A Specific Adaptation in the \( a \) Subunit of Thermoalkaliphilic \( F_1F_0 \)-ATP Synthase Enables ATP Synthesis at High pH but Not at Neutral pH Values*

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Analysis of the \( atp \) operon from the thermoalkaliphilic \( Bacillus \) sp. TA2.A1 and comparison with other \( atp \) operons from alkaliphilic bacteria reveals the presence of a conserved lysine residue at position 180 (\( Bacillus \) sp. TA2.A1 numbering) within the \( a \) subunit of these \( F_1F_0 \)-ATP synthases. We hypothesize that the basic nature of this residue is ideally suited to capture protons from the bulk phase at high pH. To test this hypothesis, a heterologous expression system for the \( F_1 \) synthase from \( Bacillus \) sp. TA2.A1 (TA2F, \( F_0 \)) was developed in \( Escherichia coli \) DK8 (\( \Delta \text{atp} \)). Amino acid substitutions were made in the \( a \) subunit of TA2F, \( F_0 \) at position 180. Lysine (\( \text{aK180} \)) was substituted for the basic residues histidine (\( \text{aK180H} \)) or arginine (\( \text{aK180R} \)), and the uncharged residue glycine (\( \text{aK180G} \)). ATP synthesis experiments were performed in ADP plus \( P_i \)-loaded right-side-out membrane vesicles energized by ascorbate-phenazine methosulfate. When these enzyme complexes were examined for their ability to perform ATP synthesis over the pH range from 7.0 to 10.0, TA2F, \( F_0 \) and aK180R showed a similar pH profile having optimum ATP synthesis rates at pH 9.0–9.5 with no measurable ATP synthesis at pH 7.5. Conversely, aK180H and aK180G showed maximal ATP synthesis at pH values 8.0 and 7.5, respectively. ATP synthesis under these conditions for all enzyme forms was sensitive to DCCD. These data strongly imply that amino acid residue Lys180 is a specific adaptation within the \( a \) subunit of TA2F, \( F_0 \) to facilitate proton capture at high pH. At pH values near the \( pK_a \) of Lys180, the trapped protons readily dissociate to reach the subunit \( c \) binding sites, but this dissociation is impeded at neutral pH values causing either a blocking of the proposed H\(^+\) channel and/or mechanism of proton translocation, and hence ATP synthesis is inhibited.

For nearly all aerobic life on earth, the \( F_1F_0 \)-ATP synthases are the major enzymes responsible for providing ATP to drive endergonic reactions of the cell. These two domain membrane-bound enzymes are found in mitochondria, chloroplasts, and bacteria, coupling protons or Na\(^+\) ions to the synthesis of ATP (1, 2). The intracellular water-soluble \( F_1 \) domain contains the catalytic sites of the enzyme, while the bulk of the hydrophobic \( F_0 \) domain is embedded in the cytoplasmic membrane and contains the functional center for the capture and translocation of protons. The bacterial \( F_1 \) domain has a stoichiometry of \( \alpha_\beta_3 \gamma_2 \delta e \) in which the catalytic nucleotide-binding sites are formed by each of the \( \beta \) subunits and the non-catalytic nucleotide-binding sites are located in the \( \alpha \) subunits (3–5). The \( F_0 \) domain consists of one \( a \) subunit, two \( b \) subunits making the stator, and a ring of 10–15 \( c \) subunits depending on the species (6–12). The coupling of ion translocation to the rotational mechanism of the \( F_0 \) c-ring forces open, loose, or tight conformational changes within the \( \beta \) subunits of the \( F_1 \) domain, driving the synthesis of ATP from ADP and inorganic phosphate (13). Conversely, the \( F_1 \) domain can hydrolyze ATP causing the reverse rotation of the \( c \)-ring and pumping protons into the bulk phase, a mechanism that is absent in aerobic alkaliphilic organisms (14–17).

With \( F_1F_0 \)-ATP synthases that operate in a neutral to acidic pH environment, proton coupling is an energetically favorable process as the proton concentration outside the cell exceeds that in the cytoplasm. For example, at pH 7.1 \( Escherichia coli \) generates an electrochemical gradient of protons (\( \Delta \mu H^+ \))\(^4\) that is in the range –200 to –210 mV, with most of the \( \Delta \mu H^+ \) being in the form of an electrical potential (\( \Delta \psi > 150 \text{ mV} \)) but with a positive contribution of the \( \Delta \phi \) (18). In bacteria that inhabit alkaline pH environments, the magnitude of the \( \Delta \mu H^+ \) is markedly lower (in the range –50 to –100 mV) because of the inverted pH gradient (acidic, alkaline out) (19–21). Despite this apparent thermodynamic problem, the aerobic alkaliphilic bacteria studied to date employ a proton-coupled ATP synthase (14–16). The apparent thermodynamic problem is based

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4 The abbreviations used are: \( \Delta \mu H^+ \), electrochemical proton gradient; \( \Delta \phi \), transmembrane electrical potential; \( \Delta \psi \), transmembrane pH gradient; TA2F, \( F_0 \); TA2F, \( F_0 \)–ATP synthase from \( Bacillus \) sp. TA2.A1 (recombinant TA2F, \( F_0 \) investigated in the present study has a hexa-histidine tag at the N-terminus of the \( b \) subunit); ECF, \( F_1 \) \( E. coli \) \( F_0 \),–ATP synthase; aK180H, aK180G, and aK180R; recombinant TA2F, \( F_0 \), where Lys180 in the \( a \) subunit was substituted with His, Gly, and Arg, respectively; RSO, right-side-out; TMH, transmembrane helix; MOPS, 4-morpholinepropanesulfonic acid; LDAO, lauryldimethylamine oxide; PMS, phenazine methosulfate; DCCD, dicyclohexylcarbodiimide; DDM, dodecyl maltoside; TPMP \(^{3+} \), methyltriphenylmethylphosphonium iodide; PEG, polyethylene glycol.
on a mechanism with a delocalized $\Delta \mu H^+$ in the bulk phase but could be overcome by a more localized proton pathway between the respiratory chain and the ATP synthase. Support for such a model has been provided by Guffanti et al. (25) who demonstrated in Bacillus firmus RAB that a respiration-derived $\Delta \mu H^+$ could drive ATP synthesis at pH 9.0, but a valinomycin-mediated potassium-diffusion potential could not.

