCP13 were isolated from the genomic library after screening with 390- and 490-bp digoxigenin (DIG)-labeled ATPase probes amplified by PCR (see below). Plasmid pCR20 was constructed by cloning a 2.0-kb PCR product containing *atp* genes into pCR2.1 (Invitrogen). All PCR products were amplified with *C. pasteurianum* genomic DNA as a template. The following primers were used for amplification of the above PCR products: BTFP1 and BTRP1 for the 390-bp product, CPC1 and CPB3 for the 490-bp product, and CPC1 and CPAL2 for the 2.0-kb PCR product (Table 1). BTFP1 and BTRP1 were degenerate primers, designed from highly conserved amino acid sequences (^{191}ERTREGND^{198} and ^{311}YVPADDLTD^{319}, respectively) of the F_{1Ec} β subunit (11, 61). CPC1 was designed from a highly conserved sequence (ARQPEA) of the *E. coli* ATP synthase c subunit, while CPAL2 and CPB3 were designed from known sequences of *atpA* and *atpF* encoding the α and b subunits of the F_1F_{oCp} complex, respectively. The reaction conditions for PCR were as follows: an initial denaturation at 94°C for 6 min followed by 25 cycles of amplification, with each cycle consisting of 60 s of denaturation at 94°C, 60 s of annealing at 45°C, and 120 s of extension at 72°C. All PCR products were sequenced after being cloned into vector pCR2.1.

RNA isolation and RT-PCR. Total RNA was isolated from whole cells of *C. pasteurianum* with a Qiagen RNeasy midi-kit. RNA was treated with RNase-free DNase I (Roche Molecular Biochemicals, Indianapolis, Ind.) prior to use in hybridization or reverse transcription (RT)-PCR experiments. For RT-PCR experiments, total RNA (2 μg) was treated with DNase I and reverse transcribed into cDNA with either Superscript (Bethesda Research Laboratories, Rockville, Md.) or Omniscript (Qiagen) reverse transcriptase according to the manufacturer's directions. The cDNA synthesized in these reactions was used as a template in PCRs to amplify different *atp* genes.

Hybridization experiments. Colony and Northern hybridization experiments were carried out with the nonradioactive Genius System (Roche Applied Science, Indianapolis, Ind.) and DIG-labeled PCR products as probes (11, 13). RNA samples were denatured with glyoxal (5) prior to use in Northern hybridization experiments.

Membrane preparation and ATPase assays. Membranes of *M. thermoacetica* and *E. coli* DH5α were prepared by breaking cells in a French press (12). Membranes of *C. pasteurianum* were prepared by a modified lysozyme method (8). Briefly, cell paste (30 to 40 g [wet weight]) was suspended in 150 ml of lysis buffer (100 mM Tris-HCl [pH 8.0], 0.5 M sucrose) containing lysozyme (2.0 mg/ml) and incubated at 37°C for 1 h. Highly viscous cell lysate obtained at this step was centrifuged at 10,000 × *g* for 10 min. The supernatant was discarded, and the pellet containing protoplasts was suspended in TMG buffer containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), DNase I, and RNase I (0.2 mg/ml each) and passed through a French press. Intact cells and cell debris were removed by centrifugation at 6,000 × *g* for 10 min, and the supernatant was centrifuged at 100,000 × *g* for 1 h. The pellet containing membranes was washed twice and then suspended in TMG buffer containing 1 mM Na ATP. The ATPase (ATP hydrolysis) activity was assayed at 37°C with a reaction mixture containing100 mM Tris-HCl (pH 8.0) and 5 mM $MgCl_2$ and with 2 mM Na ATP or Mg ATP as a substrate. The reaction was started by adding ATP and stopped by adding sodium dodecyl sulfate (SDS) to a final concentration of 1%. P_i released by ATP hydrolysis was measured colorimetrically as described previously (12).

TABLE 1. Primers for PCR and RT-PCR experiments

Primer	Gene	Sequence (5′→3′)
BTFP1	*atpD*	GA(AG)CAG(TGA)AC(TAG)(AC)G(TGA)GA(AG)GG(TAA)AA(TC)G
BTRP1	*atpD*	GTI(AG)(AG)TC(AG)TC(ATC)GC(TAC)G(AT)AC(AG)TA
CPAL2	*atpA*	CTCTTGCTGGTGGTCTTC
CPC1	*atpE*	GC(AT)CG(AT)CA(AT)CC(AT)GAAGC
CPB3	*atpF*	TAAGTTCTGCCTCATTGG
CPBTP1	*atpD*	ATAGAGCAAGAAGGATTC
CPBTP2	*atpD*	CATACAAAATCACTCCTT
CPIP1	*atpI*	AGTTATAGCAGGATTGAT
CPIP2	*atpI*	TTAATCACTTCCTTTCTT
CPAP1	*atpB*	TACTTGAAAAATTTACGC
CPAP2	*atpB*	TAGTTATTCTTCCTCTGC
CPBP1	*atpF*	GGAAGAGCTGAGAAATTA
CPBP2	*atpF*	GCTACAGCATATCTCCTA
CPEP1	*atpC*	ATCTACAACTTCTGGTGG
APEP2	*atpC*	AATCCTTTCTTCTGCTCT

Unless otherwise stated, membranes used in all ATPase assays were prepared from glucose-grown cells. For inhibition or activation studies, reaction mixtures containing the enzyme were incubated with the activator or inhibitor at 37°C for 10 min prior to ATPase assays. Each experiment was carried out in triplicate, and the reported results are the average of three independent experiments with a standard deviation of ±0% to 10%.

