Phospholipids Occupy the Internal Lumen of the c Ring of the ATP Synthase of *Escherichia coli*

Benjamin Oberfeld,[‡] Josef Brunner,[§] and Peter Dimroth*[,‡]

Institut für Mikrobiologie, and Institut für Biochemie, ETH Zürich, 8093 Zürich, Switzerland

Received November 10, 2005

ABSTRACT: The occupancy of the central cavity of the membrane-embedded c ring of the ATP synthase of *Escherichia coli* was investigated with a photo-cross-linking approach. Single cysteine mutants were created at c subunit positions 4, 8, and 11, which are oriented to the inside of the ring. These cysteines were alkylated with reagents carrying a photoactivatable substituent and illuminated. Subunit c and derivatives were then isolated and subjected to mass spectrometric analyses. The most noticeable product, which was found exclusively in irradiated samples, had a mass increase of 719 Da, consistent with a cross-link product between the substituted c subunit and phosphatidylethanolamine. Digestion with phospholipase C converted this product into one with a mass diminished by 126 Da, indicating that the phosphoethanolamine moiety was cleaved off. Hence, the cross-link forms to the diacylglycerol moiety of phosphatidylethanolamine. Control experiments showed that the subunit c−phospholipid adducts were formed in the ATP synthase complex in its natural membrane environment and were not artifacts arising from monomeric c subunits. We conclude therefore that the inner lumen of the c ring is occupied with phospholipids. No evidence was found for an extension of subunit a into this space.

ATP synthase is the universal enzyme of living cells that synthesizes ATP from ADP and phosphate under consumption of the free energy stored as an electrochemical gradient of either protons or Na^+ ions. The enzyme consists of two rotary motors, termed F_1 and F_o, that are connected by a common shaft to exchange energy with one another. In the ATP synthesis direction, the F_o motor catalyses the downhill flux of protons or Na^+ ions across the membrane and thereby generates torque. The induced rotation is transmitted by the shaft to the water-exposed F_1 motor where it drives ATP synthesis through conformational changes at the catalytic sites of the β-subunits. The structure and mechanism for the F_1 motor have been well-established (*1*−*3*), but the same level of understanding has not yet been reached for the F_o motor (*4*−*6*), mainly because a high-resolution structure of the entire F_o moiety is not available. From medium-resolution imaging, we know the overall shape of the molecule comprising the oligomeric ring of c subunits that is abutted laterally by the a subunit and the dimer of b subunits (*7*).

Subunit c of *Escherichia coli* folds as a helical hairpin within the membrane, as was shown by early photo-cross-linking experiments (*8*) and by an NMR structure of the c monomer in an organic solvent mixture (*9*). The loops of the subunits are in close proximity to the cytoplasmatic subunits γ and ϵ (*10*, *11*), whereas the N- and the C-termini are located in the periplasm. Important progress in our understanding of the F_o motor was made recently by solving the first high-resolution structure of an oligomeric c ring (*12*) and that of a K ring from the related V-ATPase family (*13*). The structure of the c_{11} ring of the Na^+-translocating ATP synthase from *Ilyobacter tartaricus* shows an hourglass-shaped cylinder consisting of a very tightly packed inner ring comprising the N-terminal helices and an outer ring of the C-terminal helices. The latter ones pack into the grooves formed by the inner helices. Helix packing and the overall shape of the ring correspond astonishing well to an earlier medium-resolution (4.5 Å) structure of the same molecule determined by electron cryomicroscopy (*14*). In the structure, the sodium ion binding sites in the middle of the membrane (*15*) are formed by side-chain oxygens of Gln32 and Glu65 of one subunit and the hydroxyl oxygen of Ser66 and the backbone carbonyl oxygen of Val63 of the neighboring subunit (*12*).

The internal surface of the c ring is very hydrophobic, suggesting that a lipid bilayer could be bound here to seal a potentially lethal hole in the membrane. This possibility would be consistent with the presence of detergent molecules in the internal lumen of the c_{11} ring structure (*12*) and with the lipid plug feature observed in the periplasmic side in reconstituted c_{11} ring crystals by atomic force microscopy (*16*). In addition, an unsolved density was found in the central cavity of the c ring in the crystal structure of the yeast F_1c_{10}, which was proposed to be originated from phospholipids (*17*). But direct evidence for the presence of lipids in c rings in their natural membrane environment is not available. In the present paper, we have addressed this issue by performing photo-cross-linking studies with the *E. coli* c ring in its

* To whom correspondence should be addressed at Institut für Mikrobiologie, ETH Zürich, Wolfgang-Pauli-Str. 10, CH-8093 Zürich, Switzerland, Tel., +41-1-6323321; fax, +41-1-6321378; e-mail, dimroth@micro.biol.ethz.ch.

‡ Institut für Mikrobiologie, Eidgenössische Technische Hochschule Zürich.

§ Institut für Biochemie, Eidgenössische Technische Hochschule Zürich.

10.1021/bi052304+ CCC: $33.50
Published on Web 01/24/2006

Table 1: Oligonucleotides Used for Site-Directed Mutagenesis[a]

oligonucleotide	sequence (5′−3′)	mutation	restriction site
Eca73f	AAA*GGTACC*CATTCTCGCTGGTGG	-	*Kpn*I
Ecb145r	AAA*TCTAGA*AGGTCCTTATGTGCTCG	-	*Xba*I
EccL4Cf	CAAACTGGAGACTG*CCATGG*AAAACTGCAATATGGATCTGCTG	cL4C	*Nco*I
EccL8Cf	CCTGAATATGGATTGCC*TGTATA*TGGCTGCCGCTGTGATGATG	cL8C	*-Bsr*GI
EccL8Cr	CATCATCACAGCGGCAGCCA*TATACA*GGCAATCCATATTCAGG	cL8C	*-Bsr*GI
EccM11CF	GGATCTGCTGTACTGTGCTG*CCGCGGT*GATGGG	cM11C	*Sac*II, *-Bsr*GI
EccM11CR	CCCATCATCA*CCGCGG*CAGCACAGTACAGCAGATCC	cM11C	*Sac*II, *-Bsr*GI
EcacF	ACCAACACT*GCTAGC*TTTTAACTGAAACAAACTGG	none	*Nhe*I

[a] The mutated bases are underlined, and the corresponding newly introduced restriction sites are shown in italics.

natural membrane environment. When the photoactivatable probes were attached to engineered cysteine residues directed to the internal lumen of the c ring, specific cross-link products were obtained upon illumination that could be identified to contain phospholipids.

MATERIALS AND METHODS

Materials. APB,[1] MBP, biotin-maleimide, PMBN, and phospholipase C (from *Bacillus cereus*) were purchased from Sigma. TFPAM-3 and TFPAM-6 were from Molecular Probes, and POPC was from Avanti Polar Lipids. Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Santa Cruz Biotechnology, Santa Cruz, CA.

Bacterial Strains and Plasmids. For routine cloning procedures, *E. coli* strain DH5α (*18*) was used as a host. Recombinant plasmids harboring mutations in the ATP synthase genes of *E. coli* were expressed in *E. coli* strain DK8 (*19*) lacking the *atp* operon (Δ*uncBEFHAGDC*). Plasmid pACWU1·2ΔCys/αHis containing the genes for a cysteine-less *E. coli* ATP synthase was a kind gift from R. K. Nakamoto. The plasmid is related to plasmid pACWU1. 2ΔCys (*20*), but includes the DNA sequence for a His-Tag instead of a FLAG-Tag at the N-terminus of the *β*-subunit (R. K. Nakamoto, personal communication).

Strain DK8 was cultivated in LB (Luria Bertani) medium or in M13 minimal medium (*21*) supplemented with thiamine (10 mg/L), asparagine, leucine, isoleucine, valine (5 mg/L each), tetracycline (20 mg/L), and ampicillin (100 mg/L). As carbon source, either 15 mM succinate or 10 mM glucose plus 1% LB was added.

For ATP synthase production with single cysteine mutants in the N-terminal region of the c subunit, plasmids pECCLcL8C (cL8C) and pECCLcM11C (cM11C) were constructed by site-directed mutagenesis applying the PCR overlap extension method (*22*). The flanking primers Eca73f and Ecb145r and the 5′ and 3′ mutagenic primers (Table 1) containing the desired mutation were used in this approach. The plasmid pACWU1. 2ΔCys/αHis served as a template. The final PCR products comprised the gene for *E. coli* subunit c (*uncE*) and parts of *uncB* and *uncF*. They were restricted with Van91I and Psp5II, using natural restriction sites of the *unc* operon, and ligated into the plasmid pACWU1·2ΔCys/αHis.

