Escherichia coli $F_1F_0$-ATP Synthase with a b/$\delta$ Fusion Protein Allows Analysis of the Function of the Individual b Subunits*5

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**Background:** The essential “stator stalk” connects the nonrotating portions of $F_1F_0$-ATP synthase.

**Results:** A b/$\delta$ fusion protein is functional and allows analyzing the role of the individual b subunits.

**Conclusion:** Understanding the function of the stator stalk is important for understanding the mechanism of ATP synthase.

**Significance:** Understanding the function of the stator stalk is important for understanding the mechanism of ATP synthase.

The “stator stalk” of $F_1F_0$-ATP synthase is essential for rotational catalysis as it connects the nonrotating portions of the enzyme. In *Escherichia coli*, the stator stalk consists of two (identical) b subunits and the $\delta$ subunit. In mycobacteria, one of the b subunits and the $\delta$ subunit are replaced by a b/$\delta$ fusion protein; the remaining b subunit is of the shorter b' type. In the present study, it is shown that it is possible to generate a functional *E. coli* ATP synthase containing a b/$\delta$ fusion protein. This construct allowed the analysis of the roles of the individual b subunits. The full-length b subunit (which in this case is covalently linked to $\delta$ in the fusion protein) is responsible for connecting the stalk to the catalytic $F_1$ subcomplex. It is not required for interaction with the membrane-embedded $F_0$ subcomplex, as its transmembrane helix can be removed. Attachment to $F_0$ is the function of the other b subunit which in turn has only a minor (if any at all) role in binding to $\delta$. Also in *E. coli* the second b subunit can be shortened to a b' type.

$F_1F_0$-ATP synthase catalyzes the final step of oxidative phosphorylation and photophosphorylation, the synthesis of ATP from ADP and inorganic phosphate. $F_1F_0$-ATP synthase consists of the membrane-embedded $F_0$ subcomplex with, in *Escherichia coli*, a subunit composition of $ab_2c_{10}$ and the peripheral $F_1$ subcomplex, with a subunit composition of $\alpha_3\beta_3\gamma_6\delta$. The energy necessary for ATP synthesis is derived from an electrochemical transmembrane proton (or, in some organisms, sodium ion) gradient. Proton flow, down the gradient, through $F_0$, is coupled to ATP synthesis on $F_1$ by a unique rotary mechanism. The protons flow through (half) channels at the interface of a and c subunits, which drives rotation of the ring of c subunits. The $c_{10}$ ring, together with $F_1$ subunits $\gamma$ and $\epsilon$, forms the rotor. Rotation of $\gamma$ in the “cylinder” formed by the $\alpha$ and $\beta$ subunits leads to conformational changes in the catalytic nucleotide binding sites on $\beta$, where ADP and $P_i$ are bound. The conformational changes result in formation and release of ATP.

Thus, ATP synthase converts electrochemical energy, the proton gradient, into mechanical energy in form of subunit rotation, and back into chemical energy as ATP. In bacteria, under certain physiological conditions, the process runs in reverse. ATP is hydrolyzed to generate a transmembrane proton gradient which the bacterium requires for such functions as nutrient import and locomotion (1–5).

To prevent co-rotation of $\alpha_3\beta_3$ with $\gamma\epsilon c_{10}$, the $\alpha_3\beta_3$ cylinder is affixed to the a subunit via the “stator stalk” (6–8). In most bacteria, the stator stalk consists of the $\delta$ subunit and two copies of b. The $\delta$ subunit has a compact globular N-terminal domain (9) and, as can be seen from the x-ray structure of its mitochondrial counterpart, the oligomycin sensitivity-conferring protein, a more extended C-terminal domain (10). The N-terminal domain binds to $\alpha_3\beta_3$. Most of the binding energy is provided by interaction with the N-terminal helix of one of the $\alpha$ subunits (11, 12). At the N terminus of each b subunit is a transmembrane helix. In at least one of the b subunits this transmembrane helix has to bind to the a subunit. Shortly after traversing the membrane there is a break in the helix of a few amino acids (13) followed by a very long cytosolic helical segment. This segment reaches to the C terminus with just one more break approximately 15–20 residues from the C terminus. Structures of the mitochondrial b subunit and of the b counterparts in a vacuolar-type ATP synthase show that b turns by about 90° at this break (10, 14). The C-terminal helix after the break of at least one of the b subunits interacts with the C-terminal domain of $\delta$ to form the stator stalk (15–17).