Solubilization of F_1F_o and F_1 from membranes of *C. pasteurianum* and *E. coli*. Washed membranes (4 to 5 mg/ml) of *C. pasteurianum* and *E. coli* were suspended in TMG buffer containing 1 mM Na ATP and 0.5 mM PMSF. For the solubilization of F_1F_o, *n*-dodecyl-β-maltoside (DM) (Anatrace, Maumee, Ohio) was added to the membrane suspension to a final concentration of 1% (wt/vol). The suspension was incubated on ice for 30 min and then centrifuged at 100,000 × *g* for 1 h. The pellet was discarded, and the supernatant containing F_1F_o was saved. To concentrate F_1F_o, the enzyme was precipitated with 60% ammonium sulfate in the presence of 1% Na cholate as described by Hicks and Krulwich (27). The precipitate containing mostly F_1F_o was dissolved in TMG buffer containing 0.02% DM and 1 mM Mg ATP, and the solution was dialyzed against three changes of the same buffer. Solubilization of F_1 from washed membranes of *E. coli* (10, 60) and *C. pasteurianum* (8) was carried out by extraction with buffer containing EDTA. Soluble F_1 ATPase of *C. pasteurianum* (F_{1Cp}) and F_{1Ec} were concentrated on YM100 membranes with an Amicon concentrator and partially purified by gel filtration on TSK gel G3000SW (TosoHaas, Montgomeryville, Pa.) by using a fast protein liquid chromatography system (Amersham Pharmacia, Piscataway, N.J.). Fractions with the highest ATPase activity were pooled, dialyzed against TMG buffer containing 1 mM Na ATP, and concentrated as described above.

In vitro reconstitution of F_1-depleted membranes with exogenously added F_1. Washed membranes of *C. pasteurianum* and *E. coli* were stripped of F_1 by repeated extractions with EDTA buffer (8, 10, 60). Stripped membranes were washed and suspended (2 mg of protein per ml) in reconstitution buffer (50 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol [vol/vol], 0.1 mM PMSF). Partially purified F_{1Cp} or F_{1Ec} was added to F_1-depleted membranes, and the mixture was incubated at 37°C for 30 min and centrifuged at 100,000 × *g* for 1 h. The pellet containing reconstituted membranes was washed twice in reconstitution buffer prior to ATPase assays. Maximum binding of F_1 to F_1-depleted membranes occurred at a ratio of 1 U of F_1 per 1 mg of F_1-depleted membranes, irrespective of their origins. This ratio was maintained in all reconstitution reactions.

Other methods. Protein was estimated by using the Lowry method as described previously (12). Polyacrylamide gel electrophoresis (PAGE) in the presence (denatured) or absence (native) of SDS was carried out as described by Laemmli (40). Western blotting experiments were carried out according to the instructions of Bio-Rad (Hercules, Calif.) (12, 13). The ATP-driven proton-pumping activities of intact and reconstituted membranes were determined by measuring the quenching of fluorescent dye acridine orange as described by Moriyama et al. (48). The reaction mixture (3.0 ml) contained 10 mM Tricine-choline (pH 8.0), 140 mM KCl, 5 mM $MgCl_2$, 1 μg of valinomycin/ml, 1 μM acridine orange, and membranes (50 to 300 μg of protein). The reactions started by adding Na ATP (1 mM) and the changes in fluorescence (emission, 530 nm; excitation, 490 nm) were recorded with a Shimadzu fluorescence spectrometer (model RF2150). Standard techniques were used for all DNA manipulation experiments (57). Oligonucleotide synthesis and DNA sequencing reactions were carried out at the Molecular Genetics Instrumentation Facility of the University of Georgia.

Nucleotide sequence accession number. The nucleotide sequence of the *atp* operon of *C. pasteurianum* has been assigned accession number AF283808 in the GenBank, EMBL, and DDBJ libraries.

RESULTS

Cloning, sequencing, and analysis of the primary structure of ATP synthase subunits encoded by the *C. pasteurianum atp* operon. Fig. 1 outlines the strategy used to clone and sequence the *C. pasteurianum atp* operon. This operon contains nine open reading frames (ORFs) (ORF1 through ORF9) within a 7.5-kb DNA fragment. The deduced amino acid sequences encoded by ORF2 to ORF9 shared between 22 and 72% identical residues with the corresponding sequences for the eight subunits of ATP synthases from several sources. The deduced amino acid sequence encoded by ORF1 had 19% residues identical to those encoded by the first gene, *atpI*, of the *E. coli* *atp* operon, indicating that it corresponded to *atpI*. The nine genes of the *C. pasteurianum atp* operon are arranged in the order *atpI*(i), *atpB*(a), *atpE*(c), *atpF*(b), *atpH*(δ), *atpA*(α), *atpG*(γ), *atpD*(β), and *atpC*(ε), an organization identical to those of the *atp* operons of many bacteria (11, 23, 55, 64).

All *atp* genes have ATG start codons. The reading frames of all *atp* genes were AT rich, with an average GC content of 32%. A putative promoter structure with the sequence 5′-GT TGAAA-N_{17}-TCTAAT-3′, resembling the *E. coli* consensus σ^{70} promoter sequence 5′-cTTGACA-N_{17-21}-TATAaT-3′, was found 48 bp upstream of *atpI*. Long intergenic regions were found between *atpB* and a*tpE* (88 bp) and between *atpE* and *atpF* (39 bp). No secondary promoter structure was apparent in these intergenic regions. The deduced molar masses (Da) of the subunits were as follows: i, 13,818; a, 25,634; c, 8,374; b, 18,466; δ, 20,702; α, 54,943; γ, 31,300; β, 50,766; and ε, 11,866. With the exception of that for the ε subunit, these masses are comparable to those of the corresponding ATP synthase subunits from other sources (11, 27, 55, 64). The F_{1Ec} ε subunit has 139 residues, while the F_{1Cp} ε subunit has 108 residues, lacking 31 residues at the carboxyl-terminal end. Deletion mutation of the *E. coli* ε subunit has shown that this segment is not necessary for the function of the enzyme (37). The natural absence of this segment at the COOH-terminal end of the *C. pasteurianum* ATP synthase ε subunit supports this finding.

