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Calcium Binding to the Subunit c of *E. coli* ATP-Synthase and Possible Functional Implications in Energy Coupling[1]

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The 8-kDa subunit c of the *E. coli* F_0 ATP-synthase proton channel was tested for Ca^{++} binding activity using a $^{45}Ca^{++}$ ligand blot assay after transferring the protein from SDS-PAGE gels onto polyvinyl difluoride membranes. The purified subunit c binds $^{45}Ca^{++}$ strongly with Ca^{++} binding properties very similar to those of the 8-kDa CF_0 subunit III of choloroplast thylakoid membranes. The N-terminal f-Met carbonyl group seems necessary for Ca^{++} binding capacity, shown by loss of Ca^{++} binding following removal of the formyl group by mild acid treatment. The dicyclohexylcarbodiimide-reactive Asp-61 is not involved in the Ca^{++} binding, shown by Ca^{++} binding being retained in two *E. coli* mutants, Asp61→Asn and Asp61→Gly. The Ca^{++} binding is pH dependent in both the *E. coli* and thylakoid 8-kDa proteins, being absent at pH 5.0 and rising to a maximum near pH 9.0. A treatment predicted to increase the Ca^{++} binding affinity to its F_0 binding site (chlorpromazine photoaffinity attachment) caused an inhibition of ATP formation driven by a base-to-acid pH jump in whole cells. Inhibition was not observed when the Ca^{++} chelator EGTA was present with the cells during the chlorpromazine photoaffinity treatment. An apparent Ca^{++} binding constant on the site responsible for the UV plus chlorpromazine effect of near 80–100 nM was obtained using an EGTA-Ca^{++} buffer system to control free Ca^{++} concentration during the UV plus chlorpromazine treatment. The data are consistent with the notion that Ca^{++} bound to the periplasimic side of the *E. coli* F_0 proton channel can block H^+ entry into the channel. A similar effect occurs in thylakoid membranes, but the Ca^{++} binding site is on the lumen side of the thylakoid, where Ca^{++} binding can modulate acid-base jump ATP formation. The Ca^{++} binding to the F_0 and CF_0 complexes is consistent with a pH-dependent gating mechanism for control of H^+ ion flux across the opening of the H^+ channel.

KEY WORDS: ATP synthase; Ca^{++} binding proteins; energy coupling; *E. coli.*

INTRODUCTION

Proton conduction through the F_0F_1 complex of bacterial, mitochondrial, and chloroplast energy coupling membranes provides the driving force for ATP formation (Ferguson, 1985), but as yet we have scant understanding of the pathway of proton movement through the F_0 subunits of the membrane sector or of how the proton flux energizes ATP formation. This report deals with a new aspect of the F_0 proton channel function in the bacterium *E. coli,* namely Ca^{++} ion binding to the 8-kDa subunit c and its possible involvement in regulating energy-linked H^+ flux. Recent findings with the chloroplast CF_0 proton channel have revealed Ca^{++} interactions with the 8-kDa subunit III protein both with the *in situ* CF_0 and with isolated subunit III. Calcium binds tightly to the isolated sub-

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unit III, shown in an assay using a $^{45}Ca^{++}$ washing procedure applied to polyvinyl difloride paper after transferring polypeptides from SDS-PAGE gels (Zakharov *et al.*, 1993). The Ca^{++} ligand-blot method reveals the high-affinity Ca^{++}-binding site of many Ca^{++}-binding proteins such as calmodulin, troponin C, etc. (Charuk *et al.*, 1990). A further analogy to many of the diverse calcium-binding proteins is that the drug chlorpromazine can be photoaffinity attached to the thylakoid 8-kDa subunit III of the CF_0 proton channel in a Ca^{++}-dependent reaction (Chiang *et al.*, 1992). Moreover, chlorpromazine and another Ca^{++}-binding site probe, calmidazolium, block H^+ conduction through the CF_0 proton channel (Chiang *et al.*, 1992; Wooten and Dilley, 1993), indicating that the Ca^{++} interaction with the CF_0 channel reveals the properties of a gating function. That Ca^{++} binding to the CF_0 can gate proton conduction into the lumen from putative sequestered domains was suggested from other experiments with thylakoids using $\Delta\tilde{\mu}_H{}^+$-linked ATP formation and H^+ uptake assays (Chiang and Dilley, 1987; Dilley, 1991).