Apart from the thermodynamic problem of synthesizing ATP at high environmental pH, the alkaliphilic ATP synthase must also solve the problem of proton capture from an alkaline environment and subsequent translocation to the binding sites on the c-ring. Sequence analysis reveals that the ATP operon gene arrangement and deduced primary structure of the ATP synthase from alkaliphilic bacteria and other eubacteria are similar (24–26). As reported by Ivey and Krulwich (24, 25), conservation of amino acid sequences is observed in the a and c subunits that are crucial for proton translocation. The E. coli a subunit is a hydrophobic protein, spanning the membrane five to six times (27, 28). It is proposed to contain aqueous channels for protons to gain access to the binding sites in subunit five to six times (27, 28).

FIGURE 1. Alignment of the C-termiinal region of the a subunit of the F$_o$F$_{1}$-ATP synthases from Bacillus species and E. coli. Conserved residues are shaded and the alkaliphile-specific residues reported by Ivey and Krulwich (24, 25) are shown in bold. Sources of amino acid sequence were as follows: Bacillus sp. TA2.A1 (25), Bacillus pseudofirmus OF4 (25), Bacillus alcalophilus (accession number AC_002570), Bacillus halodurans (accession number NC_002570), Geobacillus stearothermophilus (accession number D38059), Bacillus sp. PS3 (62), Bacillus megaterium (63), Bacillus subtilis (64), and E. coli (65). Residue numbering for Bacillus sp. TA2.A1 and E. coli are indicated above and below the alignment, respectively.

We have recently initiated studies on the F$_o$F$_{1}$-ATP synthase from the thermoalkaliphile Bacillus sp. TA2.A1 (14, 19, 26, 32, 33). Like other alkaliphilic ATP synthases, the a subunit of strain TA2.A1 also harbors a conserved lysine residue at position 180 (Bacillus sp. TA2.A1 numbering) (Fig. 1) and we hypothesize that its side chain amino group acts as a base to capture protons at high environmental pH. In this communication, we report on the development of a heterologous expression system to overproduce and purify recombinant F$_o$F$_{1}$-ATP synthase from Bacillus sp. TA2.A1 (TA2F$_o$F$_{1}$) to address this hypothesis. Various amino acid substitutions in the a subunit were introduced to replace the lysine at position 180 (viz. glycine, histidine, and arginine) and the effect of external pH on ATP synthesis was studied in ADP plus P$_i$-loaded right-side-out membrane vesicles of E. coli DK8 expressing TA2F$_o$F$_{1}$.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—E. coli DH10B (34) was used for all cloning experiments and E. coli DK8 (35), lacking the ATP synthesis genes encoding the unc operon ($\Delta\text{unc}$), was used to overproduce the F$_o$F$_{1}$-ATP synthase of Bacillus sp. TA2.A1 (TA2F$_o$F$_{1}$) and the E. coli F$_o$F$_{1}$-ATP synthase (ECF$_o$F$_{1}$). Plasmids used were pUC19 (36) for cloning, pTrc99A (Amersham Biosciences) for overexpression of TA2F$_o$F$_{1}$ enzymes, and pBWU13 (37), which carries the unc operon coding for ECF$_o$F$_{1}$. To overproduce ATP synthases, transformants of E. coli DK8 were routinely grown in 2 × YT medium (38) containing 2 g/liter glucose and 100 µg/ml ampicillin at 37 °C with shaking at 200 rpm. For complementation studies, transformants of E. coli DK8 were selected on M13 minimal media (39) supplemented with 10 µg/ml thiamine, 50 µg/ml each of alanine, valine, asparagine, and isoleucine, 100 µg/ml ampicillin, and 35 µm sucinate as a non-fermentable carbon and energy source over a pH range of 7–9.5. Control plates contained the same amount of glucose in lieu of succinate. Plates were incubated for 2–5 days at 37 °C before scoring for growth.

Construction of an Expression Plasmid for TA2F$_o$F$_{1}$—In a previous study (26), the Bacillus sp. TA2.A1 genes encoding for the subunits of the F$_o$F$_{1}$-ATP synthase were cloned in pUC8 as
three overlapping fragments in plasmids pF10 (containing \textit{atpIBEFH}), pB9 (containing \textit{atpHAGD}'), and pA3 (containing \textit{atpgDC}). To assemble the \textit{atp} genes \textit{atpIBEFH} and part of \textit{atpD}, a 2.3-kb Sall-BamHI fragment from pF10 and a 3.4-kb BamHI-Sacl fragment from pB9 were simultaneously cloned into pUC19 (36) digested with Sall and SacI to create plasmid pATPHis5. The 

