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# Purification and Characterization of the $F_1$-ATPase from *Clostridium thermoaceticum*

D. MACK IVEY AND LARS G. LJUNGDAHL*

*Department of Biochemistry, University of Georgia, Athens, Georgia 30602*

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**The $F_1$ portion of the $H^+$-ATPase from *Clostridium thermoaceticum* was purified to homogeneity by solubilization at low ionic strength, ion-exchange chromatography, and gel filtration. The last indicated the $M_r$ to be 370,000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the pure enzyme revealed four bands with $M_r$ corresponding to 60,000, 55,000, 37,000, and 17,000 in an apparent molar ratio of 3:3:1:1. The purified enzyme would bind to stripped membranes to reconstitute dicyclohexylcarbodiimide-sensitive ATPase activity. Phosphohydrolase activity, measured at 58°C, was optimal at pH 8.5. In the presence of a 1 mM excess of $Mg^{2+}$ over the concentration of ATP, the $K_m$ for ATP was 0.4 mM, and the $V_{max}$ was 6.7 μmol $min^{-1}$ $mg^{-1}$. Unlike the membrane-bound $F_1F_0$ complex, the $F_1$-ATPase was relatively insensitive to the inhibitors dicyclohexylcarbodiimide and tributyltin chloride. Both the complex and the $F_1$-ATPase were inhibited by quercetin, azide, 7-chloro-4-nitro-benz-2-oxa-1,3-diazole, and free magnesium, and both were stimulated by primary alcohols and sulfite. In whole cells, the $F_1F_0$-ATPase catalyzed the synthesis of ATP in response to a pH gradient.**

*Clostridium thermoaceticum* is an obligate anaerobic thermophile which ferments 1 mol of glucose to 3 mol of acetate (7, 16, 37). According to the present concept of the pathways involved in the fermentation, 3 or 4 mol of ATP is formed by substrate-level phosphorylation (26, 28). However, growth yields of up to 50 g (dry weight) of cells per mol of glucose have been observed, suggesting that an additional 1 or 2 mol of ATP is formed during the glucose fermentation (5). That the additional ATP is generated by phosphorylation coupled with electron transport is suggested by the presence of membrane-bound cytochrome *b* and menaquinone in the bacterium (18). In addition, the recently reported growth of *C. thermoaceticum* on $H_2$-$CO_2$ or CO as the sole source of carbon and energy (21) suggests that an alternative energy supply mechanism exists, since substrate-level phosphorylation does not occur when the bacterium synthesizes acetate from one-carbon precursors and grows autotrophically.

The final step in electron transport phosphorylation is the synthesis of ATP by the membrane-bound $H^+$-ATPase. This enzyme is composed of two moieties, the membrane-intrinsic $F_0$ and the peripheral $F_1$. The $F_1$-ATPases purified from respiratory bacteria, mitochrondria, and chloroplasts are remarkably similar in their subunit composition and sensitivity to inhibitors (17, 31). In contrast, the ATPases from the obligate anaerobes *Clostridium pasteurianum* and *Lactobacillus casei* have a simpler architecture and inhibition pattern (10, 36). This is thought to reflect the simpler function of these enzymes, i.e., the unidirectional translocation of protons out of the cell to maintain a proton motive force necessary to drive substrate accumulation and other processes. If this relationship between structure and function holds for all cells, then the presence of a complex ATPase molecule implies the presence of a respiratory mechanism for ATP synthesis.

The work presented here describes the purification from *C. thermoaceticum* of an ATPase, the subunit structure and properties of which resemble those of energy-transducing membranes of bacteria, mitochondria, and chloroplasts. Furthermore, this ATPase apparently catalyzes the synthesis of ATP, driven by a pH gradient, in whole cells of *C. thermoaceticum*.

* Corresponding author.

## MATERIALS AND METHODS

*C. thermoaceticum* (ATCC 39073) was grown at 58°C under 100% $CO_2$ as described by Ljungdahl and Andreesen (27). Cells were harvested in the late log phase and stored at −20°C until used. ATP (disodium salt). RNase, DNase, dicyclohexylcarbodiimide (DCCD), 7-chloro-4-nitro-benz-2-oxa-1,3-diazole, and quercetin were from Sigma Chemical Co., St. Louis, Mo. Tributylin chloride was from Aldrich Chemical Co., Milwaukee, Wis. Gel filtration molecular weight standards were from Pharmacia, Uppsala, Sweden, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were from Bio-Rad Laboratories, Richmond, Calif.