Despite being genetically identical, the necessity of having nonidentical contacts with the single-copy subunits $\delta$ and a makes both b subunits functionally different. It is not even clear whether both b subunits interact directly with $\delta$ or $\alpha$. The functional difference is reflected in the fact that in photosynthetic bacteria and in chloroplasts the two b subunits are also genetically nonidentical. The most obvious difference between subunits b and b' (in photosynthetic bacteria) or I and II (in chloroplasts) is that b' and II are lacking the short C-terminal helix. Another interesting variation is encountered in mycobacteria, which contain a b/$\delta$ fusion protein (18). As the C terminus of b and the N terminus of $\delta$ are not in close spatial proximity (10), they are connected by a linker region of approximately 110 residues. In addition to the fusion protein, mycobacterial ATP

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ATP synthase has a b subunit which is of the shorter b’ type, without the C-terminal helix.

In one attempt to address the structural and functional differences of the two b subunits in E. coli experimentally, two b subunits with different tags were used. When each b subunit was equipped with a different mutation that, as the b homodimer, was lethal, the heterodimer gave a functional ATP synthase, showing that one b subunit can compensate for defects of the other (17). In another attempt, hybrids of E. coli b with b and b’ of a photosynthetic bacterium were generated (19). Studies using these hybrids revealed that, at least in the cytosolic part, the b subunits prefer to form a staggered dimer over an “in register” one, with the b’ type subunit shifted toward the N terminus with respect to the b type (20). Still, many questions remain unanswered. Here, we describe a different approach. If it were possible to generate a mycobacterium-like fusion protein in E. coli, one of the b subunits would be covalently linked to δ, the other one not. This would allow us to mutate both b subunits individually to explore their function. For this purpose, we generated a hybrid fusion protein consisting of E. coli b and δ and the b/δ linker region of Mycobacterium vanbaalenii. The fusion protein was indeed functional and enabled us to do further experiments toward clarification of the roles of the two b subunits. One result was the observation that the membrane portion of the b’ type subunit alone, in the absence of the transmembrane helix of the b type subunit, is sufficient to anchor the stator stalk to the a subunit.

EXPERIMENTAL PROCEDURES

Construction of Plasmids and Strains—Alignments of the sequences of E. coli b and δ subunits with the sequence of the b/δ fusion protein of M. vanbaalenii are shown in supplemental Fig. S1. Supplemental Fig. S2 shows the DNA and protein sequence of the hybrid E. coli/M. vanbaalenii fusion protein generated in this study. Furthermore, supplemental Table S1 contains the sequences of the mutagenic oligonucleotides and a flow chart describing the development of the plasmids (supplemental Fig. S3). Step numbers given in the following refer to supplemental Fig. S3.

A DNA sequence corresponding to the b subunit of E. coli from the PpuMI site on, followed by the b/δ linker region plus the first 20 residues of the δ segment from the b/δ fusion protein of M. vanbaalenii and the δ subunit of E. coli between δE21 and the BssHII site, was synthesized and inserted into pUC57 by GenScript (Piscataway, NJ). This PpuMI/BssHII fragment was used to replace the corresponding fragment of plasmid pBWU13.4 (21), which expresses wild-type was used to replace the corresponding fragment of plasmid by GenScript (Piscataway, NJ). This PpuMI-BssHII fragment introduced between the genes for b and the fusion protein was removed and replaced by an AvrII site (plasmid pCG11.1; step 6). The b gene was transferred on a PvuI-AvrII fragment from pCG11.1 to pCG12, giving plasmid pCG13 (step 7). Then the DNA fragment encoding the C-terminal helix of the b subunit in pCG13 (residues bD141-bL156) was deleted, resulting in plasmid pCG16 (step 8). A double point mutation, ba79K/ba128D, was introduced into the gene for b in pBWU13.4 and the fusion protein gene in pCG10, giving pCG14 and pCG15, respectively (step 9).