To obtain ATP synthase with a single cysteine mutation at position 4 of the c subunit, plasmid pECCLcL4C (cE2D, cL4C) was constructed with the QuikChange Multi Site Directed Mutagenesis Kit (Stratagene) using the oligonucleotide EccL4Cf. The second mutation (cE2D) which arrived spontaneously was identified by DNA sequencing. The plasmid pECCLcL4C contains an NheI site between *uncB* and *uncE*. This is introduced by the same method with the oligonucleotide EcacF.

Molecular biology procedures were performed according to instructions of the manufacturers or as detailed by Sambrook et al. (*18*). All DNA sequences were controlled by automated sequencing performed by Microsynth (Balgach, Switzerland).

Synthesis of SH-Directed Reagents with a Photoactivatable Diazirine Group. N-[4-(3-(Trifluoromethyl)-3*H*-diazirin-3-yl)benzyl]maleimide (Dia-18) was prepared by following a modification of the Mitsunobu reaction (*23*). Briefly, a 100 mL round-bottom flask was charged with triphenylphosphine (0.85 g, 3.24 mmol) to which 20 mL of THF was added. The solution was cooled to −15 °C. Diisopropyl azodicarboxylate (0.65 g, 3.2 mmol) was added, and the reaction mixture was stirred for 5 min after which 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl]alcohol in 1 mL of THF was added. After stirring for 5 min, neopentyl alcohol (0.14 g, 1.6 mmol) and maleimide (0.31 g, 3.2 mmol) dissolved in 1 mL of THF were added. The reaction mixture was stirred overnight at room temperature, concentrated under vacuum, and subjected to a silica gel (50 g) column which was eluted with 4:1 hexane/ethyl acetate to yield 0.44 g (46%) of the title compound, a light yellowish white powder. Thin-layer chromatography (hexane/EtOAc, 2:1) $R_f = 0.46$. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 4.68$ (s, 2H), 6.73 (s, 2H), 7.15 (d, $J = 8.4$, 2H), 7.37 (d, $J = 8.4$, 2H). Mass spectrometry (electrospray ionization; solvent, CH_2Cl_2/MeOH): $m/z = 350.1$ ($[M + MeOH + Na]^+$, calcd 350.1), 322.1 ($[M - N_2 + MeOH + Na]^+$, calcd 322.1).

3-Maleimidopropionic acid, 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl ester (Dia-19) was synthesized analogously to the iodinated product as described previously (*24*). To 4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl]alcohol (43 mg, 0.37 mmol) in THF (3.7 mL) were added 3-maleimidopropionic acid (250 mg, 1.48 mmol), dicyclohexyl carbodiimide (168 mg, 0.81 mmol), $NaHCO_3$ (370 mg), and

[1] Abbreviations: APB, *p*-azidophenacyl bromide; MBP, 4-(*N*-maleimido)benzophenone; TFPAM-3, *N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-3-maleimidopropionamide; TFPAM-6, *N*-(4-azido-2,3,5,6-tetrafluorobenzyl)-6-maleimidyl hexanamide; Dia-18, *N*-[4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl]maleimide; Dia-19, 3-maleimidopropionic acid, 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl ester; THF, tetrahydrofuran; MALDI-TOF, matrix-assited laser desorption/ionization time-of-flight; MS, mass spectrometry; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; DTT, dithiothreitol; PMBN, polymyxin B nonapeptide; PEG, poly(ethylene glycol); POPC, palmitoyl oleoyl phosphatidylcholine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

4-(dimethylamino)pyridine (0.074 mL of 1 M solution in THF). After stirring overnight, the reaction mixture was diluted with ether, extracted with 1 M citric acid, and washed with water. The organic phase was dried over $MgSO_4$, concentrated under vacuum, and subjected to silica gel column chromatography (eluant, ethyl acetate/hexane 2:1) to yield 56.5 mg (57.1%) of Dia-19, a colorless, viscous liquid. ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.69$ (t, $J = 6.9$, 2H), 3.84 (t, $J = 7.0$, 2H), 5.10 (s, 2H), 6.67 (s, 2H), 7.19 (d, $J = 8.4$, 2H), 7.38 (d, $J = 8.1$, 2H). Mass spectrometry (electrospray ionization; solvent, CH_2Cl_2/MeOH): m/z = 422.1 ([M + MeOH + Na]$^+$, calcd 422.1), 390.1 ([M + Na]$^+$, calcd 390.1), 362.1 ([M − N_2 + Na]$^+$, calcd 362.1).

Photo-Cross-Linking Experiments with E. coli Cells Containing ATP Synthase with a Single Cysteine Mutant in Subunit c. Recombinant *E. coli* cells synthesizing ATP synthase with a single cysteine mutant in subunit c were grown on minimal medium with 10 mM glucose and 1% LB as carbon source at 37 °C to late logarithmic phase. Cells were collected by centrifugation and immediately afterward washed once with KPM buffer (250 mM KCl, 50 mM K-phosphate, pH 7.4, and 1 mM $MgSO_4$) containing 1 mM DTT and once with KPM buffer containing 0.1 mM DTT. The cells were centrifuged and resuspended in KPM buffer containing 0.1 mM DTT at a density adjusted to OD_{600} = 30. PMBN was added to a final concentration of 50 μM to make the outer membranes more permeable (*25*), and cross-linking reagents were added to a final concentration of 0.6 mM. After incubation for 50−120 min at room temperature, the reaction was stopped by addition of 5 mM DTT. The cells were incubated for 10−25 min at room temperature and then irradiated for 1−2 min at $\lambda > 320$ nm and 280 W (350 W mercury lamp, SUSS LH 1000 lamp house). The cells were frozen at −20 °C.

Enrichment of ATP Synthase after Photo-Cross-Linking by Sucrose Density Gradient Centrifugation. Labeling and irradiation were performed similarly to the method described above. Since more cells were required, membranes were isolated before irradiation to reduce the volume and the turbidity of the sample during light exposure. Fresh DK8/pECCLcL8C cells (4 g wet weight) were washed twice, and the cell density was adjusted to OD_{600} = 30 as described above. PMBN and Dia-18 were added to final concentrations of 5 μM and 0.6 mM, respectively, and the suspension was incubated for 45 min at room temperature to alkylate the single cysteines at position 8 of the c subunit ring of the ATP synthase. Excess cross-linking reagent was bound to DTT during a 10 min incubation with 2 mM DTT at room temperature. Cells were collected by centrifugation (10 min, 3000*g*, 4 °C), frozen in liquid N_2, and stored overnight at −80 °C. The cells were resuspended in 15 mL of resuspension buffer (5 mM Tris/HCl, pH 8.0, 10% glycerol, 0.5 mM EDTA, 1 mM DTT, and 0.1 mM diisopropylfluorophosphate) and disrupted by passage through a French pressure cell at 100 MPa. Unbroken cells and cell debris were removed by centrifugation (35 min, 27 000*g*, 4 °C), and the membranes were collected by ultracentrifugation (1 h, 200 000*g*, 4 °C). The membranes were resuspended in 9 mL of buffer containing 50 mM MOPS, pH 7.0, and 1 mM DTT, and the suspension was irradiated for 90 s as described above for the formation of photo-cross-linking products. The ATP synthase was solubilized with 1% Triton X-100, and the membrane pellet was removed by ultracentrifugation. The ATP synthase was precipitated by addition of 50 mM $MgSO_4$ and 10.5% PEG 6000. After centrifugation (15 min, 38 000*g*, 4 °C), the protein pellet was dissolved in 600 μL of buffer containing 10 mM Tris/HCl, pH 8.0, 0.1% Triton X-100, 1 mM $MgSO_4$, and 1 mM DTT. The insoluble material was removed by centrifugation, and the soluble material was placed on top of a 12-mL sucrose gradient (5−30% (w/v) sucrose, 10% glycerol, 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM $MgSO_4$, 0.1% Triton X-100, and 0.1 mM DTT). After centrifugation at 38 000 rpm for 17 h in a Beckman SW 40 TI rotor at 4 °C, fractions of 500 μL were removed carefully from the top. A control gradient was run with wild-type subunit c extracted from DK8/pBWU13 (*26*) with chloroform/methanol and purified by sucrose density gradient centrifugation.