The deduced amino acid sequences for the α, β, and c subunits of the *C. pasteurianum* ATP synthase are more conserved (between 40 and 72% identical residues) than those for the a, b, γ, δ, and ε subunits (between 22 and 39% identical residues) relative to sequences for the corresponding ATP synthases from other origins. Several motifs and residues of the highly conserved α, β, and c subunits are apparent. They include the nucleotide-binding domain or Walker motifs (^{169}G DRQTGKT^{176} in the α subunit and ^{152}GGAGVGKT^{159} in the β subunit), the so-called γ-subunit-interacting (^{383}DELSEED^{389}) region of the β subunit, the ^{43}ARQP^{46} sequence in the hydrophilic loop of the c subunit, and the DCCD-binding acidic residue E^{64} of the c subunit (1, 19, 64).

The *atp* operon of *C. pasteurianum* is transcribed into a single polycistronic mRNA. Initially, total *C. pasteurianum* RNA was subjected to Northern hybridization experiments with different ATP synthase probes, including those used in the screening of the genomic library. The hybridization signals were very weak and almost undetectable. Increasing the amount of RNA or varying the stringency of hybridization conditions had little effect on the hybridization signals. Therefore, we used more sensitive RT-PCR experiments to verify the transcripts of the *atp* operon of *C. pasteurianum*. Total RNA (2 μg) was treated with RNase-free DNase and reverse transcribed with primer CPEP2 (Table 1), designed on the basis of the 3′ end of *atpC*, the last gene of the *atp* operon (Fig. 1). The cDNA synthesized from this reaction was used as a template in PCR to amplify *atpI*, *atpB*, *atpF*, and *atpC*. These genes were targeted because their products were reported to be absent from the purified ATP synthase from *C. pasteurianum* (8, 9). The gene-specific primers used in PCR were CPIP1 and CPIP2 for *atpI*, CPAP1 and CPAP2 for *atpB*, CPBP1 and CPBP2 for *atpF*, and CPEP1 and CPEP2 for *atpC*. A control PCR was carried out for each set of primers with DNase-treated RNA as a template. PCR products of the expected length (~200 bp) were amplified in reactions with cDNA as a template (data not

TABLE 2. ATPase activities of MB and soluble F_{1Cp} or F_1F_{oCp} after extraction with EDTA and detergent[a]

Treatment	Protein (mg)	Activity[b]		Activity ($U \cdot mg^{-1}$) in the presence of[c]:	
		U	$U \cdot mg^{-1}$	DCCD (100 μM)	Na azide (1 mM)
None	9.40	0.800	0.085	0.045	0.024
Membrane + DM					
Residual membrane	4.39	0.108	0.024	0.020	0.019
Extracts (F_1F_o)	5.24	1.198	0.228	0.125	0.073
Membrane + EDTA					
Residual membrane	7.32	0.240	0.033	0.022	0.020
Extracts (F_1)	2.22	0.942	0.424	0.330	0.135

[a] For solubilization of F_1F_o, washed membranes (2 mg/ml) were suspended in TMG buffer containing Na ATP (1 mM), DM (1% [wt/vol]), and PMSF (0.1 mM); the suspension was incubated on ice for 1 h and centrifuged at 100,000 × *g* for 1 h. The supernatant containing soluble F_1F_o was collected, and the pellet containing residual membranes was washed and resuspended in TMG buffer. For solubilization of F_1, washed membranes (1 mg/ml) were subjected to similar treatments, except that the membranes were suspended in 100 mM Tris-HCl (pH 8.0)–15 mM EDTA–2 mM LiCl–1 mM ATP. EDTA was removed from the extracts by dialysis against TMG buffer containing 1 mM Na ATP. The pellet containing residual membranes was washed and resuspended in TMG buffer. ATPase assays were done with Na ATP (2 mM) as a substrate.

[b] One unit of activity is defined as 1 μmol of P_i released per min.

[c] DCCD or Na azide was added to assay mixtures, which were incubated at 37°C for 10 min prior to the start of ATPase assays by the addition of ATP.

TABLE 3. Influence of different effectors of ATPase on activities of MB F_1F_{oCp} and F_1F_{oEc}[a]

Detergent or anion	Results for membranes from:			
	C. pasteurianum		*E. coli*	
	$U \cdot mg^{-1}$	%	$U \cdot mg^{-1}$	%
None	0.080	100	0.328	100
$NaHCO_3$ (20 mM)	0.057	71	0.406	125
Na_2SO_3 (20 mM)	0.009	12	0.436	133
$Na_2S_2O_5$ (20 mM)	0.016	26	0.360	110
NaSCN (20 mM)	0.156	195	0.138	42
KOCN (20 mM)	0.102	128	0.065	20
Methanol (10%)	0.114	143	0.456	139

[a] ATPase assays were done with Na ATP (2 mM) as a substrate as described in Materials and Methods. Effectors were incubated with membranes in assay mixtures at 37°C for 10 min prior to the start of ATPase assays.

shown) but not in those with DNase-treated RNA as a template. These results suggest that the PCR products were amplified exclusively from cDNA, which was apparently synthesized from full- to partial-length polycistronic transcripts of the *atp* operon of *C. pasteurianum*, as reported for other bacteria (11, 55).