The similarities between the CF_0 8-kDa subunit III of thylakoids and the F_0 8-kDa subunit c of *E. coli* (Sebald and Hoppe, 1981) prompted us to test the *E. coli* protein in the $^{45}Ca^{++}$-binding assay. After obtaining evidence for such binding we also tested for the ability of chlorpromazine to influence transmembrane H^+ conduction in intact *E. coli* cells. These results are reported herein, with comparison made to the previously-reported Ca^{++} interaction with the chloroplast thylakoid 8-kDa subunit III (Zakharov *et al.*, 1993).

METHODS AND MATERIALS

Purification of Subunit c (III) of ATP-Synthases

The purified subunit c of *E. coli* ATP-synthase and both D61N or D61G mutant proteins were supplied by Prof. R. Fillingame (University of Wisconsin) and were stored in chloroform:methanol:water(5:5:1, v/v/v) at −75°C. Isolation and purification of subunit III of chloroplast ATP-synthase were as described previously (Zakharov *et al.*, 1993). Purified chloroplast ATP-synthase was provided by Prof. P. Gräber (University of Stuttgart, Germany). Purified TF_0 of the gram-positive thermophilic bacterium PS-3 ATP-synthase was from Prof. Y. Kagawa (Jichi Medical School, Japan). A purified bovine heart mitochondrial F_0F_1 complex was provided by Prof. Y. Hatefi, Scripps Clinic and Research Institute, La Jolla, California. The subunit c of cyanobacterial ATP-synthase was isolated from *Synechocystis 6803* using the same protocol as in the isolation of the pea chloroplast subunit III (Zakharov *et al.*, 1993) and was used without HPLC purification. The subunit c of plant mitochondrial ATP-synthase was isolated by chloroform/methanol extraction of potato tuber mitochondria as described earlier (Neuburger *et al.*, 1982).

SDS-Electrophoresis and Electroblot Polypeptide Transfer onto PVDF Membranes

SDS-electrophoresis in 16.5% polyacrylamide gels was run according to Schägger and von Jagow (1987). After electrophoresis the gels were stained with Coomassie R or were soaked 1–15 min in electroblotting buffer (17 mM Tris-H_3BO_3, pH 8.4, 20% methanol, 0.03% SDS). Semidry electroblotting from the gel onto the PVDF membrane (Bio-Rad) was accomplished using the Trans-Blot SD semidry cell (model 200/2.0, Bio-Rad) for 3 h at a current of 130 mA and an upper voltage limit of 13 V (Charuk *et al.*, 1990). PVDF rather than nitrocellulose membranes were used because nitrocellulose membranes did not bind the low-molecular-weight hydrophobic polypeptides of the F_0 strongly enough to keep the material in place during assay.

Proteins electroeluted onto PVDF membranes were subjected to the $^{45}Ca^{++}$ binding assay or, after staining with Coomassie R, to amino acid sequence analysis by the Purdue Biochemistry Department Protein Sequencing facility using a gas-phase Applied Biosystems Model 470A sequencer.

$^{45}Ca^{++}$ Binding to Polypeptides on PVDF Membranes

Calcium-binding polypeptides were detected according to Maruyama and Nomura (1984). The blot was rinsed with water, incubated 1–2 min in 5 mM EGTA, pH 7.0, three times for 10–20 sec each in assay buffer (60 mM KCl, 3 mM $MgCl_2$, 5 mM Tris, pH 8.0), then incubated for 15 min in the same buffer with 0.3 mM $^{45}CaCl_2$ (5 μCi ^{45}Ca/ml), and washed 3–4 times with 50% ethanol (2 min each). After drying, the blot was exposed to Kodak XAR-5 film for 1–2 days. For the experiment on the pH dependence of $^{45}Ca^{++}$ binding, 5 mM MES was added and the pH

was adjusted as shown. To visualize the polypeptides the blots were stained with Coomassie R after the autoradiography was accomplished.

Acid Deformylation of the N-Terminal f-Met

Samples of subunit c or III in 5% SDS were evaporated on a SpeedVac and the proteins were incubated overnight at room temperature in acid methanol (MeOH:conc. HCl, 23:1, v/v). The solvent was evaporated again and the sample was dissolved in SDS-electrophoresis sample buffer containing 2% SDS, 6 M Urea, 50 mM DTT, and 60 mM Tris-Cl^-, pH 6.7.