Phospholipase C Digestion. Cells containing ATP synthase with a single cysteine mutant in subunit c were labeled with the photo-cross-linking reagent Dia-18. The membranes were isolated and irradiated, and the ATP synthase was extracted and precipitated with PEG 6000 essentially as described above. The protein pellet was resuspended in 1 mL of buffer (1 mM Tris/HCl, pH 8.0, 0.1% Triton X-100, and 0.2 mM DTT) and mixed with an equal volume of PLC buffer (180 mM Tris/HCl, pH 7.3, 0.2% Triton X-100, 1 mM $CaCl_2$, 0.1 mM $ZnCl_2$, and 0.2 mM DTT). An aliquot of 100 μL was treated with 8.6 U phospholipase C. After incubation at 37 °C for 8 h, the reaction was stopped by freezing at −20 °C. A control sample was incubated at 37 °C without phospholipase C, and the lipase was added directly at the extraction step together with chloroform and methanol (see below).

Extraction of Subunits a and c with Organic Solvents. Subunits a and c were extracted together from *E. coli* cells with chloroform/methanol under acidic conditions. This method is similar to the method of Dmitriev et al. (*27*) but was developed independently in our group (*28*). To 80 μL of cells labeled with photo-cross-linking reagent and irradiated as described above, 20 μL of citric acid (5%, w/v) was added. The cell suspension was mixed vigorously with 1 mL of chloroform/methanol (1:1, v/v) to precipitate insoluble proteins. After centrifugation (15 800*g*, 5 min), citric acid (145 μL, 5%, w/v) was added to an aliquot (800 μL) of the supernatant. Phase separation was achieved after mixing and a short centrifugation step. The lower organic phase containing the hydrophobic subunits a and c and phospholipids was collected, and SDS (20 μL, 1%, w/v) was added. The samples were dried under vacuum and resolved in 37.5 μL of 100 mM Tris/HCl, pH 8.0.

Purification of Subunit c. Subunit c was extracted from cells or protein solutions with chloroform/methanol as described previously (*29*). It was purified by anion-exchange HPLC as described (*30*). A linear gradient of 0−72 mM ammonium acetate in chloroform/methanol/water, 4:4:1 (v/v/v) was run over a period of 30 min at 1 mL/min. Fractions (1 mL) were collected and analyzed separately. Typically, the volume of the samples was then reduced by mixing 500 μL of a fraction with 78 μL of H_2O to get a phase separation. After mixing, the organic phase was collected, and its volume was reduced to ∼50% using a stream of N_2.

MALDI-TOF Analysis. Molecular masses were determined on a Perseptive Biosystems Voyager Elite System (a MALDI-TOF instrument) in the linear mode as described earlier (*29*) with the following modification: the samples were not dried and no formic acid was used, unless otherwise indicated.

Reaction of Sulfhydryl Groups with Phospholipids. In polypropylene reaction tubes, 5.6 mM POPC and 340 mM mercaptoethanol in chloroform/methanol/water 4:4:1 containing 1.1 mM Tris/HCl, pH 8.0, were incubated at room temperature from 2 min to 22 h. To induce phase separation, the solvent ratio was adjusted to chloroform/methanol/water 5:5:3. The organic phases were separated and dried using a stream of N_2 followed by vacuum centrifugation to remove the residual mercaptoethanol. For MALDI MS analysis, the samples were resolved in chloroform/methanol/water 4:4:1 and spotted onto a layer of dihydroxybenzoic acid.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE) and Immunoblotting. SDS−PAGE was performed according to Schägger and von Jagow (*31*) with separating gels containing 13.5% acrylamide−bisacrylamide (37.5:1.0, National Diagnostics, Atlanta, GA). Silver staining was performed as described (*32*). For immunoblotting, proteins were transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Buckinghamshire, U.K.). The nitrocellulose membrane was blocked with 3% bovine serum albumin and 2.5% low-fat powdered milk in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 (TTBS) for 1.5 h and rinsed with TTBS three times. For subunit c detection, the blocked nitrocellulose membrane was incubated at room temperature overnight with rabbit anti-c serum, diluted 1:50 000. After washing three times with TTBS, it was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:4000 for 2.5 h. The peroxidase-coated protein bands were visualized using ECL Western blotting detection reagents (Amersham Biosciences). Antibodies were removed in 62.5 mM Tris/HCl, pH 6.7, 2% SDS, and 100 mM mercaptoethanol at 50 °C. The nitrocellulose membrane was blocked, and subunit a was detected with rabbit anti-a serum, diluted 1:5000, as described above. Antibodies against *E. coli* subunits a and c were a kind gift from Gabriele Deckers-Hebestreit.

ATPase Activity. The activity of the ATPase was determined by a coupled assay as described (*33*) at pH 7.5 in the presence of 0.4% lauryldimethylamine *N*-oxide. No Triton X-100 was added.

RESULTS

Construction of Single Cysteine Mutants in Subunit C of the E. coli ATP Synthase. To identify the molecules within the internal volume of the c ring in its natural membrane environment, photo-cross-linking experiments were performed. For this purpose, single cysteine residues were introduced by mutagenesis into the *E. coli* c subunit to chemically anchor photoactivatable cross-linking reagents. Residues 4, 8, and 11 of the *E. coli* c subunit were selected for mutagenesis because these are predicted to be at the inner surface of the c ring (*8*, *34*). The mutant c subunits were constructed on plasmid pACWU1·2ΔCysαHis carrying the genes for a cysteine-less ATP synthase with an N-terminal His-tag at the α-subunit, and the *unc* deletion strain *E. coli* DK8 was transformed with the mutagenized plasmids. All of the transformed *E. coli* cells synthesizing the cysteine-less ATP synthase or enzymes with the single cysteine substitutions could grow on succinate minimal medium, indicating that the mutant ATP synthases were functional.

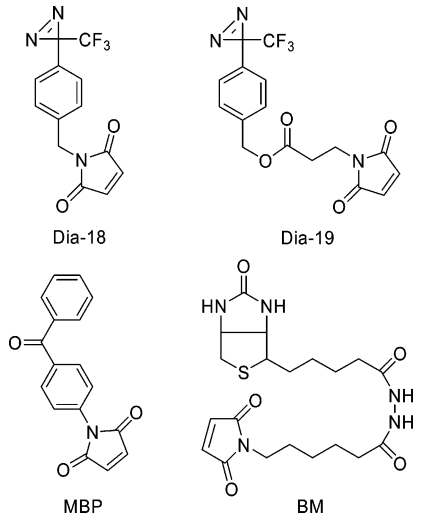

FIGURE 1: Sulfhydryl-directed reagents used in this study. MBP, 4-(*N*-maleimido)benzophenone; Dia-18, *N*-[4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzyl]maleimide; Dia-19, 3-maleimidopropionic acid; BM, biotin-maleimide.

Alkylation of SH-Groups with Photoactivatable Reagents and Cross-Link Formation. To modify the single cysteine residues introduced into the *E. coli* c subunit, two novel sulfhydryl-specific photoactivatable compounds (Dia-18 and Dia-19) were synthesized which carry aryldiazirine and maleimido groups with spacers of different length between them. In other experiments, the commercially available reagent MBP (*35*) was used, which had a maleimido residue linked to an aryl ketone group (Figure 1). Upon illumination of the aryldiazirines, N_2 is eliminated and the resulting carbenes form covalent bonds to any nearby molecule (*36*). Illumination of the aryl ketone generates a biradical which reacts with molecules in its vicinity to form a covalent bond (*37*).

In a first series of experiments, cells synthesizing ATP synthase with the cL8C mutation were labeled with either one of these reagents and illuminated. Subunit c was subsequently extracted with an organic solvent mixture and further purified by anion exchange HPLC, and subunit c-containing fractions were analyzed by MALDI MS. Cross-link products were most visible in spectra obtained from the fraction eluting 1 min before the main peak of modified and unmodified subunit c. Mass spectra obtained from these experiments are collected in Figure 2, and the reaction products assigned to the masses obtained are depicted in Table 2. Figure 2A shows the spectrum of the nonirradiated control sample and treatment with Dia-18. The main peak (II, $m/z = 8563$) corresponds to subunit c modified with Dia-18 (calculated mass = 8542). The two other peaks (I and III) could be assigned to unmodified subunit c and to the modified c subunit attached to the matrix sinapinic acid. As expected, masses corresponding to the modified c subunit are not found in the sample of Cys-less ATP synthase treated with Dia-18 (Figure 2F) or if the cL8C mutant was not incubated with Dia-18 (Figure 2G). Peak broadening in the