Stock solutions (1 mM) of DCCD, 7-chloro-4-nitro-benz-2-oxa-1,3-diazole, and tributyltin chloride were prepared by dissolving the reagents in methanol. A 20 mM stock solution of quercetin was prepared by dissolving the solid in 1 mM KOH and then adjusting the pH to 8.5 with HCl.

ATPase activity was routinely measured at 58°C in a reaction mixture containing 2 mM ATP, 1 mM $MgCl_2$, 100 mM triethanolaminehydrocloride (pH 8.5), and 1 to 5 μg of protein in a volume of 250 μl. The reaction was initiated by adding ATP and stopped after 1 or 2 min by adding 10% SDS to a final concentration of 1%. The $P_i$ released was then measured as described by Baginsky et al. (6). One unit of activity is defined as the release of 1 μmol of $P_i$ per min, and specific activity is defined as the units of activity per milligram of protein. The effect of inhibitors on ATPase activity was evaluated by incubating the ATPase in the assay mixture at 58°C with the inhibitors for 10 min before starting the reaction. Membrane protein was determined by the Lowry et al. method (29), modified to include 1% SDS (30) and with ovalbumin as the standard. Soluble protein was determined by using rose bengal as described by Elliott and Brewer (14), except that rose bengal was used at a concentration of 1 mg $ml^{-1}$. In this case, the standard was a

TABLE 1. Purification of $F_1$-ATPase from 90 g of cells of *C. thermoaceticum*

| Step | Protein (mg) | Activity | | Yield (%) |
|---|---|---|---|---|
| | | U[a] | U $mg^{-1}$ | |
| Washed membranes | 390.00 | 136.0 | 0.35 | 100 |
| Low-ionic-strength wash | 25.00 | 17.0 | 0.68 | 13 |
| DEAE Bio-Gel | 8.00 | 16.0 | 2.05 | 12 |
| Sepharose CL-6B | 2.10 | 10.0 | 4.74 | 7 |
| DEAE Bio-Gel | 0.14 | 1.7 | 12.00 | 1 |

[a] Enzyme units are in micromoles per minute.

protein solution whose concentration was determined as described previously (30).

Kinetic parameters were estimated from rate-versus-substrate curves by nonlinear regression analysis with a modified version of the HYPER program of Cleland (11). Free $Mg^{2+}$ and $MgATP^{2-}$ levels were estimated by successive approximation using the BASIC language program of Kohlbrenner and Cross (23) and the stability constants given by Adolfsen and Moudrianakis (1).

**Preparation of membranes.** Frozen cells were thawed and suspended in 2 volumes of 50 mM Tris hydrochloride (pH 7.6)–1 mM $MgCl_2$–10% glycerol (buffer A). After the addition of a few crystals of DNase and RNase, the suspension was passed through an Aminco French pressure cell at 12,000 to 15,000 $lb/in^2$. Debris and intact cells were removed by centrifugation at 30,000 × *g* for 1 h. The supernatant was centrifuged at 100,000 × *g* for 2 h, and the pellet was washed once with buffer A to give crude washed membranes.

**Solubilization and purification of $F_1$-ATPase.** Washed membranes were suspended in low-ionic-strength buffer (1 mM Tris hydrochloride [pH 7.6], 0.5 mM EDTA, 10% glycerol) at a protein concentration of 2 mg $ml^{-1}$, incubated overnight at 10°C, and then centrifuged at 100,000 × *g* for 2 h. The supernatant, which contained the soluble $F_1$-ATPase, was adjusted to contain 50 mM Tris hydrochloride (pH 8.5) and 1 mM $MgCl_2$.

