The structure of the membrane integral rotor ring of the proton translocating F$_1$F$_0$ ATP synthase from spinach chloroplasts was determined to 3.8 Å resolution by x-ray crystallography. The rotor ring consists of 14 identical protomers that are symmetrically arranged around a central pore. Comparisons with the c$_{14}$ rotor ring of the sodium translocating ATPase from Ilyobacter tartaricus show that the conserved carboxylates involved in proton or sodium transport, respectively, are 10.6 – 10.8 Å apart in both c ring rotors. This finding suggests that both ATPases have the same gear distance despite their different stoichiometries. The putative proton-binding site at the conserved arginine in the adjacent subunit returns the carboxylate to its initial conformation.

ATP synthases found in the energy-transducing membranes of bacteria, mitochondria, and chloroplasts catalyze ATP synthesis and ATP hydrolysis coupled with transmembrane proton or sodium ion transport. The enzymes are multi-subunit complexes composed of an extra-membranous catalytic F$_1$ domain and an interconnected integral membrane F$_0$ domain. The hydrophobic F$_1$ domain consists of five different polypeptides with a stoichiometry of a$_3$b$_2$g$_6$. Detailed structural information obtained with the mitochondrial enzyme (1–3) in combination with biochemical (4), biophysical (5), and single molecule studies (6–9) revealed that synthesis or hydrolysis of ATP in the F$_1$ domain is accomplished via a rotary catalytic mechanism. In addition to information on the catalytic mechanism, structure analysis and single molecule studies of the mitochondrial or the chloroplast F$_1$ complex have also unraveled the molecular mechanism of several F$_1$-specific inhibitors (10–14).

Less detailed information is available on the integral membrane F$_0$ domain, which consists of three different polypeptides (a, b, and c) and mediates the transfer of protons or sodium ions across the membrane. Subunits a and b were shown to reside at the periphery of a cylindrical complex formed by multiple copies of the c subunit (15–18). The number of c subunits in the cylindrical subcomplex shows substantial variation in different organisms. Ten protomers are found in ATP synthases from yeast, Escherichia coli and Bacillus PS3 (19–21), 11 in Ilyobacter tartaricus, Propionigenium modestum, and Clostidium paradoxum (22–24), 13 in the thermoalkalophilic Bacillus TA2.TA1 (25), 14 in spinach chloroplasts (26), and 15 in the cyanobacterium Spirulina platensis (27). The structure of isolated subunits a, b, and c from E. coli has been studied by mutagenesis analysis and by NMR spectroscopy in a mixed solvent that was suggested to mimic the membrane environment (28–32). These studies showed that subunit a folds with five membrane-spanning helices. The fourth of these helices directly interacts with subunit c and contains a conserved arginine (Arg$^{211}$), which is thought to be involved in proton transfer (33). Subunit b, which is present in two copies in the intact F$_{a,b}$, contains a single transmembrane helix. Cross-linking data support a direct interaction of the two copies of the b subunit (29). Subunit c was studied at two different pH values to obtain the protonated and deprotonated form of a conserved carboxylate (Asp$^{61}$ in E. coli) that was shown to be essential for proton transfer (34). NMR spectroscopy revealed that the isolated c subunit consists of two long hydrophobic membrane spanning segments connected by a short hydrophilic loop (30, 35). This loop is located close to the γ and ε subunit on the F$_1$ side of the membrane (36, 37). Low resolution x-ray crystallography, cryoelectron microscopy, and atomic force microscopy showed that the membrane-spanning helices of the multiple copies of subunit c in the intact F$_{a,b,c}$ complex are tightly packed in two concentric rings (19, 22, 26). Atomic resolution of the c ring was recently provided for the Na$^+$-translocating F-type ATPase from I. tartaricus (38) and the related Na$^+$-translocating V-type ATPase from Enterococcus hirae (39). Rotation of the c ring was demonstrated by cross-linking (18), fluorescence studies (40), and single molecule visualization (41, 42). Based on the structural and biochemical information on F$_1$, F$_0$, different mechanical models have been proposed describing how the...
rotation of the c ring is coupled to the rotation of the F₁ rotor subunits. This rotation in turn drives sequential conformational shifts at the three catalytic β subunits that result in ATP synthesis (43–45). Vice versa hydrolysis of ATP in the F₁ domain is thought to drive rotation of the γεc₁₀₋₁₅ subcomplex and transports protons or sodium ions across the membrane.

Here we describe the crystal structure of the chloroplast c₁₄ rotor, which is the first structure of an isolated c ring rotor from a proton driven ATPase. The structure was solved by molecular replacement using a tetradecameric search model that was generated from a monomer taken from the *I. tartaricus* c₁₁ structure. The imposition of noncrystallographic symmetry restraints during refinement substantially improved electron density and structure determination.