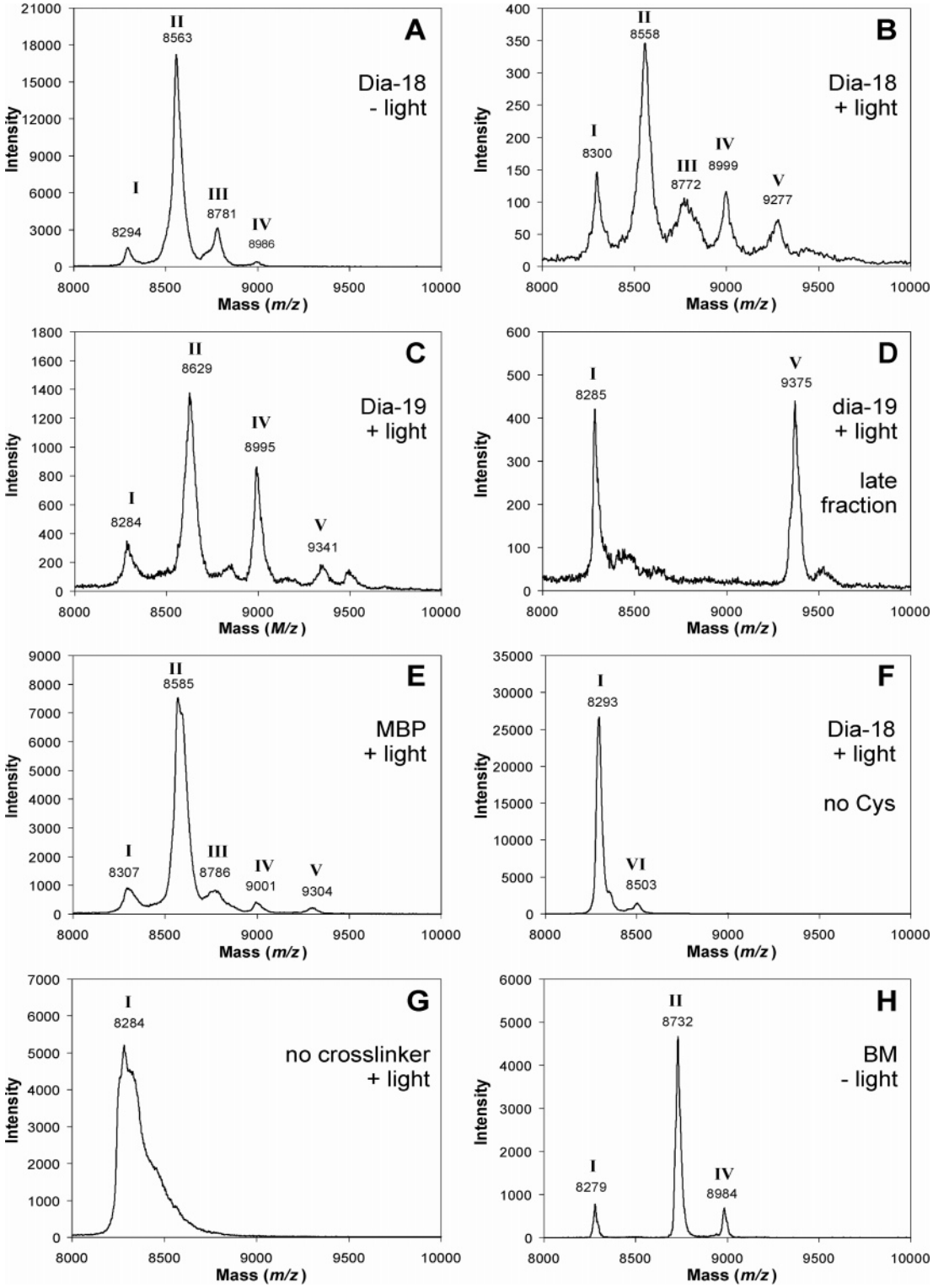

FIGURE 2: MALDI mass spectra of modified subunit c and cross-link products purified from *E. coli* cells containing ATP synthase with a single cysteine mutant at position 8 of subunit c. (A) *E. coli* cells containing F_1F_o ATP synthase with a single cysteine mutation at position 8 of the c subunit were treated with the SH-directed photoactivatable reagent Dia-18. Modified subunit c and cross-link products were extracted from the cells with chloroform/methanol and purified by anion exchange HPLC. (B) Procedure is as described in panel A, but cells were irradiated (λ > 320 nm) after modification. (C and E) *Procedures* are as described in panel B, but the SH-directed photoactivatable reagents Dia-19 and MBP were used, respectively. (D) Procedure is as described in panel C, but a later HPLC fraction eluting after the labeled subunit c was analyzed. (F) Procedure is as described in panel B, but with wild-type subunit c (without cysteine). (G) Procedure is as described in panel B, but no cross-linking reagent was used. (H) Procedure is as described in panel A, but cells were grown on LB medium, harvested, washed, frozen at −20 °C, thawed, and treated with biotin-maleimide (BM). Modified subunit c and cross-link products were extracted from the cells with chloroform/methanol. The sample was dried, resolved in chloroform/methanol/formic acid 1:1:1 and directly subjected to MALDI-TOF analysis without HPLC purification. The assignments of the peaks are given in Table 2. I, unmodified subunit c; II, subunit c modified with sulfhydryl-specific reagent; III, subunit c modified with sulfhydryl-specific reagent with sinapinic acid (matrix peak); IV, subunit c bound to phospholipid; V, subunit c modified with SH-directed photoactivatable reagent cross-linked to phospholipid; VI, unmodified subunit c with sinapinic acid (matrix peak).

Table 2: Assignment of the Masses Recorded in MALDI MS Spectra (Figure 2 and Figure 3)[b]

identified product	calculated mass	mass found (m/z)
Figure 2A		
cL8C, unlabeled (I)	8275	8294
cL8C, labeled with Dia-18 (II)[b]	8542	8563
cL8C, labeled with Dia-18, sinapinic acid (matrix peak) (III)	8766	8781
cL8C linked to phospholipid (IV)	8993	8986
Figure 2B		
cL8C, labeled with Dia-18, cross-linked with phospholipid (V)	9260	9277
Figure 2C		
cL8C, labeled with Dia-19 (II)[b]	8614	8629
cL8C, labeled with Dia-19, cross-linked with phospholipid (V)	9332	9341
Figure 2D		
cL8C, labeled with Dia-19, cross-linked with phosphatidylglycerol (V)	9363	9375
Figure 2E		
cL8C, labeled with MBP (II)	8552	8585
cL8C, labeled with MBP, sinapinic acid (matrix peak) (III)	8776	8786
cL8C, labeled with MBP, cross-linked with phospholipid (V)	9270	9304
Figure 2F		
wild-type subunit c, unlabeled (I)	8285	8293
wild-type subunit c, unlabeled, sinapinic acid (matrix peak) (VI)	8509	8503
Figure 2H		
cL8C, labeled with biotin-maleimide (II)	8726	8732
Figure 3A		
cL8C bound to phospholipid, PLC digest (phosphoethanolamine group removed) (VIII)	8870	8883
cL8C, labeled with Dia-18, cross-linked with phospholipid, PLC digest (phosphoethanolamine group removed) (VIII)	9137	9136
Figure 3C		
cL4C, unlabeled (I)	8261	8278
cL4C, labeled with Dia-18 (II)[b]	8528	8540
cL4C, labeled with Dia-18, sinapinic acid (matrix peak) (III)	8752	8756
cL8C linked to phospholipid (IV)	8979	8979
cL4C, labeled with Dia-18, cross-linked with phospholipid (V)	9246	9247
Figure 3D		
cM11C, unlabeled (I)	8257	8285
cM11C, unlabeled, sinapinic acid (matrix peak) (VI)	8481	8488
cM11C, labeled with Dia-18 (II)[b]	8524	8549
cL8C linked to phospholipid (IV)	8975	8995
cM11C, labeled with Dia-18, cross-linked with phospholipid (V)	9242	9273

[a] All masses are given for protonated molecular ions $[M + H]^+$ and for palmitoyl vaccenoyl phosphatidyl ethanolamine (PE 16:0, 18:1) as the bound phospholipid, if not otherwise indicated. PLC, phospholipase C. [b] Masses calculated without N_2 (N_2 is easily lost during MALDI MS).

latter spectrum is probably due to the oxidation of the cysteine SH-groups. These control experiments thus show that Dia-18 reacts specifically with the single cysteine residue at position 8 of subunit c. The most interesting additional peak found after illumination is that with $m/z = 9277$ (V, Figure 2B). This mass is in good agreement with a cross-link product of the modified c subunit with phosphatidylethanolamine, the most abundant phospholipid in *E. coli*. Phosphatidylethanolamine with fatty acid side chains of 16 and 18 C atoms (PE 16:0, 18:1), which are the most prevalent in *E. coli* (*38*), has a calculated mass of 717.5 Da. The observed mass difference between the modified subunit c (peak II) and the cross-link product V is 719 Da. Specific photo-cross-link products were also found in parallel experiments with the cL8C mutant and the cross-linking reagents Dia-19 or MBP (peak V in Figure 2C,E), and the masses were shifted in accordance to the changes in the molecular masses of the cross-linking reagents. Hence, subunit c carrying a photoactivatable residue exposed to the inner lumen of the c ring became cross-linked to phospholipid upon illumination, indicating that phospholipids are present inside the c ring in its natural membrane environment.

