Published in final edited form as:

Biochemistry. 2011 June 21; 50(24): 5497–5506. doi:10.1021/bi2005009.

Mutations in a helix-1 motif of the ATP synthase c-subunit of *Bacillus pseudofirmus* OF4 cause functional deficits and changes in c-ring stability and mobility on SDS-PAGE

Jun Liu[‡], Oliver J. Fackelmayer[‡], David B. Hicks[‡], Laura Preiss^{||}, Thomas Meier^{||}, Eric A. Sobie[‡], and Terry A. Krulwich^{‡,*}

[‡] Department of Pharmacology and Systems Therapeutics, Mount Sinai School of Medicine, 1 G. Levy Place, New York, New York 10029, USA

^{||} Max-Planck Institute of Biophysics, Department of Structural Biology, Max-von-Laue-Str. 3, 60438, Frankfurt am Main, Germany

Abstract

The ATP synthase of the alkaliphile *Bacillus pseudofirmus* OF4 has a tridecameric c-subunit rotor ring. Each c-subunit has an AxAxAx motif near the center of the inner helix, where neutralophilic bacteria generally have GxGxGxG. Here, we studied the impact of four single and six multiple Ala-to-Gly chromosomal mutations in the A16xAxAx22 motif on the capacity for non-fermentative growth and, for most of the mutants, on ATP synthesis by ADP + P_i-loaded membrane vesicles at pH 7.5 and 10.5. SDS-PAGE analyses of the holo-ATP synthases were used to probe stability of the mutant c-rotors and mobility properties of the c-rotors as well as the monomeric c-subunits that are released from them by trichloroacetic acid treatment. Mutants containing an Ala16-to-Gly mutation exhibited the most severe functional defects. On SDS-PAGE, most of the mutant c-monomers exhibited increased mobility relative to the WT c-subunit, but among the intact c-rings, only Ala16-to-Gly containing mutants exhibited significantly increased mobility relative to the WT c-ring. The hypothesis that these c-rings have a decreased c-subunit stoichiometry is still untested but the functional impact of an Ala16-to-Gly mutation clearly depended upon additional Ala-to-Gly mutation(s) and their positions. The double A16/20G mutant exhibited a larger functional deficit than both the A16G and A16/18G mutants. Most of the mutant c-rings showed *in vitro* instability relative to wild-type (WT) c-ring. However, the functional deficits of mutants did not correlate well with the extent of c-ring stability loss, so this property is unlikely to be a major factor *in vivo*.

OXPPOS1 by the extremely alkaliphilic *Bacillus pseudofirmus* OF4 at high pH and low bulk PMF has long been hypothesized to depend upon particularly effective kinetic trapping of protons near the membrane surface as they emerge from proton-pumping respiratory chain complexes(1). Evidence suggests that such sequestration of protons near the surface may allow protons to reach the proton-coupled F₁F₀-ATP synthase (hereafter referred to as ATP synthase) of alkaliphiles before they fully equilibrate with the bulk medium that can be

*To whom correspondence should be addressed. terry.krulwich@mssm.edu. Telephone: 212-241-7280. Fax: 212-996-7214.

[‡]This work was supported by research grants GM28454-26 (to TAK), R01 HL076230 (to EAS), and by P50 GM071558 (to TAK and EAS) from the National Institutes of Health, and the Cluster of Excellence "Macromolecular Complexes" at the Goethe University Frankfurt (DFG Project EXC 115) (to TM) and the DFG Collaborative Research Center (SFB) 807 (to TM).

¹Abbreviations: CM, chloroform/methanol; DDM, dodecyl β-D-maltoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; dMW, difference between apparent and formula molecular weight; DTT, Dithiothreitol; LS, lauroyl sarcosine; OG, octyl β-D-glucoside; OXPPOS, oxidative phosphorylation; PMF, proton-motive force; PMSF, phenylmethanesulfonyl fluoride; TCA, trichloroacetic acid; WT, wild-type.

above pH 11 (1–4). Additionally, successful function of the alkaliphile ATP synthase at high pH depends upon specific adaptations of both the respiratory chain complexes and the ATP synthase (5–9). Adaptations of the ATP synthase to function at alkaline pH were found in the membrane-embedded F_o complex (5, 8–11). In the c -subunit of alkaliphiles, which forms the c_{13} rotor ring, two specific motifs were described. The PxxExxP motif is located in the outer (C-terminal) helix and contains the conserved, proton-binding residue (Glu54 in *B. pseudofirmus* OF4) (12). The AxAxAx motif is in the inner (N-terminal) helix of extremely alkaliphilic *B. pseudofirmus* OF4 in place of the GxGxGxG motif of most neutralophiles (9); less extreme alkaliphiles have motifs with 2–3 Ala residues instead of the full four substitutions in *B. pseudofirmus* OF4 (8, 9) (Figure 1A). Initial studies of a panel of single, double, triple and quadruple mutants in the alanine motif primarily focused on the quadruple mutant, which shows a major deficit on malate growth and ATP synthesis at pH 10.5; the synthetic deficits were smaller at pH 7.5 and the mutant enzyme exhibited coupled ATPase activity, with latency similar to the WT enzyme and sensitivity to dicyclohexylcarbodiimide inhibition (8). It was concluded that changes in the AxAxAx motif might exert their effects via modulation of inter-subunit interactions (13) that could in turn affect the pK_a of the proton-binding Glu54 (8).

In the high-resolution crystal structure of the tridecameric rotor ring of the *B. pseudofirmus* OF4 ATP synthase (14) the overall shape of the complex was found to be slightly different from other rotor rings. The *B. pseudofirmus* OF4 c_{13} -ring is flared at the cytoplasmic end but more straight at the periplasmic end so that this c -ring lacks the pronounced hour-glass shape of other high-resolution crystal structures of c - (or K -) rings (14–17). The shape of the c_{13} -ring reflects structural effects imposed by the AxAxAx and PxxExxP motifs of *B. pseudofirmus* OF4 (Figure 1) (14). The AxAxAx motif is located in the middle of the inner helix, near the ion-binding glutamate and in the area of the c -ring that is most tightly packed. Like the GxSxGxS motif of thermoalkaliphile *Caldalkalibacillus thermarum* TA2.A1, it is hypothesized to play a role in distancing the individual c -subunits from one another more than has been found in other known c -ring structures, thus widening the c -ring diameter (10, 14). Here, we examined in greater detail than in the earlier study, a panel of Ala-to-Gly mutants in the alkaliphile AxAxAx motif that includes all four single mutants, an expanded panel of four double mutants (relative to one double mutant in the earlier study) as well as a triple and quadruple mutant. The first major goal was to determine whether the functional impact of Ala mutations on non-fermentative growth and ATP synthase activity correlated primarily with the number of Ala-to-Gly changes in a given mutant, i.e. single, double, triple or quadruple, or strongly depended on the positions of mutations in particular mutants. The second major goal was to take advantage of the exceptional stability of the *B. pseudofirmus* OF4 c -ring (3, 14, 18) to determine whether Ala-to-Gly mutations in the AxAxAx motif change that stability and, if so, whether such changes correlated with changes in functional capacity. Stability was probed using analyses on SDS-PAGE gels, which also revealed unanticipated changes in the mobility properties of some of the mutant c -rings and of c -monomers that dissociate from the mutant c -rings upon TCA treatment.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Mutant Construction and Growth Conditions