Solubilization of F_1F_{oCp} and F_{1Cp} from membranes by extraction with detergent and EDTA. Washed membranes (9.4 mg of protein) of *C. pasteurianum* were extracted with DM and EDTA to solubilize F_1F_o and F_1, respectively. The total ATPase activities solubilized from membranes by DM (1.198 μmol of $P_i \cdot min^{-1}$) and EDTA (0.942 μmol of $P_i \cdot min^{-1}$) were found to be higher than that of the same amount of untreated membranes (0.080 μmol of $P_i \cdot min^{-1}$) (Table 2). A similar increase in the activity of soluble F_1F_{oCp} was observed with other detergents, e.g., Triton X-100 (1% [vol/vol]) and octylglucopyranoside (1% [wt/vol]). The increase in the ATPase activity of F_1F_o after solubilization from membranes by DM has been reported for other bacteria (27). The increase in the activity of F_{1Cp} after solubilization from membranes by EDTA is unusual, but a similar finding has been reported for ATPase in *E. coli* mutants with abnormalities in the functions of the F_o moiety of the enzyme complex (39). DCCD and Na azide inhibited both membrane-bound (MB) and soluble F_1F_{oCp} or F_{1Cp} (Table 2). The levels of inhibition by DCCD (100 μM) were 53% for MB F_1F_{oCp} and 55% for soluble F_1F_{oCp}. The levels of inhibition by Na azide (1 mM) were 72% for MB F_{1Cp} and 68% for soluble F_{1Cp}. In contrast, the levels of inhibition of MB F_{1Ec} (or F_1F_{oEc}) at the above concentrations of DCCD and Na azide were between 92 and 98%. A higher tolerance of MB and soluble F_1F_{oCp} or F_{1Cp} to inhibition by DCCD and Na azide is not unusual, as similar results were reported for several gram-positive bacteria (3, 26, 28, 32, 49). A comparison of the ATPase activities of DM- and EDTA-extracted membranes with that of untreated membranes indicated that about 87 and 70% of ATPase activities were solubilized from membranes by DM and EDTA, respectively (Table 2). The residual ATPase activities of DM- and EDTA-extracted membranes were more tolerant to inhibition by DCCD or Na azide than that of untreated membranes; therefore, these activities could be attributed to other types of ATPases.

Low level of the F_1F_o complex in *C. pasteurianum* membranes. Washed membranes of *C. pasteurianum* had a low level of ATPase activity, 0.082 μmol · $min^{-1} \cdot mg^{-1}$, about one-fourth that of *E. coli* membranes, 0.328 μmol · $min^{-1} \cdot mg^{-1}$. To test whether the enzyme was latent or not, washed membranes of *C. pasteurianum* were subjected to treatments that are known to activate MB F_1F_o. Limited proteolysis with trypsin had no effect, while treatment with detergents or anions had a marginal effect on the activity of MB F_1F_{oCp} (Table 3), suggesting that the level of MB F_1F_{oCp} could be low; this notion is further supported by the poor transcription of the *atp* genes of *C. pasteurianum*, as the *atp* transcripts were almost undetectable on Northern blots. For further verification, whole-cell extracts of *C. pasteurianum*, *E. coli*, and *M. ther-*

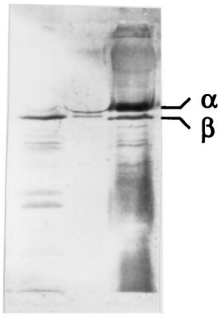

FIG. 2. Immunoblots of whole-cell extracts (50 μg of protein) from *E. coli* (Ec), *C. pasteurianum* (Cp), and *M. thermoacetica* (Mt) after reaction with antibodies against purified F_{1Mt}. Proteins were separated by SDS-PAGE (10% acrylamide) and transblotted onto polyvinylidene difluoride membranes prior to the immunoreactions.

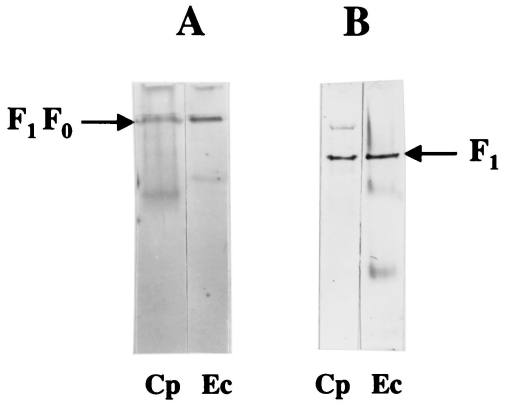

FIG. 3. Immunoblots of soluble F_1F_o (A) and F_1 (B) from *C. pasteurianum* (Cp) and *E. coli* DH5α (Ec) after reactions with antibodies against purified F_{1Mt}. (A) Soluble F_1F_{oCp} after precipitation with ammonium sulfate from DM extracts (1.0% [wt/vol]) of *C. pasteurianum* membranes (30 μg) and soluble F_1F_{oEc} in DM extracts (1.0% [wt/vol]) of *E. coli* membranes (30 μg). (B) Partially purified F_{1Cp} (30 μg) and F_{1Ec} (30 μg). Proteins were separated by native PAGE (7.5% acrylamide) and transblotted onto polyvinylidene difluoride membranes prior to immunoreactions.

moacetica were subjected to Western blotting experiments with antibodies raised against purified F_{1Mt}. The antibodies reacted with the α and β subunits of F_{1Cp} and F_{1Mt} but only with the β subunit of F_{1Ec} (Fig. 2), indicating the antigenic similarity of the latter subunit in the three bacteria. Strong antigenic similarity of the β subunit of F_1 has also been reported for other bacteria (42, 53, 60). The β subunit of F_{1Mt} was more like the β subunit of F_{1Cp} (73% identical residues) than the β subunit of F_{1Ec} (68% identical residues). The intensity of the immunosignal corresponding to the β subunit was much stronger in *E. coli* extracts than in *C. pasteurianum* extracts (Fig. 2). Since the level of the β subunit reflects the amount of the F_1F_o complex, these results support a low level of F_1F_o in *C. pasteurianum*.