The peaks numbered IV ($m/z \sim 9000$ Da) were found in all samples containing the cL8C mutation and either one of the three maleinimide-linked cross-linkers despite their different masses. A product with this mass was routinely not found if the Cys-less ATP synthase was incubated with a cross-linking reagent. In the examples shown here, the product was not found in the absence of cross-linking reagents, but in other experiments, the product was also found after purification of subunit c derivatives from *E. coli* cells containing the cL8C mutation in the absence of any cross-linking reagent (data not shown). The peak was found in irradiated and also in nonirradiated samples (Figure 2A,B). Please note that the relative intensity of peak IV depends on the fraction from the HPLC column selected for MALDI analysis and there was no consistent increase in the peak upon irradiation as apparent from the samples selected. Hence, this product is not formed by photo-cross-linking and the cross-linkers are not part of it. This conclusion was corroborated by finding a peak with the same mass after incubation of the *E. coli* cells with the cL8C mutation with biotin-maleinimide without irradiation (Figure 2H). The mass of peak IV perfectly reflects the mass of subunit c plus one

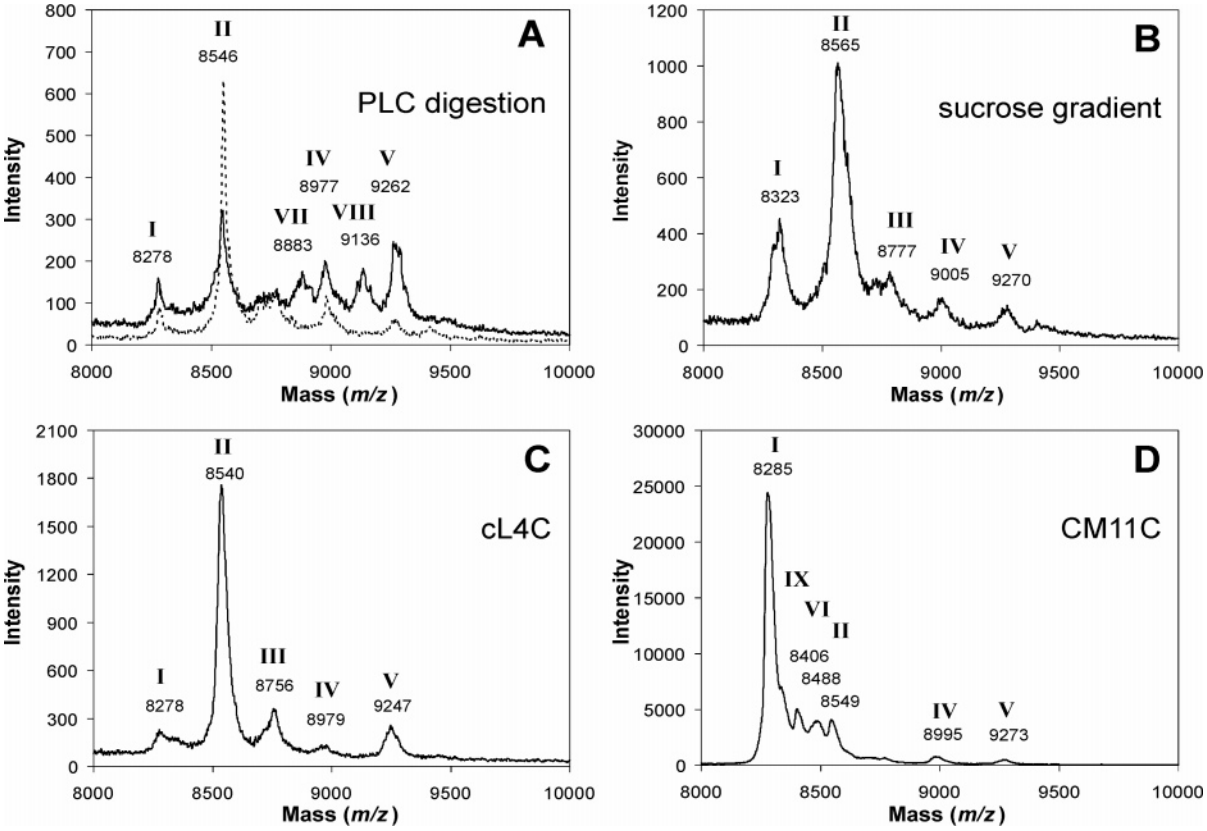

FIGURE 3: MALDI mass spectra of modified subunit c and cross-link products purified from *E. coli* cells containing ATP synthase with a single cysteine mutant at position 4, 8, or 11 of subunit c. (A and B) *E. coli* cells containing F_1F_o ATP synthase with a single cysteine mutation at position 8 of the c subunit were treated with the SH-directed photoactivatable reagent Dia-18. Membranes were isolated and irradiated (λ > 320 nm). ATP synthase was extracted from the membrane with 1% Triton-X100 and concentrated by PEG precipitation. (A) The sample was treated with phospholipase C (solid line). A control sample was incubated without phospholipase (dashed line). Masses are given for the phospholipase C-treated sample. (B) The sample was loaded on sucrose density gradient. A spectrum obtained from the pooled most active fractions is shown. (C and D) *E. coli* cells containing F_1F_o ATP synthase with a single cysteine mutation at position 4 (C) or 11 (D) of subunit c were treated with the SH-directed photoactivatable reagent Dia-18 and irradiated (λ > 320 nm). The assignments of the peaks are given in Table 2. I, unmodified subunit c; II, subunit c modified with cross-linking reagent; III, subunit c modified with cross-linking reagent with sinapinic acid (matrix peak); IV, subunit c bound to phospholipid; V, subunit c modified with SH-directed photoactivatable reagent cross-linked to phospholipid; VI, unmodified subunit c with sinapinic acid (matrix peak); VII, subunit c bound to diacylglycerol; VIII, subunit c modified with SH-directed photoactivatable reagent cross-linked to diacylglycerol; IX, unidentified peak.

phosphatidylethanolamine. Such a product might be formed by the addition of the cysteine SH group of subunit c to the double bond of a fatty acid side chain forming a stable thioether bond. It is known from literature that thiols can add to olefins via a free radical chain mechanism (*39*). Oxygen or lipid peroxides can act as initiators for this reaction. To test if such a reaction might occur under the applied conditions, the phospholipid POPC and mercaptoethanol were incubated from 2 min to 22 h in the same organic solvent mixture used for extraction of subunit c. Mercaptoethanol was chosen as a sulfhydryl group-containing model substance, since it is more soluble in organic solvents and easier to remove than cysteine. A peak with the mass of the addition product of POPC and mercaptoethanol (838.6 Da) was found (data not shown), indicating a reaction of a sulfhydryl group with the double bond of a fatty acid.

Phospholipase C Digestion. For further analyses of the products appearing in mass spectroscopy as peak V and IV, respectively, the samples were digested with phospholipase C. This enzyme catalyses the hydrolysis of the ester bond between diacylglyceride and the polar headgroup of a phosphoglyceride (*40*). By photo-cross-linking, subunit c could be either bound to the diacylglycerol moiety or to the polar headgroup of the phospholipid molecule. In the first case, the digestion product should have a mass of 9137 Da and, a new peak with $m/z = 9136$ was indeed found (peak VIII, Figure 3A). In accordance with the second possibility, the digestion product should have a mass of 8683 Da. Clear evidence for a product of this size was not obtained, but some intensity was seen in this mass range with or without phospholipase C digestion, potentially masking a small peak. These results confirm the cross-link formation between subunit c and phospholipid, and further indicate that the linkage occurs to the diacylglycerol moiety of the phospholipid. The peak found at $m/z = 8883$ (peak VII) might correspond to the digestion product of the subunit c−lipid conjugate (peak IV) mentioned above.