The WT strain is a derivative of alkaliphilic *B. pseudofirmus* OF4 811M that has an EcoRI site introduced into *atpB* (8). Three new double mutants of the c -subunit, A16/18G, A18/22G and A20/22G, were constructed as described previously for the other mutants (8). The primers used for the mutations in this study are available upon request. Homologous recombination introduced the mutation into the chromosomal *atp* locus. Appropriate PCR products encompassing the *atp* operon were sequenced by Genewiz, Inc. (South Plainfield, NJ) and verified to have only the desired mutation(s). For growth experiments, the WT and

mutant strains were grown on malate-containing MYE medium or on glucose-containing GYE medium at pH 7.5 and 10.5, with duplicate samples in 2–3 independent experiments (8). Determinations of growth of the full mutant panel on malate, assessed by the A_{600} at 14 hours in 2 ml of culture in 15 ml tubes were carried out at the two pH values as described before (8), with the WT strain and the F_0 deletion strain ($\Delta F_0, \Delta atpB-F$) as the positive and negative controls. Appropriate dilutions were made to give A_{600} readings in the linear range of 0.3–0.5. In the earlier study that focused on the quadruple mutant, two A16/18/20/22G mutant strains were included; the mutant designated A4G-1 in the earlier study was used here for the data shown in Table 1. For several mutants, growth on MYE medium at pH 10.5 was monitored over time during growth using 50 ml of culture in 250 ml flasks, grown with shaking 250 rpm at 30°C in comparison with the same positive and negative controls used for the single point growth experiments in tubes; for the growth curve experiments, the inocula for all the strains were pre-grown on GYE medium at pH 10.5.

Isolation of Everted Membrane Vesicles, Assays of their Octylglucoside- Stimulated ATPase Activity, and Assessment of the Membrane Content of ATP Synthase Protein

Everted membrane vesicles were prepared from the WT and three new double mutants using the protocol that was previously described (8). Protein content for this and other experiments was determined by the method of Lowry *et al.* (19). OG-stimulated ATPase assays were carried out as described earlier (8). The amount of ATP synthase β -subunit found in everted membrane vesicles expressing mutant ATP synthases was compared to the amount in vesicles expressing the WT enzyme as an assessment of relative ATP synthase content of the everted vesicles. Vesicles equivalent to 1.6 μ g of protein were fractionated on 11% SDS-PAGE mini-gels (20), and transferred electrophoretically to nitrocellulose membranes. Immunoblot analyses of the β -subunit were carried out as in an earlier study (8). For purpose of quantitation, image analysis was performed using ImageJ 1.40 software (<http://rsbweb.nih.gov/ij/>).

Assays of ATP Synthesis by ADP + P_i -Loaded Right-Side-Out (RSO) Membrane Vesicles

WT and mutant strains were grown to an A_{600} of 0.5–0.6 on MYE medium, 30°C, at pH 7.5 because some of the mutant strains did not exhibit significant growth at pH 10.5. Right-side-out (RSO) ADP + P_i -loaded membrane vesicles were prepared and assayed as described in an earlier study at pH 7.5 and 10.5 (8). Briefly, the RSO vesicles were prepared and loaded with ADP + P_i in buffer at pH 8.3. Energization with 10 mM ascorbate and 0.1 mM phenazine methosulfate was initiated upon dilution of the loaded vesicles into the assay buffer at either pH 7.5 or 10.5 at room temperature. This was followed by a 10 second reaction period, with continuous vigorous aeration, before the reactions were stopped by rapid transfer of the reaction mix to tubes with 50 μ l of ice-cold 30% perchloric acid. After neutralization, the ATP content was determined by the luciferin-luciferase method (8).

Purification of His-Tagged ATP Synthases

To facilitate the purification of the *Bacillus pseudofirmus* OF4 ATP synthase, an 18 nucleotide sequence encoding six histidines was placed in the chromosomal *atp* operon immediately after the ATG initiation codon of the β -subunit of the ATP synthase. Briefly, a β -subunit deletion strain was first constructed by introducing a spectinomycin cassette in place of part of *atpGD* (nucleotides 7851–8511 with *atp* operon numbering referring to the deposited sequence of the region, AF330160). Then the replaced region was reintroduced but with the additional 18 nucleotide sequence. For the construction of His-tagged mutants, a gene encoding a His-tagged β -subunit was introduced in the ΔF_0 ($\Delta atpB-F$) strain and mutations were then constructed in that background in the usual way (9).

For the expression of ATP synthase, all His-tagged strains, except for the A16/18/20/22G mutant, were cultured on MYE medium with 10% Luria-Bertani broth (1 g tryptone, 0.5 g yeast extract, 1 g NaCl per liter) at pH 7.5 rather than higher pH because many of the mutants grew poorly on malate at pH 10.5. The A16/18/20/22G mutant was grown on glucose, pH 10.5, because of its poor malate growth at either pH 7.5 or 10.5. The growth substrate (malate vs. glucose) and pH (7.5 vs. 10.5) did not affect the properties of the WT *c*-ring on SDS-PAGE gels (unpublished results). Because the A16G mutant grew on malate at pH 10.5, we also checked the properties of its purified ATP synthase when the strain was grown on malate at pH 10.5. We observed no difference in either *c*-ring or *c*-monomer properties on SDS-PAGE (data not shown). Strains were grown for 4–6 hours at 37 °C with 225 rpm shaking until the A_{600} reached 1.4–1.6. His-tagged ATP synthase was purified as described in detail elsewhere (5) except that cells from 12 liters of culture were harvested for purification. Each strain was grown on 2 separate occasions and each set was then used in a separate purification procedure. Where indicated, samples were precipitated with 10% TCA.

Purification of the *c*-Ring

The *c*-ring was partially purified as described previously (21). Briefly, 1 mg of purified ATP synthase was mixed with 1% of LS and incubated for 10 minutes at 65°C. After cooling to room temperature, $(\text{NH}_4)_2\text{SO}_4$ was added to 65% saturation. The mixture was incubated for 20 minutes at room temperature and centrifuged for 15 minutes at room temperature. The supernatant was filtered through a PVDF 0.22 μm filter unit (Millipore) and dialyzed overnight against 10 mM Tris-HCl, pH 8.0 at 4°C. The sample was concentrated on an ultra-4.5 kDa filter (Millipore). All of the samples contained the *c*-ring and the δ and *b* subunits.