The soluble $F_1$-ATPase was applied to a DEAE Bio-Gel column (1.5 by 20 cm; Bio-Rad) previously equilibrated with 50 mM Tris-hydrochloride (pH 8.6)–1 mM $MgCl_2$–10% glycerol (buffer B). After application of the enzyme, the column was first washed with 2 volumes of buffer B, and the enzyme was then eluted with a 200-ml linear gradient of KCl (0.1 to 0.3 M) in buffer B. Fractions containing ATPase were pooled, and ammonium sulfate was added to 50% saturation. The precipitate, which contained ATPase, was recovered by centrifugation and dissolved in 2 ml of buffer B. This solution was applied to a column (1.5 by 90 cm) of Sepharose CL-6B equilibrated with buffer B. This buffer was used to elute the enzyme. The fractions with activity were pooled and applied to a second DEAE Bio-Gel column (0.5 by 5 cm), which was operated like the first column. ATPase eluted from this column was judged to be pure by various criteria, including gel filtration and gel electrophoresis.

The purified $F_1$-ATPase is stable for several weeks at 10°C in buffer containing glycerol. It can be stored for long periods at 10°C in buffer containing 50% saturated ammonium sulfate.

**Electrophoresis and $M_r$ determinations.** Analytical PAGE was preformed with the alkaline buffer system (pH 8.9) of Brewer and Ashworth (9). Gels were stained for protein with 0.04% Coomassie brilliant blue G250 in 3.5% (wt/vol) perchloric acid. SDS-PAGE was by the modified Laemmli procedure (24) with a 4% acrylamide stacking gel and a 12.6% acrylamide separating gel.

The $M_r$ of the $F_1$-ATPase was determined by gel filtration with a column (1.5 by 60 cm) of Bio-Gel A 1.5m with 0.1 M KCl in buffer B as solvent. The standards used were aldolase (158,000), catalase (232,000), ferritin (440,000), and thyroglobulin (669,000). The $M_r$ of the subunits was estimated by SDS-PAGE with the low-molecular-weight standards from Bio-Rad.

**Binding of purified ATPase to stripped membranes.** Stripped membranes were prepared by washing them with low-ionic-strength buffer until the ATPase specific activity was less than 10% of the original value. They were then dissolved in buffer A to a concentration of protein of 10 mg $ml^{-1}$ and stored at −20°C until used. A 1-mg sample of stripped membranes was incubated with $F_1$-ATPase ranging from 0 to 0.65 U at 58°C, and after 10 min the solutions were diluted to 1 ml with buffer A. Membranes were collected by ultracentrifugation at 100,000 × *g* for 2 h and dissolved in 1 ml of buffer A. Assays for ATPase activity, protein, and DCCD sensitivity were as described above.

**Synthesis of ATP by whole cells.** Cells of *C. thermoaceticum* were suspended in 100 mM sodium phosphate buffer (pH 7.5) to give an absorbance at 600 nm of about 2 (approximately 8 mg of cells [wet weight] per ml) and kept on ice. A 1-ml suspension was incubated at 58°C for 5 min and then acidified as indicated below with 6 N HCl. At regular intervals, 100-μl samples were pipetted onto 25 μl of ice-cold perchloric acid. After 20 min on ice, the samples were neutralized with 75 μl of 1 M KOH, and 50-μl samples were assayed for ATP with the luciferin-luciferase system of Kimmich et al. (20). Alternatively, whole cells were extracted by boiling, and ATP in the extracts was measured photometrically (4). The results of separate experiments agreed to within an error of 2 to 5%.

## RESULTS

**Purification of $F_1$-ATPase from *C. thermoaceticum*.** A representative purification from 90 g of cells is shown in Table 1. The yield varied from preparation to preparation, depending primarily on the efficiency of the solubilization step. The purified enzyme formed a single band on electrophoresis in nondenaturing gels, and activity stains for ATPase revealed a single band, the position of which corresponded to that of the purified enzyme. Glycerol was included in all buffers to prevent rapid inactivation of the soluble enzyme. Methanol (20% vol/vol) can be substituted for the glycerol with no apparent loss of stability of the enzyme. Unlike the $F_1$-ATPase from the extreme thermophile PS3 (41), the *C. thermoaceticum* enzyme was not stable in dissociating agents such as SDS or urea. In the presence of glycerol or methanol the ATPase was not cold labile. Purification by the anaerobic techniques of Yamamoto et al. (38) had no apparent effect on activity.