The Cross-Linking Products between Subunit c and Phospholipids Are Copurified with the ATP Synthase. To exclude the possibility that the found c subunit conjugates originated from subunit c which was not assembled in whole F_1F_o ATP synthase, we separated the holoenzyme from monomeric subunit c by gel filtration before analysis. For this purpose, the cells containing the cL8C mutation were treated with the cross-linking reagent Dia-18 and illuminated as described. The ATP synthase was then extracted from the membrane with 1% Triton X-100 and purified by sucrose

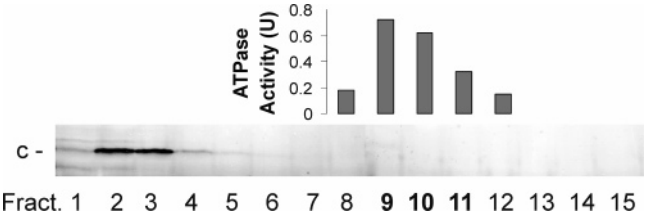

FIGURE 4: Fractionation of ATP synthase or monomeric subunit c from *E. coli* by sucrose density centrifugation. Purified subunit c was loaded on top of a sucrose gradient. After centrifugation, fractions (500 μL) were removed carefully from the top and analyzed by SDS−PAGE. Gels were stained with silver. Fractions 1−15 from 25 are shown. Monomeric subunit c was mainly found near the top of the gradient in fractions 2 and 3 and not in fractions 9−11 (written in bold letters), where most of the ATPase activity was found in a gradient carried out under the same conditions containing ATP synthase.

density gradient centrifugation. The fractions with most of the ATPase activity (nos. 9−11) were pooled, and the c subunit was extracted with chloroform/methanol and subjected to MALDI-TOF analysis. The results shown in Figure 3B indicate that the cross-link product of modified subunit c with phospholipid (designed peak V) is present in these samples. Monomeric subunit c subjected to sucrose density gradient centrifugation in parallel was found exclusively near the top of the gradient (fractions 2 and 3) but not in the ATPase-containing fractions (nos. 9−11) (Figure 4) according to its small size. Hence, the cross-link product reflects the presence of phospholipids within the central cavity of the c ring of the ATP synthase and is not an artifact arising from monomeric c subunits.

Investigations with Other Cysteine Mutants of Subunit C. Similar experiments as those described above were also performed with the mutants c L4C and c L11C, and the mass spectra are shown in Figure 3C−D. In the case of the mutant c L4C (Figure 3C), nearly all c subunits became modified by the maleinimide-containing cross-linking reagent Dia-18 (peak II), and this compound was cross-linked to phospholipids upon illumination (peak V). In the case of mutant c L11C, only a small fraction of the c subunits was modified with this cross-linking reagent (Figure 3D) reflecting poor accessibility of the cysteine at this more deeply membrane-embedded location. Accordingly, only a small peak of the cross-linking product to phospholipid (peak V) was found. Peak IX found between the unmodified c subunit and that with the attached cross-linker could not be identified.

Phospholipid Species inside the Cavity. The three major phospholipids in *E. coli* are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL), where PE represents about 70−80% of the total phospholipid. The predominant fatty acids present in cytoplasmic membrane lipids are palmitic (hexadecanoic) acid, palmitoleic (*cis*-9-hexadecanoic) acid, and *cis*-vaccenic (*cis*-11-octadecanoic) acid. The majority of phospholipids contains a saturated fatty acid and an unsaturated fatty acid (*38*). The observed mass difference of 715 ± 15 Da between peaks II and V matches that of PE containing a palmitic acid and a *cis*-vaccenic acid ($[M + H]^+ = 718.5$ Da) or that of the sodium ion adduct of PE containing a palmitic acid and a palmitoleic acid ($[M + Na]^+ = 712.5$ Da). Lipids are often detected as sodium ion-bound molecules in MALDI MS analysis (*41*). However, the peaks in the MALDI mass spectra are relatively broad and might contain more than one phospholipid species.

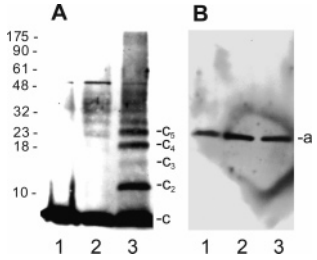

FIGURE 5: Analysis of cross-link product formation between subunits c and a. Cells harboring ATP synthase with a cysteine at position 8 of subunit c (cL8C) or with wild-type subunit c were labeled with MBP and irradiated. Subunits a and c were extracted with chloroform/methanol under acidic conditions and subjected to SDS−PAGE and Western blotting as described in Materials and Methods. (A) Antiserum specific to *E. coli* subunit c was used to detect subunit c and its cross-link products. Lane 1, wild-type subunit c (without cysteine) with irradiation; lane 2, cL8C without irradiation; lane 3, cL8C with irradiation. In lane 3, multimers of subunit c can be detected. The masses (in kDa) of marker proteins are indicated at the left side. Note that subunit c runs at a lower apparent mass than the real mass (8.3 kDa). (B) Same as panel A, but an antiserum specific to *E. coli* subunit a was used. Only subunit a, but no cross-link products, is visible.

The small mass difference between the polar headgroups of PE and PG (31 Da), in addition to the variability of the fatty acid composition, makes it impossible to distinguish between PE and PG on the basis of the observed masses. But PG in contrast to PE has a negative net charge and therefore is expected to elute from anion exchange HPLC column at higher salt concentrations. The product shown in Figure 2B,C,E (peak V) elutes slightly earlier then the main part of labeled subunit c (peak II) and could therefore be PE linked to the labeled c subunit. In a sample treated with Dia-19, a peak at $m/z = 9375$ was found to elute 4−5 mL later than the labeled subunit c (Figure 2D). The observed mass difference to peak V of the early eluting fraction (Figure 2C) of 34 Da matches perfectly to the difference between PE and PG.

The third phospholipid, CL, has a mass of 1196−1308 Da. Therefore, subunit c labeled with Dia-18 linked to CL would have a mass of 9736−9850 Da. A peak in this mass range was never observed. These results indicate that PE, as well as PG but not CL, is present in the central cavity of the c ring.

Photo-Cross-Linking Reagents at the inside of the c Ring React with Subunit c but Not with Subunit a. In some reports in the literature, it was proposed that subunit a, or part of it, is located in the inside of the c ring (*42*). To investigate this possibility, we used *E. coli* cells with the c L8C mutation and labeled this residue with either one of six different photoactivatable cross-linking reagents (MBP, Dia-18, Dia-19, APB, TFPAM-3, and TFPAM-6). The cells were then irradiated for cross-link formation, and subunits c and a were extracted together with chloroform/methanol at acidic pH. To exclude the formation of disulfide bonds, the samples were treated for 3 min with 5% mercaptoethanol at 95 °C. They were then analyzed by SDS−PAGE and Western blotting using antiserum against subunit c and subunit a. Similar results were obtained with all different cross-linking reagents, and a representative example, where the c subunits were labeled with MBP, is shown in Figure 5. When the blot was developed with antiserum against subunit c, a ladder of subunit c multimers was seen reaching from c_2 to c_5 and

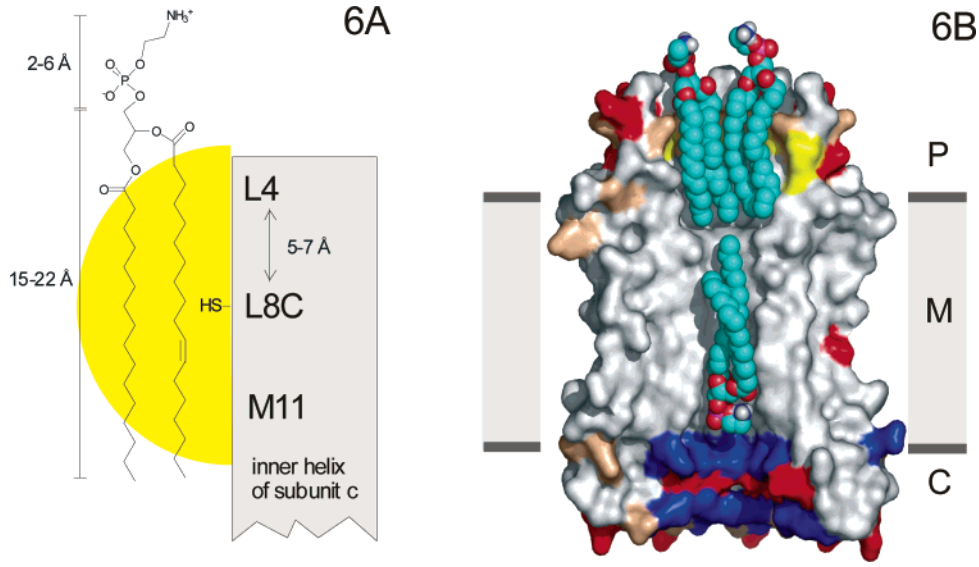

FIGURE 6: Localization of phospholipids inside the c ring. (A) Proposed topology of phospholipids at the periplasmic side of the central cavity of the *E. coli* c ring. The hydrophobic chains of the fatty acid residues are located inside the c ring, whereas the hydrophilic head of the molecule protrudes to the periplasm. The estimated range of the cross-linking reagent Dia-18 linked to the cysteine at position 8 is indicated by the yellow circle. (B) Model of c ring filled with lipids. Phosphatidylethanolamine molecules were fitted by eye into the homology model of the *E. coli* c_{10} ring (*12*). Only five of 10 c subunits are shown. Phospholipids are shown in cyan, and the residue L8 is shown in yellow. The surface is colored light-gray for apolar, yellowish-brown for polar, red for acidic, and blue for basic residues. P, periplasm; M, membrane; C, cytoplasm. The picture was created with PyMOL (*45*). Coordinates for POPE molecules were taken from www.ucalgary.ca/~tieleman/download.html (*46*).