**EXPERIMENTAL PROCEDURES**

**Purification of CF₁F₀ from Spinach Thylakoid Membranes**—Thylakoid membranes were prepared from spinach plants grown in the local greenhouse according to Strotmann et al. (46). Briefly, the membranes were washed twice in a medium containing 20 mm sodium pyrophosphate pH 7.4, 2 mM DTT, and 0.002% (w/v) PMSF to reduce contamination by ribulose-1,5-bisphosphate carboxylase. After each washing step thylakoid membranes were precipitated by centrifugation at 3000 × g. The membranes were resuspended in 400 mM sucrose, 20 mM Tricine, pH 7.4, 5 mM magnesium chloride, 0.002% (w/v) PMSF, and 50 mM DTT. CF₁F₀ was solubilized from the membranes by the addition of 1% (w/v) sodium cholate and 2% (w/v) β-dodecamaltoside (DDM). The suspension was stirred on ice for 15 min and sonicated with a Branson sonifier (duty cycle, 50%). Unsolubilized material was removed by centrifugation at 100,000 × g. All of the steps were carried out at 4 °C. Further purification of CF₁F₀ was achieved by adding ammonium sulfate to the supernatant to a concentration of 48% (w/v). The resulting ammonium sulfate pellet was dissolved in 10 ml of 50 mM Tricine, pH 8.0, 4% (w/v) glycine, 10% (w/v) glycerol, 5 mM magnesium chloride, 5 mM DTT, 0.002% (w/v) PMSF, and 0.1% (w/v) DDM and loaded on the top of a sucrose gradient that was generated by freezing a solution of 600 mM sucrose, 50 mM Tricine, pH 8.0, 0.1% (w/v) DDM, 5 mM DTT, and 0.002% (w/v) PMSF in polyallomer tubes (2.5 × 8.9 cm; Beckman, Krefeld, Germany) and thawing them slowly at 4 °C. The samples were centrifuged for 24 h at 75,000 × g at 4 °C. The fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and those containing CF₁F₀ were pooled and further purified by anion exchange chromatography on POROS HQ20 (Applied Biosystems Inc., Foster City, CA) as described (47), using a buffer containing 50 mM Tricine, pH 8.0, 10% (w/v) glycine, 4% (w/v) glycerol, 5 mM magnesium chloride, 5 mM DTT, 0.002% (w/v) PMSF, and 0.1% (w/v) DDM. Purity of the protein was checked by SDS gel electrophoresis. Purified CF₁F₀ was concentrated by Amicon Ultra-100 filter (Millipore Corporation, Billerica, MA) to a volume of ~50 μl. The buffer was changed to 25 mM BisTrisPropane, pH 6.5, 10% (v/v) glycerol, 4% (w/v) glycine, 2 mm magnesium chloride, 1 mm EDTA, pH 7.0, 0.002% (w/v) PMSF, 0.02% (w/v) sodium azide, and 35 mm C-HEGA-10 by several concentration steps on the Amicon filtrator followed by dilution in the buffer mentioned above.

**Analytical Methods**—Protein concentrations were determined using the bicinchoninic acid method (Pierce) using bovine serum albumin as standard. The proteins were analyzed by SDS-PAGE on 15% polyacrylamide gels by the Laemmli system (48). After electrophoresis, the proteins were detected by silver staining according to Heukeshoven and Dernick (49).

**Crystallization and Data Collection**—The crystals were grown by micro batch or hanging drop vapor diffusion at 15 °C in a crystallization buffer containing 30% (v/v) polyethylene glycol 400, 100 mm sodium acetate, pH 4.6, 100 mm cadmium chloride, and 100 mm lithium chloride (50). ADP at 1 mm was added to the protein solution in an attempt to stabilize the F₁F₀ complex. In a typical experiment, 2 μl of the purified CF₁F₀ holoenzyme at 10 mg/ml were mixed with an equal volume of the crystallization buffer. In micro batch trials mixed droplets were covered by mineral oil, and in hanging drop trials droplets were equilibrated against 400 μl of reservoir solution containing the crystallization buffer. A precipitate formed shortly after set-up of crystallization trials. Initial crystals appeared after 4 days and grew to the size of 0.1 × 0.1 × 0.1 mm. The crystals belonged to space group C2 (β = 104.7°) with unit cell dimensions *a* = 128.6 Å, *b* = 90.0 Å, *c* = 124.9 Å, and a Matthews coefficient of 3.64 Å³/Da corresponding to a solvent plus detergent content of 66% (v/v). The crystals were harvested in nylon loops and immediately transferred to polyethylene glycol 400 as a cryo-protectant. The crystals were flash frozen in a stream of nitrogen at a temperature of 100 K. The data were collected at the ID14-2 Beamline at the European Synchrotron Radiation Facility (Grenoble, France). The crystals showed anisotropic diffraction up to 3.3 Å. The native data were collected to 3.8 Å resolution using a wavelength of 0.933 Å and an oscillation range of 0.5°.

