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Purification and Characterization of the F_1 ATPase from *Bacillus subtilis* and Its Uncoupler-Resistant Mutant Derivatives

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The F_1 ATPase of *Bacillus subtilis* BD99 was extracted from everted membrane vesicles by low-ionic-strength treatment and purified by DEAE-cellulose chromatography, hydrophobic interaction chromatography, and anion-exchange high-performance liquid chromatography. The subunit structure of the enzyme was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the absence and presence of urea. In the absence of urea, the α and β subunits comigrated and the ATPase was resolved into four bands. The mobility of the β subunit, identified by immunoblotting with anti-β from *Escherichia coli* F_1, was altered dramatically by the presence of urea, causing it to migrate more slowly than the α subunit. The catalytic activity of the ATPase was strongly metal dependent; in the absence of effectors, the Ca^{2+}-ATPase activity was 15- to 20-fold higher than the Mg^{2+}-ATPase activity. On the other hand, sulfite anion, methanol, and optimally, octylglucoside stimulated the Mg^{2+}-ATPase activity up to twice the level of Ca^{2+}-ATPase activity (specific activity, about 80 μmol of P_i per min per mg of protein). The F_1 ATPase was also isolated from mutants of *B. subtilis* that had been isolated and characterized in this laboratory by their ability to grow in the presence of protonophores. The specific activities of the ATPase preparations from the mutant and the wild type were very similar for both Mg^{2+}- and Ca^{2+}-dependent activities. Kinetic parameters (V_{max} and K_m for Mg-ATP) for octylglucoside-stimulated Mg^{2+}-ATPase activity were similar in both preparations. Structural analysis by polyacrylamide gel electrophoresis and isoelectric focusing indicated that the five F_1 subunits from ATPase preparations from the mutant and wild-type strains had identical apparent molecular weights and that no charge differences were detectable in the α and β subunits in the two preparations. Thus, the increased ATPase activity that had been observed in the uncoupler-resistant mutants is probably not due to a mutation in the F_1 moiety of the ATPase complex.

We have initiated a study of the ATPase in the gram-positive aerobic species *Bacillus subtilis* as part of an overall effort to delineate the bioenergetic properties of this organism and its uncoupler-resistant mutant derivatives. Less is known about the ATPase in bacilli than the ATPase found in *Escherichia coli* and in eucaryotic organelles. The opportunity to characterize the ATPase in *B. subtilis*, for which the genetics are highly developed, makes this effort a potentially fruitful complement to the very extensive genetic and biochemical studies that have been conducted on the enzyme from *E. coli* (7, 8, 22).

This laboratory has isolated a series of *B. subtilis* mutants by their ability to grow in the presence of micromolar concentrations of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (9). At suboptimal values of the proton motive force, the mutants synthesized more ATP via oxidative phosphorylation than the wild type (9). Uncoupler resistance was not due to the exclusion or degradation of CCCP or to an altered proton permeability of the coupling membrane or to a diminished ability of CCCP to enhance that permeability. Of particular interest in connection with the current study was the finding that membrane vesicles of the mutants had somewhat elevated ATPase activity (50 to 75%) relative to the wild type, an elevation that was reflected in vivo by the greater rate of ATP depletion during starvation in the mutant cells than in the wild type. Although the increased ATPase activity is probably not related directly to the special bioenergetic properties of the mutants, it was of interest to determine the basis for the increase since it might offer an avenue of investigation into the catalytic mechanism or the biogenesis of this complicated enzyme. Thus, part of the current study of the *B. subtilis* F_1 was to determine whether the increased ATPase activity in the uncoupler-resistant strains resulted from a mutation in a subunit of the F_1 ATPase. Evidence is presented that F_1 is unaltered in the mutants.

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MATERIALS AND METHODS

Materials. The following items were bought from the indicated companies: acrylamide, methylene bisacrylamide, Coomassie blue R-250, immunoblot assay kit, and urea from Bio-Rad Laboratories; phenyl agarose from Bethesda Research Laboratories; ATP from Sigma Chemical Co.; DEAE-cellulose DE-52 from Whatman; and the Mono Q anion exchange column from Pharmacia Fine Chemicals.

Organisms and growth conditions. *B. subtilis* BD99 and the CCCP-resistant mutants AG1A3 and AG2A were grown in Spizizen salts medium (24), pH 7.0, supplemented with 0.1% yeast extract and L-histidine, threonine, and L-tryptophan, each at 50 μg/ml. DL-Malate (50 mM) was used as the carbon source. All strains were grown in the absence of CCCP, but were checked for resistance and appropriate markers. For isolation of the F_1 ATPase, cells were grown in 16-liter carboys, washed, and stored at −70°C as a pellet until needed. The medium for washing contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH

7.5), 10 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM *p*-aminobenzamidine, and 0.05 mM L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK).