possibly higher order multimers. These products were not formed in control samples without irradiation or if ATP synthase without the cysteine mutation in subunit c was used. If the blot was developed with antiserum against subunit a, only a single band representing this polypeptide became visible and no cross-linking products with higher molecular weight could be identified. These data therefore argue against the hypothesis that part of subunit a is located within the central cavity of the c ring and are congruent with the finding that this space is filled with phospholipids.

DISCUSSION

The high-resolution structure of the c_{11} ring of the Na^+-translocating ATP synthase from *I. tartaricus* (*12*) provided new insights into the function of this motor, but for a comprehensive mechanism, structural details of the residual F_o parts still have to be elucidated. A peculiar feature of the c_{11} ring is a central cavity which must undoubtedly be sealed in the natural membrane environment to avoid a collapse of the membrane potential. In the c_{11} ring structure, the internal surface is covered with hydrophobic amino acids in its central and periplasmic positions. It contains hydrophilic amino acids near the cytoplasmic surface forming the interface with the γ-subunit. It has been pointed out that the hydrophobic surface inside the c_{11} ring could be covered with a lipid bilayer providing a perfect seal against undesirable ion fluxes through the membrane. A hydrophobic internal surface has also been implicated for the *E. coli* c_{10} ring from a homology model that is based on the *I. tartaricus* c_{11} ring structure. The model is roughly consistent with early biochemical data showing that residues on one helical face of the N-terminal inner helix (L4, L8, M11, V15, and L19) are all hydrophobic and accessible to the hydrophobic photoactivatable reagent 3-(trifluoromethyl)-3-(iodophenyl)diazirine (*8*). It was also shown that the single cysteine mutants L8C, M11C, and V15C form disulfide cross-linked homodimers in the presence of an oxidant (*34*). This result is consistent with the exposure of these cysteines toward the inside of the ring where they would come close to each other facilitating disulfide bond formation.

On the basis of these data, we reasoned that the amino acids L4, L8, and M11 are oriented toward the inside of the *E. coli* c ring. These were therefore individually mutated to cysteines to chemically anchor photoactivatable probes. In this report, we have shown that the single cysteine mutants were modified with the probes in the natural membrane environment and that phospholipids became covalently attached upon illumination, indicating that these molecules do indeed cover the internal lumen of the c ring. These observations are easily reconciled with the presence of detergent molecules in the cavity of the *I. tartaricus* c ring structure (*12*) and with the lipid plug found at the periplasmic surface of reconstituted c_{11} rings by atomic force microscopy (*16*).

The proportion of c subunits which became cross-linked to phospholipids was relatively small. This was not unexpected, since the photoactivated probe will react with any nearby molecule. This could be an amino acid of the same c subunit forming an intramolecular cross-link or a water molecule if the reactive part of the cross-linking reagent is oriented toward the periplasm. Part of the probes also form intermolecular c subunit cross-links (Figure 5), and part of the reagents Dia-18 or Dia-19 could be photolyzed to the unreactive linear diazo products (*36*). We also note that the relative peak areas in MALDI MS spectra may not reflect the true ratios between the different molecules present in the sample.

The cavity of the *I. tartaricus* c ring has a diameter of 20−25 Å at the periplasmic side and a diameter of 14 Å in the central part. Assuming that one phospholipid molecule

occupies ~40 Å^2 (as found for lipid monolayers of distearic (dioctadecanoic) phosphoethanolamine (*43*)), there is enough space for 7−10 phospholipid molecules in the periplasmic leaflet and for 2−3 phospholipid molecules in the cytoplasmic leaflet of the putative bilayer within the c ring. In *E. coli*, the number of c subunits per ring is most probably 10 (*44*). Therefore, the internal lumen of the *E. coli* c ring is expected to be somewhat smaller and containing fewer phospholipid molecules than that of the *I. tartaricus* c_{11} ring.

Figure 6A shows a model of the topology of a phospholipid molecule inside the c ring. The hydrophobic chains of the fatty acids are assumed to be located inside the cavity, whereas the hydrophilic head of the molecule protrudes to the periplasm. The positions of the aliphatic chains are in accord with the finding that the mutant L8C labeled with Dia-18 formed cross-link products with the diacylglycerol moiety but not with the ethanolamine part of the molecule. The protrusion of the polar heads of the phospholipids outside the c ring is in accord with atomic force microscopy of the reconstituted *I. tartaricus* c ring showing a lipid plug that protrudes 7 ± 3 Å from the periplasmic surface (*16*).

In accordance with recent structural data, a phospholipid bilayer is present in the central cavity of the K ring of the V-ATPase from *Enterococcus hirae* (*13*). The K ring is much larger than the c ring. Its central cavity has a diameter of 54 Å and is surrounded by 20 transmembrane helices, while the central cavity of the c ring from *I. tartaricus* has a diameter of 14 Å in the central part and is surrounded by 11 transmembrane helices. At present, there is no positive evidence for a bilayer inside the c ring. However, the hydrophobic internal surface which is sufficiently large to accommodate such a bilayer and the presence of phospholipids in the periplasmic portion of the internal cavity argue in favor of this possibility. Additionally, it is reasonable that assembly of the c ring proceeds around a core of lipids covering its internal lumen. In Figure 6B, a homology model of the *E. coli* c_{10} ring (*12*) filled with phospholipids, is shown.

ACKNOWLEDGMENT

The authors thank C. von Ballmoos for help, U. Matthey for advice, H. Rechsteiner and T. Egger for excellent technical assistance, and G. Cook for critical reading of the manuscript. G. Deckers-Hebestreit is acknowledged for supplying antibodies, K. Diederichs for providing the PDB file of the homology model of the *E. coli* c10 ring, and R. K. Nakamoto for supplying pACWU1.2ΔCys/αHis.