Expression and Purification of TA2F\textsubscript{1}Fo—DK8 harboring plasmid pATPHis5 was grown to an \(A_{600}\) of 0.35 to 0.4. The culture was induced with \(1 \text{ mM isopropyl-} \beta\text{-d-thiogalactopyranoside (IPTG)}\) and incubation continued for 5 h. Cells were harvested, washed with pre-cooled resuspension buffer A (20 mM sodium phosphate, pH 8.0, 2 mM MgCl\(_2\)), and resuspended in the same buffer. Phenylmethylsulfonyl fluoride was added to 0.1 mM, and the cells were disrupted by two passages through a French pressure cell at 20,000 psi. Pancreatic DNaseI was added to 0.1 mg/ml, and the mixture was kept on ice for 1 h until viscosity decreased. Lysate was cleared of debris by centrifugation at \(8,000 \times g\) for 10 min and the inverted membrane vesicles were pelleted from the supernatant at \(180,000 \times g\) for 1 h at 4 °C. Inverted membrane vesicles were washed in resuspension buffer A, followed by another wash in resuspension buffer A containing 1% (w/v) sodium cholate. To extract TA2F\textsubscript{1}Fo, from the cytoplasmic membrane, membrane vesicles were diluted to 5 mg of protein/ml in solubilization buffer (20 mM sodium phosphate, pH 8.0, 2 mM MgCl\(_2\), 10% glycerol, 2% dodecyl maltoside (DDM), 500 mM NaCl, 10 mM imidazole) and incubated at room temperature with gentle stirring for 1 h. The non-solubilized material was removed by ultracentrifugation (180,000 \(\times g\), 1 h, 4 °C), and the supernatant was incubated with gentle agitation for 45 min at room temperature with a slurry of Nickel-Sepharose High Performance (Amersham Biosciences), that was previously washed with water and equilibrated with IMAC buffer (20 mM sodium phosphate, pH 8.0, 2 mM MgCl\(_2\), 10% glycerol, 0.05% DDM, 500 mM NaCl) containing 10 mM imidazole. To remove contaminating proteins, the resin was washed with IMAC buffer containing 40 mM imidazole, and TA2F\textsubscript{1}Fo was eluted with IMAC buffer supplemented with 150 mM imidazole. To remove excess salt, the eluted TA2F\textsubscript{1}Fo was precipitated with 15% (w/v) polyethylene glycol (PEG) 6000 overnight on ice. The precipitate was pelleted by centrifugation at 54,000 \(\times g\) for 20 min at 4 °C and resuspended in resuspension buffer B (20 mM Tris-HCl, pH 8.0, 2 mM MgCl\(_2\), 10% glycerol, 0.05% DDM). The PEG-precipitated enzyme was applied to a POROS 50 HQ (Applied Biosystems) anion-exchange column, which was equilibrated with resuspension buffer B. Bound proteins were eluted with twelve column volumes of a linear gradient of 0–600 mM NaCl in the same buffer containing 0.05% DDM. The ATPas-containing fractions were pooled and concentrated to 30 mg/ml using Amicon Ultra centrifugal filter devices (molecular weight cutoff (MWCO), 100,000). The concentrated sample was then applied to a Superose 6 (Amersham Biosciences) gel filtration column and fractionated with 20 mM Tris-HCl, pH 8.0, 2 mM MgCl\(_2\), and 100 mM NaCl buffer containing 0.05% DDM. The eluted TA2F\textsubscript{1}Fo-containing fractions were pooled and concentrated to 10 mg/ml using Amicon Ultra centrifugal filter devices (MWCO, 100,000). Native TA2F\textsubscript{1}Fo was purified from \textit{Bacillus} sp. TA2A1 as described previously (14).

Preparation of RSO ADP plus Pi-loaded Membrane Vesicles—Plasmid transformants of \textit{E. coli} DK8 were grown to an \(A_{600}\) of 0.25, before addition of 1 mM IPTG to induce expression. RSO membrane vesicles were prepared from mid-log phase cultures (\(A_{600}\) of 1.1) by previously described methods (41, 42), with the exception that cellular debris and unbroken spheroplasts were removed by low speed centrifugation at 480 \(\times g\), 15 min, instead of 730 \(\times g\), 30 min. Spheroplasts were prepared in a hypertonic buffer (33 mM Tris-HCl, pH 8.0, 22% sucrose, 20 mM EDTA) containing 1.0 mg/ml lysozyme and gently stirred at room tem...
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perature for 40 min. Spheroplasts were pelleted by centrifugation at 11,600 × g, resuspended with an 18-gauge needle in 100 mM potassium phosphate buffer (pH 7.8) containing 20 mM MgSO₄, 20% sucrose (pH 7.8), and 5 mg/ml DNasel and lysed by osmotic shock, by diluting 100-fold into 50 mM potassium phosphate buffer (pH 7.8) containing 5 mM ADP. The suspension was stirred for 15 min at room temperature before the addition of 10 mM EDTA and 15 mM MgSO₄, stirring for 15 min at room temperature between steps. The suspension was centrifuged at 22,300 × g, and the pellet was resuspended in pre-cooled 100 mM potassium phosphate buffer (pH 7.8) containing 10 mM EDTA and 5 mM ADP. Cellular debris and unbroken spheroplasts were removed by low speed centrifugation (480 × g, 15 min). RSO membrane vesicles were harvested by centri-fuging the supernatant at 180,000 × g for 45 min and resuspending in 100 mM potassium phosphate buffer (pH 7.8) containing 2 mM MgCl₂ and 10% glycerol. Estimates of the fraction of vesicles in the opposite orientation using these methods vary, but they generally range from 10 to 20% (43, 44). To confirm vesicle orientation, NADH oxidation activity was measured in RSO membrane vesicles and compared with the same activity in inverted membrane vesicles. NADH (final concentration 0.25 mM) oxidation activity was monitored continuously at 340 nm.