**Data Integration, Scaling, and Structure Determination**—Programs of the CCP4 suite (51) were used for data reduction and structure determination. The data were integrated with MOSFLM (52), scaled, and merged with SCALA (53), and the amplitudes were estimated using TRUNCATE (54). The data collection statistics are summarized in Table 1. The details of the structure determination are given below.

**Model Building and Refinement**—Model building and refinement were done using COOT (55) and REFMAC (56), respectively. To reflect the tetradecameric noncrystallographic symmetry (NCS), appropriate restraints were applied, and all of the chains were treated equally. The complete ring was used as a single group for TLS refinement. SFCHECK (57) was used for evaluation of the data and atomic model.

**Electrostatic Potentials Calculations**—We manually expanded the final crystallographic model to a complete structure with all amino acids. All previously omitted side chains were added in the most plausible rotamer conformation. To relax any clashes, we refined this model in REFMAC (r = 33% and *R* <sub>free</sub> = 36%). The web-based program *H* + (58) was used to calculate *pK*<sub>a</sub> values Glu<sup>61</sup> with the following parameters: salin-
Structure of Chloroplast F-ATPase Rotor Ring

**RESULTS**

Structure Determination—Analysis of the crystals by SDS gel electrophoresis (Fig. 1) showed that they contain the membrane integral c ring rotor of the chloroplast ATP synthase, but F₁ subunits and subunits a, b, and b' of the membrane integral F₀ domain are lost during the crystallization process. We solved the structure of the chloroplast c₁₄ ring rotor at 3.8 Å resolution by molecular replacement. Initial trials using c monomers from *L. tartaricus* (PDB code 1yce) or *E. coli* (PDB codes 1a91 and 1c99) as search molecules were unsuccessful. The self-rotation function of the diffraction data calculated with MOLREP (60) (supplemental Fig. S1) and atomic force microscopy studies (26) suggest that the chloroplastic c ring rotor consists of 14 identical copies. Thus we built hypothetical c₁₄ rings from the backbone of the *Ilyobacter* monomer (1yce) (38) and used these as search models. We tested a large number of initial models of tetradecameric rings generated by shell scripts varying the radii and the relative orientations of poly-Ala monomer models derived from the *Ilyobacter* structure (38). Residues to the C-terminal side of the kinked area (corresponding to Ser71 in 1yce) were removed, because this region varies between the structures from *Ilyobacter* and *Enterococcus*. Each multimeric starting model was used in molecular replacement with MOL-REP, followed by rigid body and restrained refinement steps with REFMAC, while monitoring the contrast value from MOL-REP and the R_free from REFMAC. To test the suitability of a model for model building, monomers in the complex were randomly deleted, and PHASER (61) was used to replace them. The optimum values obtained for the radius and angles were used to generate a tetradecameric ring with side chains of the spinach chloroplast c subunit included according to the Chainsaw procedure. The structure was improved in several rounds of model building with COOT and refinement with REFMAC. All of the monomers were treated equally using NCS-averaged electron density maps in COOT and by applying strict NCS restraints in REFMAC. This way, we averaged the structures of the 14 monomers and improved the structural precision by a factor of 3.7 (which is the square root of 14). The refinement statistics are summarized in Table 1.

As already indicated in the analysis of the crystals by SDS-PAGE, the electron density maps also showed no indication that any further subunits of the F₁F₀ ATP synthase are present in the crystal. Furthermore, the crystal packing does not accommodate any subunit of the F₁ subcomplex. Crystalization of the isolated c ring starting from intact F₁F₀ has also been described recently by Varco-Merth *et al.* (62), even though no crystallization conditions were given in this reference. However, cell parameters suggest that conditions were similar to those obtained earlier by our group (50).