**Molecular weight and subunit composition.** As determined by gel filtration, the $M_r$ of $F_1$-ATPase was about 370,000. SDS-PAGE revealed four bands, with $M_r$s corresponding to 60,000, 56,000, 37,000, and 17,500. The ratios of the peak areas of densitometric scans of the gels suggest a molar ratio of 3:3:1:1 for the polypeptides in the native enzyme (Fig. 1).

**Binding of $F_1$-ATPase to stripped membranes.** Purified $F_1$-ATPase could bind to membranes from which most of the $F_1$ has been removed (Fig. 2A). The reconstituted activity was inhibited by DCCD, and the extent of inhibition resembled that observed for the enzyme in native membranes (Fig. 2B).

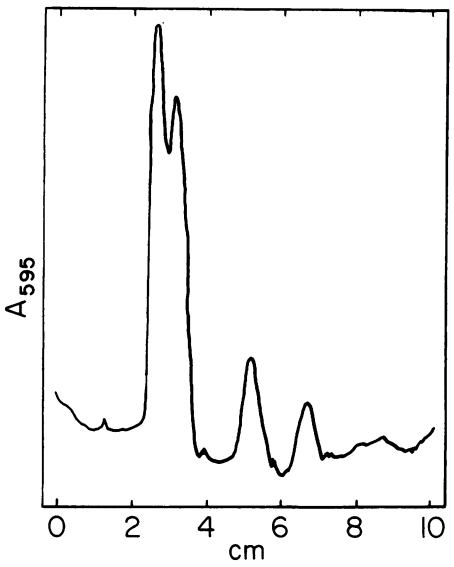


FIG. 1. SDS-PAGE of purified $F_1$-ATPase from *C. thermoaceticum*. Electrophoresis of 40 μg of enzyme was performed as described previously (24) with 12.6% acrylamide. The gel was stained for protein with Coomassie blue, and the intensity of staining was estimated densitometrically. The four subunits have apparent $M_r$s of 60,000, 55,000, 37,000, and 17,000, and the apparent ratio of subunits is 3:3:1:1.

**Kinetics.** $F_1$-ATPase had optimal phosphohydrolase activity at around pH 8.5. At this pH, at 58°C and in the presence of excess $MgCl_2$, the $K_m$ for $MgATP^{2-}$ was 0.4 mM, and the $V_{max}$ was 6.7 μmol $min^{-1}$ $mg^{-1}$. Free $Mg^{2+}$ inhibited phosphohydrolase activity both by raising the $K_m$ for $MgATP^{2-}$ and by lowering the $V_{max}$. The inhibition constant $K_i$ is 0.43 mM, and *$K'_i$ is 1.00 mM, as determined from plots of* $K_m/V_{max}$ and $1/V_{max}$ against concentrations of free $Mg^{2+}$ ranging from 0.1 to 3 mM.

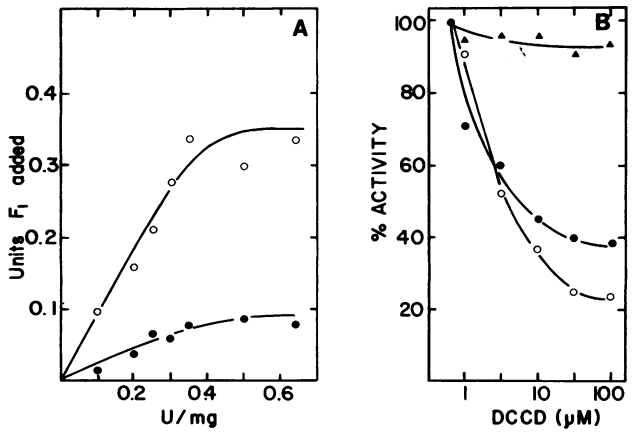


FIG. 2. Reconstitution of DCCD-sensitive ATPase activity. (A) $F_1$-ATPase was incubated with stripped membranes as described above, and the reconstituted membranes were assayed for ATPase activity in the assay mixture (○) and in the assay mixture plus 20 μM DCCD (●). (B) Samples (5-μg each) of (○) native membranes, (●) reconstituted membranes, and (▲) $F_1$-ATPase were treated with the concentrations of DCCD shown for 10 min at 58°C before the start of the reaction.