An AvrII site was inserted upstream of the fusion protein gene in pCG15, resulting in plasmid pCG15.1 (step 10). An AvrII-AflII fragment was transferred from pCG15.1 to pCG11.1, replacing the fusion protein gene with a fusion protein gene containing the ba79K/ba128D double point mutation (step 11). The resulting plasmid was named pCG20. In pCG20 the DNA sequence corresponding to the transmembrane helix of the fusion protein (bN2-bM30) was deleted (step 12), giving plasmid pCG21. In pCG21 the DNA segment corresponding to the C-terminal helix of the b subunit (bD141-bL156) was removed, resulting in plasmid pCG22 (step 13).

For the construction of pCG23, an AvrII site was inserted into pBWU13.4 between the genes for b and δ, giving plasmid pCG23.00 (step 14). Then the DNA segment encoding the transmembrane helix of the b subunit (bN2-bM30) was deleted, resulting in plasmid pCG23.0 (step 15). Finally, the PflM1-AvrII segment of pCG20 was removed and replaced by the PflM1-AvrII segment of pCG23.0, thereby replacing the full-length b subunit gene with a b subunit gene from which the portion encoding the transmembrane helix was deleted (step 16), giving plasmid pCG23. For protein expression, plasmids were transformed into strain DK8 (22), which has the ATP synthase operon removed from its chromosome.

Isolation of Inverted Membrane Vesicles, Functional Analysis of Mutant Strains, Determination of F1F0 Content in E. coli Membranes, and Analysis of Possible Fusion Protein Proteolysis—E. coli strain DK8 harboring wild-type or mutated plasmids was aerobically cultivated at 37 °C in LB medium containing 100 μg/ml ampicillin until it reached an absorbance at 590 nm of ~1.5. Inverted membrane vesicles were prepared as described (23). Growth of strains expressing wild-type or mutant ATP synthase in limiting glucose (24), ATPase activities (25), and ATP-driven H+ pumping (26) were determined as described previously, except that ATPase activities and H+ pumping were determined at 23 °C instead of 42 °C. The relative amount of mutant F1F0 in the membranes was estimated via Western blotting, using an anti-β antibody (Agrisera, Vännäs, Sweden). The staining intensity was quantified using a Photodyne imaging system and ImageJ acquisition software (National Institutes of Health). The anti-β antibody used to detect possible fusion protein hydrolysis was custom-made by Pocono Rabbit Farm and Laboratory (Canadensis, PA), using bN134–156 (27) as antigen.
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RESULTS

Design of the Hybrid E. coli/M. vanbaalenii b/δ Fusion Protein—Fig. 1 gives an overview of the b/δ fusion protein of M. vanbaalenii. Portions corresponding to b and δ are highlighted; they are connected by a linker of approximately 110 residues. Secondary structure predictions show that the b portion has two helix breaks, one immediately downstream of the transmembrane helix, the other upstream of the C-terminal helix of about 15 residues. The presence of the C-terminal helix and the overall length of the cytosolic portion of the b subunit segment classify it as a full-length b subunit (i.e. b type, not b’; see the Introduction). The presence of a full-length b portion in the fusion protein was expected, as the b subunit found in mycobacteria in addition to the fusion protein is of the shorter b variety. In the present study, we intended to construct a hybrid fusion protein consisting of E. coli b and δ segments plus a mycobacterial b/δ linker region, to see whether such a fusion protein would be incorporated into E. coli ATP synthase.