**Structure of the Chloroplast c₁₄ Ring**—The final model of the chloroplast c ring rotor consists of 14 identical protomers that are symmetrically arranged around a central pore (Fig. 2). Each protomer consists of two membrane spanning α-helices that are connected by a short loop. The N-terminal helices are tightly packed on the inside, whereas the C-terminal helices are located at the periphery of the ring. The overall density of the c₁₄ rotor corresponds to a barrel-shaped complex with an external diameter of 58 Å and internal diameters of 25 Å (top) and 38 Å (bottom), respectively. The cylinder has a waist at the con-

---

**TABLE 1**

Data collection and crystallographic analysis

| Data processing | 19.8-3.8 (4.0-3.8) |
|-----------------|-----------------|
| Resolution (Å)  | 19.8-3.8 (4.0-3.8) |
| No. of reflections | 12655 (1852) |
| Multiplicity of observations | 2.6 (2.5) |
| I/σl | 4.1 (3.3) |
| Completeness (%) | 92.7 (92.2) |
| Rmerge (%) | 8.5 (24.8) |
| Space group | C2 |
| Unit cell parameters (Å) | a = 128.6, b = 90.0, c = 124.9, α = γ = 90°, β = 104.7° |

---

**FIGURE 1.** Subunit composition of purified spinach chloroplast F₁F₀ and of protein crystals obtained. The purified chloroplast F₁F₀ was denatured in SDS and separated on 12% polyacrylamide gel that was silver-stained for the detection of individual subunits. The positions of subunits are indicated on the left. Left lane, purified chloroplast F₁F₀. Right lane, crystal dissolved in SDS gel buffer.
served carboxylate Glu61 (i.e., at the middle of the complex) with a minimal external diameter of 47 Å. The total height of the complex is 65 Å. According to typical residues located at the membrane interphase (63), the position of the membrane is approximately located between residues Arg50 and Asn78, corresponding to a bilayer thickness of 39 Å. The electron density in the 2$\langle F_o \rangle - F_c$ map and the NCS-averaged 2$\langle F_o \rangle - F_c$ map is well defined for all main chain atoms from Pro3 to Pro79 for each protomer (for 2$\langle F_o \rangle - F_c$ map; Fig. 3). Side chains are well defined for all residues except Asn2, Leu4, Leu15, Ile22, Gln28, Gln34, Glu37, Glu44, Lys48, Ile49, Met46, Leu74, and Asn78. All of the residues fall in the allowed regions of the Ramachandran diagram as defined by PROCHECK (64); 76.6% are located in the most favored regions, whereas the remaining 23.4% are found in the additional allowed zones. Significant additional electron density, which probably corresponds to bound lipids or detergent molecules, was observed in the central cavity of the c ring at residues Ala16 to Val26 (Fig. 3). Localization of lipids or detergents at this part of the rotor ring is further supported by the hydrophobic character of the residues lined up in this area of the c$_{14}$-cylinder (Ala12–Val16). Nevertheless, precise interpretation of this density was not possible at the present resolution of the diffraction data. Final parameters of refinement and model stereochemistry are summarized in Table 1.

**Structure of the Putative Proton-binding Site at the Conserved Carboxylate Glu61**—Mutagenesis and inhibitor studies revealed that a conserved carboxylate in the C-terminal helix (Asp61 in *E. coli* and Glu61 in spinach) that is thought to undergo cycles of protonation and deprotonation plays an essential role in proton transfer across the integral membrane domain of H$^+$-translocating F-ATPases. Models of the c ring that are based on the solution NMR structure of the *E. coli* c subunit monomer have placed the conserved carboxylate in the periphery of the ring but in a shielded position where it packs between the helices of adjacent protomers pointing toward the N-terminal transmembrane helix at the interior of the c ring rotor (35, 65). Reversible deprotonation of the conserved carboxylate during proton transport is thought to introduce a transiently charged residue in the c ring. Hence, a shielded location of the carboxyl group in the complex seems essential to avoid an energetically unfavorable exposure of the charge toward the hydrophobic phase of the membrane. Cysteine-cysteine cross-linking studies supported the buried location of the carboxylate in the c ring rotor (66). However, in our structure of the chloroplast c$_{14}$ ring, the conserved carboxylate points toward the periphery. This conformation is in agreement with the structure of the Na$^+$-transporting F-ATPase from *I. tartaricus*. To remove model bias in the structure of the chloroplast c$_{14}$ ring caused by using the atomic model of the *I. tartaricus* monomer for the construction of the search model, the side chains of the c$_{14}$-cylinder (Ala12–Val16) are realigned to the positions in the *E. coli* c$_{14}$-cylinder.
Expression of the chloroplast helicase (34, 35) and ATPase (36, 37) was performed in the expression system of the E. coli host strains BL21 (DE3) and DH5α using pET28a and pET28a−ΔC as expression vectors, respectively. The expression was induced with antibiotics in the presence of 1 mM IPTG and 50 μg/ml of ampicillin and 1 μg/ml of kanamycin according to the manufacturer’s instructions. The bacterial cultures were grown at 37°C. The recombinant proteins were purified by affinity chromatography using nickel–nitrilotriacetic acid resin (NucPro, Bio-Rad, Hercules, CA). The purified proteins were concentrated in the presence of 10 mM imidazole and 100 mM NaCl using Amicon concentrator 1000-mL centrifugal filters (Millipore, Billerica, MA). The purified proteins were stored at −80°C. The specific activities of the recombinant proteins were determined by NADPH-dependent oxidation of NADH and expressed as nmol of NADPH oxidized per minute per milligram of protein. The specific activities of the recombinant proteins were determined by NADPH-dependent oxidation of NADH and expressed as nmol of NADPH oxidized per minute per milligram of protein. The specific activities of the recombinant proteins were determined by NADPH-dependent oxidation of NADH and expressed as nmol of NADPH oxidized per minute per milligram of protein. The specific activities of the recombinant proteins were determined by NADPH-dependent oxidation of NADH and expressed as nmol of NADPH oxidized per minute per milligram of protein.
model of the yeast mitochondrial F-ATPase (19). Comparison of the crystal structure of the chloroplast \( c_{14} \) complex to the rotor rings of the Na\(^+\)-transporting ATPases reveals that all rotor complexes form a barrel-shaped cylinder. However, the diameters of these rotary rotors differ because of the varying number of protomers in the cylindrical complexes. The undecameric ring of \( I. tartaricus \) shows an external diameter of 50 Å at its boundaries and of 40 Å in the middle at the Na\(^+\)-binding site. The decameric ring of \( E. hirae \) consists of protomers with four membrane-spanning helices and has an external diameter of 80 and 68 Å at the conserved Glu\(^{139}\) in helix 4.

