Direct Interaction of Subunits a and b of the F0 Complex of Escherichia coli ATP Synthase by Forming an ab2 Subcomplex*

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The addition of a His8 tag to the N terminus of subunit a of the F0 complex of the Escherichia coli ATP synthase allowed the purification of an ab2 subcomplex after solubilization of membranes with n-dodecyl-β-D-maltoside and subsequent nickel-nitriilotriacetic acid affinity chromatography. After co-reconstitution of the ab2 subcomplex with purified subunit c, passive proton translocation rates as well as coupled ATPase activities after binding of F1 were measured that were comparable with those of wild type F0. The interaction between subunits a and b, which has been shown to be stoichiometric and functional, is not triggered by any cross-linking reagent and therefore reflects subunit interactions occurring within the F0 complex in vivo.

The membrane-bound ATP synthase (F0F1) catalyzes the synthesis of ATP during oxidative phosphorylation or photosynthesis in a reaction that is driven by an electrochemical gradient generated by electron transport systems. ATP synthases share high homology with respect to the mechanism of catalysis and ion translocation as well as the mode of coupling. In Escherichia coli, the ATP synthase is built up of two structurally and functionally distinct entities, the peripheral F1 part (α3β3γδε) and the membrane-embedded F0 complex (αb2γδε). In F1, subunits a and b are alternately arranged in a hexamer surrounding the centrally located subunit γ, which extends from the lower part of the hexamer to form a 4.5-nm stalk region. Subunit ε is in tight contact to subunit γ in the lower part of F1, and both subunits are involved in the coupling between F1 and F0. Subunit δ is located near the top of the hexagon. During catalysis, the three β subunits carrying the catalytic sites are forced into asymmetry by eccentric rotation of the γε subcomplex, resulting in different binding affinities for the nucleotides (for reviews see Refs. 1–4).

During ATP synthesis, the rotation of subunits γ and ε is driven by proton translocation through F0. Both subunits are known to contact the subunit c oligomer at the cytoplasmic surface of F0 (2), and during coupled catalysis, a γεcε subcomplex rotates relative to the remainder of the F0F1 complex driven by successive protonation/deprotonation of amino acid residue CAsp-61 located in the second transmembrane helix of subunit c (3, 5). Because of the rotational movement of the central stalk, a second stalk is necessary for the stabilization of the F0F1 complex, which is built up at least of the two copies of subunit b (6, 7). During rotational catalysis, elastic torque is generated, which is thought to be stored in the two stalks, and as a consequence drives ATP synthesis in F1 or proton pumping in F0. Good candidates for elastic deformation are the intertwined helices of subunit γ as a torsional spring and the parallel helices of the subunit b dimer topped by subunit δ and bottomed by subunit a serving as a parallelogram-like spring (3).

Dividing the subunits of the ATP synthase into structural elements of rotor and stator, there is general agreement that the αβγδε complex and subunit δ of F1 as well as subunits α and b of F0 belong to the stator (1, 3). Thereby, the subunit b dimer was shown to be a prerequisite for the binding of F1 to F0, and close proximity between subunit b and subunits α, β, and δ of F1 was very well documented arguing in favor of direct interactions (8). Within the F0 complex, subunits a and b are located outside the ring-like subunit c oligomer (9) and cross-linking between both subunits has been observed but mostly without defining the contact sites (2, 10–13).

In this study, the purification of a stable ab2 subcomplex isolated by Ni-NTA affinity chromatography after addition of a His8 tag to the N terminus of subunit a is described, clearly demonstrating strong interactions between both F0 subunits without further manipulation. The ab2 subcomplex was reconstituted together with purified subunit c into phospholipid vesicles and exhibited passive proton translocation rates and DCCD-sensitive coupled ATPase activities after rebinding of F1 comparable with those of wild type F0.

EXPERIMENTAL PROCEDURES

Construction of Mutants and Growth Conditions—All of the plasmids were derived from plasmid pBWU13 (aptBEFHAGDC) (14). His tag insertions in subunits a and b were introduced by deoxynucleotidyl-directed mutagenesis using the double PCR technique by Ho et al. (15). To generate a N-His6 (pWS3) and b N-His5 (pWS5), five additional histidine residues were inserted behind aHis271 of subunit a to obtain a His11 tag at the C terminus of subunit a. The plasmids were expressed in E. coli strain DKS (16) lacking the atp operon (aptBEFHAGDC) by growth on minimal medium with glycerol as carbon source (17).