Isolation of everted membranes. The following procedures were carried out at 4°C. Washed cells were suspended in the wash medium supplemented with a small amount of DNase and passed through a precooled French pressure cell at 20,000 lb/in^2. The supernatant from low-speed centrifugation (15,000 × *g*, 10 min) was centrifuged at 300,000 × g_{max} for 1 h to recover the membranes. The membranes were washed with 20 mM Tricine (pH 8.0)–100 mM NaCl–0.1 mM PMSF–1 mM *p*-aminobenzamidine–0.05 mM TPCK. For ATPase assays and gel electrophoresis, the membranes were suspended in 0.4 M sucrose–20 mM Tricine (pH 8.0)–15 mM NaCl and stored at −70°C. For isolation of the F_1 ATPase, the washed membranes were not stored and were instead treated immediately as described below.

Extraction and purification of the F_1 ATPase. Unless otherwise noted, the following procedures were carried out at room temperature. Washed membranes were suspended to 1 to 2 mg of protein per ml in a low-ionic-strength medium consisting of 50 mM sucrose buffered with 2 mM Tricine titrated to pH 7.5 with solid Tris (12, 26), plus the protease inhibitors PMSF (0.1 mM) and TPCK (0.05 mM). The protease inhibitor *p*-aminobenzamidine was omitted at this step, since its presence may prevent release of F_1 (4). The suspension was stirred for 15 to 20 min, followed by ultracentrifugation (300,000 × g_{max}, 1 h). The supernatant was brought to 20 mM Tris sulfate–2 mM EDTA–1 mM ATP–3 mM sodium azide–1 mM *p*-aminobenzamidine and loaded onto a DEAE-cellulose (DE-52) column (2.5 by 20 cm) previously equilibrated with the same solution.

The solutions for DEAE-cellulose and phenyl agarose chromatography contained 20 mM Tris sulfate, 2 mM EDTA, 1 mM ATP, 3 mM sodium azide, 0.1 mM PMSF, 1 mM *p*-aminobenzamidine, and 0.05 mM TPCK; the solutions for Mono Q high-performance liquid chromatography (HPLC) contained 20 mM Tris sulfate, pH 8.0 and 2 mM EDTA. The DE-52 column was washed with several bed volumes of 0.1 M ammonium sulfate, and F_1 was eluted with 0.3 M ammonium sulfate. The DEAE eluate (100 to 200 ml) was concentrated to 10 to 20 ml by ultrafiltration with a YM-10 membrane at 35 lb/in^2 and then loaded onto a phenyl agarose column (2 by 15 cm) equilibrated with 0.3 M ammonium sulfate. This column was sequentially washed (2 to 3 bed volumes) with 0.3 M and 0.2 M ammonium sulfate, and the bulk of the ATPase activity was eluted with the buffered solution lacking ammonium sulfate. The phenyl agarose low-ionic-strength eluate was subjected to two to three cycles of dilution (10-fold) in Tris sulfate–EDTA and concentration by ultrafiltration to lower the concentration of PMSF, which, due to its limited solubility in water and its UV absorbance, interfered with the subsequent HPLC purification steps.

Mono Q anion-exchange chromatography was performed in a Shimadzu LC-6A HPLC system. The column (0.5 by 5 cm) was equilibrated with Tris sulfate–EDTA, and then 2 to 4 ml of the partially purified ATPase was loaded on the column. The column was washed with 10 to 20 ml of buffer, followed by a similar wash with 90 mM ammonium sulfate, which eluted residual ATP. An ammonium sulfate gradient of 100 to 180 mM (20 ml) was used to elute F_1. The flow rate was 1 ml/min. The enzyme eluted at about 120 mM ammonium sulfate. The ATPase activity eluted in one major peak, followed by a second smaller peak of activity that was electrophoretically less pure than the major peak, although the specific activity of the second peak was not substantially less than that of the major peak. Fractions from the major peak were pooled, diluted, and concentrated by ultrafiltration to lower the ammonium sulfate concentration and then reloaded onto the Mono Q column. This preparation was eluted as described above, and ATPase fractions were analyzed by polyacrylamide gel electrophoresis (PAGE) with sodium dodecyl sulfate (SDS) and urea. Peak fractions containing just five bands were pooled and adjusted to 20 mM Tricine, pH 8.0, 2 mM EDTA, 1 mM ATP, and 3 mM sodium azide by dilution and ultrafiltration. *Bacillus firmus* RAB F_1 (10) and spinach CF_1 (12) were purified by published methods.

ATPase assay. Hydrolysis of ATP was measured spectrophotometrically as released P_i by the method of LeBel et al. (15) as described by Jagendorf (12). Reactions were carried out at 37°C for 3 min. Protein content of samples was estimated by the method of Lowry et al. (17), with bovine serum albumin as the standard.