Although the \( \text{Na}^+ / \text{H}^+ \) transporter \( I. tartaricus \) ring has a pronounced waist in the middle of the complex, the \( E. hirae \) rotor complex is more like the chloroplast \( c_{14} \) ring with a less pronounced concave curvature of the cylindrical outer surface. In a similar way, the backbone structure of the yeast mitochondrial decameric ring features only a slight curvature. This structure has an external diameter of 34 Å at the conserved carboxylate residue (Asp\(^{61}\)) that is located approximately in the middle of the complex. Both the Na\(^+\)- and the H\(^+\)-transporting ATPases have their conserved carboxylate side chains close to the outer surface of the cylinder in the helices forming the peripheral ring of the complex. Analysis of the conserved residues in the Na\(^+\)-transporting F-ATPase from \( I. tartaricus \) and in the H\(^+\)-translocating F-ATPases from spinach chloroplasts and yeast mitochondria shows that the carboxylates are 10.6–10.8 Å apart in all c ring rotors. Providing that this value reflects an intrinsic constant of the rotor complexes, we propose that the diameter of any c-multimer can be calculated from the number of c subunits in the complex or the stoichiometry of any unknown c ring complex can be estimated from its diameter. The identical distance of the conserved carboxylates in adjacent protomers of Na\(^+\)- and H\(^+\)-transporting ATPases implies that despite their different stoichiometries, they all may have the same gear distance. Furthermore, this figure might reflect the (electrostatic) boundary to which the transport of a single sodium ion or proton can dislocate a single protomer in the complex.

Comparison of Proton- and Sodium-binding Sites in F-ATPases—Comparison of the Na\(^+\)-binding site of the \( I. tartaricus \) ATPase to the H\(^+\)-binding site at the conserved carboxylate of the spinach chloroplast ATPase suggests that a shift in the ion specificity might be related to the hydrophobicity or to the hydrogen bonding potential of side chains adjacent to the conserved carboxylate (supplemental Table S1). In the chloroplast enzyme residues Leu\(^{57}\), Phe\(^{59}\), Ala\(^{62}\), and Leu\(^{63}\) provide a hydrophobic shell around the conserved Glu\(^{61}\) carboxylate. Alanine in position 62 is found in the c subunit of all H\(^+\)-translocating F-ATPases, whereas Na\(^+\)-translocating enzymes hold a polar serine or threonine in the equivalent position. Similarly, the hydrophobic side chain of Leu\(^{63}\) in the chloroplast c ring or various hydrocarbon chains found in the same position in other H\(^+\)-translocating enzymes are substituted by an invariant threonine in Na\(^+\)-translocating ATPases. Furthermore, leucine or hydrocarbon side chains at position 57 of H\(^+\)-ATPases are also substituted by polar residues (Asp, Gln, and Ser) in most c subunits transporting sodium ions. In addition, substitution of valine, which is found in most Na\(^+\)-transporting c rings at a position equiva-

alent to residue 59 in the chloroplast enzyme, by a more bulky hydrophobic residue like leucine or phenylalanine seems advantageous to promote proton translocation.