ATP Formation in Intact *E. coli* Cells

E. coli cells (strain AP_1) were grown in NaCl-Trypton medium (7 g/liter NaCl, 13 g/liter Trypton) to mid-log phase. Base-to-acid driven ATP formation was measured following Maloney *et al.* (1974) using intact cells which were starved to reduce the cytosolic ATP level. Cells harvested by centrifugation were washed with 0.12 M Tris-HCl, pH 8.0, and resuspended in minimal volume of this buffer. After incubation for 10 min at 37°C, EDTA was added to a final concentration of 0.5 mM and cells were diluted 20-fold into starvation medium (20 mM α-methylglucoside, 20 mM Na arsenate, 1 mM KCN, 30 mM Tris-HCl, pH 7.0, 50 mM NaCl, 20 mM $(NH_4)_2SO_4$, 7 mM $MgSO_4$, 0.3 M KCl) and incubated for 2 h at 37°C. After starvation, cells were washed with 0.1 M NaP_i, pH 8.0, 0.2 M KCl, then with 0.2 M NaP_i, pH 8.0, and resuspended in minimal volume of 0.2 M NaP_i, pH 8.0. Cell samples were adjusted to 10^{11} cells per ml with the pH 8.0 medium and subjected to a 14-min incubation with or without the UV and 30 μM chlorpromazine treatments as indicated in the tables. The ATP formation step was started by a 20-fold dilution of the cells in the above pH 8.0 media into the acid stage with 0.2 M NaP_i, pH 5.0. Valinomycin or nonactin (10 μM final concentration) were added after 10 sec. After 1 min at 23°C 0.2 ml of 30% $HClO_4$ was added to 0.8 ml of cells, and the mixture was incubated in ice for 10 min, then 0.3 ml 2N KOH was added to solubilize the cell walls and release the cytosolic contents. The pellet was removed by centrifugation, and the supernatants were frozen and kept until ATP concentration was measured by the firefly bioluminescence method (Chiang and Dilley, 1987).

In some experiments the concentration of free Ca^{++} in the medium used for the UV photoaffinity labeling of chlorpromazine was controlled by using an "EGTA Ca^{++} buffer" prepared according to Bers *et al.* (1994). The free Ca^{++} concentration of the buffers used was checked with a Fura-2 fluorescence assay system (Grynkiewicz *et al.*, 1985) using an SLM DMX-1000 fluorimeter.

RESULTS

Detection of $^{45}Ca^{++}$ Binding with 8-kDa F_0 and CF_0 Subunits

The Ca^{++} binding capacity of the proteins was tested by the $^{45}Ca^{++}$-ligand blot assay essentially as described by Maruyama and Nomura (1984). Only high-affinity Ca^{++}-binding sites can be detected in this assay, because the membranes are incubated with $^{45}CaCl_2$ in the presence of 10-fold excess of $MgCl_2$ and 60-fold excess of KCl followed by 3–4 washings of the membrane by 50% ethanol.

Figure 1 shows a comparison of $^{45}Ca^{++}$ binding to the *E. coli* F_0 subunit c and the thylakoid subunit III. Panel A is the Coomassie R-stained PVFD paper and Panel B is the $^{45}Ca^{++}$ autoradiogram of the material transferred to the PVFD paper. Both 8-kDa F_0 subunits gave strong $^{45}Ca^{++}$ binding. The stoichiometry of Ca^{++} binding to the subunits c or III is difficult to specify