Functional Assays—ATP hydrolysis activity was measured using a spectrophotometric ATP-regenerating assay at 45 °C. The assay mixture contained 100 mM Bis-Tris propane (various pH values), 2 mM MgCl₂, 3 mM phosphoenolpyruvate, 0.25 mM NADH, 0.57 units/ml pyruvate kinase, 3.2 units/ml lactate dehydrogenase, and 2.5 mM ATP. The reaction was initiated by the addition of enzyme into 1 ml of assay mixture, and the rate of NADH oxidation was monitored continuously at 340 nm using a Cary 50 (Varian) spectrophotometer. Approximately 21 μg of recombinant TA2F₁Fₒ was used for measurements. The activity that hydrolyzed 1 μmol of ATP per min is defined as 1 unit.

ATP synthesis in inverted membrane vesicles was carried out by the method of Tomashke et al. (45) at 37 °C in 1 ml of 10 mM MOPS/Tris-HCl (pH 8.0) buffer containing 2 mM MgCl₂ with stirring. Approximately 0.5 mg of membrane vesicles were incubated with stirring at 37 °C for 2 min, followed by incubation in the presence of 2.5 mM NADH for 2 min. When performing inhibition experiments, 100 μM DCCD was added 1 min prior to the addition of NADH. ATP synthesis was initiated with the concurrent addition of 0.75 mM ADP and 2.5 mM potassium phosphate (pH 8.0). At various time intervals, 100-μl aliquots were removed and transferred to 400 μl of stop solution (1% trichloroacetic acid, 2 mM EDTA). Each sample was diluted 500-fold in water prior to the measurement of ATP (see below).

ATP synthesis in ADP (5 mM) plus P₇ (50 mM)-loaded RSO membrane vesicles was based on the protocol of Tsuchiya (46). Membrane vesicles were diluted 20-fold to a concentration of 0.5 mg/ml into prewarmed (35 °C) 25 mM Bis-Tris propane buffer (various pH values) containing 0.25 mM sucrose, 5 mM MgCl₂, and 200 mM K₂SO₄. When performing inhibition experiments, membranes were incubated with 100 μM DCCD for 2 min prior to dilution. The reaction was initiated within 10 s of dilution by energizing the system with 20 mM potassium ascorbate and 0.1 mM phenazine methosulfate (ascorbate- PMS) with aerated vesicles. Aliquots were removed at various time points, transferred to precooled stop solution (30% perchloric acid, 9 mM Na₂EDTA), and incubated on ice for 20 min. Denatured protein was removed by centrifugation (13,000 × g for 5 min), and the supernatant neutralized by the addition of 1 M NaOH plus 0.5 M NaCO₃, followed by snap freezing. Salt was removed from the frozen supernatant by rapid thawing and centrifugation for 5 min at 13,000 × g. To ensure residual perchloric acid did not inhibit subsequent ATP measurements, each sample was diluted 50-fold in water.

Using the diluted samples from inverted and RSO membrane vesicle preparations, the amount of ATP was determined by the luciferin-lucerase assay as described previously (14). To measure ATP, each sample was diluted into 400 μl of Tris acetate buffer (50 mM Tris acetate, pH 7.8, 2 mM EDTA, 50 mM MgCl₂) in a luminometer tube. 50 μl of luciferin-lucerase reagent (Sigma) was added to the tube, and the fluorescence monitored with a chemiluminometer (FB 12 luminometer; Berthold). The amount of ATP synthesized was calculated from a standard curve performed on the day of each set of ATP measurements. For each individual experimental set, the presence of background ATP was measured using non-energized vesicles and subtracted from total ATP measured.

TPMP⁺ Accumulation in RSO Membrane Vesicles—The membrane potential (Δψ) generated by ascorbate-PMS energization of RSO membrane vesicles over the pH range 7–10 was determined by measuring the accumulation of [³H]methyltritylphenylphosphonium iodide (TPMP⁺) (30–60 Ci/mmol) using filtration assays (0.45-μm cellulose-acetate filters) (47). A value of 2.2 μl of intravesicular volume per mg of protein was used (48). RSO membrane vesicles (0.6–0.9 mg/ml) were energized with ascorbate-PMS under identical conditions used for measuring ATP synthesis in RSO membrane vesicles (see above) and the accumulation of [³H]TPMP⁺ (1 μM final concentration) measured after 1 min incubation at 35 °C. The Δψ was calculated from the Nernst equation (Δψ = 61 × log [TPMP]₅₀/[TPMP]₀). Nonspecific TPMP⁺ binding was estimated from RSO membrane vesicles that had been treated with either CCCP or a combination of valinomycin and nigericin (10 μM each) for 10 min. The results reported are the mean values of three biological replicates and the standard error of the mean associated with these measurements is shown.

SDS-PAGE and Immunoblotting—TA2F₁Fₒ preparations were routinely analyzed on 16% SDS-polyacrylamide gels in the presence of 0.1% SDS using the buffer system of Laemmli (49). Polyepitope bands were visualized using either Simply Blue® Safe Stain (Invitrogen) or Coomassie Brilliant Blue. During immunoblotting, RSO membrane vesicles were subjected to 14% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting onto a polyvinylidene difluoride membrane ensuring efficient transfer by including 0.02% SDS in the running buffer. Detection was achieved using a penta-His antibody conjugate (Qiagen) directed against the hexa-histidine tag of the β subunit of the recombinant TA2F₁Fₒ. The antibody-specific bands were visualized using the SuperSignal® West Pico chemiluminescence system.
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Protein Assay—Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Sigma) with bovine serum albumin as the standard.