SDS-PAGE Analyses

SDS-PAGE was carried out as described previously (20). Proteins were visualized by silver staining (22). A Criterion apparatus (Bio-Rad, Hercules, CA) was used in the gels shown in Figure 3 and for the gels shown in Figures 4 and 5, a larger gel (Bio-Rad Protean II) was used.

Determination of Molecular Masses of the Monomeric *c*-Subunits by MALDI-TOF-MS

100 μl of purified *B. pseudofirmus* OF4 ATP synthase (300–400 μg) was mixed with 1 ml of 1:1 (v/v) chloroform/methanol (CM). Phase separation was initiated by addition of 200 μl of 10 mM Tris-HCl, pH 8.0 with the *c*-subunit extracted into the organic solvent. The organic phase was evaporated using a UNIVAPO 150H Vacuum Concentrator (UniEquip) and the dry pellet was stored at -20°C . For mass analysis of *c*-subunits by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), the pellet was dissolved in CM and directly used for mass analysis either at the Functional Genomics Center Zürich (FGCZ), Switzerland or at the Max-Planck-Institute of Biophysics, Germany.

Quantitative Analyses of *c*-Monomer Bands on SDS-PAGE Gels

Quantitative analysis of band intensity was performed using custom software. A high-resolution TIFF image of the gel was first inverted so that the protein bands appeared as bright rather than dark regions. This was followed by calculation of the average intensity in each band. In addition to these basic calculations, procedures were followed to ensure the consistency and quantitative accuracy of the comparisons. The average intensity of each band was computed over an equal area, was corrected for background intensity, and normalized to a specified standard in the lane containing the protein molecular weight standards. All software for gel analysis was written in the scientific programming language MatlabTM (The Mathworks, Natick, MA).

Structure Figures

The structure figures were created with PyMol (23).

RESULTS

Ala-to-Gly Mutations Affect the Amount of ATP Synthase Found in the Membrane

A panel of ten mutants (Table 1), which encompassed single, double, triple and quadruple mutants, was used to evaluate the effects of mutations in the AxAxAx motif of the *c*-subunit on the amount of ATP synthase found in the membrane. The ATP synthase content of membranes was assessed by β -subunit content, and the total ATPase activity was assayed under OG-stimulated conditions (OG-ATPase). The Ala-to-Gly mutations led to reductions of ATP synthase β -subunit content in everted membrane vesicles as compared to the content of WT vesicles in all strains tested except for the A20/22G mutant. The results for each of the mutants in comparison with the WT control are shown in Table 1A, which divides the mutant panel into three groups based on % of WT β -subunit content: Group 1, with only 42–51%; Group 2, with 59–67%, and Group 3, with 76–82%. No mutants with an Ala16-to-Gly change were found in Group 3. The two mutants in Group 3 that had the highest β -subunit contents of the mutant panel were A18/22, which had two Ala-to-Gly mutations in the alanine motif oriented toward the same neighbor subunit, and A20/22G, which had two Ala-to-Gly mutations oriented differently (Figure 1B). The total ATPase activities in the mutant vs. WT vesicles were also reduced and were $\leq 81\%$ of the WT value, generally correlating well with the β -subunit levels (Table 1A).

Ala-to-Gly Changes in the AxAxAx Motif Affect Function of the ATP Synthase

Function of the ATP synthase in supporting non-fermentative growth on malate in MYE (malate-yeast extract) medium was assessed for the full mutant panel in single point experiments. For the values shown in Table 1A, the A_{600} reached after 14 hours of growth at pH 10.5 and 7.5 was corrected for the amount of growth exhibited by parallel cultures of the ΔF_0 mutant (negative control) and compared to the WT value (Table 1); under these conditions, the WT growth was similar at pH 7.5 and 10.5. This was consistent with results obtained in earlier continuous culture experiments in which growth at pH 10.5 on malate was comparable to that at pH 7.5 in spite of a much lower protonmotive force at pH 10.5 (24). For the seven mutants that had been studied earlier, the new growth data were comparable to the prior results (8). Many of the mutants exhibited growth deficits in malate growth at pH 10.5 relative to WT growth but those deficits were comparable in magnitude to the deficit in membrane β -subunit content for those same mutants. Thus for these mutants, no functional deficit could be inferred beyond that accounted for by reduced enzyme levels in the membrane, e.g. for the single A18G, A16G and A22G mutants. The three mutants whose loss of non-fermentative growth capacity stood out were the A16/20G, A16/18/20/22G mutants of Group 1 and the A16/20/22G mutant of Group 2 (boxed in Table 1A), all of which exhibited a % WT $A_{600}/14$ hr metric that was more than 35% points lower than the % WT β -subunit found for that mutant. For each of those mutants, the non-fermentative growth deficit observed at pH 7.5 was significantly less severe than at pH 10.5, i.e. 46% vs. 9% for A16/20G, 9% vs. –2% for the quadruple mutant A16/18/20/22, and 38% vs. 16% for A16/20/22G. The three mutants in Group 3 also exhibited significant loss of non-fermentative growth capacity at pH 10.5 relative to the deficits in β -subunit, i.e. 19–25% points lower, and exhibited no loss of non-fermentative growth capacity at pH 7.5 (Table 1A). Although the data are not shown, we used the same growth protocol to obtain single point A_{600} data for growth of the panel of mutants on GYE, glucose-containing medium, at both pH 10.5 and 7.5. All the mutants exhibited glucose growth at a % WT A_{600} that was equal to or higher than predicted by the β -subunit content of the mutant, at both pH 7.5 and 10.5.

The particularly severe defects in some of the mutants with an Ala16-to-Gly change indicated that one or more additional mutations were important determinants of the functional deficits. In addition, the severity depended upon location of the additional mutation(s). For example, the single A16G mutant did not have a significant functional deficit relative to the % β -subunit in the membrane, nor did the double A16/18G mutant, while the A16/20G mutant exhibited a major deficit in malate growth at pH 10.5 (Table 1A). Since the two double mutants, A16/18G and A16/20G, had comparable % β -subunit profiles, we conducted growth experiments over time in MYE medium at pH 10.5 to compare their growth phenotypes in greater detail. As shown in Figure 2, growth of the negative control, the ΔF_o strain, which could only utilize the limited fermentable components of the yeast extract that is in the MYE medium, leveled off at an $A_{600} = 0.56$, whereas the WT grew to an $A_{600} = 1.8$. The A16/18G double mutant grew at the same rate as WT after cessation of fermentative growth of the negative control but growth of A16/18G subsequently leveled off earlier than WT, at $A_{600} = 1.6$. Relative to the A16/18G mutant, the A16/20G mutant exhibited a deficit in both the rate of growth and its final growth point, $A_{600} = 1.2$. We note that the growth rates observed here during the non-fermentative growth phase were slower than observed in earlier studies on the WT strain because the pre-growth of these cells was on glucose-containing medium, which reduces the levels of respiratory chain complexes and ATP synthase, but supports comparable pre-growth of all the strains. Although not shown, we also conducted a similar set of growth experiments on single mutants A16G and A22G, whose % β -subunit profiles were comparable. There was no significant difference in the growth rates of the two strains during the 8–11 hour period of the experiment, but growth after 14hrs of the A22G was consistently a little higher than that of the A16G mutant, at 80% vs. 74% of WT growth.