To increase the chances of obtaining a functional enzyme, we used BLAST alignments (28) to identify the mycobacterial species whose linker region might match best with the E. coli b and δ segments. For both segments, of 17 mycobacterial species, in the group with the highest degree of similarity to E. coli was M. vanbaalenii (supplemental Fig. S4). A good match between the linker region and the δ segment appears to be especially important, as the linker has to connect the C terminus of b with the N terminus of δ. Based on the available information (see the Introduction), it is the C terminus of δ that interacts with the C terminus of b, whereas the N terminus of δ is more remote. Thus, it is highly likely that the linker region has contacts with the δ segment of the fusion protein. Similar considerations caused us to use in the δ portion of the hybrid fusion protein the first 20 residues from M. vanbaalenii instead of those from E. coli. According to secondary structure predictions (Fig. 1), this 20-residue segment from M. vanbaalenii forms an α-helix (corresponding to helix 1 in E. coli δ). However, there is very little similarity between both species in this helix (unless gaps are introduced). It is interesting to note that BLAST detected weak similarities between E. coli b and the linker region of some of the mycobacterial species, suggesting that the linker region might have evolved via duplication of the b gene.

Enzymatic Activity of E. coli ATP Synthase with a b/δ Fusion Protein—Table 1 gives a list of strains generated in the course of this study. pCG10/DK8 should express (b/δ) ATP synthase which has b and δ subunits replaced by the hybrid E. coli/M. vanbaalenii b/δ fusion protein (for the designation of the different ATP synthase constructs, see Table 1). Plasmid pCG11 contains, in addition to the gene encoding the fusion protein, the normal gene for b. Thus, strain pCG11/DK8 should be able to express b(δ) ATP synthase, with a heterodimeric stalk consisting of one copy of b and one copy of the fusion protein. ATP synthase activity of the resulting enzymes was tested in vivo via the ability to support oxidative phosphorylation by determining growth yields in limiting glucose. To assess ATP hydrolysis activity, inverted membrane vesicles were prepared and ATPase activity and ATP-driven proton pumping were assayed. The most important result was that the hybrid E. coli/M. vanbaalenii b/δ fusion protein is actually functional. Growth rates in limiting glucose were between one half and two thirds of that of the wild-type control, ATPase activities close to 60%, and ATP-driven proton-pumping similar to wild-type (Table 2 and Fig. 2). Western blots indicated that the amount of ATP synthase on the membranes was decreased by 20–30% (Table 2), suggesting that replacing b and δ by the fusion protein reduced expression and/or stability of the enzyme. It should be noted that in overproducing strains, such as pBWU13.4/DK8 and its derivatives, ATP-driven H⁺ pumping can be impaired to a certain extent before a drop-off is observed in the respective assay. Taking this provision into consideration, it appeared that presence of the fusion protein resulted in a slight impairment of enzymatic activity. However, there should be no doubt that the mutant enzymes are competent to perform catalysis by the normal rotational mechanism. Interestingly, the presence of the additional b subunit did not seem to be required, as (b/δ) and b(b/δ) ATP synthase exhibited similar activities (Table 2).

Homodimer Formation by the Fusion Protein?—For the photosynthetic cyanobacterium Synechocystis PCC 6803 it was shown that the b and b’ subunits cannot form homodimers, only a heterodimer (29). Therefore, in all likelihood, in mycobacteria the b segment of the b/δ fusion protein forms a heterodimer with the additional b (or, more precisely, b’) subunit. However, as E. coli b naturally forms a homodimer, in the case described here it appears possible that the b segment of the hybrid b/δ fusion protein might do the same, which could explain why (b/δ) ATP synthase without an additional b subunit is active. The alternative explanation for the activity of the (b/δ) enzyme would be that a single fusion protein is sufficient to take over the role of the stator stalk.

To address this issue, we generated a bA79K/bA128D double mutation in the b segment of the hybrid fusion protein in pCG10/DK8, resulting in strain pCG15/DK8 which should express (bA79K/A128D/δ) ATP synthase. Each mutation individually has been shown to prevent or, at least, to impede b dimerization (30–33). As control, we introduced the bA79K/bA128D double mutation into the wild-type enzyme, creating strain pCG14/DK8. As in E. coli b dimerization is essential for binding to F₁ (31), we could, as expected, neither detect any membrane-bound F₁ (specifically, the β subunit) nor any ATP synthase activity in pCG14/DK8 (Table 2) because of a lack of assembly of bA79K/A128D/δ ATP synthase. Also, strain pCG15/DK8 showed no membrane-bound F₁ and no ATP synthase activity, demonstrating that (bA79K/A128D/δ) ATP synthase could not be assembled either. We will show below (see e.g. pCG20/DK8) that the bA79K/bA128D double mutation does not prevent expression of a correctly folded b/δ fusion protein. Thus, failure to assemble (bA79K/A128D/δ) ATP synthase indicated that a fusion protein monomer could not replace a complete stator stalk, strongly suggesting that dimerization of the b
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TABLE 1
ATP synthase expression strains used in this study