REFERENCES

1. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Structure at 2.8 Å resolution of F_1-ATPase from bovine heart mitochondria, *Nature 370*, 621−628.
2. Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K. (1997) Direct observation of the rotation of F_1-ATPase, *Nature 386*, 299−302.
3. Boyer, P. D. (1993) The binding change mechanism for ATP synthase−some probabilities and possibilities, *Biochim. Biophys. Acta 1140*, 215−250.
4. Capaldi, R. A., and Aggeler, R. (2002) Mechanism of the F_1F_0-type ATP synthase, a biological rotary motor, *Trends Biochem. Sci. 27*, 154−160.
5. Dimroth, P., von Ballmoos, C., Meier, T., and Kaim, G. (2003) Electrical power fuels rotary ATP synthase, *Structure (London) 11*, 1469−1473.
6. Angevine, C. M., Herold, K. A., and Fillingame, R. H. (2003) Aqueous access pathways in subunit a of rotary ATP synthase extend to both sides of the membrane, *Proc. Natl. Acad. Sci. U.S.A. 100*, 13179−13183.
7. Mellwig, C., and Böttcher, B. (2003) A unique resting position of the ATP-synthase from chloroplasts, *J. Biol. Chem. 278*, 18544−18549.
8. Hoppe, J., Brunner, J., and Jørgensen, B. B. (1984) Structure of the membrane-embedded F_0 part of F_1F_0 ATP synthase from *Escherichia coli* as inferred from labeling with 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine, *Biochemistry 23*, 5610−5616.
9. Girvin, M. E., Rastogi, V. K., Albildgaard, F., Markley, J. L., and Fillingame, R. H. (1998) Solution structure of the transmembrane H^+-transporting subunit c of the F_1F_0 ATP synthase, *Biochemistry 37*, 8817−8824.
10. Watts, S. D., Zhang, Y., Fillingame, R. H., and Capaldi, R. A. (1995) The gamma subunit in the *Escherichia coli* ATP synthase complex (ECF_1F_0) extends through the stalk and contacts the c subunits of the F_0 part, *FEBS Lett. 368*, 235−238.
11. Hermolin, J., Dmitriev, O. Y., Zhang, Y., and Fillingame, R. H. (1999) Defining the domain of binding of F_1 subunit ϵ with the polar loop of F_0 subunit c in the *Escherichia coli* ATP synthase, *J. Biol. Chem. 274*, 17011−17016.
12. Meier, T., Polzer, P., Diederichs, K., Welte, W., and Dimroth, P. (2005) Structure of the rotor ring of F-Type Na^+-ATPase from *Ilyobacter tartaricus*, *Science 308*, 659−662.
13. Murata, T., Yamato, I., Kakinuma, Y., Leslie, A. G., and Walker, J. E. (2005) Structure of the rotor of the V-Type Na^+-ATPase from *Enterococcus hirae*, *Science 308*, 654−659.
14. Vonck, J., von Nidda, T. K., Meier, T., Matthey, U., Mills, D. J., Kühlbrandt, W., and Dimroth, P. (2002) Molecular architecture of the undecameric rotor of a bacterial Na^+-ATP synthase, *J. Mol. Biol. 321*, 307−316.
15. von Ballmoos, C., Meier, T., and Dimroth, P. (2002) Membrane embedded location of Na^+ or H^+ binding sites on the rotor ring of F_1F_0 ATP synthases, *Eur. J. Biochem. 269*, 5581−5589.
16. Meier, T., Matthey, U., Henzen, F., Dimroth, P., and Müller, D. J. (2002) The central plug in the reconstituted undecameric c cylinder of a bacterial ATP synthase consists of phospholipids, *FEBS Lett. 505*, 353−356.
17. Stock, D., Leslie, A. G. W., and Walker, J. E. (1999) Molecular architecture of the rotary motor in ATP synthase, *Science 286*, 1700−1705.
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
19. Klionsky, D. J., Brusilow, W. S., and Simoni, R. D. (1984) In vivo evidence for the role of the epsilon subunit as an inhibitor of the proton-translocating ATPase of *Escherichia coli*, *J. Bacteriol. 160*, 1055−1060.
20. Kuo, P. H., Ketchum, C. J., and Nakamoto, R. K. (1998) Stability and functionality of cysteine-less F_0F_1 ATP synthase from *Escherichia coli*, *FEBS Lett. 426*, 217−220.
21. Kaim, G., and Dimroth, P. (1995) A double mutation in subunit c of the Na^+-specific F_1F_0-ATPase of *Propionigenium modestum* results in a switch from Na^+ to H^+-coupled ATP synthesis in the *Escherichia coli* host cells, *J. Mol. Biol. 253*, 726−738.
22. Horton, R. M., Cai, Z. L., Ho, S. N., and Pease, L. R. (1990) Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction, *BioTechniques 8*, 528−535.
23. Walker, M. A. (1995) A high-yielding synthesis of *N*-alkyl maleimides using a novel modification of the Mitsunobu reaction, *J. Org. Chem. 60*, 5352−5355.
24. Weber, T., and Brunner, J. (1995) 2-(Tributylstannyl)-4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzyl alcohol−a building-block for photolabeling and cross-linking reagents of very high specific radioactivity, *J. Am. Chem. Soc. 117*, 3084−3095.
25. Wada, W., Long, J. C., Zhang, D., and Vik, S. B. (1999) A novel labeling approach supports the five-transmembrane model of subunit a of the *Escherichia coli* ATP synthase, *J. Biol. Chem. 274*, 17353−17357.
26. Moriyama, Y., Iwamoto, A., Hanada, H., Maeda, M., and Futai, M. (1991) One-step purification of *Escherichia coli* H^+-ATPase (F_0F_1) and its reconstitution into liposomes with neurotransmitter transporters, *J. Biol. Chem. 266*, 22141−22146.
27. Dmitriev, O. Y., Altendorf, K., and Fillingame, R. H. (2004) Subunit a of the *E. coli* ATP synthase: reconstitution and high-resolution NMR with protein purified in a mixed polarity solvent, *FEBS Lett. 556*, 35−38.

28. von Ballmoos, C. (2005) Moleculare interactions along the ion pathway of F_1F_0 ATP synthase, Ph.D. Thesis, Eidgenössische Technische Hochschule (ETH) Zürich, Zürich, Switzerland.
29. von Ballmoos, C., Appoldt, Y., Brunner, J., Granier, T., Vasella, A., and Dimroth, P. (2002) Membrane topography of the coupling ion binding site in Na^+-translocating F_1F_0 ATP synthase, *J. Biol. Chem. 277*, 3504−3510.
30. Kluge, C., and Dimroth, P. (1994) Modification of isolated subunit c of the F_1F_0-ATPase from *Propionigenium modestum* by dicyclohexylcarbodiimide, *FEBS Lett. 340*, 245−248.
31. Schägger, H., and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal. Biochem. 166*, 368−379.
32. Nesterenko, M. V., Tilley, M., and Upton, S. J. (1994) A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels, *J. Biochem. Biophys. Methods 28*, 239−242.
33. Laubinger, W., and Dimroth, P. (1987) Characterization of the Na^+-stimulated ATPase of *Propionigenium modestum* as an enzyme of the F_1F_0 type, *Eur. J. Biochem. 168*, 475−480.
34. Jones, P. C., Jiang, W., and Fillingame, R. H. (1998) Arrangement of the multicopy H^+-translocating subunit c in the membrane sector of the *Escherichia coli* F_1F_0 ATP synthase, *J. Biol. Chem. 273*, 17178−17185.
35. Tao, T., Lamkin, M., and Scheiner, C. J. (1985) The conformation of the C-terminal region of actin: a site-specific photocrosslinking study using benzophenone-4-maleimide, *Arch. Biochem. Biophys. 240*, 627−634.
36. Brunner, J. (1993) New photolabeling and crosslinking methods, *Annu. Rev. Biochem. 62*, 483−514.
37. Turro, N. J. (1978) *Modern Molecular Photochemistry*, pp 372−377, Benjamin/Cummings, Menlo Park, CA.
38. Kadner, R. J. (1996) Cytoplasmic membrane, in *Escherichia coli and Salmonella typhimurium*: *Cellular and Molecular Biology* (Neidhardt, F. C., Curtiss, R. I., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., Eds.) pp 58−87, American Society of Microbiology Press, Washington, DC.
39. Stacey, F. W., and Harris, J. F. (1963) Formation of carbon-hetero atom bonds by free radical chain additions to carbon−carbon multiple bonds, *Org. React. 13*, 150−376.
40. Zwaal, R. F., and Roelofsen, B. (1974) Phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Bacillus cereus*, *Methods Enzymol. 32*, 154−161.
41. Al-Saad, K. A., Zabrouskov, V., Siems, W. F., Knowles, N. R., Hannan, R. M., and Hill, H. H., Jr. (2003) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of lipids: ionization and prompt fragmentation patterns, *Rapid Commun. Mass Spectrom. 17*, 87−96.
42. Seelert, H., Dencher, N. A., and Müller, D. J. (2003) Fourteen protomers compose the oligomer III of the proton-rotor in spinach chloroplast ATP synthase, *J. Mol. Biol. 333*, 337−344.
43. Majewski, J., Kuhl, T. L., Kjaer, K., Gerstenberg, M. C., Als-Nielsen, J., Israelachvili, J. N., and Smith, G. S. (1998) X-ray synchrotron study of packing and protrusions of polymer−lipid monolayers at the air−water interface, *J. Am. Chem. Soc. 120*, 1469−1473.
44. Jiang, W., Hermolin, J., and Fillingame, R. H. (2001) The preferred stoichiometry of c subunits in the rotary sector of *Escherichia coli* ATP synthase is 10, *Proc. Natl. Acad. Sci. U.S.A. 98*, 4966−4971.
45. DeLano, W. L. (2002), *The PyMOL Moleculare Graphics System*, DeLano Scientific, San Carlos, CA (www.pymol.org).
46. Tieleman, D. P., and Berendsen, H. J. (1998) A molecular dynamics study of the pores formed by *Escherichia coli* OmpF porin in a fully hydrated palmitoyloleoylphosphatidylcholine bilayer, *Biophys. J. 74*, 2786−2801.

BI052304+