The capacity for ATP synthesis at pH 10.5 and 7.5 was assayed in ADP + P_i -loaded vesicles from all five mutants containing an Ala16-to-Gly mutation and at least one mutant in each of the three Groups of Table 1. The same three mutants that exhibited particularly poor malate growth at pH 10.5 synthesized the least ATP at pH 10.5 in an *in vitro* assay in which they were compared to the WT strain. A16/20G and A16/18/20/22G of Group 1 synthesized the least ATP, at 53% and 20% of the WT amount respectively, and A16/20/22G of Group 2 synthesized ATP at 64% of the WT amount, but only the deficit in synthesis by the quadruple mutant was low compared to its % β content in the membrane. The other mutants that were examined, single mutants A16G, A18G, and A20G and double mutant A16/18G, all synthesized ATP at 70–79% of the WT amount, values that were higher than their % β content in some cases (Table 1A).

Ala-to-Gly Change(s) in the AxAxAx Motif Affect the c-Ring Stability

After purification of the WT enzyme, the *c*-ring remains intact during SDS-PAGE (5, 18). Treatment of *c*-rings with TCA dissociated more than 94% of the oligomer into monomeric *c*-subunits (Figure 3A), as assessed quantitatively in lauroyl sarcosine-extracted preparations (data not shown). We used the total amount of *c*-subunit observed in TCA-treated preparations as an approximate measure of the total *c*-subunit on the gel in order to ascertain whether the *c*-rings of any of the mutants dissociated substantially during extraction or purification. In such instances, the total amount of *c*-subunit would be significantly lower than the WT level although the same amount of purified ATP synthase protein was loaded on the gel for each strain. Two mutants, A16/18G and A16/18/20/22G, exhibited major reductions in the intensity of the *c*-monomer bands in the TCA-treated samples that represented loss of ~70% of the total *c*-subunit relative to the WT amount (Fig. 3A, Table 1B, left). The single A16G mutant and triple A16/20/22G mutants, which also included an Ala16-to-Gly change, were at the low end of the remaining mutants with respect to total *c*-monomer on the SDS-PAGE. On the other end of the range, the A22G and A20/22G

mutants exhibited total *c*-monomer contents that were close to or comparable to the WT preparation.

The next question addressed was the relative amounts of *c*-monomer released by the mutants during SDS-PAGE in the absence of TCA treatment, under conditions where such release is minor or undetectable with WT preparations. Although the A16/18G and A16/18/20/22G mutants were already depleted in *c*-subunit because of loss during extraction/purification, most of the remaining *c*-ring dissociated during SDS-PAGE. There was only very little residual *c*-ring in the A16/18G double mutant and no apparent *c*-ring remaining in the A16/18/20/22G mutant and release of *c*-monomer clearly occurred from both these mutant preparations as well as from the triple mutant without TCA (Figure 3B). For the A16/20/22G and A16/18/20/22G mutants, there was more *c*-monomer released in samples not treated with TCA than was observed in those treated with TCA, probably because some *c*-monomer was lost during TCA extraction. Except for the single A18G mutant, all the mutants consistently exhibited some *c*-ring dissociation in the absence of TCA treatment. Apart from the mutants that included an A16G change, the most impressive dissociation was observed in the A20/22G mutant. An estimate of the stability of the mutant relative to WT *c*-rings that takes into account the amount of *c*-ring on the gel and the extent of its dissociation to monomer on SDS-PAGE without TCA treatment is shown in Table 1B (right) in three categories (S=stable, U=unstable, VU=very unstable) that are defined in the Table legend. All three of the mutants containing an Ala16-to-Gly mutation and that had exhibited the largest functional defects, i.e., the A16/20G double mutant, triple mutant A16/18/20G and quadruple mutant A16/18/20/22G, were in the VU rotor category. However, the double mutants A16/18G and A20/22G, which have much smaller functional deficits, were also in the VU category. A22G was the mutant with the most stable *c*-ring, based on both the total *c*-subunit found on SDS-PAGE after TCA treatment of the samples (Table 1B, left) and the categorization based on release of *c*-monomer during SDS-PAGE without TCA treatment (Table 1B, right).

Seven Ala-to-Gly Mutant *c*-Monomers Exhibit Anomalous SDS-PAGE Mobility

We observed that the *c*-subunits of some mutants ran faster on SDS-PAGE than WT *c*-subunits (Figure 3A,B). Using a large format 15% gel (see Experimental Procedures), it was possible to resolve three different sizes on SDS-PAGE (Figure 4). The apparent MW of *c*-subunit monomers was determined by calibration of the gels using low-molecular weight polypeptide standards. The values for the difference between apparent molecular weight vs. formula molecular weight (dMW) were calculated as described by Rath *et al.* (25) (and see footnote to Table 2). The *c*-monomers of three single mutants (A18G, A20G and A22G) migrated almost the same as those from WT monomer; seven mutant *c*-monomers showed a faster migration profile than the WT *c*-monomers. The *c*-subunits from the A16G, A18/22G and A20/22G mutants ran slightly faster than WT *c*-subunits and those from the A16/18G, A16/20G, A16/20/22G and A16/18/20/22G mutants migrated much faster than WT *c*-subunits (Table 2). In order to verify that the *c*-subunits were intact, their masses were determined by MALDI-TOF-MS (Table 2). The measured masses all correlated with the ones calculated from the amino acid sequences of the corresponding *c*-subunit. These results indicated that all mutant *c*-subunits were intact, which excluded the possibility that some of anomalous migration behavior of the *c*-subunits on SDS-PAGE is caused by degradation.

All Ala16-to-Gly Containing Mutant *c*-Rings Exhibit Increased Mobility on SDS-PAGE Relative to the WT *c*-Ring

The gel patterns observed for the mutants revealed differences in *c*-ring migration compared to WT *c*-ring migration for several of them (Figure 3B). The A16/18/20/22G mutant had no clear *c*-ring band, and both the A16/18G and A16/20G double mutants and the A16/20/22G

triple mutant showed weak *c*-ring bands relative to the WT *c*-ring band. The single A16G mutant exhibited two apparent bands close to where the WT *c*-ring band was observed. The upper one was at the same position of the WT band and the other was a modestly lower band. The double A16/18G and A16/20G mutants appeared to have at least a trace of both forms. The remaining mutants, with no Ala16-to-Gly change, had *c*-ring bands of approximately the same position as the WT *c*-ring band and similar band intensities, except for the A20/22G mutant, which exhibited lower intensity. Because of the increased mobility of *c*-ring bands in mutants containing an Ala16-to-Gly change, we used lauroyl sarcosine (LS) to extract the *c*-rings from the ATP synthase preparations purified from the WT strain and all five Ala16-to-Gly containing mutants. The LS extracts were then analyzed on large format 10% SDS-PAGE gels (Figure 5). The A16G mutant exhibited two obvious bands in the region where the WT *c*-ring was observed: a less intense band with comparable migration to the WT band and a more intense band below it. Trace amounts of the band co-migrating with the upper A16G band were observed for the two double mutants, supporting the inference from the SDS-PAGE analyses before partial purification of the *c*-rings by LS extraction (Figure 3B). The upper band was at the same position as the WT *c*-ring, while the lower band suggested a smaller apparent size (Figure 5). The other mutants that had an Ala16-to-Gly mutation, A16/20/22G and A16/18/20/22G, displayed single *c*-ring bands of a size comparable to the lower species in the single A16G mutant.