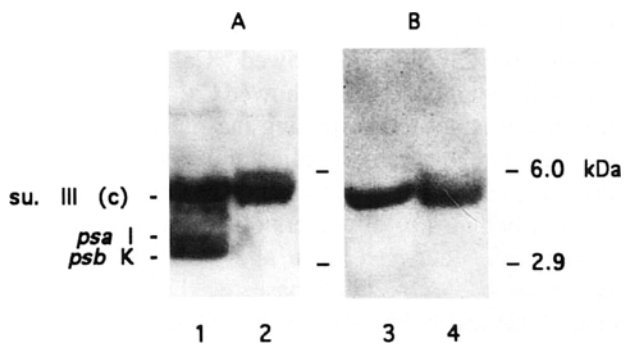

Fig. 1. Ca^{++} binding to the subunit c of *E. coli* and subunit III of pea chloroplast ATP-synthases. (A) Coomassie-stained PVDF membrane. (B) Autoradiograph. The purified subunit c and the polypeptides of the *n*-butanol extract of thylakoids were transferred, after SDS-PAGE, onto a PVDF membrane, and the calcium binding capacity was tested as in Materials and Methods. After autoradiography, the membrane was stained by Coomassie R. Lanes 1, 3—the polypeptides of the *n*-butanol extract of thylakoids; lanes 2, 4—*E. coli* subunit c. The bars on the right-hand side indicate the position of protein standards of 2.9 and 6.0 kDa.

with confidence using this assay, in part because we must assume 100% of the 8-kDa protein loaded on the SDS-PAGE gel gets transferred to the PVFD paper and in part because we do not know if any $^{45}Ca^{++}$ is washed off the PVFD paper in the washing step. Within those constraints, our best estimate of the binding stoichiometry is about one Ca^{++} per five subunit III at pH 7.0. However, we do not presume that this is a "hard number" for the *in situ* situation. Perhaps further NMR studies can clarify this, following the initial work of Moody *et al.* (1987), who gave evidence for one lanthanide binding site (lanthanides are excellent Ca^{++} binding site probes) per an approximate trimer.

The thylakoid subunit III preparation used for Fig. 1 was a partially purified fraction (the *n*-butanol extract of thylakoids, cf. Zakharov *et al.*, 1993) which contains two additional hydrophobic thylakoid membrane polypeptides, the psaI and psbK gene products. The psaI and psbK gene products have been reported as constituents of PS1 and PS2 preparations, respectively (Ikeuchi *et al.*, 1990; Murata *et al.*, 1988). Neither the psaI or psbK polypeptide binds $^{45}Ca^{++}$ in this assay system, nor is there any reason to believe they should do so, but they provide a type of control to emphasize the selectivity of the $^{45}Ca^{++}$ binding assay (cf. below for additional comments on this point). The 8-kDa hydrophobic subunit c (III) polypeptides are known to run slightly below the position of the 6-kDa protein standard used (bovine trypsin inhibitor).

Further evidence that the $^{45}Ca^{++}$-binding assay has a high degree of specificity can be seen in Fig. 2 for which a preparation of the isolated thylakoid CF_0CF_1 ATP-synthase complex was used. Clearly none of the other three CF_0 subunits showed $^{45}Ca^{++}$ binding and only the β subunit of the CF_1 showed Ca^{++} binding. The latter result is not too surprising inasmuch as the β subunit has divalent cation binding sites (Hochman and Carmeli, 1981). However, this Ca^{++}-binding band could be ascribed also to oligomers of 8-kDa subunit III, whose position between the α and β subunits of *Thermus thermophilus* ATP-synthase (Zakharov and Kuzmina, 1992) and chloroplast ATP-synthase (S. D. Zakharov, unpublished data) was identified by a [^{14}C-DCCD] labeling pretreatment of membranes before enzyme extraction (data not shown).

The identity and purity of the *E. coli* F_0 subunit c was verified in our hands by carrying out six cycles of amino acid sequence determination after acid deformylation of the protein (cf. Materials and Methods). The results showed the expected sequence (Met-Glu-Asn-Leu-Asn-Met . . .) with yields in the cycles

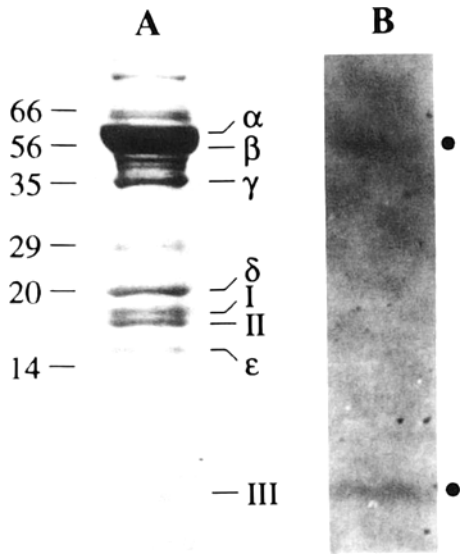

Fig. 2. Calcium binding to the subunits of the chloroplast CF_0-CF_1 ATP-synthase. The purified ATP-synthase was run on SDS-PAGE gels, electroblotted onto PVDF membrane, and Ca^{++}-ligand blot assayed as in Materials and Methods. After radioautography the membrane was stained by Coomassie Blue R. (A) Stained blot membrane. (B) Radioautograph. Position and numbers of protein molecular weight standards are shown on the left side; closed circles show the position of calcium-binding subunits.