Proton Translocation in the c Ring Rotor—Current models of proton transport in F-ATPases suggest that either a single access channel or two-half channels located in the a subunit or at the a-c interphase are engaged in proton transport to and from the conserved carboxylate in the middle of the c ring (44, 45). During proton transfer the central carboxylate is thought to be transiently exposed to a conserved arginine in the a subunit stator located at the periphery of the c ring rotor. Exposure of the charged arginine to the carboxyl side chain will lower the \( pK_a \) of the acidic residue resulting in the release of the proton from the conserved carboxylate. Crystals of the chloroplast \( c_{14} \) ring were obtained at pH 4.6. Hence, the structure reflects the protonated form of the chloroplast c ring rotor, assuming a \( pK_a \) of 8.5 for the acidic residue as described for the Glu\(^{65}\) carboxylate in the c566A mutant of the \( I. tartaricus \) enzyme (67). Protonation of Glu\(^{61}\) in the crystal is further supported by the web-based program \( H^+ + \), which computes \( pK_a \) values of ionizable groups in macromolecules on the basis of structural coordinates. Using this program an average value of 7.3 ± 1.3 was calculated for Glu\(^{61}\) in the c ring rotor. In the protonated conformation the conserved Glu\(^{61}\) carboxylate is located at the periphery of the c ring but largely shielded from the external hydrophobic membrane phase. The Glu\(^{61}\) side chain conformation is stabilized by hydrogen bonding with Tyr\(^{66}\), with the backbone carbonyl of Phe\(^{59}\) in an adjacent protomer and with Thr\(^{64}\) in the same protomer. We propose that upon exposure to the charged Arg\(^{189}\) of the a subunit, the conformation of the Glu\(^{61}\) side chain changes to another rotamer and becomes fully exposed to the periphery of the ring because of the deprotonation of the acidic group, which affects hydrogen bonding to the Phe\(^{59}\) main chain carbonyl (see Fig. 6 for illustration). Reprotonation of Glu\(^{61}\) by the conserved arginine returns the carboxylate to the initial conformation, which is largely shielded from the hydrophobic membrane. This mechanism does not require substantial reorientation of the C-terminal helix of subunit c to form contact with the arginine in the subunit a stator. This idea contradicts previous models based on the \( E. coli \) solution NMR structure and cysteine-cysteine cross-linking studies (35, 65). But how are the protons transported to and from the conserved Glu\(^{61}\)? The structure of the chloroplast \( c_{14} \) ring shows no apparent intrinsic proton transport channel within the protomers, neither from the thylakoid lumen (P-side) nor from the stroma (N-side), to provide access to the putative proton-binding site at the conserved carboxylate. Nevertheless, it cannot be ruled out that dynamic fluctuation of the protein might open a transient pathway that is not evident in the static crystal structure. However, based on accessibility studies with hydrophilic probes (68), it seems more likely that an aqueous access channel formed by the membrane-spanning helices 2–5 of subunit a provides access to the conserved arginine from the P-side of the membrane. Proton transport along this channel reprotonates the arginine in the a subunit, which in turn protonates the conserved carboxylate Glu\(^{61}\) in the c subunit. Transfer of the protons from the proton-binding site at Glu\(^{61}\) to the N-side of the membrane is supposed to occur via an aqueous access channel.
formed at the α-ε interphase. Modification of genetically engineered cysteines by Ag\(^{+}\) ions and various thiolate-reactive reagents in the *E. coli* c subunit rotor suggests that residues at positions 57, 58, 62, and 65 are involved in this pathway, and residues 63 and 64 were moderately affected (69). With the exception of the substitutions causing moderate effects, all residues identified in these studies are located at the periphery in the chloroplast c\(_{14}\) rotor and are readily accessible from the external phase (supplemental Fig. S2). However, none of the residues identified in these studies is charged or polar and has the potential to provide side chain hydrogen bonding. Thus it seems reasonable to assume that a water wire for proton transport is reloaded from the lumen side of the thylakoid membrane when a transmembrane proton gradient drives ATP synthesis and results in a counter-clockwise rotation of the c ring.

Acknowledgments—We thank the staff of Beamline ID14 at the European Synchrotron Radiation Facility in Grenoble for help and Elisabeth Straßmann for help with the manuscript.