DISCUSSION

In this study, a panel of ten Ala-to-Gly mutants in the AxAxAx motif of the inner helix of the ATP synthase *c*-ring of alkaliphilic *B. pseudofirmus* OF4 was used to probe the relationship of functional effects to the number and position of the mutations. We also explored whether functional effects of the mutations correlated with changes in physical properties of the *c*-rings that could be inferred from behavior of the mutant enzymes or rotor-enriched fractions in SDS-PAGE analyses. A general finding was that all ten mutations reduced the amount of holo-enzyme in the membrane, as assessed by the amount of membrane-associated β -subunit, and/or reduced the capacity for non-fermentative growth on malate at high pH. Another general finding was that all of the mutant *c*-rings, except for the A22G mutant *c*-ring, exhibited instability relative to the WT *c*-ring in one or both indicators of instability from the SDS-PAGE analyses (Table 1B). Both GxxxG and AxxxA motifs have been proposed to stabilize helix-helix interactions in membrane proteins (26–28). Work by Serrano *et al.* (29) has indicated that alanine has a greater stabilizing effect than glycine when present in the middle of α -helices. The AxAxAx motif of *B. pseudofirmus* OF4 is located at the interface of the inner helices of two *c*-subunits. The interaction of thirteen such subunits to form a c_{13} -ring structure is mediated in part by the AxAxAx packing motif (14). Alanine residues at positions 16 and 20, together with the alanine residues at 18 and 22 from the next helix, form the hydrophobic contact area of two adjacent helices (Figure 1B). When glycine is substituted for one of the alanine residues, the contact surface is reduced and may become partially disrupted (Figure 6). This type of disruption is likely to account for the loss of stability observed here. However, although the *c*-rings of the three most functionally deficient mutants, A16/20G, A16/20/22G and A16/18/20/22G, were in the very unstable (VU) group, the correlation between stability and function was not strong overall, e.g. single mutant A20G exhibited a significant deficit in malate growth relative to the membrane β -content whereas A22G did not, but both mutant *c*-rings were stable (S). It appears likely that much of the stability loss among the mutants, compared to the WT strain, has an impact upon extraction of the enzyme from the membrane and/or during fractionation on SDS-PAGE, but is not a property that has a major impact *in vivo*.

It was notable that the magnitude of functional deficits observed in the vesicle assays of ATP synthesis were lower than those for the same mutants in the single point growth assays

on malate at pH 10.5 (Table 1A). This is likely to reflect, in part, the more vigorous aeration during the 10 second synthesis period than in the single point growth assays in shaken tubes. The growth curve experiments conducted in flasks over time were also conducted under more highly aerated conditions than the single point assays and the two mutants whose growth is shown in Figure 2 exhibited a smaller growth deficit relative to the WT strain under the more aerated conditions. The ATP synthesis assays have an additional difference from malate growth assays that could result in more robust synthesis by mutant strains than expected from their malate growth relative to the WT strain. In the ATP synthase assays the electron donor, ascorbate (added together with PMS), feeds directly into the proton-pumping Cta (cytochrome *caa3*) cytochrome oxidase that plays a crucial role in oxidative phosphorylation by *B. pseudofirmus* OF4 (30, 31). By contrast, during *in vivo* growth on malate there are multiple upstream steps in electron transfer, e.g. the dehydrogenase steps, which could limit the relative rate of electron delivery. Differences in the efficacy of delivery of electrons to the respiratory chain and the availability of the final electron acceptor, oxygen, likely account for observations such as the retention by the quadruple mutant of ATP synthesis capacity (20% of WT vs. its 50% of WT β -subunit content) as opposed to its failure to grow on malate at pH 10.5 (Table 1A). Another striking observation of the same type is the absence of a deficit in ATP synthesis for the triple A16/20/22G mutant in the *in vitro* assay, relative to membrane β -content, while its growth on malate was very significantly affected at pH 10.5 and even affected at pH 7.5. The observation that an ATP synthase that appears fully functional in the *in vitro* assay poorly supports malate growth highlights the complexity of *in vivo* OXPHOS, where issues of electron delivery, competition for protons with the antiporter(s) involved in pH homeostasis and other challenges of this central physiological system are in play. This greater complexity may increase sensitivity of the cells to nuanced compromises in function of the ATP synthase that are undetected under the *in vitro* assay conditions.

The functional assays of the expanded mutant panel in this study clearly showed that the greatest deficits in function were found in mutants containing an Ala16-to-Gly mutation together with at least one additional Ala-to-Gly mutation. Concomitantly, SDS-PAGE analyses of purified holo-ATP synthases from the mutant panel revealed greater mobility of *c*-rings from all of the Ala16-to-Gly containing mutants. Furthermore, the single A16G and to a lesser extent the double mutants in this sub-set also showed a minor band with the WT *c*-ring mobility that was extracted with LS along with the more mobile band. We hypothesize that the Ala16-to-Gly mutants have a c_{12} -ring, but that minor amounts of a c_{13} -ring are retained in those mutants that still have at least two Ala residues of the AxAxAxA motif. A *c*-ring with mostly a c_{12} -subunit stoichiometry would account for the increased mobility. It would also be consistent with the modestly but reproducibly lower in $A_{600}/14$ hrs reached by the A16G mutant during malate growth at pH 10.5 as compared to the A22G mutant, 74% vs. 80% of WT growth, and the lower final A_{600} values shown in Figure 2 for both the A16/18G and A16/20G mutants compared to the WT strain. At the high medium pH of 10.5, the effective protonmotive force is lower than optimal and a *c*-ring with higher stoichiometry, which is directly related to the H^+ taken up/ATP synthesized, would support more ATP synthesis than a *c*-ring with a lower stoichiometry (3, 32). It is possible that the apparent band that retains WT mobility in some of the mutants has a c_{12} rather than the WT c_{13} stoichiometry, i.e. that the apparent c_{13} component is an artifact. Some monomeric *c*-subunit that dissociated from the *c*-ring during purification might have stuck to a smaller c_{12} -ring to produce a ring that has the same mobility as a WT *c*-ring. Such a *c*-ring plus monomer artifact has been described in studies of the heterologously-expressed *c*-subunit from the *I. tartaricus* ATP synthase (33). However, if the alkaliphile *c*-ring is similarly sticky, it is difficult to explain why only the *c*-rings of the A16G (and perhaps, to a minor extent, A16/18G and A16/20G) mutants have the putative “stuck c_1 ” when other mutants release *c*-monomer but do not exhibit doublet rings. We consider it more likely that during