SDS-PAGE, two-dimensional gel electrophoresis, and immunoblotting. Denaturing PAGE was performed in a Laemmli system (14) as described elsewhere (3, 10). Where indicated, urea was included in the resolving but not the stacking gel. No urea was present in the sample buffer. For two-dimensional gel electrophoresis, gel strips from the first dimension (either native 6% polyacrylamide gels or isoelectric focusing gels) that had been stored at −20°C were equilibrated twice for 15 min each in 15 ml of 0.12 M Tris hydrochloride (pH 6.8)–4% SDS–5 mM dithiothreitol and were sealed on the surface of the stacking gel of the second-dimension gel with 1% agarose–0.12 M Tris hydrochloride (pH 6.8)–2% SDS–0.1% bromophenol blue. To identify the subunits, purified F_1 was electrophoresed in a side well. Electrophoresis was carried out overnight at 50 V. Electrophoretic transfer and immunoblotting were carried out as recommended by Bio-Rad, except that bovine serum albumin replaced gelatin as the blocking agent. The monospecific antibody against the β subunit of the F_1 ATPase from *E. coli* was a generous gift of Nathan Nelson, Roche Institute of Molecular Biology.

Isoelectric focusing. Isoelectric focusing was performed in a Bio-Rad horizontal electrophoresis cell at 25°C. The polyacrylamide gel contained 4.3% acrylamide, 0.24% bisacrylamide, 12.5% glycerol, 8 M urea, 2% Nonidet P-40, and ampholytes (Pharmacia). For equilibrium isoelectric focusing, the gel contained 0.3 ml each of pH 4 to 6.5 and 5 to 8 ampholytes per 10 ml, while nonequilibrium gels contained 0.23 ml of pH 2.5 to 5, 0.17 ml of pH 5 to 8, and 0.2 ml of pH 8 to 10.5 ampholytes per 10 ml. The gels were polymerized with 0.1% TEMED (*N,N,N′,N′*-tetramethylethylenediamine) and 0.03% ammonium persulfate. Samples were prepared for electrophoresis by the addition of solid urea to 8 M, followed by additions of stock solutions of Nonidet P-40, ampholytes, and dithiothreitol to final concentrations of 2%, 2.5%, and 50 mM, respectively. The gels were prefocused at 800 V for 1 h prior to sample application in the middle of the gel. Equilibrium running conditions, as illustrated in Fig. 4, were 4.5 h at 1,000 V, while nonequilibrium conditions were 1.5 h at 1,000 V. After focusing, the pH gradient was measured with a surface pH electrode. The gels were fixed in 10% trichloroacetic acid–33% ethanol for 1.5 h, followed by 0.5 h in 5% in 5% trichloroacetic acid–33% ethanol and overnight in destaining solution (40% methanol–7% acetic acid). The gels were stained for 2 h in freshly prepared 0.2% Coomassie blue R-250 in 30% methanol–10% acetic acid and destained in 40% methanol–7% acetic acid.

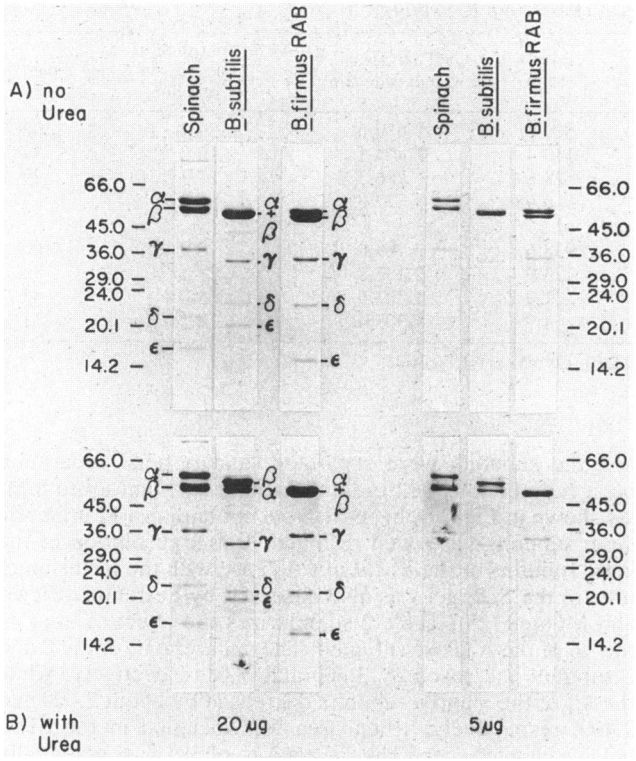

FIG. 1. Analysis of subunit structure of the F_1 ATPase from *B. subtilis* by SDS-polyacrylamide gradient gel electrophoresis in the absence or presence of urea. The F_1 ATPases from *B. subtilis*, purified as described in the text, from *B. firmus* RAB, and from spinach chloroplasts were subjected to 10–15% polyacrylamide–SDS gradient gel electrophoresis. Left, 20 µg of ATPase; right, 5 µg of ATPase. (A) No urea included in the gel; (B) 4 M urea included in the resolving gel. Molecular weight markers (10^3) and subunits are indicated.