estimated at 130, 64, 89, 74, 44, and 24 pmol, respectively. No other amino acid peaks of significance over background noise were detected in any of the six cycles.

Modification of Amino Acid Candidates for Ca^{++} Binding

Oxygen atoms, normally seven in number, are the preferred coordination ligands for Ca^{++} binding in proteins (Strynadka and James, 1989). In the subunit c (III) proteins, oxygens could be contributed by the N-terminal formyl group, carboxyl groups including that of the C-terminal carboxyl residue, carbonyl group oxygen of Asn or Gln, and main-chain peptide-bond carbonyl oxygen. Water oxygens also can contribute coordination sites in some Ca^{++} binding proteins (Strynadka and James, 1989; McPhalen *et al.*, 1991).

Deformylation of the N-terminal Met groups of both the *E. coli* subunit c and the thylakoid subunit III in acidic MeOH resulted in loss of $^{45}Ca^{++}$ binding (Fig. 3). The deformylated proteins ran with only a slightly decreased M_r on the SDS-PAGE gels (lanes 1 and 2 of Fig. 3 show the stained bands of subunit c

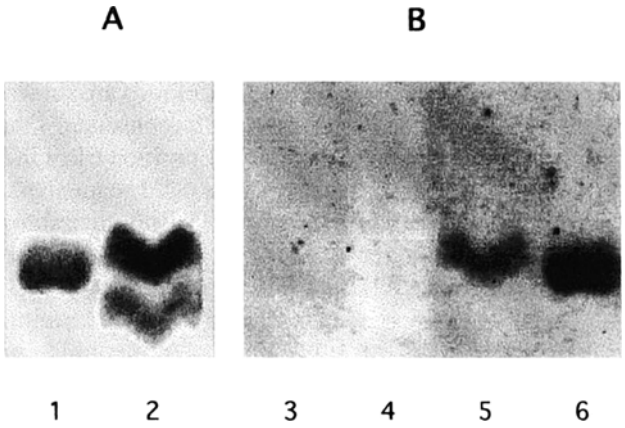

Fig. 3. The effect of N-terminal Met-1 deformylation on the Ca^{++} binding capacity of the subunits c and III of *E. coli* and higher plant chloroplast ATP-synthases. (A) Coomassie-stained PVDF-membrane. (B) Autoradiograph of PVDF membrane after the calcium-ligand blot assay. The de-blocking of the formyl group was carried out with the purified subunit c of *E. coli* ATP-synthase and polypeptides of *n*-butanol extract of thylakoids, resuspended in MeOH/conc. HCl (v/v 23:1). After 24 h incubation at room temperature the solvent was evaporated, and polypeptides were solubilized in SDS-PAGE buffer (cf. Materials and Methods) and tested in the Ca^{++}-ligand blot assay as described in Materials and Methods. Lanes 1, 3, 5—*E. coli* subunit c; lanes 2, 4, 6—*n*-butanol extract of thylakoids. Lanes 1, 2, 3, 4—deformylated polypeptides; lanes 5, 6—untreated polypeptides. The radioautogram and the Coomassie-stained gel were scanned using a ScanJet 11cx (Hewlett-Packard) and the program Desk-Scan II, and printed on LX Jet Series Glossy Paper HP 51636H using a Desk Writer 550C printer 9 (Hewlett-Packard).