c-ring assembly, both a minor fraction of rings with the native c_{13} stoichiometry is formed along with the major fraction with the lower stoichiometry. The putative c_{13} fraction is only formed in strains that have A16G mutations and that retain at least two alanine residues from the AxAxAx motif. The adequacy of two amino acid residues with side chains, in place of side chain-less glycines to support a c_{13} -ring has precedent in the extended diameter of the c_{13} -ring of thermoalkaliphilic *C. thermarum* TA2.A1, which has two larger residues (serines) together with two glycines in the GxSxGxS sequence in the middle of its inner *c*-subunit helix (Figure 1A) (10). At present, however, the hypothesized change in *c*-subunit stoichiometry in the A16-to-Gly containing subset of mutants is strictly a hypothesis. We have not yet been able to obtain images or otherwise directly test the hypothesis that the Ala16-to-Gly mutants contain *c*-rings with a mostly or entirely c_{12} rather than the WT c_{13} stoichiometry, because of diverse technical challenges. We are continuing to seek ways to overcome these challenges.

Additional mutations in an Ala16-to-Gly background promoted dominance of the band with increased mobility, without further altering mobility (Figure 5). The results of this study show that the position(s) of the additional mutation(s) had a major effect on the functional properties of the particular mutants. For example, the A16/18G and A16/20G double mutants had *c*-rings of comparable mobility but the functional deficits of the A16/18G mutant were significantly less severe than those of the A16/20G mutant in growth experiments (Figure 2) and ATP synthesis experiments (Table 1A). Different combinations of mutations presumably affect the shape of the rings in different ways and at different depths of the ring, which could account for their different impacts on function.

Finally we note that the monomeric *c*-subunits from seven of the mutants that were released from the *c*-rings, e.g. upon treatment with TCA, exhibited a range of increased mobility on SDS-PAGE that differed from that of the WT *c*-subunit (Figure 4). Similar behavior has been documented by others (25, 34). Rath *et al.* (25) concluded that the anomalous mobility of a set of mutant hairpin protein segments derived from CFTR on SDS-PAGE resulted from subtle changes in the protein folds that altered the detergent-binding properties of individual mutant proteins, leading to their altered mobility. Again the mobility change among the AxAxAx mutants, relative to the WT mobility, was particularly pronounced in mutants with an Ala16-to-Gly change. The single A16G mutant was the only single mutant that exhibited a significant mobility change and the multiple mutants that included an Ala16-to-Gly change were the most affected in the panel. But both the A18/22G and A20/22G mutants showed consistent indications of modestly increased mobility relative to the WT monomer on SDS-PAGE (Figure 4; Table 2). It is impressive that the mutant hairpin-like *c*-subunits retain structural information in SDS-PAGE that correlate with changes in function of the larger ATP synthase nano-machine in the complex physiological process of OXPHOS.

Acknowledgments

We thank Julian Langer (Max-Planck Institute of Biophysics, Germany) for the special efforts required to obtain a MALDI-TOF-MS analysis result for the A16/18/20/22G mutant *c*-subunit.

References

1. Krulwich TA. Alkaliphiles: 'basic' molecular problems of pH tolerance and bioenergetics. *Mol Microbiol.* 1995; 15:403–410. [PubMed: 7783613]
2. Bränden M, Sanden T, Brzezinski P, Widengren J. Localized proton microcircuits at the biological membrane-water interface. *Proc Natl Acad Sci USA.* 2006; 103:19766–19770. [PubMed: 17172452]

3. Hicks DB, Liu J, Fujisawa M, Krulwich TA. F₁F₀-ATP synthases of alkaliphilic bacteria: lessons from their adaptations. *Biochim Biophys Acta*. 2010; 1797:1362–1377. [PubMed: 20193659]
4. Mulikidjanian AY, Heberle J, Cherepanov DA. Protons @ interfaces: implications for biological energy conservation. *Biochim Biophys Acta*. 2006; 1757:913–930. [PubMed: 16624250]
5. Fujisawa M, Fackelmayer O, Liu J, Krulwich TA, Hicks DB. The ATP synthase a-subunit of extreme alkaliphiles is a distinct variant. *J Biol Chem*. 2010; 285:32105–32115. [PubMed: 20716528]
6. Ivey DM, Krulwich TA. Two unrelated alkaliphilic *Bacillus* species possess identical deviations in sequence from those of other prokaryotes in regions of F₀ proposed to be involved in proton translocation through the ATP synthase. *Res Microbiol*. 1992; 143:467–470. [PubMed: 1448623]
7. Krulwich, TA.; Hicks, DB.; Swartz, TH.; Ito, M. Bioenergetic adaptations that support alkaliphily. In: Gerday, C.; Glansdorff, N., editors. *Physiology and Biochemistry of Extremophiles*. ASM Press; Washington, DC: 2007. p. 311–329.
8. Liu J, Fujisawa M, Hicks DB, Krulwich TA. Characterization of the functionally critical AXAXAXA and PXXEXXP motifs of the ATP synthase c-subunit from an alkaliphilic *Bacillus*. *J Biol Chem*. 2009; 284:8714–8725. [PubMed: 19176524]
9. Wang Z, Hicks DB, Guffanti AA, Baldwin K, Krulwich TA. Replacement of amino acid sequence features of a- and c-subunits of ATP synthases of alkaliphilic *Bacillus* with the *Bacillus* consensus sequence results in defective oxidative phosphorylation and non-fermentative growth at pH 10.5. *J Biol Chem*. 2004; 279:26546–26554. [PubMed: 15024007]
10. Matthies D, Preiss L, Klyszejko AL, Müller DJ, Cook GM, Vonck J, Meier T. The c₁₃ ring from a thermoalkaliphilic ATP synthase reveals an extended diameter due to a special structural region. *J Mol Biol*. 2009; 388:611–618. [PubMed: 19327366]
11. McMillan DG, Keis S, Dimroth P, Cook GM. A specific adaptation in the a subunit of thermoalkaliphilic F₁F₀-ATP synthase enables ATP synthesis at high pH but not at neutral pH values. *J Biol Chem*. 2007; 282:17395–17404. [PubMed: 17434874]
12. Arechaga I, Jones PC. The rotor in the membrane of the ATP synthase and relatives. *FEBS Lett*. 2001; 494:1–5. [PubMed: 11297723]
13. Vonck J, von Nidda TK, Meier T, Matthey U, Mills DJ, Kühlbrandt W, Dimroth P. Molecular architecture of the undecameric rotor of a bacterial Na⁺-ATP synthase. *J Mol Biol*. 2002; 321:307–316. [PubMed: 12144787]
14. Preiss L, Yildiz Ö, Hicks D, Krulwich TA, Meier T. A new type of proton coordination in an F₁F₀-ATP synthase rotor ring. *PLoS Biol*. 2010; 8:e1000443. [PubMed: 20689804]
15. Meier T, Polzer P, Diederichs K, Welte W, Dimroth P. Structure of the rotor ring of F-Type Na⁺-ATPase from *Ilyobacter tartaricus*. *Science*. 2005; 308:659–662. [PubMed: 15860619]
16. Murata T, Yamato I, Kakinuma Y, Leslie AG, Walker JE. Structure of the rotor of the V-Type Na⁺-ATPase from *Enterococcus hirae*. *Science*. 2005; 308:654–659. [PubMed: 15802565]
17. Pogoryelov D, Yildiz Ö, Faraldo-Gomez JD, Meier T. High-resolution structure of the rotor ring of a proton-dependent ATP synthase. *Nat Struct Mol Biol*. 2009; 16:1068–1073. [PubMed: 19783985]
18. Hoffmann J, Sokolova L, Preiss L, Hicks DB, Krulwich TA, Morgner N, Wittig I, Schagger H, Meier T, Brutschy B. ATP synthases: cellular nanomotors characterized by LILBID mass spectrometry. *Phys Chem Chem Phys*. 2010; 12:13375–13382. [PubMed: 20820587]
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951; 193:265–275. [PubMed: 14907713]
20. Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem*. 1987; 166:368–379. [PubMed: 2449095]
21. Meier T, Matthey U, von Ballmoos C, Vonck J, Krug von Nidda T, Kühlbrandt W, Dimroth P. Evidence for structural integrity in the undecameric c-rings isolated from sodium ATP synthases. *J Mol Biol*. 2003; 325:389–397. [PubMed: 12488103]
22. Nesterenko MV, Tilley M, Upton SJ. A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. *J Biochem Biophys Methods*. 1994; 28:239–242. [PubMed: 8064118]