RESULTS

Extraction of the F_1 ATPase. The chloroform procedure for extraction of the F_1 ATPase (16) from everted membrane vesicles was used in the early stages of this work. However, this method was inconsistent in its ability to extract ATPase activity and also resulted in preparations with contaminants that proved difficult to remove. We therefore turned to the low-ionic-strength method of extraction (26) and found that it extracted about 40 to 50% of the membrane ATPase activity in one treatment; subsequent treatments solubilized little additional activity. This extraction was less efficient than had previously been found in studies with alkalophilic *B. firmus* RAB (10), in which 70 to 80% of the other membrane ATPase activity was extracted by low ionic strength. Nonetheless, the method gave sufficient yields to enable us to purify the enzyme to apparent homogeneity.

Purification of the F_1 ATPase. Crude extracts were chromatographed on DEAE-cellulose, and after washing, the ATPase was eluted with 0.3 M ammonium sulfate. The eluate was concentrated by ultrafiltration and loaded immediately on a phenyl agarose column. Washes containing 0.3 or 0.2 M or no ammonium sulfate were applied sequentially to the column; the latter two fractions contained ATPase activity. Although both active fractions could be further purified, the low-ionic-strength eluate had somewhat more activity and a higher specific activity, so this fraction was used in subsequent purification steps. The low-ionic-strength eluate was concentrated to a small volume and subjected to anion-exchange HPLC. When analyzed on SDS-urea-polyacrylamide gels, the preparation at this stage was resolved into six bands, five of which appeared to correspond to authentic F_1 subunits, while the sixth, with an M_r between those of the α and γ subunits, was assumed to be a contaminant. To remove this contaminant, a second HPLC run was carried out in which the fractions were analyzed both for ATPase activity and for subunit composition. Peak fractions yielded essentially pure material, while considerable activity was sacrificed that contained the troublesome contaminant. SDS-PAGE discussed in more detail below, showed that a small amount of the contaminant remained in the preparation (Fig. 1A). In an SDS-urea gel system, this contaminant resolved into two components whose intensity was less than the δ or ε subunits (Fig. 1B). At least one of these polypeptides was a proteolytic fragment of an authentic F_1 subunit, as judged by protein immunoblotting with an anti-β antibody (Fig. 2) and by a two-dimensional gel electrophoretic procedure (native PAGE in the first dimension, followed by denaturing electrophoresis in the second dimen-

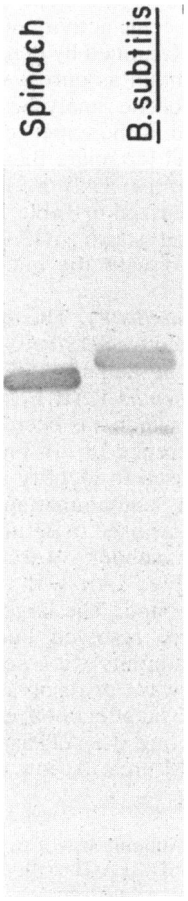

FIG. 2. Identification of the β subunit of *B. subtilis* F_1 by protein immunoblotting. The subunits of *B. subtilis* F_1 (2 µg) and spinach CF_1 (2 µg) were separated on SDS–urea–10 to 15% polyacrylamide gels, transferred to nitrocellulose, and blotted with anti-β from *E. coli* F_1. The *B. subtilis* F_1 subunit reacting with anti-β has lower mobility than the β subunit of CF_1; when compared with the Coomassie-stained gel of Fig. 1B, these data show that the β subunit of *B. subtilis* F_1 has lower mobility (i.e., higher M_r) than the α subunit of *B. subtilis* F_1 in SDS-urea-polyacrylamide gels.