Except for pBWU13.4/DK8, all strains listed were generated in this study. The second through fourth columns indicate whether the expressed enzyme contains b, the b/δ fusion protein (b/δ), and/or δ. bAC and (b/δ) stand for a b subunit and a b/δ fusion protein, respectively, with the N-terminal transmembrane helix missing. bAC indicates a b subunit with the C-terminal helix removed, effectively converting it into a b' type subunit. A79K and A128D are point mutations inserted to prevent b dimerization (see "Results"). The last column gives the designation used for the expressed ATP synthase.

| Strain         | b | (b/δ) | δ | ATP synthase designation |
|----------------|---|-------|---|--------------------------|
| pBWU13.4/DK8   | b |       | δ | bδ or wild-type (21)     |
| pCG10/DK8      | b | (b/δ) |   |                          |
| pCG11/DK8      | b | (b/δ) |δ |                          |
| pCG12/DK8      | b |       | δ |                          |
| pCG13/DK8      | b | (b/δ) |δ |                          |
| pCG14/DK8      | b | (b/δ) |δ |                          |
| pCG15/DK8      | b | (b/AC) |δ |                          |
| pCG16/DK8      | b | (b/AC) |δ |                          |
| pCG20/DK8      | b |       |δ |                          |
| pCG21/DK8      | b | (b/AC) |δ |                          |
| pCG22/DK8      | b | (b/AC) |δ |                          |
| pCG23/DK8      | b | (b/AC) |δ |                          |

TABLE 2
Functional properties of E. coli strains expressing ATP synthase containing the b/δ fusion protein

Strain pBWU13.4/DK8, expressing wild-type ATP synthase, served as positive control. Strain pUC118/DK8, expressing no ATP synthase, was the negative control. Growth yield in limiting glucose was determined via the turbidity of the liquid culture (measured as absorbance at 590 nm). For evaluation, in each experiment the value of the positive control was set at 100%, the value of the negative control as 0%. The relative content of ATP synthase in membrane preparations was measured by quantitative evaluation of Western blots using antibodies against the β subunit. The staining intensity observed for the wild-type sample was set to 100%; the background staining intensity observed for negative control (pUC118/DK8) was set to 0%. Wild-type ATP synthase contributes 25–30% of the total protein in membrane preparations of pBWU13.4/DK8 (21). The ATPase activity of membrane preparations was measured via the concentration of released inorganic phosphate. ATP-driven proton pumping was determined by quenching of the fluorescence of acridine orange (AO). All given numbers are the averages of at least three independent determinations ± S.D.