REFERENCES

1. Abrahams, J. P., Leslie, A. G., Lutter, R., and Walker, J. E. (1994) *Nature* 370, 621–628
2. Meinz, R. I., Walker, J. E., and Leslie, A. G. (2001) *Cell* 100, 331–341
3. Bowler, M. W., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2007) *J. Biol. Chem.* 282, 14238–14242
4. Duncan, T. M., Bulfyin, V. V., Zhou, Y., Hutcheon, M. L., and Cross, R. L. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 10964–10968
5. Sabbatt, D., Engelbrecht, S., and Junge, W. (1996) *Nature* 381, 623–625
6. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (1997) *Nature* 386, 299–302
7. Itoh, H., Takahashi, A., Adachi, K., Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (2004) *Nature* 427, 465–468
8. Rondellez, Y., Tresset, G., Nakashima, T., Kato-Yamada, Y., Fujita, H., Takeuchi, S., and Noji, H. (2005) *Nature* 433, 773–777
9. Masaite, T., Koyama-Horibe, F., Oiwa, K., Yoshida, M., and Nishizaka, T. (2008) *Nat. Struct. Mol. Biol.* 15, 1326–1333
10. Abrahams, J. P., Buchanan, S. K., Van Raaij, M. J., Fearnley, I. M., Leslie, A. G., and Walker, J. E. (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93, 9420–9424
11. Groth, G. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99, 3464–3468
12. Bowler, M. W., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2006) *Proc. Natl. Acad. Sci. U.S.A.* 103, 8846–8849
13. Gledhill, J. R., Montgomery, M. G., Leslie, A. G., and Walker, J. E. (2007) *Proc. Natl. Acad. Sci. U.S.A.* 104, 13632–13637
14. Meiss, E., Konno, H., Groth, H., and Hisabori, T. (2008) *J. Biol. Chem.* 283, 24594–24599
15. Birkenhager, R., Hoppert, M., Deckers-Hebestreit, G., Mayer, F., and Altendorf, K. (1995) *Eur. J. Biochem.* 230, 58–67
16. Singh, S., Turina, P., Bustamante, C. J., Keller, D. J., and Capaldi, R. (1996) FEBs Lett. 397, 30–34
17. Jiang, W., and Fillingame, R. H. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95, 6607–6612
18. Jones, P. C., Hermolin, J., Jiang, W., and Fillingame, R. H. (2000) *J. Biol. Chem.* 275, 31340–31346
19. Stock, D., Leslie, A. G., and Walker, J. E. (1999) *Science* 286, 1700–1705
20. Jiang, W., Hermolin, J., and Fillingame, R. H. (2001) *Proc. Natl. Acad. Sci. U.S.A.* 98, 4966–4971
21. Mitome, N., Suzuki, T., Hayashi, S., and Yoshida, M. (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101, 12159–12164
22. Stahlberg, H., Müller, D. J., Suda, K., Fotiadiis, D., Engel, A., Meier, T., Matthey, U., and Dimroth, P. (2001) *EMBO Rep.* 2, 229–233
23. Meier, T., Matthey, U., von Ballmoos, C., Vonck, J., Krug von Didda, T., Kühlbrandt, W., and Dimroth, P. (2003) *J. Mol. Biol.* 325, 389–397
24. Meier, T., Ferguson, S. A., Cook, G. M., Dimroth, P., and Vonck, J. (2006) *J. Bacteriol.* 188, 7759–7764
25. Meier, T., Morgner, N., Matthies, D., Poganyolov, D., Keis, S., Cook, G. M., Dimroth, P., and Brutschi, B. (2007) *Mol. Microbiol.* 65, 1181–1192
26. Seelert, H., Poetsch, A., Dencher, N. A., Engel, A., Stahlberg, H., and Müller, D. J. (2000) *Nature* 405, 418–419
27. Poganyolov, D., Yu, J., Meier, T., Vonck, J., Dimroth, P., and Muller, D. J. (2005) *EMBO Rep.* 6, 1040–1044
28. Dmitriev, O. Y., Altenedorf, K., and Fillingame, R. H. (2004) FEBs Lett. 556, 35–38
29. Dmitriev, O., Jones, P. C., Jiang, W., and Fillingame, R. H. (1999) *J. Biol. Chem.* 274, 15598–15604
30. Girvin, M. E., Rastogi, V. K., Abildgaard, F., Markley, L. J., and Fillingame, R. H. (1998) *Biochemistry* 37, 8817–8824
31. Valiyaveetil, F. I., and Fillingame, R. H. (1998) *J. Biol. Chem.* 273, 16241–16247
32. Wada, T., Long, J. C., Zhang, D., and Vík, S. B. (1999) *J. Biol. Chem.* 274, 17353–17357
33. Valiyaveetil, F. I., and Fillingame, R. H. (1997) *J. Biol. Chem.* 272, 32635–32641