RESULTS

Expression of TA2F₁Fo in E. coli—The atp operon, coding for TA2F₁Fo, with a hexa-histidine tag at the N terminus of the β subunit, was cloned into the expression plasmid pTrc99A, and expressed in the unc deletion mutant E. coli DK8. TA2F₁Fo was extracted from E. coli membranes and purified via a three step purification procedure. SDS-PAGE analysis of the purified recombinant enzyme identified seven subunits (viz. α, β, γ, δ, b, ε, and c) that corresponded to those of the native F₁Fo purified from Bacillus sp. TA2.A1 (Fig. 2A, lanes 1 and 2). The α subunit was difficult to visualize in both the native and recombinant TA2F₁Fo enzyme purified from E. coli membranes and purified via a three step purification procedure. SDS-PAGE analysis of the purified recombinant enzyme identified seven subunits (viz. α, β, γ, δ, b, ε, and c) that corresponded to those of the native F₁Fo purified from Bacillus sp. TA2.A1. The α subunit was difficult to visualize in both the native and recombinant TA2F₁Fo-ATP synthase using the staining methods employed here. However, chloroform-methanol extraction as previously described (14) demonstrated that the α subunit was present in both our preparations (data not shown). Moreover, ATP synthase was inhibited by DCCD confirming that the ATP synthase was indeed coupled (see Fig. 3C). A unique feature of the TA2F₁Fo enzyme purified from Bacillus sp. TA2.A1 is its specific blockage in ATP hydrolysis activity, and this activity can be stimulated >15-fold with 0.4% LDAO (14). Like the native TA2F₁Fo, the purified recombinant enzyme was blocked in ATP hydrolysis activity, with a specific activity of 0.7 units/mg protein (Fig. 2B). This ATPase activity could be stimulated 25-fold with 0.4% LDAO (Fig. 2B), corresponding to a specific activity of 17.6 units/mg protein, similar to that observed for the native TA2F₁Fo (14). The pH profile of the recombinant TA2F₁Fo enzyme was comparable to that of the native TA2F₁Fo enzyme showing a maximum rate of ATP hydrolysis activity at pH 7.5 (Fig. 2C). To determine whether the TA2F₁Fo enzyme could complement E. coli DK8 Δatp, the strain harboring the entire atp operon of Bacillus sp. TA2.A1 (i.e. DK8/pATPHis5) was plated onto M13 minimal medium containing succinate as the sole carbon and energy source over the pH range 7–9.5. As a positive control, E. coli DK8 was transformed with plasmid pBWU13 (37) encoding for ECF₁Fo. The positive control grew on either glucose or succinate over the pH range 7–8.5. E. coli DK8 harboring TA2F₁Fo grew on glucose but no growth was observed on succinate over the pH range tested. Hence, the atp operon encoding for TA2F₁Fo was unable to produce an ATP synthase in the host cells with proper function to support growth of E. coli DK8 on non-fermentable carbon sources over the pH range 7–9.5.

ATP Synthesis in E. coli Inverted Membrane Vesicles—Inverted membrane vesicles were prepared from DK8, DK8-expressing ECF₁Fo, and DK8-expressing TA2F₁Fo (Fig. 3). ATP synthesis was energized by the addition of NADH 2 min prior to initiating ATP synthesis with ADP and P_i. Inverted membrane vesicles of DK8 containing ECF₁Fo synthesized ATP at a rate of 403 ± 17 nmol of ATP/min/mg protein and the majority of this activity was inhibited by the addition of DCCD (Fig. 3A). The rate of ATP synthesis observed here agrees well with previous reports, which showed the synthesis rate of ECF₁Fo in inverted membrane vesicles to be 520 nmol ATP/min/mg protein at pH 7.5 (45). Inverted membrane vesicles of DK8 synthesized ATP at a rate of 174 ± 22 nmol of ATP/min/mg protein that was not inhibited by DCCD, suggesting that this ATP synthesis was via some endogenous route as these vesicles do not contain ECF₁Fo (Fig. 3B). It is noteworthy that this level of ATP synthesis is similar to the rate of ATP synthesis in ECF₁Fo in the presence of DCCD (Fig. 3A). Inverted membrane vesicles of DK8 containing TA2F₁Fo showed an ATP synthesis rate of 486 ± 19 nmol of ATP/min/mg protein and DCCD inhibited this rate to similar levels seen for ECF₁Fo (Fig. 3C). These data indicated that the TA2F₁Fo enzyme was functional in membrane vesicles of E. coli DK8 and therefore we proceeded to make various mutations in the α subunit of this enzyme for functional studies.

Expression of aK180 Mutants in E. coli DK8—To characterize the role of the conserved lysine residue at position 180 (Bacillus sp. TA2.A1 numbering) in the α subunit of the TA2F₁Fo enzyme (identified in Fig. 1), three amino acid substitutions were made in pATPHis5 (viz. aK180H, aK180G, aK180R). Mutant constructs were expressed in E. coli DK8, and aK180H, aK180G, and aK180R were solubilized and purified to determine their biochemical properties. To examine if the aK180 mutant complexes assembled properly, SDS-PAGE analysis was used to compare these to TA2F₁Fo (Fig. 4). SDS-PAGE analysis revealed that all mutant aK180 complexes were assembled in the E. coli membranes and functional assays showed that the ATPase activity (LDAO-stimulated) of these mutant forms was comparable to the recombinant TA2F₁Fo complex.
The apparent $K_m$ for ATP and Mg$^{2+}$ were also in the range of the values reported for the native TA2F1Fo enzyme (data not shown). The pH profile (pH 6.5–9.0) of each $aK180$ mutant was determined and each mutant had a pH optimum of ~7.5 (data not shown), indicating that the substitution of the lysine residue for histidine, glycine, and arginine did not change the pH profile of the mutant enzyme in the ATP hydrolysis direction. None of the $aK180$ mutants were able to complement $E. coli$ DK8 for growth on minimal media containing succinate as the sole carbon source and energy source.