| Strain         | ATP synthase expressed | Growth yield in limiting glucose | Relative content of ATP synthase in membranes | Membrane ATPase activity | ATP-driven H+ pumping |
|----------------|------------------------|---------------------------------|---------------------------------------------|--------------------------|----------------------|
|                | % of wild-type         | % of wild-type                  | units/mg                                   | % of wild-type           | % AO quenching       |
| pBWU13.4/DK8   | Wild-type, bδ          | 100                             | 100                                        | 2.52 ± 0.35              | 100                  | 88 ± 4               |
| pUC118/DK8     | None                   | 0                               | 0                                          | 0.05 ± 0.05              | 2                    | 0                    |
| pCG10/DK8      | (b/δ)                  | 47 ± 8                          | 70 ± 14                                    | 1.45 ± 0.20              | 58                   | 85 ± 5               |
| pCG11/DK8      | b(b/δ)                 | 63 ± 6                          | 78 ± 11                                    | 1.54 ± 0.14              | 51                   | 83 ± 9               |
| pCG12/DK8      | (b/AN/δ)               | 0 ± 2                           | 0 ± 5                                      | 0.05 ± 0.03              | 2                    | 0                    |
| pCG13/DK8      | b(b/AN/δ)              | 62 ± 5                          | 75 ± 11                                    | 1.61 ± 0.19              | 64                   | 85 ± 6               |
| pCG14/DK8      | b(b/A79K/A128D/δ)      | 1 ± 2                           | 0 ± 3                                      | 0.01 ± 0.01              | 0                    | 0                    |
| pCG15/DK8      | b(b/A79K/A128D/δ)      | 1 ± 2                           | 1 ± 5                                      | 0.03 ± 0.05              | 1                    | 0                    |
| pCG16/DK8      | b(b/AN/A79K/A128D/δ)   | 40 ± 4                          | 63 ± 6                                     | 1.34 ± 0.09              | 53                   | 63 ± 4               |
| pCG20/DK8      | b(b/AN/A79K/A128D/δ)   | 13 ± 4                          | 21 ± 5                                     | 0.33 ± 0.06              | 13                   | 25 ± 5               |
| pCG21/DK8      | b(b/AN/A79K/A128D/δ)   | 20 ± 4                          | 23 ± 5                                     | 0.38 ± 0.12              | 15                   | 25 ± 3               |
| pCG22/DK8      | b(b/AN/A79K/A128D/δ)   | 17 ± 6                          | 20 ± 4                                     | 0.32 ± 0.03              | 13                   | 23 ± 2               |
| pCG23/DK8      | b(b/AN/A79K/A128D/δ)   | 0 ± 1                           | 0 ± 4                                      | 0.02 ± 0.01              | 1                    | 0                    |

Segment of the hybrid fusion protein was responsible for the enzymatic activity of the (b/δ) ATP synthase, which does not contain an extra b subunit in addition to the fusion protein. The δ portion of the second fusion protein, which is not involved in binding to αβ3, can obviously be accommodated in such a way that it does not interfere with the enzymatic activity. Western blotting with anti-β antibody show that the δ portion of the second fusion protein is not proteolytically removed (see supplemental Fig. S5). The observation that the fusion protein can form a functional dimer also means that in b(b/δ) ATP synthase the stator stalk might contain either a fusion protein homodimer or a b/fusion protein heterodimer.

The Function of Each of the b Subunits—The finding that in E. coli dimerization of b is required for binding to F1 (31) certainly suggests that both b subunits are contributing to this function. On the other hand, in chloroplasts and some bacteria the C-terminal helix of b, which appears to be most directly involved in the contacts to δ (10, 15–17), is missing, indicating that at least in those organisms one of the b subunits might play a less prominent role in binding of δ and F1. Similarly, it is not known whether both b subunits are required for binding to a. To answer the question of the role of each b subunit, we first generated strain pCG12/DK8 which contains the gene for the hybrid b/δ fusion protein (but not that for the additional b subunit), with the DNA sequence encoding the transmembrane helix of the fusion protein deleted. As expected, without the possibility to anchor the fusion protein (independent whether monomeric or dimeric) to a, (b/AN/δ) ATP synthase fails to assemble. Neither membrane-bound F1 nor any ATP synthase activity could be detected. In contrast, activity could be restored when the b gene was added to pCG12, to give pCG13. Strain pCG13/DK8 expressed a functional b(b/AN/δ) ATP synthase, which showed a level of expression (and/or stability) and activity very similar to the b(b/δ) enzyme (Table 2), demonstrating that a single transmembrane helix is sufficient to anchor the stator stalk to the α subunit and F1α. As mentioned above, in mycobacteria the b subunit contained in the fusion protein is of the full-length b type, whereas the additional b subunit is of the shorter b' variety. To test whether it is possible to omit the C-terminal helix in the additional b subunit in E. coli ATP synthase containing the b/δ fusion protein, thereby essentially generating a b' subunit, we engineered plasmid pCG16. This plasmid would encode bAC(b/AN/δ) ATP synthase, with a fusion protein without transmembrane helix and with an additional b subunit with residues b141–156 missing. Secondary structure predictions (data not
shown) indicated that in this construct the long cytoplasmic helix would have its helical character preserved until its terminus; and indeed, strain pCG16/DK8 gave a functional b^{AC}(b^{A79K/A128D}/δ) ATP synthase, with overall slightly lower expression/assembly and activities than the (b^{AN}/δ) enzyme, which maintained the C-terminal helix of the second b subunit (Table 2).