34. Hoppe, J., and Sebald, W. (1984) *Biochim. Biophys. Acta* 768, 1–27
35. Rastogi, V. K., and Girvin, M. E. (1999) *Nature* 402, 263–268
36. Watts, S. D., Tang, C., and Capaldi, R. A. (1996) *J. Biol. Chem.* 271, 28341–28347
37. Tsunoda, S. P., Aggeler, R., Yoshida, M., and Capaldi, R. A. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 898–902
38. Meier, T., Polzer, P., Diederichs, K., Welte, W., and Dimroth, P. (2005) Science 308, 659–662
39. Murata, T., Yamato, I., Kakinuma, Y., Leslie, A. G., and Walker, J. E. (2005) Science 308, 654–659
40. Diez, M., Zimmermann, B., Börsch, M., König, M., Schweinberger, E., Steigmiller, S., Reuter, R., Felekyan, S., Kudryavtsev, V., Seidel, C. A., and Gräber, P. (2004) Nat. Struct. Mol. Biol. 11, 135–141
41. Pänke, O., Gumbiowski, K., Junge, W., and Engelbrecht, S. (2000) FEBS Lett. 472, 34–38
42. Nishio, K., Iwamoto-Kihara, A., Yamamoto, A., Wada, Y., and Futai, M. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 13448–13452
43. Junge, W., Sabbert, D., and Engelbrecht, S. (1996) Ber. Bunsenges Phys. Chem. 100, 2014–2019
44. Dimroth, P., Wang, H., Grabe, M., and Oster, G. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 4924–4929
45. Fillingame, R. H., Angevine, C. M., and Dmitriev, O. Y. (2003) FEBS Lett. 555, 29–34
46. Strotmann, H., Hesse, H., and Edelmann, K. (1973) Biochim. Biophys. Acta 314, 202–210
47. Groth, G., and Schirwitz, K. (1999) Eur. J. Biochem. 260, 15–21
48. Laemmli, U. K. (1970) Nature 227, 680–685
49. Heukeshoven, J., and Dernick, R. (1999) Electrophoresis 9, 28–32
50. Büchner, C. (2004) Crystallisation and Structure Analysis of Chloroplast FoF1-ATPase. Ph. D. dissertation, University of Düsseldorf, Düsseldorf, Germany
51. Collaborative Computational Project, Number 4. (1994) Acta Crystallogr. D Biol. Crystallogr. 50, 760–763
52. Leslie, A. G. W. (1992) Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography, No. 26, CCP4 and ESF-EAMCB, Daresbury, UK
53. Evans, P. R. (1993) Proceedings of the CCP4 Study Weekend. Data Collection and Processing, pp. 114–122, Daresbury Laboratory, Warrington, UK
54. French, G. S., and Wilson, K. S. (1978) Acta Crystallogr. Sect. A 34, 517–525
55. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132
56. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. D Biol. Crystallogr. 53, 240–255
57. Vaguine, A. A., Richelle, J., and Wodak, S. J. (1999) Acta Crystallogr. D Biol. Crystallogr. 55, 191–205
58. Gordon, J. C., Myers, J. B., Folta, T., Shoja, V., Heath, L. S., and Onufriev, A. (2005) Nucleic Acids Res. 33, W368–371
59. DeLano, W. L., (2002) The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos, CA, U.S.A.
60. Vagin, A., and Teplyakov, A. (1997) J. Appl. Crystallogr. 30, 1022–1025
61. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) J. Appl. Crystallogr. 40, 658–674
62. Varco-Merth, B., Fromme, R., Wang, M., and Fromme, P. (2008) Biochim. Biophys. Acta 1777, 605–612
63. Palldottir, H., and Hunte, C. (2004) Biochim. Biophys. Acta 1666, 2–18
64. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
65. Dmitriev, O. Y., Jones, P. C., and Fillingame, R. H. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 7785–7790
66. Jones, P. C., Jiang, W., and Fillingame, R. H. (1998) J. Biol. Chem. 273, 17178–17185
67. von Ballmoos, C., and Dimroth, P. (2007) Biochemistry 46, 11800–11809
68. Angevine, C. M., Haid, K. A., Vincent, O. D., and Fillingame, R. H. (2008) J. Biol. Chem. 282, 9001–9007
69. Steed, P. R., and Fillingame, R. H. (2009) J. Biol. Chem. 283, 12362–12372
70. Baker, E. N., and Hubbard, R. E. (1984) Prog. Biophys. Mol. Biol. 44, 97–179
71. Luo, P., and Baldwin, R. L. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 4930–4935