TABLE 1. Purification of the F_1 ATPase from *B. subtilis*

Strain	Step	Protein (mg)	ATPase activity[a] (μmol of P_i/min)	Sp act (μmol of P_i/min per mg of protein)	Yield (%)
Wild-type	Tris-Tricine extract[b]	611.1	2,680.6	4.3	(100)
	DEAE-cellulose column	140.4	1,404.1	10.0	54
	Phenyl agarose column	28.8	786.3	27.3	29
	Anion-exchange on Mono Q (HPLC)[c]	0.95	77.8	81.9	3
Uncoupler-resistant AG2A	Tris-Tricine extract[b]	938.6	2,769.0	2.9	(100)
	DEAE-cellulose column	123.9	2,187.8	28.1	79
	Phenyl agarose column	21.9	1,203.9	55.0	43
	Anion-exchange on Mono Q (HPLC)[c]	3.9	303.4	78.3	11

[a] ATPase activity was measured in 20 mM Tricine (pH8.0)–5 mM ATP–2.5 mM $MgCl_2$–30 mM octylglucoside.
[b] About 100 g (wet weight) of cells was used for extraction of the ATPase.
[c] After two chromatographic runs on the Mono Q column.

sion) (not shown); the latter procedure also indicated that a band migrating between the β and α subunits was also a proteolytic product of an F_1 subunit. Evidence of proteolysis was found even though a cocktail of three protease inhibitors was present throughout the purification procedure except during the HPLC runs, as described in Materials and Methods. Further chromatographic steps of the purified ATPase did not increase the specific activity of the enzyme or change its composition, as determined by denaturing gel electrophoresis. The effort to obtain as pure a sample as possible, as judged by electrophoretic analysis and specific ATPase activity, was achieved at the expense of yields of F_1 preparations normally found (compare these results, for example with those for *B. firmus* RAB F_1 [10]). The purification procedures are summarized in Table 1 for the wild type and the uncoupler-resistant strain AG2A. Similar results (not shown) were obtained with the uncoupler-resistant strain AG1A3.

SDS-PAGE of *B. subtilis* F_1. The subunit structure of *B. subtilis* F_1 was analyzed by SDS-PAGE. It was of interest to compare the subunits of the *B. subtilis* ATPase with those of another bacillus, *B. firmus* RAB F_1 (10), and with spinach chloroplast F_1 (CF_1), which has been extensively characterized (reviewed in reference 18). In Fig. 1, a large amount of protein (20 μg) was used to identify all five subunits of the ATPase as well as any contaminating polypeptides, while a small amount (5 μg) was used to optimize resolution of the α and β subunits. Four subunits were observed in standard Laemmli-type gels (Fig. 1A), with the α and β subunits migrating as a single band. The large subunits of the other ATPases were clearly resolved under these conditions. Although the large subunits were separable by isoelectric focusing, alternative ways of resolving them in an SDS gel system were sought. The effect of urea on subunit migration was tested by subjecting the ATPase to a transverse urea gradient from 0 to 8 M urea. At low concentration (about 1 M), the subunits were separated, and optimal separation occurred at about 4 M urea, remaining constant up to 8 M. As shown in Fig. 1B, in an SDS gel containing 4 M urea, the large subunits were well resolved. Better resolution of the large subunits of the *B. subtilis* ATPase with the inclusion of urea in the SDS gel was also observed by Serrahima-Zieger and Monteil (23). Table 2 summarizes the effect of urea on the M_r of the ATPase subunits. The apparent M_r of the β and ε subunits increased by 3,600 and 600, respectively, while the M_r of the γ and δ subunits decreased by about 2,600 and 2,400, respectively, when urea was included in the SDS-polyacrylamide gel. The migration of α was essentially unaffected by the presence of urea. Urea also affected the migration of different subunits of the *B. firmus* RAB and spinach ATPases, although none as dramatically as the β subunit of *B. subtilis* F_1. Most affected were the α and δ subunits of *B. firmus* RAB F_1 (decreases of 1,500 and 1,600, respectively) and the γ and δ subunits of spinach CF_1 (a decrease of 1,700 and an increase of 1,800, respectively). Although urea only slightly affected the migration of the β subunit of *B. firmus* RAB F_1, increasing its M_r by about 600, when coupled with the decrease in the M_r of the α subunit, the two subunits ran as a single band in the presence of urea (Fig. 1B.).

TABLE 2. Subunit M_rs of *B. subtilis* F_1 as determined by SDS-PAGE with and without urea

Subunit	Mean M_r ± SD in SDS-PAGE		Change with urea
	Without urea (n = 4)	With urea (n = 5)	
α	50,000 ± 700	50,200 ± 900	+200
β	50,000 ± 700	53,600 ± 900	+3,600
γ	34,400 ± 900	31,800 ± 1,100	−2,600
δ	23,600 ± 400	21,200 ± 100	−2,400
ε	19,500 ± 900	20,100 ± 1,300	+600

The slowest-migrating subunit of *B. subtilis* F_1 migrated between the α and β subunits of spinach CF_1, while the second slowest band migrated just under the β subunit of CF_1. This provided a convenient way of determining the identity of the large subunits by protein immunoblotting, with spinach CF_1 as the reference protein. The band of *B. subtilis* F_1 reacting with anti-β of *E. coli* F_1 had a lower mobility than the β subunit of CF_1 (Fig. 2). This indicates that the β subunit of *B. subtilis* F_1 is the slowest-migrating band of the protein, i.e., it has the highest apparent molecular weight. Although it is rare for the β subunit to have a higher apparent M_r than the α subunit, immunoblotting experiments by Dunn et al. (5) indicated that in energy-transducing membranes of *B. subtilis* the band reacting with a monoclonal antibody against the β subunit of *E. coli* F_1 had a higher molecular weight than the band reacting with anti-α.