The results obtained so far might be interpreted in such a way that one b subunit is responsible for binding of the b dimer to δ and F_{α}, and the other one for binding to α and F_{Ψ}. However, all we showed above was that one transmembrane helix was sufficient to anchor the b dimer to δ, and that the transmembrane helix of the b+ type subunit, which is less (or not at all) involved in binding of δ, could fulfill this function. The question remained whether it would be possible to obtain a functional b^{AN}(b/δ) ATP synthase, where the single transmembrane helix would belong to the b subunit that is also responsible for binding to δ (which in the case here means the fusion protein).

Unfortunately, b^{AN}(b/δ) ATP synthase could not be used directly to address this question, as it would allow again formation of a fusion protein dimer. However, it should be possible to circumvent this problem if introduction of the bA79K/bA128D double mutation in just one of the b subunits, in this case the b subunit that forms part of the fusion protein, would allow assembly of a functional ATP synthase; and indeed, strain pCG20/DK8 expressed an active b(b^{A79K/A128D}/δ) enzyme (Table 2 and Fig. 2), with a fusion protein carrying the bA79K/bA128D double mutation and a wild-type b subunit. This observation indicated that the mutations bA79K and bA128D completely prevent b dimerization only if they are present in both subunits. If they are present in only one of the b subunits, as here, they appear to destabilize the enzyme, resulting in a 75–80% decrease in the amount of ATP synthase detected on membranes. After correction for this loss in yield, the enzyme molecules present on the membranes had activities comparable with those of the parent b(b/δ) ATP synthase.

As with the (b/δ) enzyme, it was possible to remove from b(b^{A79K/A128D}/δ) ATP synthase first the transmembrane helix of the fusion protein, giving b(b^{AN}/b^{A79K/A128D}/δ) ATP synthase (expressed by strain pCG21/DK8), and then the C-terminal helix of the b subunit, resulting in b^{AC}(b^{AN}/b^{A79K/A128D}/δ) enzyme (strain pCG22/DK8), without significant effect on the enzymatic activity (Table 2 and Fig. 2). However, it was not possible to transfer the single transmembrane helix to the b segment of the fusion protein under preservation of function. Attempts to express b^{AN}(b^{A79K/A128D}/δ) ATP synthase (strain pCG23/DK8), which would contain a heterodimer of full-length fusion protein and an additional b subunit without transmembrane helix, resulted in no membrane-bound F_{ψ} and no ATP synthase activity (Table 2 and Fig. 2). Taken together, the results show that it is possible to generate a functional ATP synthase with a single transmembrane helix in the stator stalk; this transmembrane helix has to be on the b+ type subunit, the b subunit which is less (or not at all) involved in binding to δ.

**DISCUSSION**

The main goal of the present study was to identify the role of each of the two b subunits in the stator stalk of *E. coli* ATP synthase. For this purpose, it was necessary to design an enzyme that allowed differentiation between the two genetically identical b subunits. We were able to achieve this aim by attaching one of the b subunits covalently to δ, creating a b/δ fusion protein as encountered naturally in mycobacteria. This fusion protein can still dimerize, and the fusion protein homodimer gives a functional stator stalk. Dimerization could be prevented directly by generating the double mutation bA79K/bA128D in the b segment of the fusion protein, or incorporation of the fusion protein dimer into ATP synthase could be prevented by removing the N-terminal transmembrane helix. Both the b^{AN}/b^{A79K/A128D}/δ and the b^{AN}/δ fusion protein require formation of a heterodimer with an additional b subunit to give a stator stalk that is assembled into a functional enzyme. Obviously, a b homodimer cannot give an active ATP synthase as no δ subunit is present.