F_1F_{oCp} has a more complex subunit composition than previously reported. The F_{1Cp}, F_1F_{oCp}, F_{1Ec}, and F_1F_{oEc} complexes solubilized from membranes were subjected to native PAGE and Western blotting experiments with antibodies raised against purified F_{1Mt} (12). Immunosignals of F_{1Cp} and F_1F_{oCp} corresponded to the signals of F_{1Ec}, and F_1F_{oEc}, respectively (Fig. 3), suggesting that these complexes have similar masses and perhaps similar compositions. The antibodies against purified F_{1Mt} demonstrated the presence of the α and β subunits of F_1F_{oCp} (Fig. 2). To identify other subunits of F_1F_{oCp}, antibodies against purified subunits γ, ε, a, b, and c of F_1F_{oEc} and those against a synthetic peptide (^{1}MSENQNVA RRYARALFNIARE^{20}) designed on the basis of the NH_2-terminal end of the δ subunit of F_{1Mt} were used (11). Nine residues of this peptide (^{8}RRYAXALXNIA^{19}) were identical to the corresponding sequence at the NH_2-terminal end of the F_{1Cp} δ subunit. Figure 4A to F show that the antibodies against each of the purified ATP synthase subunits reacted with the corresponding subunits of the F_1F_{oCp} complex, demonstrating the presence of all eight subunits typically found in bacterial ATP synthases. The interactions between the antigen and the corresponding antibodies were much weaker for the subunits of F_{oCp} than the subunits of F_{1Cp}, a result which is not unusual, as reported for other bacteria (60). A comparison of the molar masses of the F_1F_{oCp}, F_1F_{oEc}, and *M. thermoacetica* F_1F_o subunits, as shown on Western blots, indicated the expected similarities in the masses for subunits α, β, γ, δ, and c from the three bacteria and the expected differences in the masses for subunits a, b, and ε.

F_1F_{oCp} is activated by thiocyanate and inhibited by sulfite. MB F_1F_{oCp} responded differently to anions than did MB F_1F_{oEc}. Sulfite (SO_3^{2-}) and bisulfite ($S_2O_5^{2-}$) activated MB F_1F_{oEc} but inhibited MB F_1F_{oCp} (Table 3). On the other hand, thiocyanate and cyanate activated F_1F_{oCp} but inhibited F_1F_{oEc} (Table 3 and Fig. 5). Thiocyanate treatment did not release F_1 from *C. pasteurianum* membranes, suggesting that the activation was not due to the solubilization of the enzyme. The activation of F_1F_{oCp} by thiocyanate or cyanate and its inhibition by sulfite or bisulfite were reproducible with different preparations of membranes from cells grown on either glucose or glycerol as a carbon source. Soluble F_1F_{oCp} and soluble F_{1Cp}

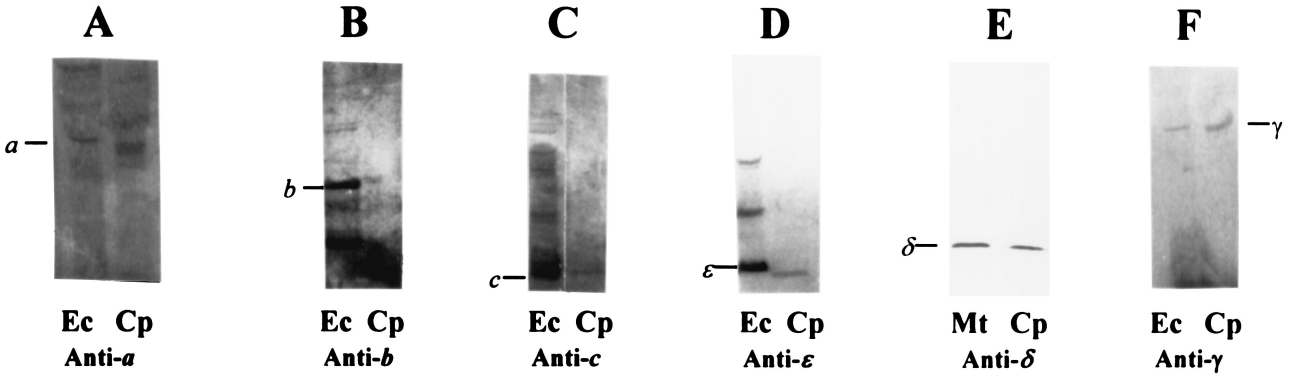

FIG. 4. Immunoblots of concentrated DM extracts of *C. pasteurianum* membranes (Cp, 60 μg of protein) and whole-cell extracts of *E. coli* (Ec, 30 μg of protein) and *M. thermoacetica* (Mt, 30 μg of protein) after reactions with antibodies against ATP synthase subunits a (A), b (B), c (C), and ε (D) of F_1F_{oEc}, subunit δ of F_{1Mt} (E), and purified γ of F_{1Ec} (F). DM extracts of *C. pasteurianum* membranes were concentrated by precipitation with ammonium sulfate as described in Materials and Methods. Proteins were separated by SDS-PAGE (10% acrylamide) and transblotted onto polyvinylidene difluoride membranes prior to immunoreactions.