TABLE 2. Effects of inhibitors on the membrane bound and purified $F_1$-ATPase from *C. thermoaceticum*

| Inhibitor[a] | Concn (μM) | % Activity[b] | |
|---|---|---|---|
| | | $F_1F_0$-ATPase | $F_1$-ATPase |
| DCCD | 1 | 75 | 89 |
| | 5 | 53 | 82 |
| Tributyltin chloride | 1 | 53 | ND[c] |
| | 5 | 13 | 82 |
| | 50 | 0 | 92 |
| 7-Chloro-4-nitro-benz-2-oxa-1,3-diazole | 5 | 76 | 86 |
| | 50 | 33 | 32 |
| Quercetin | 5 | 54 | 89 |
| | 50 | 17 | 63 |
| Azide | 50 | 97 | 59 |
| | 500 | 51 | 45 |

[a] A 5-μg sample of either washed membranes ($F_1F_0$-ATPase) or purified $F_1$-ATPase was incubated in 0.25 ml of regular assay mixture with inhibitor at 58°C for 10 min before the start of the reaction.

[b] The control is enzyme activity in the absence of inhibitors. For inhibitors dissolved in methanol, the control contained the appropriate volume of methanol.

[c] ND, Not determined.

**Inhibition.** The ATPase was examined for sensitivity to several inhibitors known to affect the ATPases of other organisms (25) (Table 2). The membrane $F_1F_0$-ATPase was extremely sensitive to DCCD and tributyltin chloride, whereas these chemicals had little effect on the $F_1$-ATPase. Both inhibitors are believed to act by binding to the $F_0$ moiety. The bioflavinoid quercetin inhibited both the $F_1F_0$ and the $F_1$-ATPase, as did sodium azide and 7-chloro-4-nitro-benz-2-oxa-1,3-diazole.

**Effects of ions and alcohols on $F_1$-ATPase.** As is the case with other ATPases (2, 3, 13), metal cations and anions have a striking effect of the *C. thermoaceticum* ATPase (Table 3). $Mg^{2+}$ complexed with ATP is required for activity, but a $Mg^{2+}$/ATP ratio of above 1:2 was inhibitory (Fig. 3). Neither $MnATP^{2-}$ nor $CaATP^{2-}$ could fully substitute for $MgATP^{2-}$ as the substrate for ATPase (data not shown). $Na^+$ and $K^+$ showed only slight inhibition of activity in the presence of $Mg^{2+}$. Anion effects ranged from mild inhibition (by nitrate) to large stimulation (by sulfite). Sulfite did not protect the enzyme from inhibition by $Mg^{2+}$ (Fig. 3), as has been reported for *Alcaligenes faecalis* (2).

Stimulation of the ATPases of mitochondria, chloroplasts, and bacteria by alcohols has been reported (19, 22, 32, 34).

TABLE 3. Effects of ions on the activity of $F_1$-ATPase from *C. thermoaceticum*[a]

| Addition | Concn (mM) | Activity (%) |
|---|---|---|
| None | | 100 |
| $NaNO_3$ | 20 | 72 |
| $Na_2SO_4$ | 20 | 106 |
| $Na_2CO_4$ | 20 | 166 |
| $Na_2SO_3$ | 20 | 446 |
| NaCl | 100 | 74 |
| KCl | 50 | 94 |
| | 100 | 84 |

[a] A 5-μg sample of $F_1$-ATPase was incubated with the salts indicated at 58°C for 10 min before starting the reaction.