Both constructs, b(b^{AN}/δ) and b(b^{A79K/A128D}/δ) ATP synthase, represent excellent tools to study function and location of the individual b subunits. Using these tools, we were able to show that the role of one of the b subunits is binding of the b dimer to δ and F_{α}; the other one is responsible for binding to the a subunit and F_{Ψ}. In species which express two different b subunits, binding to δ seems to be the responsibility of the full-length b+ type subunit, whereas the shorter b- type subunit is required for binding to a. With b binding to δ and b+ to a, the findings explain why dimerization of the b subunit is required for a functional stator stalk.

It is worthwhile to compare these results with the outcome of a very recent study of the interactions of the individual b subunits with other F_{α} and F_{Ψ} subunits, using cysteine-cysteine cross-linking (34). One finding of that study was that it was the same b subunit that made contact with both δ and a. Obviously,
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*FIGURE 3. Model of the possible arrangement of the transmembrane helices in E. coli ATP synthase.* The figure shows helix 2 of 4 of the 10 c subunits in blue; helix 2 is located on the outside of the c ring. 4 of the 5 of the a subunits are shown in green; the transmembrane helices of the two b subunit are colored red. The view is from the cytoplasmic side, where the F_{0} subcomplex is located. The arrow gives the direction of rotation of the c ring during ATP synthesis. The proposed arrangement of the helices is presented under “Discussion.”

the presence of cross-links indicates that two subunits are spatially close, but says nothing about the functional requirement of this contact. Similarly, the absence of cross-links does not necessarily mean that two subunits are distant. Combining the results of both studies, it appears most likely that actually both b subunits make contact with a. The interaction of a with the b’ type subunit, which is not (or less) involved in binding to δ, is the functionally important one and would alone provide sufficient binding energy to fixate the stator stalk to F_{0}, during rotational catalysis. The b type subunit, which interacts primarily with δ, is close to a so that it can be cross-linked (34); however, the additional binding energy due to this interaction is not required. Such an arrangement would be in line with previous results that suggest that the wild-type stator stalk is “overengineered” to ensure efficient coupling of proton translocation and catalysis (35).

Fig. 3 shows a possible arrangement of the transmembrane helices of F_{0}. The spatial relationship between transmembrane helices 2 to 5 in subunit a, and between helix 4 of a and helix 2 of one of the c subunits was suggested based on cross-linking experiments (36, 37). This arrangement of a and c allows the interaction between aR210 and cD61 that is essential for proton translocation through F_{0} (8, 38–40). Cysteine-cysteine cross-linking studies indicated that also the membrane portion of b forms a dimer (13). Single molecule fluorescence resonance energy transfer experiments showed that, when seen from the cytosolic side (as in Fig. 3), relative to the c ring the b dimer is located clockwise of a (41), which puts b close to transmembrane helices 3 and 4 of a. This orientation would stabilize the interactions between a and the b dimer during rotational catalysis by pushing a and b2 together instead of tearing them apart (41). It should be noted that a very recent cross-linking study (42) places the b dimer on the other side of a, close to helices 2 and 5. Such an arrangement would be destabilized during rotation.

The observation that a cysteine at the N terminus of b can be cross-linked to a cysteine at the C terminus of a c subunit suggests that one of the b helices is close to the c ring (43). Based on the finding that maximally 50% of b were involved in the b/c cross-link (43), it appears likely that the membrane portion of the other b subunit is more remote from the c ring. The conclusion obtained above, that probably both b subunits bind to a, would also require that only one of the b subunits can be close to the c ring. The assignment of b (and not b’) as the helix that is more distant from the c ring (Fig. 3) is tentative; it is based on the observation that in mitochondrial ATP synthase the single b subunit, which is of the full-length b type with a C-terminal helix, appears to be on the side of the stator stalk that points away from the c ring (44). This question could be addressed in a b/c cross-linking experiment as in Ref. 43, using as a tool one of the ATP synthase constructs with “differentiated” b subunits generated in this study.

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