Preparative Procedures—F1, F0, and subunit c were purified as described previously (18–20). Proteoliposomes were prepared according to Okamoto et al. (21) with the following modifications. E. coli lipids (Avanti Pro Lipid) present in chloroform were dried under a stream of argon and redissolved in buffer by sonication at a concentration of 30 mg/ml. The weight ratio of phospholipid to protein was 1:2.66. Dialysis

1 The abbreviations used are: Ni-NTA, nickel-nitriilotriacetic acid; a N-His5, His5 tag introduced at the N terminus of subunit a; C-His6, His6 tag at the C terminus of subunit a; b N-His6, His6 tag at the N terminus of subunit b; DCCD, N,N'-dicyclohexylcarbodiimide; DM, n-dodecyl-β-D-maltoside; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
was carried out at 4 °C for 40 h changing the buffer once. For reconstitution of subunit c in lipids prior to the removal of the organic solvent. K+ loading of proteoliposomes was carried out as described previously (22) with the following modifications. Proteoliposomes (usually 180 μl) and 0.45 mM K2SO4 in 66 mM sodium phosphate buffer, pH 7.0, were mixed in a ratio of 2:1, and the sonication/freezing (liquid nitrogen)/thawing cycle was repeated twice.

Purification of ab2 Subcomplex—For purification of the ab2 subcomplex, everted membrane vesicles were prepared at 4 °C as described by Schneider and Altendorf (19) using 0.1 mM TES/NaOH, pH 7.0, 40 mM e-aminocaproic acid, 250 mM sucrose, 0.25 mM EGTA, 20 mM magnesium acetate, 5 mM p-aminobenzamidine, and 1 mM dithiothreitol as buffer system (23). For the removal of F1, membranes were washed once with each of the buffers described previously (23). Subsequently, membranes were incubated overnight in 1 mM Tris/HCl, pH 7.5, 0.5 mM EDTA, and 10% (v/v) glycerol, collected by centrifugation, and washed twice with 10% (v/v) sodium deoxycholate (19) before resuspension in 50 mM Tris/HCl, pH 8.0, and 10% (v/v) glycerol.

For solubilization, membranes (10 mg/ml) were stirred with 0.8% (w/v) n-dodecyl-β-D-maltoside (DM) (Anasazi) for 1–2 h on ice. After centrifugation, 150 mM NaCl and 10 mM imidazole were added to the supernatant prior to incubation with Ni-NTA-agarose (Qiagen) equilibrated in 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 0.05% (w/v) DM, and 10 mM imidazole. Supernatant and Ni-NTA-agarose were mixed in amounts corresponding to 9–15 mg of membrane protein/ml gel matrix and incubated by end-over-end shaking for 1–2 h at 4 °C. Subsequently, the loaded gel matrix was packed into a column and washed with 10 volumes of buffer and with 10 volumes of the same buffer containing 20 mM imidazole. To allow subsequent ammonium sulfate precipitation of the protein and reconstitution into liposomes by dialysis, the detergent was exchanged by washing the column with 10 volumes of 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 10% (v/v) glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 1% (w/v) sodium cholate, and 20 mM imidazole. Finally, the ab2 subcomplex was eluted with the same buffer containing 100 mM imidazole, precipitated with 35% (w/v) ammonium sulfate (19), and resuspended in the cholate-containing buffer without imidazole.

Assays—Protein concentrations were determined with the BCA assay (Pierce) used as recommended by the supplier. Proteins were separated by SDS-PAGE and detected by silver staining (17). Densitometric analyses of band intensities of silver-stained SDS-gels were performed with ImageQuant (Amersham Biosciences). Immunoblotting was performed according to Birkenhager et al. (24). Passive proton translocation rates were measured (25) using 2 μM valinomycin for induction of the diffusion potential, and the initial rates were calculated according to Dmitriev et al. (26). DCCD-sensitive ATPase activities of F1-reconstituted proteoliposomes were determined as described previously (27).

RESULTS

Characterization of Mutant Strains Carrying His Tag-modified F0 Complexes—His tags were introduced at either the N or the C terminus of subunit α and at the N terminus of subunit β for purification of F0 subunits. The ability of each of the His tag constructs to grow on the non-fermentable carbon source succinate served as a sensitive test for the function of the ATP synthase in vivo, revealing that all of the mutant strains showed wild-type-like growth (tested for 2 days at 37 °C; data not shown). Nevertheless, an immunoblot analysis of intact cells revealed that the amount of F0 subunits synthesized, particularly that of subunit c, is low in strain DK8/pWS4 carrying the His tag at the C terminus of subunit α (Fig. 1). Therefore, the a C-His5 construct was not used for further experiments, although an ab2 subcomplex could also be prepared from this construct (data not shown). As expected, because of the presence of six additional histidine residues, a slight increase of the apparent molecular masses was observed (Figs. 1 and 2). Everted membrane vesicles prepared from mutant strains carrying subunit a N-His6 or subunit b N-His6 showed ATPase activities (6–7 μmol P1·min⁻¹·mg⁻¹), which are approximately a factor of 2 lower as those observed for wild type membrane vesicles, although the immunodecoration of F0 subunits in intact cells did not reveal such differences in the amount of protein present (Fig. 1).