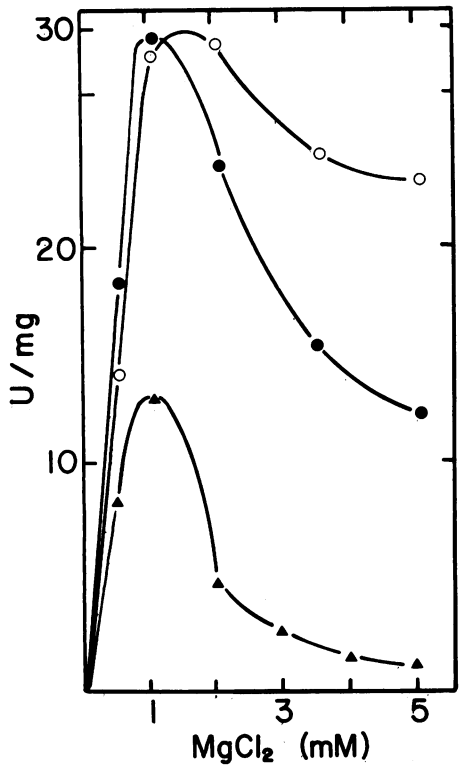


FIG. 3. Effects of sulfite and ethanol on the inhibition of ATPase by $Mg^{2+}$. Purified $F_1$-ATPase (5 μg) was incubated in solution containing 100 mM triethanolaminehydrochloride (pH 8.5) and the concentrations of $MgCl_2$ shown. Symbols: (▲) control, (○) with 30 mM sodium sulfite, (●) with 1 mM ethanol. The reaction was started by adding ATP to 2 mM.

Alcohols strongly stimulated the ATPase in *C. thermoaceticum* (Table 4), with a rough correlation between the extent of stimulation and the number of carbons in the primary alcohol. Like sulfite, ethanol did not protect the enzyme from inhibition by $Mg^{2+}$ (Fig. 3). However, 1 M ethanol raised both the inhibition constants $K_i$ and $K'_i$ for $MgCl_2$ (data not shown), suggesting that at least part of the stimulation is due to decreased binding and inhibition by $Mg^{2+}$.

**ATP synthesis in cells of *C. thermoaceticum* driven by a pH gradient.** An artificially imposed proton motive force, composed primarily of a pH gradient, was created in whole cells by subjecting them to a pH jump. When cell suspensions of *C. thermoaceticum* at pH 7.5 were acidified to a pH of 4.8, a transient increase in the ATP concentration occurred (Fig. 4). A smaller pH jump, from 7.5 to 6.7, did not result in significant synthesis of ATP. Pretreatment of cells with DCCD prevented ATP synthesis in response to the large pH jump.

TABLE 4. Effects of alcohols on the activity of $F_1$-ATPase from *C. thermoaceticum*

| Alcohol[a] | Concn (mM) | Enzyme activity[b] |
|---|---|---|
| None | | 1.18 |
| Methanol | 380 | 1.44 |
| Ethanol | 380 | 1.55 |
| 1-Propanol | 380 | 2.52 |
| 1-Butanol | 380 | 3.80 |
| 1-Hexanol | 75 | 4.38 |
| 1-Heptanol | 75 | 5.13 |

[a] $F_1$-ATPase was incubated with alcohol at 58°C for 10 min before starting the reaction.

[b] Units (micromoles of $P_i$ released per minute) per milliliter.

## DISCUSSION

The $F_1$-ATPase from *C. thermoaceticum* was purified and found to resemble the complex ATPases of energy-transducing membranes. The enzyme has four types of subunits; the $M_r$s and ratios of the subunits suggest an $\alpha_3\beta_3\gamma\delta$ architecture. This pattern is similar to that found for enzymes isolated from several aerobic organisms. These enzymes consist of two types of major subunits and one to three types of minor subunits (31). It is possible that a fifth subunit is lost during isolation and purification. However, isolation of $F_1$ with chloroform, as described by Beechey et al. (8), and subsequent purification also resulted in the isolation of an enzyme with four types of subunits. In addition, the enzyme purified by diffferent methods, for instance, in which one of the chromatography steps is replaced by glycerol gradient centrifugation, still contained four types of subunits. The purified enzyme can bind to stripped membranes, indicating that a fifth subunit is not required for the interaction of $F_1$ with $F_0$. In several bacteria, the smallest subunit appears to be a natural inhibitor of ATPase, resulting in latent activity which is manifested by treatments which cause dissociation of the subunit. We did not observe latency in the *C. thermoaceticum* enzyme; neither heat nor trypsin treatment caused an increase in activity of either the membrane-bound or soluble enzyme.

The complex subunit structure of the *C. thermoaceticum* ATPase distinguishes it from those purified from nonrespiratory anaerobes. The *C. pasterurianum* enzyme contains three types of subunits with $M_r$s of 65,000, 57,000, and 43,000 in a molar ratio of 2:1:2 (10). The recently purified ATPase from *L. casei* is a hexamer of only one type of subunit, $M_r$ 43,000 (36).