ATPase activity of *B. subtilis* F_1. The hydrolytic activity of the purified ATPase was studied as a function of the divalent cation requirement for activity. The magnesium ion-dependent ATPase activity was very low, 2 to 3 μmol of P_i per min per mg of protein. This was not entirely unexpected, since the specific Mg^{2+}-ATPase activity of everted membrane vesicles of *B. subtilis* is also quite low (9). On the other hand, the calcium ion-dependent ATPase activity of the purified F_1 was substantial, about 35 to 40 μmol of P_i per min

TABLE 3. ATPase activities of the F_1 ATPases from *B. subtilis* wild type and uncoupler-resistant strain AG2A

Cation[a]	Effector[b]	ATPase sp act[c] (μmol of P_i/min per mg of protein)	
		Wild type	AG2A
Mg^{2+}	None	2.3 ± 0.2 (4)	2.6 ± 0.4 (4)
Mg^{2+}	Sodium sulfite	42.6 ± 1.2 (2)	39.9 ± 0.2 (2)
Mg^{2+}	Methanol	50.7 ± 2.8 (4)	46.8 ± 1.4 (4)
Mg^{2+}	Octylglucoside	83.9 ± 7.7 (8)	78.4 ± 6.4 (8)
Ca^{2+}	None	40.9 ± 4.2 (8)	36.3 ± 6.8 (8)

[a] The assay mixture contained 20 mM Tricine (pH 8.0)–5 mM ATP–2.5 mM $MgCl_2$ with and without effector or 20 mM Tricine (pH 8.0)–10 mM ATP–10 mM $CaCl_2$.

[b] Concentrations were 50 mM sodium sulfite, 25% (vol/vol) methanol, or 30 mM octylglucoside.

[c] Results are means ± standard deviation; the number of trials is shown in parentheses.

per mg of protein. High Ca^{2+}-ATPase activity and low Mg^{2+}-ATPase activity were also encountered in ATPase assays of purified *B. firmus* RAB F_1 (10), and it was found that certain agents dramatically stimulated the Mg^{2+}-ATPase (but not the Ca^{2+}-ATPase) activity of that F_1. Exposing *B. subtilis* F_1 to the same agents had similar stimulatory effects on Mg^{2+}-ATPase activity (Table 3). Whereas methanol was the most potent effector of *B. firmus* RAB F_1 activity, octylglucoside optimally stimulated the Mg^{2+}-ATPase activity of *B. subtilis* F_1, increasing activity 30- to 36-fold, to twice that of the Ca^{2+}-ATPase activity. Although somewhat less stimulatory, sulfite and methanol were also strong activating agents, elevating the ATPase activity 15- to 20-fold. Octylglucoside and methanol inhibited (25 to 50%) the Ca^{2+}-ATPase activity of F_1, while sulfite slightly (10 to 15%) increased the Ca^{2+}-ATPase activity.

Comparison of the ATPases from wild-type and uncoupler-resistant strains. As described in the Introduction, we have isolated mutants of *B. subtilis* that can grow in the presence of the protonophore CCCP and have elevated levels of ATPase activity (9). The F_1 ATPase from two of the three mutants, AG1A3 and AG2A, was purified. The ATPases from the two mutants and the wild type had similar specific activities and identical apparent subunit molecular weights.

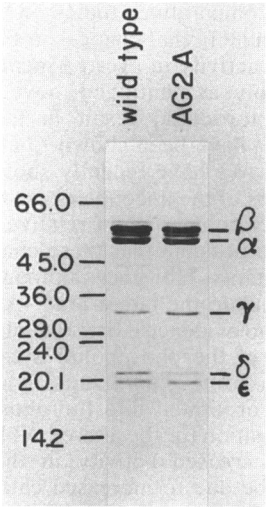

FIG. 3. SDS-urea-polyacrylamide gradient gel electrophoresis of the F_1 ATPase from *B. subtilis* wild type and uncoupler-resistant mutant AG2A.