### Table 1

| F0 preparation      | Initial rate of H⁺ uptake (μmol H⁺·min⁻¹·mg⁻¹) | DCCD-sensitive ATPase activity (μmol P1·min⁻¹·mg⁻¹) |
|---------------------|-----------------------------------------------|-----------------------------------------------|
| Control             | 6.8                                           | 0.0                                           |
| DK8/pBWU13 (wild type) | 25.8 (19.0)                                  | 10.2 (10.2)                                   |
| DK8/pWS3 (a N-His6) | 25.8 (19.0)                                  | 11.2 (11.2)                                   |
| DK8/pWS5 (b N-His6) | 9.4 (2.6)                                    | 7.0 (7.0)                                     |

The values given in parentheses are those obtained after subtraction of the control values. Control, liposomes without added protein.
Reconstitution of His Tag-modified F₀ Complexes—In both cases, the a N-His₅ and the b N-His₅ construct, solubilization of membranes as described by Schneider and Altdorf (19) allowed the isolation of F₀ complexes comparable with wild type (data not shown). The F₀ complexes were incorporated into liposomes prepared from E. coli lipids and assayed for passive proton translocation by imposing a K⁺/valinomycin diffusion potential. The resulting initial rates of H⁺ uptake were comparable for F₀ complexes prepared from both the a N-His₅ construct and the wild type strain, whereas the net initial rate of F₀ isolated from membranes containing b N-His₅ amounts only to −15% of that value (Table I, compare values in parentheses). In addition, after the binding of F₁ to F₀-containing proteoliposomes, DCCD-sensitive coupled ATPase activity was again comparable for a N-His₅ and wild type while b N-His₅ exhibited only 60–70% ATPase activity (Table I). The results obtained indicate that the additional histidine residues at the N terminus of subunit a have no influence on the function of F₀ in proton translocation and F₁ binding. However, an addition of histidine residues to the N terminus of subunit b affects at least the reconstitution of the protein complex into phospholipid vesicles, since growth of cells and ATPase activities in native membranes remained unchanged.

Purification of ab₂ Subcomplex—Incubation of F₁-depleted everted membrane vesicles of the a N-His₅ construct with 9.8% DM resulted in solubilization of >90% of the F₀ subunits present. Surprisingly, the use of Ni-NTA affinity chromatography allowed the purification of an ab₂ subcomplex instead of single subunit a carrying the His tag, whereas subunit c was completely removed during the washing steps prior to elution of the protein with 100 mM imidazole (Fig. 2). The protein complex obtained is homogeneous with the exception of a protein with an apparent molecular mass of 15,000 present in low variable amounts (Fig. 2), which could be identified by immunoblot analysis as a degradation product of subunit b (Fig. 1B, compare lane 6). The quantification of band intensities of subunits a and b in silver-stained SDS gels at different protein concentrations revealed almost identical densitometric proportions in both wild type F₀ and the purified subcomplex (data not shown). Therefore, the stoichiometry of both proteins remains unchanged during the purification process, resulting in the isolation of an ab₂ subcomplex.

Reconstitution of F₀ Subcomplexes—For the reconstitution of an F₀ complex, functional in both passive proton translocation and coupled ATPase activity after binding of F₁, all of the three F₀ subunits are necessary (17, 19, 28). Therefore, also for the ab₂ subcomplex, passive proton translocation (Fig. 3) and functional binding of F₁ (Table II) could only be observed after co-reconstitution with subunit c, whereas the rates measured either for reconstituted ab₂ subcomplex or subunit c alone (even in the presence of a 6-fold excess) were comparable with those of plain liposomes.

To obtain maximum rates of proton translocation and ATPase activity, the amounts of subunit c necessary for co-reconstitution are higher as calculated (Table II and Fig. 3). Therefore, co-reconstitution was performed with varying amounts of subunit c. A possible explanation might be that subunit c is present in different conformations after chloroform/methanol extraction and that it cannot completely refold after incorporation into the lipid environment or during assembly with the other F₀ subunits. A second explanation might be that subunit c is integrated into the lipid bilayer in different orientations so that only half of the protein is suitable for reconstitution of F₀. Furthermore, the relatively low amount of ab₂ subcomplex present in the proteoliposomes can only be saturated with subunit c in the presence of an excess of proteolipid. In addition, because of the extreme hydrophobicity of subunit c, a correct determination of the protein concentration is generally difficult.