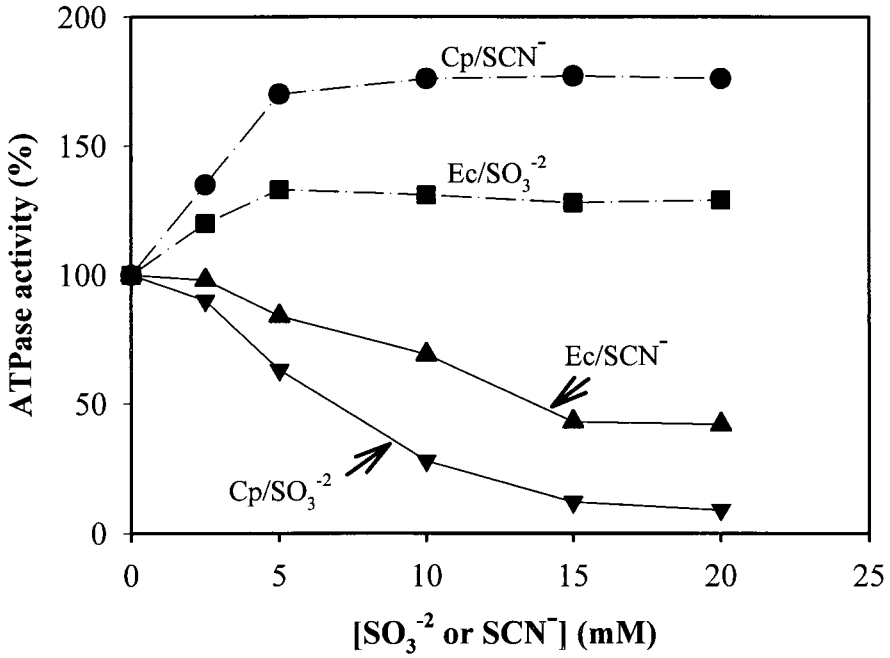

FIG. 5. Effect of NaSCN and Na_2SO_3 on ATPase activities of MB F_1F_{oCp} and F_1F_{oEc}. Washed membranes were incubated with NaSCN or Na_2SO_3 at the indicated concentrations in ATPase assay reaction mixtures at 37°C for 10 min prior to ATPase assays. The ATPase activities of untreated membranes of *C. pasteurianum* (0.080 μmol of $P_i \cdot min^{-1} \cdot mg^{-1}$) and *E. coli* (0.333 μmol of $P_i \cdot min^{-1} \cdot mg^{-1}$) were taken as 100% for calculation of the percent activation or inhibition of ATPase activity by NaSCN or Na_2SO_3. Symbols: ●, activation of MB F_1F_{oCp} by NaSCN; ■, activation of MB F_1F_{oEc} by Na_2SO_3; ▲, inhibition of MB F_1F_{oCp} by Na_2SO_3; ▼, inhibition of MB F_1F_{oCp} by Na_2SO_3.

also exhibited activation or inhibition by oxyanions similar to that of MB F_1F_{oCp} (Table 4). The thiocyanate activation or sulfite inhibition of F_1F_{oCp} at variable concentrations of the substrate (Mg ATP) followed Michaelis-Menten enzyme kinetics (Fig. 6). Double-reciprocal plots of the inhibition of ATPase activity versus fixed substrate concentrations ($[SO_3^-]$) were found to be noncompetitive with respect to Mg ATP (data not shown). The K_m for Mg ATP was 0.62 ± 0.03 mM (mean and standard deviation). A similar K_m in the presence of SCN^- or SO_3^{2-} suggested that the affinity of the enzyme for the substrate (Mg ATP) was not affected by either of these anions, a finding previously reported by others (16, 30, 50, 62). The K_is for sulfite inhibition, calculated from plots of the inhibition of ATPase activity versus $[SO_3^-]$ (Dixon plots; data not shown) were 3.1 and 2 mM in the presence and absence of 5 mM NaSCN, respectively. The increase in the K_i for the inhibitory anion in the presence of the activating anion is a normal response of F_1F_o ATPase also reported by others (16). It has been suggested that both activating and inhibiting anions compete for the same regulatory site(s) of F_1F_o complexes (30, 31, 44, 62). Accordingly, thiocyanate was found to antagonize the effect of sulfite on F_1F_{oCp} or vice versa, and sulfite was found to antagonize the effect of thiocyanate on F_1F_{oEc} or vice versa (data not shown).

TABLE 4. Effect of anions on ATPase activities of MB and soluble F_{1Cp} or F_1F_{oCp}[a]

Anion (mM)	Results for:					
	Membranes		Soluble F_{1Cp}[b]		Soluble F_1F_{oCp}[c]	
	$U \cdot mg^{-1}$	%	$U \cdot mg^{-1}$	%	$U \cdot mg^{-1}$	%
None	0.092	100	0.428	100	0.162	100
KSCN (10)	0.185	202	0.831	190	0.458	283
KOCN (10)	0.120	131	0.599	140	0.210	130
Na_2SO_3 (10)	0.015	16	0.128	30	0.040	25

[a] Anions were incubated in assay mixtures at 37°C for 10 min prior to the start of ATPase assays.
[b] Soluble F_{1Cp} was partially purified from EDTA extracts by gel filtration as described in Materials and Methods.
[c] Soluble F_1F_{oCp} was obtained from DM extracts of membranes after precipitation with ammonium sulfate as described in Materials and Methods.

Activation of F_1F_{oCp} by sulfhydryl reagents. Sulfhydryl compounds are known to activate F_1F_o from plants and photosynthetic bacteria (29, 51, 52). Here we show that the sulfhydryl compounds dithiothreitol (DTT), β-mercaptoethanol (β-ME), and cysteine activated both MB and soluble F_1F_{oCp} or F_{1Cp} (Table 5 and Fig. 7). None of these compounds had any effect on F_1F_{oEc} (data not shown). The activation of F_1F_{oCp} by sulfhydryl reagents increased in the following order: cysteine < ME < DTT. Maximum activation of the enzyme occurred in the presence of 0.2 mM β-ME and 1 mM DTT or cysteine. At higher concentrations, all sulfhydryl compounds were inhibitory, and the inhibition was significantly higher with β-ME than with DTT or cysteine (Fig. 7). The activation of soluble F_{1Cp} by sulfhydryl compounds was comparable to that of MB or soluble F_1F_{oCp} (Table 5), suggesting that F_1 is the target of thiol activation. Thiol activation of F_1 was previously reported only for enzymes from chloroplasts and cyanobacteria (29, 51, 52).