**ATP Synthesis of $aK180$ Mutants in $E. coli$ DK8 Inverted Membrane Vesicles**

Inverted membrane vesicles of DK8 containing $aK180H$, $aK180G$, and $aK180R$ were prepared with an internal pH of 8.0. Time course ATP synthesis assays energized by NADH were conducted independently for each set of vesicles with or without the addition of the specific ATPase inhibitor DCCD at pH 8.0 (Fig. 5). $aK180H$ and $aK180G$ showed ATP synthesis rates of 973 ± 11 and 735 ± 8 nmol of ATP/min/mg protein, respectively (Fig. 5, A and B). These rates were 1.5- to 2-fold higher than for the TA2F1Fo enzyme (see Fig. 3C). With the $aK180R$ enzyme, a rate of ATP synthesis similar to TA2F1Fo was observed (i.e. 386 ± 11 nmol of ATP/min/mg protein) (Fig. 5C). The addition of DCCD caused a significant inhibition of ATP synthesis in all $aK180$ mutants (Fig. 5, A–C).

These experiments were performed at an external pH of 8.0 that mimics the intracellular environment of the enzyme using inverted membrane vesicles (i.e. $F_1$ on the outside). In order to assess the effect of external pH on ATP synthesis, we prepared ADP plus Pi-loaded RSO membrane vesicles where the $F_1$ portion was on the inside of the vesicles (maintained at pH 7.5–8.0) and the $F_o$ (i.e. a subunit) could be exposed to different external pH values (e.g. pH 7.0–10.0). $E. coli$ has been shown to grow at an external pH of 8.7 (50, 51), and experiments have been performed previously with RSO membrane vesicles at external pH values of 2.5–8.5 (46, 52).

**ATP Synthesis of TA2F1Fo and $aK180$ Mutants in ADP plus Pi-loaded RSO Membrane Vesicles**

RSO membrane vesicles were prepared using a standard $E. coli$ procedure (41, 42) from each strain with an internal pH of 7.5–8.0 containing 5 mM ADP and 50 mM potassium phosphate. While the protocol is designed to generate RSO membrane vesicles, it has been esti-
mated that 10–20% of these vesicles may in fact be orientated opposite to the direction intended (43, 44). Importantly, in terms of this study, the fraction orientated opposite to the intended direction should be constant from *E. coli* mutant to mutant. A comparison of NADH oxidation activity at 340 nm between the RSO membrane vesicle and inverted membrane vesicle preparations revealed that while we could detect significant NADH oxidation (880 nmol NADH/min/mg protein) by the inverted membrane vesicles, there was negligible activity by the RSO membrane vesicles. On the basis of this data, the vesicles are in the correct orientation (i.e., RSO).

To assess proper subunit assembly within RSO membrane vesicles and TA2F1Fo expression, an SDS-PAGE of RSO membrane vesicles revealed that all forms of the TA2F1Fo were assembled and an immunoblot of the purified proteins was performed which targeted the hexa-histidine tag on the N terminus of the β subunit. Expression was equal across all strains (Fig. 6).

ATP synthesis in RSO membrane vesicles was energized using a number of standard methods (e.g., valinomycin-induced K⁺ diffusion potential, NADH oxidation, or ascorbate-PMS). Ascorbate-PMS was the most effective energization source for these experiments. This was validated by measuring the accumulation of [³H]triphenylmethylphosphonium iodide in RSO membrane vesicles of ECF1Fo and TA2F1Fo under identical conditions used to measure ATP synthesis over the pH range 7–10. The membrane potential over this pH range, ranged from −66 to −75 mV (interior negative) for RSO membrane vesicles of ECF1Fo and at pH 10 the Δψ was −73 ± 4 mV. RSO membrane vesicles of TA2F1Fo generated a Δψ of −69 to −83 mV over the pH range tested and at pH 10 the Δψ was −81 ± 8. These data demonstrate that the external pH, during the course of the incubation, did not have a significant effect on the ability of RSO membrane vesicles to generate a significant Δψ that could be used to drive ATP synthesis. Furthermore, the Δψ values reported here are in good agreement with previous reports using RSO membrane vesicles of *E. coli* energized via the oxidation of ascorbate-PMS (i.e., −75 mV over the pH range 7–8.5) (47, 53). The Δψ generated under these conditions has been shown to be sufficient to drive energetic processes (e.g., lactose, proline, and serine transport) (54).

ATP synthesis experiments were initially performed with DK8 membrane vesicles, and DK8 membrane vesicles harboring ECF1Fo and TA2F1Fo, at an external pH of 8.0 using ascorbate-PMS energization. These experiments showed ECF1Fo

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**FIGURE 5.** ATP synthesis of aK180 mutants in *E. coli* inverted membrane vesicles. A–C, time course ATP synthesis assays at pH 8.0 and 37 °C with 0.5 mg of inverted membrane vesicles using the ATP synthesis inverted membrane vesicle assay described under “Experimental Procedures.” Membranes were preincubated for 2 min with 2.5 mM NADH with stirring before the reaction was initiated using 0.75 mM ADP and 2.5 mM Pi. Open circles, with no DCCD; closed circles, a 1-min preincubation with 100 μM DCCD. A, aK180H; B, aK180G; C, aK180R. Each point is the result of three biological replicates, and the standard error associated with this measurement is shown.