### DISCUSSION

A strong interaction between subunits a and b of F₀, which has been favored by dividing the subunits of the ATP synthase into structural elements of rotor and stator, has now been unequivocally demonstrated by the purification of a stable ab₂ subcomplex. Surprisingly, the addition of a His tag to the N terminus of subunit a as well as to the N terminus of subunit b and the C terminus of subunit a (data not shown) only allowed the purification of a stable ab₂ subcomplex. Single His-tagged subunits could not be purified, although several detergent mix-

![Fig. 3. Passive proton translocation of ab₂ subcomplex reconstituted into E. coli lipid vesicles (corresponds to the second experiment of Table II). The ab₂ subcomplex and subunit c were reconstituted in amounts according to the stoichiometry a:b:x = 1:2:10 (F₀, 27 μg; ab₂, 12 μg; c, 15 μg) with different amounts of subunit c as indicated by the index “x-times” (c 1x corresponds to 15 μg, e. g. one stoichiometric unit). Passive proton translocation was measured using a K⁺/valinomycin diffusion potential for energization. Control, plain liposomes.

### TABLE II

Passive proton translocation of reconstituted ab₂ subcomplex and coupled ATPase activity after binding of F₁

| Subunit composition | Initial rate of H⁺ uptake | DCCD-sensitive ATPase activity |
|---------------------|---------------------------|-----------------------------|
|                     | 1st experiment | 2nd experiment | 1st experiment | 2nd experiment |
| Control             | 2.5 μmol H⁺ min⁻¹ mg⁻¹ | 1.2 | 0 | 0 |
| c 6x                | 3.0 | 1.1 | 0 | 2 |
| F₀                  | 21.2 | 18.0 | 13.0 | 12.1 |
| ab₂                 | 4.4 | 2.6 | 0.7 | 0.0 |
| ab₂ + c 0.5x        | 13.5 | 10.1 | 7.0 | 19.8 |
| ab₂ + c 1x          | 23.5 | 16.0 | 17.4 | 17.4 |
| ab₂ + c 4x          | 24.5 | 24.8 | 15.8 | 15.6 |

Reconstitution was performed as described in Fig. 3. Two experiments are summarized, which were performed with independent protein preparations. Control, liposomes without added protein.
tures were tested for solubilization, pointing toward a strong interaction between subunits a and b. Whether each of the two copies of subunit b interact individually with subunit a or whether one of the b subunits is in direct contact with subunit a while the second one is bound via the well established subunit b dimer (see Ref. 1) remains to be elucidated.

A first hint for a possible interaction between both subunits has been indicated years ago by the mutation of a-Pro-240 to alanine or leucine, which suppresses the effects observed by mutation G69D (29). However, the evaluation of second site suppressors can be delusive without an additional approach demonstrating direct interaction, because mutations can cause long distance conformational changes (30). Early data on chemical cross-linking of F0,F1 only indicated the proximity of subunits a and b whereas recently, cross-linking within region b-Pro-28 to b Gly-43 of cysteine-substituted subunit b with subunit a was demonstrated (10, 13). However, the counterparts in subunit a remained unidentified.

In addition, a review, a cross-link between the N terminus of subunit b (bN2C) and residues aG227C or aL228C of subunit a has been reported previously (2). The formation of the ab2 subcomplex isolated in this study is not triggered by any cross-linking reagent and therefore reflects subunit interactions occurring within the F0 complex in vivo. Nevertheless, a determination of distinct contact sites between subunits a and b is still of great interest.

Studies on solubilization and purification of F0 allowed the isolation of several F0 complexes in addition to single subunits (17, 19, 28). In each case, after the addition of the missing subunit(s), the subcomplexes can be co-reconstituted to form an F0 complex functional in proton translocation and F1 binding.

The strong interaction between subunits a and b as part of the stator has been demonstrated in this study. The formation of a stable ab2 subcomplex seems reasonable, because both subunits belong to the stator part of the F0 complex and are supposed to withstand mechanical torque built up during rotational catalysis. In addition, subunits a and c cooperatively catalyze proton translocation during ATP synthesis/hydrolysis (3, 5, 31). The interaction of both subunits was shown by purification of a stable ac10 subcomplex (19) as well as by cross-linking between subunit a and at least one copy of subunit c, revealing that the C-terminal helix of subunit c and the penultimate helix of subunit a pack close enough to interact during functional catalysis (5, 32). However, the isolation of a subcomplex consisting of subunits b and c has not yet been achieved, although disulfide bond formation between the N terminus of subunit b (bN2C) and the C terminus of subunit c (cV78C) indicate a close proximity of both subunits (33) arguing for only a reasonably weak interaction between stator and rotor at the subunit b-c interface (see Ref. 10).

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