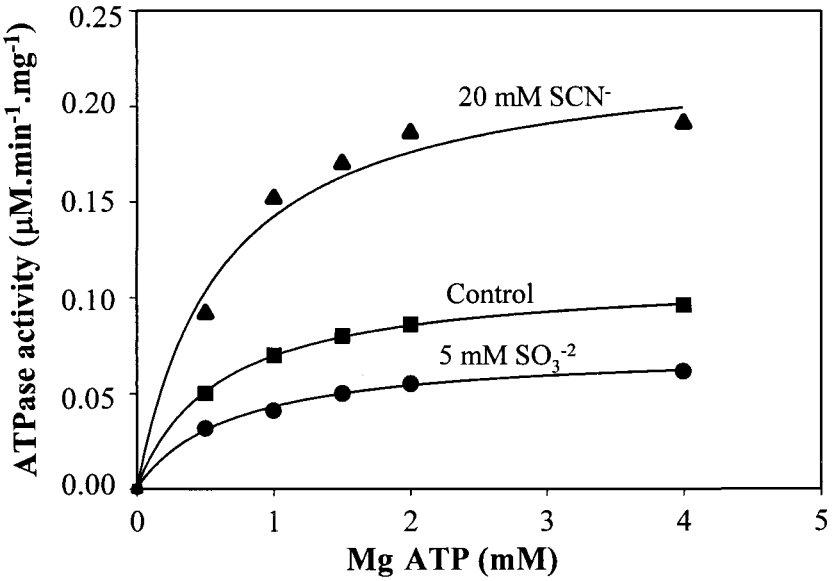

FIG. 6. ATPase activity of *C. pasteurianum* membranes at various concentrations of substrate (Mg ATP) in the absence (■) or presence of 20 mM NaSCN (▲) or 5 mM Na_2SO_3 (●).

The F_1 and F_o portions of the F_1F_{oCp} and F_1F_{oEc} complexes are not functionally compatible. Inhibition studies indicated that both MB F_1F_{oCp} and soluble F_1F_{oCp} are relatively tolerant to inhibition by DCCD compared to Na azide (Table 2). Washed membranes of *C. pasteurianum* failed to generate a proton gradient from ATP hydrolysis (data not shown). A general interpretation of these results is that ATP hydrolysis by F_1 could be uncoupled from proton translocation through F_o. To further investigate this notion, F_1-depleted membranes of *C. pasteurianum* (F_{oCp}) and *E. coli* (F_{oEc}) were reconstituted with F_1 from the above bacteria. DCCD-sensitive ATPase activity and ATP-driven proton pumping were observed only with reconstituted membranes consisting of F_{1Ec} and F_{oEc} (data not shown) and not with other combinations when F_1 and F_o were from different bacteria. Previously, we reported similar results for F_1 and F_o of the ATP synthases from *E. coli* and the gram-positive anaerobic bacterium *M. thermoacetica* (10). These results suggest that the F_1 and F_o portions of the ATP synthases from *E. coli* and gram-positive anaerobic bacteria are not functionally compatible, as previously reported (10).

TABLE 5. Effect of sulfhydryl reagents on ATPase activities of MB and soluble F_{1Cp} and F_1F_{oCp}[a]

Sulfhydryl reagent (mM)	Results for:					
	Membranes		F_{1Cp}		F_1F_{oCp}	
	U · mg^{-1}	%	U · mg^{-1}	%	U · mg^{-1}	%
None	0.078	100	0.436	100	0.156	100
DTT (2)	0.104	143	0.628	144	0.217	139
Cysteine (2)	0.105	141	0.610	140	0.212	136
β-ME (0.5)	0.096	124	0.545	125	0.193	124

[a] Sulfhydryl reagents were added to assay mixtures containing membranes, soluble F_{1Cp}, or soluble F_1F_{oCp}, and mixtures were incubated at 37°C for 10 min prior to the start of ATPase assays. Solutions of sulfhydryl reagents were prepared fresh before each experiment. Soluble F_1F_{oCp} and soluble F_{1Cp} were obtained as described in Materials and Methods.

DISCUSSION

This report is a correction of an earlier report that the functionally active F_1F_{oCp} complex consists of only four subunits instead of the eight commonly found in most bacteria. Here we have shown that F_1F_{oCp} had a composition similar to that of *E. coli* ATP synthase. The *atp* operon of *C. pasteurianum* consists of nine genes, like the *atp* operon of *E. coli*. The RT-PCR experiments confirmed the presence of all *atp* genes, including those encoding a, b, δ, and ε subunits, which were previously not found in purified F_1F_{oCp} (8, 9). Native PAGE analyses (Fig. 3) of intact F_1 and F_1F_o complexes suggested similar molar masses for F_{1Cp} and F_{1Ec} or F_1F_{oCp} and F_1F_{oEc}. Western blotting experiments with antibodies against the corresponding subunits of the F_1F_{oEc} and *M. thermoacetica* F_1F_o complexes confirmed the presence of all eight ATP synthase