23. De Lano, WL. The PyMOL molecular graphics system. DeLano Scientific; San Carlos, CA: 2002.
24. Sturr MG, Guffanti AA, Krulwich TA. Growth and bioenergetics of alkaliphilic *Bacillus firmus* OF4 in continuous culture at high pH. *J Bacteriol.* 1994; 176:3111–3116. [PubMed: 8195065]
25. Rath A, Glibowicka M, Nadeau VG, Chen G, Deber CM. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proc Natl Acad Sci U S A.* 2009; 106:1760–1765. [PubMed: 19181854]
26. Curran AR, Engelman DM. Sequence motifs, polar interactions and conformational changes in helical membrane proteins. *Curr Opin Struct Biol.* 2003; 13:412–417. [PubMed: 12948770]
27. Rath A, Deber CM. Surface recognition elements of membrane protein oligomerization. *Proteins.* 2008; 70:786–793. [PubMed: 17729275]
28. Walters RF, DeGrado WF. Helix-packing motifs in membrane proteins. *Proc Natl Acad Sci U S A.* 2006; 103:13658–13663. [PubMed: 16954199]
29. Serrano L, Neira JL, Sancho J, Fersht AR. Effect of alanine versus glycine in alpha-helices on protein stability. *Nature.* 1992; 356:453–455. [PubMed: 1557131]
30. Quirk PG, Hicks DB, Krulwich TA. Cloning of the *cta* operon from alkaliphilic *Bacillus firmus* OF4 and characterization of the pH-regulated cytochrome *caa3* oxidase it encodes. *J Biol Chem.* 1993; 268:678–685. [PubMed: 7678007]
31. Gilmour R, Krulwich TA. Construction and characterization of a mutant of alkaliphilic *Bacillus firmus* OF4 with a disrupted *cta* operon and purification of a novel cytochrome *bd*. *J Bacteriol.* 1997; 179:863–870. [PubMed: 9006044]
32. Meier T, Morgner N, Matthies D, Pogoryelov D, Keis S, Cook GM, Dimroth P, Brutschy B. A tridecameric c ring of the adenosine triphosphate (ATP) synthase from the thermoalkaliphilic *Bacillus* sp. strain TA2.A1 facilitates ATP synthesis at low electrochemical proton potential. *Mol Microbiol.* 2007; 65:1181–1192. [PubMed: 17645441]
33. Meier T, Yu J, Raschle T, Henzen F, Dimroth P, Müller DJ. Structural evidence for a constant *c*₁₁ ring stoichiometry in the sodium F-ATP synthase. *FEBS J.* 2005; 272:5474–5483. [PubMed: 16262688]
34. Wehbi H, Rath A, Glibowicka M, Deber CM. Role of the extracellular loop in the folding of a CFTR transmembrane helical hairpin. *Biochemistry.* 2007; 46:7099–7106. [PubMed: 17516627]

Figure 1.

(A) Alignment of the *c*-subunit of the ATP synthase from several alkaliphiles and neutralophiles. The organisms shaded are the alkaliphilic *Bacillus* species. Residue numbers are given for the *B. pseudofirmus* OF4 (top) and the *I. tartaricus* *c*-subunits (bottom). The alanines of the alkaliphile AxAxAxA are shaded with colors corresponding to those in the (B) panel and PxxExxP motifs and the RQPE periplasmic loop region are in grey. The two series of a distinct thermoalkaliphile motif are shaded in grey, as they play roles in packing of the *C. thermarum* ring (10). The open boxes show the conserved GxGxGxG motif in the inner helix of selected neutralophiles. *In order to maximize the size of the motif areas, residues were deleted from the N-terminal end of the longest *c*-subunits, 5, 6 and 10, respectively, from the subunits of *E. coli*, *S. platensis*, and *I. tartaricus*. (B) The location of the AxAxAxA motif in the *c*-ring structure of *B. pseudofirmus* OF4 as shown from the membrane plane. The four alanines are shown in different colors. The red spheres represent the bound H₂O molecules (14). The zoomed panel shows the *c*-ring on the level of the ion binding site perpendicular to the membrane as viewed from the cytoplasm and the alanines displayed with the same color coding.

Figure 2.

Comparison of the growth of the A16/18G and A16/20G mutants malate-containing medium at pH 10.5. Growth of the two double mutants was compared to the WT strain and to the ΔF_0 strain, under conditions described in Experimental Procedures, with readings of the A_{600} recorded hourly for 15 hours and a final point taken at 25 hours.

Figure 3.