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**FIGURE 6.** Amounts of TA2F1Fo and aK180 mutants in *E. coli* RSO membrane vesicles. RSO membrane vesicles (25 μg of protein) were separated by polyacrylamide gel electrophoresis (14%) in the presence of 0.1% sodium dodecyl sulfate. Proteins were transferred to a polyvinylidene difluoride membrane in the presence of 0.02% sodium dodecyl sulfate and then immunoblotted using a penta-His antibody conjugate to detect the hexa-histidine tag of the β subunit. Lane 1, TA2F1Fo; lane 2, aK180R; lane 3, aK180H; lane 4, aK180G.
performed ATP synthesis at a rate of 1.66 ± 0.12 nmol of ATP/min/mg protein with most of this activity being inhibited by DCCD (Fig. 7A). RSO membrane vesicles of DK8 vesicles showed low rates of ATP synthesis both in the absence and presence of DCCD (0.25 ± 0.11 and 0.12 ± 0.01 nmol of ATP/min/mg protein, respectively) (Fig. 7B). The rate of ATP synthesis for DK8 membrane vesicles containing TA2F1Fo was 1.14 ± 0.08 nmol of ATP/min/mg protein, and this rate was almost completely abolished by DCCD (Fig. 7C).

Having confirmed that the experimental system was reproducible and in agreement for E. coli ATP synthesis rates published (46), time course ATP synthesis assays were conducted independently over a pH range from 7.0 to 10.0 for each set of RSO membrane vesicles (Fig. 8). ECF1Fo showed high ATP synthesis activity at pH 7.0 and 7.5 (6.23 ± 0.12 and 5.52 ± 0.14 nmol ATP/min/mg protein, respectively) (Fig. 8A). As the external pH was increased, the rate of ATP synthesis decreased dramatically and no ATP synthesis was measurable at pH > 8.5 (Fig. 8A). RSO membrane vesicles of DK8 had no detectable levels of ATP synthesis over the entire pH range studied (Fig. 8B). The ATP synthesis pH profile of TA2F1Fo was opposite to ECF1Fo with a pH optimum between 9.0–9.5 and no significant rates of ATP synthesis observed below pH 8.0 (Fig. 8C). All of the ATP synthesis under these conditions was sensitive to DCCD (Fig. 8C). aK180R had a pH profile for ATP synthesis (all DCCD sensitive) that was comparable to that of TA2F1Fo (Fig. 8F). When the K180 in the α subunit was substituted for either histidine or glycine, the pH profile of the TA2F1Fo enzyme was shifted toward the neutral pH range with ATP synthesis rates peaking at pH 8.0 and pH 7.5 for aK180H and aK180G, respectively (Fig. 8, D and E).

Based on the observation that the aK180H and aK180G mutants exhibited a similar pH profile to ECF1Fo for ATP synthesis, we tested the ability of these constructs to complement growth of strain DK8 Δatp over the pH range 7–9.5 on M13 minimal medium containing succinate as the sole carbon and energy source. Under no conditions was complementation (i.e. growth) observed on succinate-containing medium (data not shown).

DISCUSSION

The ATP synthases from bacteria, mitochondria, and chloroplasts are highly conserved enzymes that have evolved to capture and translocate protons or sodium ions from the bulk phase to drive rotation of an oligomeric c-ring coupled to the synthesis of ATP at the catalytic β subunits. In bacteria, two different classes of F-type ATP synthases have been reported, those that are coupled to protons and another class that employ sodium ions (1). In those ATP synthases that use sodium ions, a specific sodium-binding motif has been identified in the c subunit (viz. Ψ^28, E^61, and S^62) (7, 55–58). For those bacteria growing at alkaline pH, very few protons (e.g. pH 10 [H^+] = 1 × 10^{-10} M) exist in the bulk phase and therefore it would seem disadvantageous to couple membrane-bound bioenergetic processes to proton translocation. The inverted pH gradient in these bacteria leads to an overall low electrochemical potential of protons and the ATP synthase works against a large ΔpH (i.e. 2 pH units) to synthesize ATP. Despite these apparent ther-
could be termed a facultative alkaliphile (i.e. pH 7.5–10) during growth on fermentable carbon sources (19), but an obligate alkaliphile during growth on non-fermentable carbon sources (i.e. >pH 9.0). In the current study, we hypothesized that the ATP synthase in strain TA2.A1 may be better adapted to function at high pH versus neutral pH values thus providing an explanation for the lack of growth at pH values below 9 on non-fermentable carbon sources.

To address the above hypothesis we developed a heterologous system to express the TA2F1Fo enzyme in E. coli DK8 (Δatp) for functional studies and used ADP plus Pi-loaded RSO membrane vesicles of E. coli to measure ATP synthesis. In the absence of a genetic system for Bacillus sp. TA2.A1, the recombinant TA2F1Fo system enabled us to make various amino acid substitutions to the invariant lysine residue at position 180 of the a subunit. Mutations were created substituting aK180 for either histidine (pKₐ = 6.1), arginine (pKₐ = 12.84), or glycine and the effect of external pH on ATP synthesis was studied. TA2F1Fo synthesized ATP between external pH values of 8.5 to 9.5 that was DCCD-sensitive. A similar pH profile was observed for aK180R. When histidine was substituted for lysine 180, the pH profile of the enzyme was much broader (pH 7–9.5) and shifted into the neutral pH range (pH optimum of 8). The aK180G mutant exhibited a pH profile that was comparable to that of E. coli. On the basis of these findings, we propose that a residue with a strong base, such as lysine or arginine, is ideally suited to function at alkaline pH, but is inefficient at pH values well below its pKₐ in solution (e.g. 10.3 for lysine). A residue at position 180 without a base in its side chain, such as glycine, was on the other hand favorable for ATP synthesis at neutral pH but did not support ATP synthesis at high pH. Histidine with a pKₐ in the neutral range supported ATP synthesis at pH values from neutral to alkaline. It should be pointed out, that in general, there was a poor quantitative correlation between the pH maximum of ATP synthesis activity and the pKₐ of the residue at position 180. This was particularly evident for histidine where significant ATP synthesis activity was observed at pH 9.0. However, the pKₐ values assumed here are for the residues in solution and the ionization states under physiological conditions often differ, particularly in a membrane environment (60). For example, Cymes et al. (60) revealed that histidine could participate in proton transfer reactions over the broad pH range 6 to 9 when engineered in the α-helical lining of the transmembrane pore of the nicotinic acetylcholine receptor.