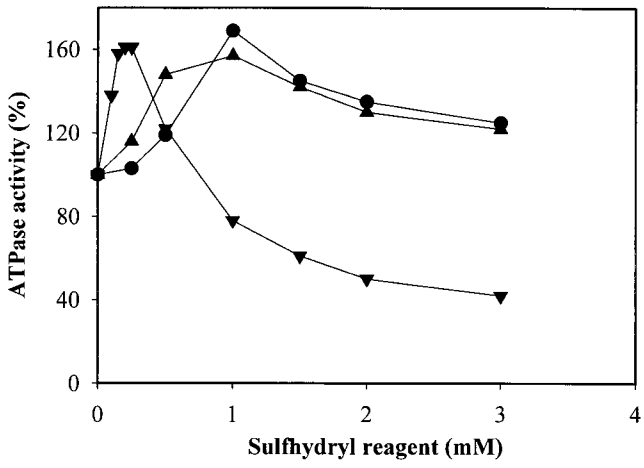

FIG. 7. Activation of MB F_1F_{oCp} by sulfhydryl reagents. Symbols: ●, DTT; ▼, cysteine; ▲, β-ME.

subunits in F_1F_{oCp}. Apparently, the missing subunits were lost during the purification of the F_1F_{oCp} complex reported by Clarke and coworkers (8, 9), a finding which is not unusual for anaerobic bacteria (11, 12, 56). One may speculate that the conditions used in the purification steps were too harsh for the integrity of the enzyme complex, e.g., excessive use of chromatograpic steps, absence of protease inhibitors in purification buffers, and use of sonication for the solubilization of F_1F_o from membranes. In most instances, F_1F_o complexes have been purified under milder conditions with fewer chromatographic steps (12, 21, 27, 41, 56).

The low level of activity of MB F_{1Cp} could have been influenced by F_o, as reported for *E. coli* mutants with a $Gly^{213} \rightarrow$ Cys substitution in the highly conserved fourth transmembrane helix (TMH4) of the a subunit of ATP synthase (39). TMH4 of the ATP synthase a subunit is the crucial segment of the subunit that interacts with the c subunit during proton translocation through membrane-integral F_o of the F_1F_o complex (14, 18, 33, 63). Analysis of the primary structure of the a subunit of F_1F_{oCp} revealed unusual features in its TMH4. It contains two Cys residues, Cys^{160} and Cys^{165}, that are complementary to Gly^{208} and Gly^{213} in the corresponding segment of the ATP synthase a subunit from *E. coli* and other sources. Recently, by site-directed mutagenesis it was shown that the replacement of Gly^{213} with Cys in the a subunit inhibited the growth of *E. coli* on succinate, increased the tolerance to DCCD inhibition, and blocked the ATP-driven proton-pumping activity of the membranes (33, 39). These properties resemble those of the MB F_1F_{oCp} complex.

F_1F_{oCp} exhibits unusual responses to oxyanions. Sulfite has been shown to be a strong activator (2, 6, 15–17, 27, 30–32, 44) and thiocyanate has been shown to be a strong inhibitor (7, 30, 31) of F_1F_o ATP synthase, findings that were also demonstrated in this study for F_1F_{oEc}. In contrast, sulfite inhibited and thiocyanate activated F_1F_{oCp}, and they were competitive with respect to each other but noncompetitive with respect to ATP (Fig. 6). It is not clear whether the two anions compete for the same or a different regulatory site(s) in the enzyme complex.

Thiol activation of F_1F_o was previously reported only for the enzymes in plants and photosynthetic bacteria (29, 51, 52). The activation was shown to occur because of the reduction of an intrapeptide disulfide bond between two cysteine residues within the γ subunit of the enzyme complex (47). Analysis of the primary structure of the subunits of the F_1F_{oCp} complex revealed the presence of six Cys residues, four in the α subunit and one each in the β and γ subunits. The possibility of the formation of intra- or interpeptide disulfide bonds between these Cys residues was investigated by homology modeling based on the structure of the bovine F_1 ATPase (1). Measurement of the relative distances between the Cys residues indicated a close proximity between Cys^{47} and Cys^{73} (within 6 Å) of the F_{1Cp} α subunit, while the distances between other Cys residues, including those of the β and γ subunits, were much longer (data not shown). Therefore, the possibility of disulfide bond formation between Cys^{47} and Cys^{73} of the F_{1Cp} α subunit is reasonable. In a database search, Cys residues complementary to Cys^{47}, Cys^{73}, and Cys^{193} were found to be present in the α subunit of F_1 ATPase from two other clostridial species, *C. acetobutylicum* (GenBank accession no. G972520) and *Clostridium perfringens* (GenBank accession no BAB81895). It is not known whether F_1F_o complexes from these bacteria are subject to thiol modulation.

DCCD inhibits the activity of F_1F_o by reacting with a key carboxylate residue (Glu or Asp) in the second transmembrane helix of the c subunit (19, 20, 24). It has been shown that the binding of DCCD to the carboxylate residue is strongly influenced by the regions of the two helices of the c subunit (20, 24) as well as by the regions of the a subunit (TMH4) that interact with this residue (19, 63). The above factors might contribute to the weaker binding of DCCD to the c subunit of F_1F_{oCp}, resulting in increased tolerance to the inhibitor.

Unlike most aerobic bacteria and some anaerobic bacteria, e.g., *M. thermoacetica*, *Moorella thermoautotrophica*, or *A. woodii*, *C. pasteurianum* lacks the ability to synthesize ATP from ATP synthase via chemiosmosis (25, 43). *C. pasteurianum* is a fermentative anaerobic bacterium. Depending on growth conditions, several fermentative bacteria, e.g., *Salmonella enterica* serovar *Typhimurium* (22), *Enterococcus hirae* (36), and *Lactobacillus acidophilus* (38), utilize ATP synthase exclusively in the direction of ATP hydrolysis to regulate cytoplasmic pH. A similar function might be attributed to the F_1F_{oCp} ATP synthase, as suggested by Harris (25). The unusual domain structure (TMH4) of the ATP synthase a subunit might have some regulatory role in the ATP synthase function of the enzyme complex, similar to that observed in *E. coli* mutants (33, 39). However, the physiological relevance of this possibility could be questioned without mutational changes in the clostridial enzyme.

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