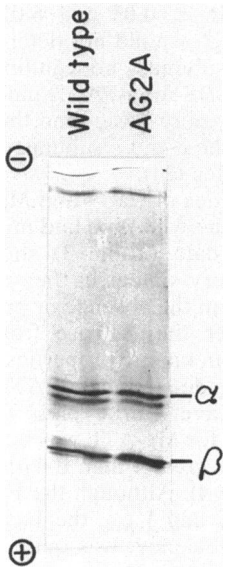

FIG. 4. Isoelectric focusing of the F_1 ATPase from *B. subtilis* wild type and uncoupler-resistant mutant AG2A. Isoelectric focusing was carried out as described in Materials and Methods. The pH gradient (pH 5 to 8.5) was measured by a surface pH electrode immediately after the end of focusing. Each lane contained 20 μg of ATPase.

A more extensive comparison was carried out between the F_1 ATPase from AG2A and the wild type.

The subunit M_rs of the subunits of the ATPases from both AG2A and the wild type were identical (Fig. 3). Slight differences in subunit content (e.g., AG2A F_1 was somewhat deficient in δ subunit content relative to the wild type F_1) were considered to be normal variations found in F_1 preparations. The SDS-urea-polyacrylamide gels indicated that both preparations had a small amount of a proteolytic fragment migrating between the β and α subunits.

The preparations were also analyzed by isoelectric focusing. Under equilibrium conditions with a pH gradient of about 5 to 8.5, the small subunits migrated to the cathode end of the gel while the β and α subunits migrated toward the anode and were well resolved from one another. The major α band was accompanied by a minor band with a lower pI; this band was identified by two-dimensional gel electrophoresis as the band that migrated between the β and α subunits in SDS-urea-polyacrylamide gels (Fig. 1B). The β and α subunits from both the wild-type and mutant F_1 preparations were resolved into components of identical isoelectric points (Fig. 4). The pIs determined for the α and β subunits of *B. subtilis* F_1 were 6.25 ± 0.04 and 5.90 ± 0.04, respectively (mean ± standard deviation; $n = 3$). As a point of interest, the isoelectric points of the α and β subunits of *B. firmus* RAB F_1 were also measured in the same gels; these were

TABLE 4. Kinetic parameters for octylglucoside-stimulated Mg^{2+}-ATPase activity of the F_1 ATPases from *B. subtilis* wild type and uncoupler-resistant strain AG2A[a]

F_1 source	K_m (mM)	V_{max} (μmol of P_i/min per mg of protein)	V_{max}/K_m
Wild type	0.77 ± 0.10	108.5 ± 7.9	140.9
AG2A	0.95 ± 0.09	122.2 ± 6.3	128.6

[a] Means ± standard deviation for three trials.

estimated to be 6.16 ± 0.03 and 6.07 ± 0.04 (n = 3), respectively. Although we did not determine the isoelectric points of the small subunits, nonequilibrium isoelectric focusing followed by SDS-urea-PAGE indicated that the γ, δ, and ε subunits were more basic than the α and β subunits. This contrasts with the δ and ε subunits of *E. coli* F_1, which are acidic polypeptides (24).

The specific activities of Ca^{2+}- and Mg^{2+}-dependent ATP hydrolysis for both the wild-type and mutant ATPases were determined. These data (Table 3) showed that the two preparations were very similar in their ability to hydrolyze Ca-ATP or Mg-ATP in the absence or presence of effectors. To examine whether the ATPase from the mutant had undergone a change in kinetic properties, the kinetic parameters of octylglucoside-stimulated ATPase activity were determined. Lineweaver-Burke plots of the kinetic data revealed that the K_m for Mg-ATP was between 0.75 and 0.85 mM and the V_{max} was greater than 100 μmol of P_i per min per mg of protein (Table 4). Although the F_1 from AG2A had a somewhat higher K_m and V_{max}, the V_{max}/K_m ratio, an indication of catalytic efficiency, was quite similar to the value determined for the wild type.

DISCUSSION

To our knowledge, this work represents the first preparation of the F_1 ATPase from *B. subtilis* containing all five subunits typical of this class of ATPases. An earlier report on *B. subtilis* indicated that the ATPase contained just two subunits, corresponding to the α and β subunits (23). Those authors found that additional bands were absent in the preparations when the protease inhibitor PMSF was included in the purification procedures. In our hands, however, inclusion of PMSF and two additional protease inhibitors did not reduce the number of subunits that were evident in denaturing PAGE. Subunit composition and specific ATPase activity were not changed by further chromatographic steps subsequent to anion-exchange HPLC. We conclude that the five prominent bands observed in SDS-urea-polyacrylamide gels correspond to the five types of subunits of other F_1 ATPases.