SDS-PAGE analyses of the purified ATP synthase from WT and mutant strains. One μg of purified protein was loaded for each lane. The purified enzyme was resolved on 11% polyacrylamide gels and stained with silver. Samples were precipitated with TCA (A) or not treated (B); the outline in panel B highlights the *c*-ring bands. In panel A, the total *c*-subunit (as % of WT) that was observed on SDS-PAGE is shown under the lane showing the fractionation of each strain. It was calculated using the equation: Total *c*-subunit as % of WT = [amount of *c*-monomer in mutant TCA sample]/[amount of *c*-monomer in WT TCA sample] $\times$ 100%. The values are the average of determinations from two independent protein preparations, with $\pm$ SD. The intensity of *c*-monomer was quantified by MATLAB analysis as described under Experimental Procedures.

Figure 4.

SDS-PAGE analyses of *c*-monomers from WT and mutant strains. The purified proteins treated with TCA were resolved on a 15% polyacrylamide gel and stained with silver as described under Experimental Procedures. The protein loaded in each lane was adjusted in order to give approximately comparable-staining bands. The protein amount ranged from 2.5–7.5 μ g. The gel image was cropped to show only the region of the *c*-monomer bands.

Figure 5.

SDS-PAGE analyses of the *c*-rings in lauroyl sarcosine extracts of the ATP synthases from WT and mutant strains including an A16G change. The purified proteins treated with lauroyl sarcosine were resolved on a large format 10% polyacrylamide gel and stained with silver, as described under Experimental Procedures. The protein loaded in each lane was adjusted in order to produce bands with approximately comparable staining. The protein amount loaded ranged from 0.5-1.1 μ g. The gel image was cropped to show only the region of the *c*-ring bands.

Figure 6.

Model of the contact area among three inner helices of the *B. pseudofirmus* OF4 c_{13} -ring (14). (A) WT and (B) A16G mutant. The space required for a Gly residue (G16) is less than that for an Ala residue (A16) with a methyl side chain, resulting in less tight and suboptimal packing upon an A16G mutation in the c -subunit. The model is based on the structure of the c_{13} -ring, pdb code: 2x2v. Different subunits are shown in blue, yellow and green. The A16G mutation is highlighted in red. The mutation A16G was introduced in PyMol without subsequent energy minimization or stereochemical optimization.

Membrane Content and Function of the ATP Synthase with Different Ala-to-Gly Mutations and Stability of the Mutant *c*-Rings

Table 1

Strains	A				B			
	β -subunit content (% of WT) ^a	OG- stimulated ATPase activity (% of WT) ^c	Malate growth, A ₆₀₀ at 14hrs, (% of WT) ^d		<i>in vitro</i> ATP synthesis (% of WT) ^e		<i>c</i> -ring stability	
			pH 10.5	pH 7.5	pH 10.5	pH 7.5	Total <i>c</i> -subunit % of WT ^f	<i>c</i> -ring stability on SDS-PAGE ^g
WT	100	100	100	100	100	100	100	S
	Group 1: 42–51%							
A18G	51±22 ^b	61±1 ^b	65±2	69±2	71±14	63±12	69±7	S
A16/18G	42±10	51±5	36±1	50±2	70±1	67±6	21±5	VU
A16/20G	47±2 ^b	52±10 ^b	9±0.1	46±1	53±10	87±4	64±7	VU
A16/18/20/22G	50±10 ^b	52±14 ^b	–2±1	9±1	20±8 ^b	81±14 ^b	29±1	VU
	Group 2: 59–67%							
A16G	65±6 ^b	65±0.4 ^b	62±1	84±2	79±3	88±11	63±10	U
A22G	59±22 ^b	77±3 ^b	69±2	78±3	ND	ND	95±10	S
A16/20/22G	67±1 ^b	62±9 ^b	16±0.1	38±1	64±7	86±14	57±5	VU
	Group 3: 76–82%							
A20G	76±9 ^b	72±2 ^b	57±1	79±3	ND	ND	79±8	S
A18/22G	79±5	81±1	58±1	77±1	ND	ND	76±5	U
A20/22G	82±29	78±9	57±2	84±0.1	72±14	77±6	84±14	VU

^a An equal amount of everted vesicle membrane protein from preparations from WT and mutant cells was loaded on SDS-PAGE gels. The amount of the ATP synthase β -subunit detected on immunoblots was quantitatively analyzed as described under Experimental Procedures. These and other values in the Table are shown $\pm$ SD.

^b The membrane levels and OG-stimulated ATPase activity of these mutants were determined in the previous study (8).

^c The OG-stimulated ATPase activity of WT strain was 1.35±0.05 U/mg membrane protein x min.

^d The capacity for growth on malate, expressed as %WT, was assessed from the A₆₀₀ of 2 ml cultures of WT and mutant strains grown for 14 hours on malate-containing medium minus the A₆₀₀ of parallel cultures of the ΔF_0 mutant parent strain that is negative for non-fermentative growth (8).

^e ATP synthesis assays were conducted on ADP + P_i-loaded, RSO membrane vesicles from the WT and seven of the mutants, upon energization with ascorbate-PMS, as described previously (8). During the 10 seconds of incubation with the electron donor, the reaction mixtures were continuously aerated by vortex mixing. WT strain had an ATP synthesis activity of 6.25±1.5 nmoles ATP/mg protein and 4.44±1 nmoles ATP/mg protein at pH 7.5 and 10.5, respectively.

^fThe total *c*-subunit of purified ATP synthases from each mutant, as % of WT, was quantified by the *c*-subunit observed on SDS-PAGE after treatment of the synthase with TCA as described under Experimental Procedures (and see Fig. 3).

^g*c*-ring stability was estimated using the following calculation: (total *c*-subunit monomer in untreated, purified ATP synthase samples/monomer in TCA-treated samples)×100%. Three broad categories are used: S = stable (values 3–10); U = unstable (values 35–36); VU = very unstable (60 - ~100) relative to a WT value of 0.5 for dissociation to monomer without TCA treatment.

Table 2

Formula MW, Measured Masses (MALDI-MS), Apparent MW and Mobility Changes of *c*-Monomer from WT and Mutant Strains.

<i>c</i> -monomer	Formula MW(Da) [*]	Measured masses (Da) [≠]	Apparent MW(Da)	Mobility change (dMW,%) [‡]
WT	6957	6955	5596	-19.6±2.1
A16G	6943	6942	5238	-24.6±2.1
A18G	6943	6942	5533	-20.3±1.5
A20G	6943	6942	5549	-20±1.8
A22G	6943	6942	5568	-19.8±1.6
A16/18G	6929	6928	4746	-31.5±1.9
A16/20G	6929	6928	4737	-31.6±2
A18/22G	6929	6928	5122	-26.1±1.4
A20/22G	6929	6928	5184	-25.2±1.6
A16/20/22G	6915	6914	4701	-32±1.5
A16/18/20/22G	6901	6904	4640	-32.8±1.3

^{*} Formula MW is estimated by Gene runner software.

[≠] The reported masses are after subtraction of the N-terminal formylation.

[‡] dMW=(apparent MW-formula MW)/formula MW x 100%. Values are shown ± SD. Negative values for a dMW indicate faster migration on SDS-PAGE (i.e., a lower apparent MW) than expected (25).