Inhibition data provide more evidence of complexity (Table 2). The sensitivity of the enzyme to tributyltin chloride is shared by other complex $F_1F_0$-ATPases, but distinguishes it from the *C. pasteurianum* enzyme, which is not inhibited by organotins. Agents such as azide, quercetin, and 7-chloro-4-nitro-benz-2-oxa-1,3-diazole, which react with the $F_1$ portion of complex ATPases, also inhibited the *C. thermoaceticum*

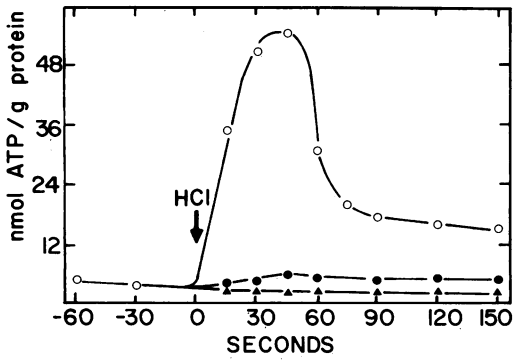


FIG. 4. Synthesis of ATP by whole cells of *C. thermoaceticum*. The concentration of intracellular ATP was measured in response to the following conditions: (○) pH jump from 7.5 to 4.8, (●) pH jump from 7.5 to 6.7, (▲) pH jump from 7.5 to 4.8 after treatment with 10 μM DCCD for 5 min at 58°C.

ATPase. We feel that the inhibition pattern and the complexity of the *C. thermoaceticum* ATPase suggest that it can function as a proton motive force-driven ATP synthase.

Experiments with whole cells support this proposal. The concentration of ATP in whole cells rose in response to a large pH jump (7.5 to 4.8), whereas a smaller pH gradient did not drive significant ATP synthesis (Fig. 4). The rise in internal ATP was abolished by DCCD, suggesting that ATP synthesis is catalyzed by the $F_1F_0$-ATPase.

In its response to ions, the *C. thermoaceticum* ATPase resembles many other ATPases (2, 3, 13). $Mg^{2+}$ is required in low concentrations, suggesting that $MgATP^{2-}$ is the substrate, but at higher concentrations $Mg^{2+}$ partially inhibits its activity. It appears to act as a mixed inhibitor, with a $K_i$ of 0.43 mM and a $K'_i$ of 1.00 mM. It may be that $Mg^{2+}$ acts both by competing with $MgATP^{2-}$ at the active site and by binding to a site distinct from the active site. Such a mechanism has been proposed to explain $Mg^{2+}$ inhibition in *A. faecalis* (2).

The mechanism of stimulation by sulfite and alcohols is unclear. In *A. faecalis*, sulfite appears to function by preventing inhibition by free $Mg^{2+}$. This appears not to be the case here, since sulfite stimulates even when the $Mg^{2+}$ concentration is below inhibitory levels. Alcohols are known to stimulate chloroplast coupling factors from spinach (35) and *Chlamydomonas reinhardii* (22). In spinach chloroplasts, alcohols seem to function by enhancing the release of tightly bound ADP. In *C. reinhardii*, stimulation is related to changes in the enzyme's sensitivity to inhibition by $Mg^{2+}$ and ADP. In *C. thermoaceticum*, we have observed slight changes in the ATPase's response to $Mg^{2+}$, although the effects are not sufficiently pronounced to explain the large stimulation. We are currently investigating the effects of alcohol on bound nucleotides.

The properties of the ATPase of *C. thermoaceticum* suggest that this bacterium can synthesize ATP by using a proton motive force. Peck and Odom (33) have speculated that a hydrogen cycling mechanism similar to that reported for *Desulfovibrio* sp. might be employed by *C. thermoaceticum* and other acetogens for generating a proton motive force. Drake's demonstration of hydrogenase in extracts of *C. thermoaceticum* (12) supports this speculation, as does the presence of multiple types of ferredoxin (15, 40) and rubredoxin (39) in this bacterium.

## ACKNOWLEDGMENTS

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