According to accessibility studies for hydrophilic probes, glycine 218 of E. coli, which corresponds to aK180 of TA2F1Fo, is proposed to lie at the entrance of the periplasmic access channel for protons to gain access to the binding sites on the c-ring (29, 30). A possible explanation for the observations described above is that Lys180 with its high pKₐ is ideally suited for proton acquisition from the bulk phase at high pH. Furthermore, at pH values near the pKₐ of the side chain amino group of Lys180, the captured proton could easily dissociate again and continue its journey through the channel to the c subunit binding sites. At pH values well below the pKₐ of lysine 180, however, the trapped proton would largely be retained on its amino group and this would cause either a blocking of the proposed proton channel and/or mechanism of proton translocation, and there-
fore ATP synthesis is inhibited. Consistent with this hypothesis is the lack of ATP synthesis in TA2F,Fo (i.e. aK180) and aK180R enzymes at pH values less than 8.0. A glycine at position 180 can obviously not contribute to proton acquisition from an alkaline environment, nor will it act as a permanent proton trap and hence, with this residue ATP synthesis is most favorable in the neutral pH range. The model presented above is corroborated by observations made with the ATP synthase of E. coli whereby an E. coli aG218K mutant was able to grow on a fermentable carbon source like glucose, but unable to grow on succinate at neutral pH values (61). The effect of higher pH on the growth of this mutant on succinate was not tested.

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REFERENCES

1. Dimroth, P., von Ballmoos, C., and Meier, T. (2006) EMBO Rep. 7, 276–282
2. Yoshida, M., Muneyuki, E., and Hisabori, T. (2001) Nat. Rev. Mol. Cell. Biol. 2, 669–677
3. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) J. Mol. Biol. 239, 327–333
4. Guffanti, A. A., Fuchs, R. T., Schneier, M., Chiu, E., and Kruwlich, T. A. (1985) Arch. Biochem. Biophys. 239, 327–333
5. Guffanti, A. A., Fuchs, R. T., Schneier, M., Chiu, E., and Kruwlich, T. A. (1984) J. Biol. Chem. 259, 2971–2975
6. Ivey, D. M., and Kruwlich, T. A. (1992) Res. Microbiol. 143, 467–470
7. Ivey, D. M., and Kruwlich, T. A. (1991) Mol. Gen. Genet. 229, 292–300
8. Keis, S., Kaim, G., Dimroth, P., and Cook, G. M. (2004) Biochim. Biophys. Acta 1676, 112–147
9. Valiyaveetil, F. I., and Fillingame, R. H. (1998) J. Biol. Chem. 273, 16241–16247
10. Stahlberg, H., Müller, D. J., Suda, K., Fotiadis, D., Engel, A., Meier, T., and Müller, D. J. (2005) EMBO Rep. 6, 1040–1044
11. Stahlberg, H., Müller, D. J., Suda, K., Fotiadi, D., Engel, A., Meier, T., Matthey, U., and Dimroth, P. (2001) EMBO Rep. 2, 229–233
12. Stock, D., Kaim, G. A. W., and Walker, J. E. (1999) Science 286, 1700–1705
13. Seelert, H., Dencher, N. A., and Müller, D. J. (2003) J. Mol. Biol. 333, 337–344
14. Boyer, P. D. (1997) Annu. Rev. Biochem. 66, 717–749
15. Cook, G. M., Keis, S., Morgan, H. W., von Ballmoos, C., Matthey, U., Kaim, G., and Dimroth, P. (2003) J. Biol. Chem. 278, 4442–4449
16. Ivey, D. M., and Kruwlich, T. A. (1990) J. Biol. Chem. 265, 20547–20554
17. Hoffmann, A., and Dimroth, P. (1990) Eur. J. Biochem. 194, 423–430
18. Dimroth, P., and Cook, G. M. (2004) Adv. Microb. Physiol. 49, 175–218
19. Kashket, E. R. (1985) Annu. Rev. Microbiol. 39, 219–242
20. Olsson, K., Keis, S., Morgan, H. W., Dimroth, P., and Cook, G. M. (2003) J. Biol. Chem. 278, 461–465
21. Hoffmann, A., and Dimroth, P. (1991) Eur. J. Biochem. 201, 467–473
22. Sturr, M. G., Guffanti, A. A., and Kruwlich, T. A. (1994) J. Bacteriol. 176, 3111–3116
23. Guffanti, A. A., Chiu, E., and Kruwlich, T. A. (1985) Arch. Biochem. Biophys. 239, 327–333
24. Ivey, D. M., and Kruwlich, T. A. (1992) Res. Microbiol. 143, 467–470
25. Ivey, D. M., and Kruwlich, T. A. (1991) Mol. Gen. Genet. 229, 292–300
26. Keis, S., Kaim, G., Dimroth, P., and Cook, G. M. (2004) Biochim. Biophys. Acta 1766, 112–147
27. Valiyaveetil, F. I., and Fillingame, R. H. (1998) J. Biol. Chem. 273, 16241–16247