ATP hydrolysis catalyzed by *B. subtilis* F_1 is strongly metal dependent and subject to stimulation by a number of potent effectors. Sulfite may stimulate the hydrolysis of Mg-ATP by removal of free Mg^{2+}, an inhibitor of ATPase activity, or by interaction with the F_1 to prevent binding of free Mg^{2+} (1); the effects of methanol and octylglucoside may relate to the release of the ε subunit, an ATPase inhibitor (2, 26), and to hydrophobic and hydrophilic interactions of the ATPase with the detergent (1, 2, 19, 26). A partial explanation for the metal dependence of spinach CF_1 was provided by kinetic data revealing a low K_i for free Mg^{2+} and a high K_i for free Ca^{2+} (11). This type of analysis may be useful in understanding the metal dependence of ATPases of bacilli, since a similar pattern of low (but inducible) Mg^{2+}- and high Ca^{2+}-ATPase activities have been observed with *B. firmus* RAB F_1 (10). In any event, the low Mg^{2+}-ATPase activity (in the absence of effectors) may be of some physiological consequence in these obligately aerobic organisms by preventing or at least attenuating ATP hydrolysis during transient reductions in the proton motive force that could result from substrate deprivation.

An examination of the subunit composition of *B. subtilis* F_1 by SDS-PAGE revealed some interesting effects of urea on the migration of some of the subunits. Most pronounced was the change in the M_r of the β subunit, which increased by about 3,600 in the presence of urea. Urea has been shown by Feinstein and Moudrianakis (6) to affect the migration of certain subunits of the mitochondrial F_1. These authors used urea analogs and electrophoretic buffers of different pHs to show that the decrease in M_r observed with β was probably due to hydrophobic interactions with urea, while the increased M_r of δ may have resulted from hydrogen bonding with urea. At present, we do not know the mechanism causing the decreased mobility of the β subunit of *B. subtilis* F_1, although by analogy with that work, it may also be due to hydrogen bonding of β with urea. The β subunit of *B. firmus* RAB F_1 can be differentiated from the homologous subunit of *B. subtilis* by the lack of a significant effect of urea on its migration. Relative to the α subunits of *E. coli* F_1 and spinach CF_1, the α subunits from both bacilli were much smaller, by about 5,000 to 6,000 daltons.

One aspect of this work was to examine the F_1 from uncoupler-resistant mutants to determine whether the increased ATPase activity exhibited by these strains is due to alterations in the F_1 moiety of the ATPase. F_1 preparations from the wild-type and mutant strains were identical in subunit molecular weights. In addition, no changes in net charge were observed in the α and β subunits of either preparation. Of course, we cannot rule out point mutations in these subunits that would not involve a change in the charge of the molecule, nor do we know whether the small subunits have undergone point mutations.

The catalytic properties of the preparations were similar, showing the same metal dependence, i.e., high Ca^{2+}-ATPase activity and low Mg^{2+}-ATPase activity which was stimulated by the effectors sulfite, methanol, and octylglucoside. The kinetic parameters for octylglucoside-stimulated Mg^{2+}-ATPase activity were determined to be substantially the same in the preparations from the wild-type and the mutant AG2A. As noted below, changes in ATPase activity could be due to alterations in the F_0 moiety of the ATPase complex. One method of evaluating that possibility is to reconstitute F_1-depleted membranes with homologous or heterologous F_1s and determine whether the activity of the enzyme is different in the two systems. Unfortunately, we have not been able to carry out this type of experiment because of the lack of resolution we obtain with our low-ionic-strength treatment of the membranes (only about 50% of the ATPase activity is extracted by this procedure).

In general, the comparative studies on the ATPase preparations make it unlikely that changes in F_1 are the cause of increased ATPase activity in everted membrane vesicles of the mutants. Possibly, as mentioned above, a mutation in the F_0 membrane sector could result in increased activity; perturbations of F_0 have been shown to affect the catalytic activity of F_1 (19). We have recently shown that the membrane phospholipids of the uncoupler-resistant mutants have an altered fatty acid composition relative to the wild type (13). It is thus also possible that these changes activate the ATPase of the mutants. However, although free fatty acids are known to stimulate the latent Mg^{2+}-ATPase activity of spinach CF_1 (17), no evidence exists that changes in the fatty acid substitutents of the phospholipids cause such activation. Indeed, data from experiments in which exogenous fatty acids are incorporated into the phospholipids do not support that explanation for the altered ATPase activity (13).

Alternatively, increased activity in the membranes of mutants may not be due to increased catalytic turnover of the F_1-F_0 complex but instead may reflect an increase in the number of ATPase complexes residing in the membrane. It has been observed that a CCCP-resistant mutant of *E. coli*

exhibited aberrant subcellular localization of an elongation factor, suggestive of alterations in protein processing or translocation (18). It would therefore be of interest to examine, by quantitative immunochemical techniques, whether the *B. subtilis* mutants show such an increase in the number of ATPase molecules or other membrane-associated proteins.

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