and III, respectively), with the psaI and psbK proteins running ahead of the 8-kDa subunit III. Neither of the latter proteins showed $^{45}Ca^{++}$ binding (lanes 3 and 4). The native subunit c (lane 6) and subunit III (lane 5) polypeptides, with the f-met present, gave $^{45}Ca^{++}$ binding as already shown in Fig. 1. The MeOH used in the formyl group deblocking experiment could methylate the carboxyl groups in the proteins, and if so, that effect rather than the loss of the formyl group on the N-terminal Met may have caused the loss of Ca^{++} binding. To test this we subjected some of the deformylated thylakoid protein dissolved in triethylamine to 2 min, 15 min, and 2 h of incubation at pH 10, conditions adequate to hydrolyze carboxy–methyl linkages (Terwilliger and Clarke, 1981). Calcium binding was not observed in any of the pH 10-treated samples, although strong Coomassie staining bands were present and the control protein sample with the formyl group present (also treated at pH 10 with triethylamine) gave good Ca^{++} binding (data not shown).

On the other hand, derivatizing the Glu61 of the thylakoid subunit III with DCCD had no effect on $^{45}Ca^{++}$ binding (Zakharov *et al.*, 1993). The DCCD treatment was not carried out with the *E. coli* subunit c protein. However, additional evidence that the Asp61 of that protein is not essential for Ca^{++} binding comes from the finding that two of the Asp61 mutations obtained from the Fillingame laboratory, D61G and D61N, showed $^{45}Ca^{++}$ binding comparable to the control subunit c (Fig. 4).

pH Dependence of Ca^{++} Binding

The pH dependence of Ca^{++} binding to the *E. coli* and thylakoid proteins was studied by treating samples of the proteins (electroblotted onto PVDF membranes) with solutions of different pH during the incubation with $^{45}Ca^{++}$. Figure 5 shows that the $^{45}Ca^{++}$ binding is absent at pH 5.0 and it increases in a curvelinear fashion for both polypeptides. The result suggests that there is an ionizable group(s) in subunit c and subunit III necessary for Ca^{++} binding and which is completely protonated at pH 5.0 and dissociates as the pH is raised. However, the curve rises with increasing pH too slowly to be consistent with a single ionizable group being responsible for the formation of the Ca^{++} binding site. The origin of this effect is not understood, but it could be caused by the conditions of protein aggregation and or interaction of the protein with the PVDF paper being somewhat variable.

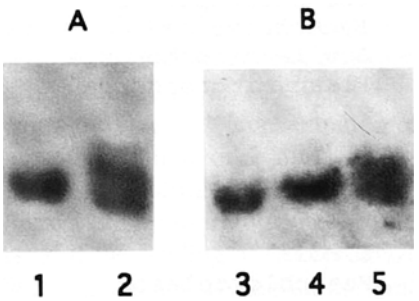

Fig. 4. Ca^{++} binding to the mutated subunit c protein from two *E. coli* mutants. Point mutations were generated in the subunit c with Asp61 → Asn (MM441) or Asp61 → Gly substitution (Fillingame *et al.*, 1984). The purified proteins (Miller *et al.*, 1989) were tested for $^{45}Ca^{++}$ binding as in Materials and Methods. (A) Coomassie stained gel. (B) $^{45}Ca^{++}$ autoradiograph. Lanes 1, 4—subunit c from the Asp61 → Gly mutant. Lane 2, 5—subunit c from mutant Asp61 → Asn. Lane 3—subunit c from the wild type.

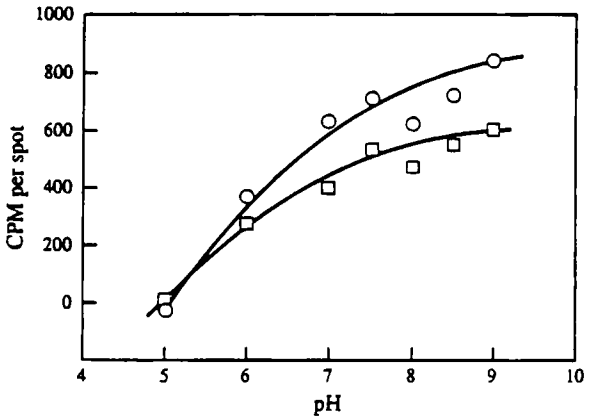

Fig. 5. pH dependence of Ca^{++} binding to subunits c and III of *E. coli* and chloroplast ATP-synthase. The pieces of PVDF membrane after electroblotting with similar spots of 8-kDa subunits were incubated at different pH according to the Ca^{++}-ligand blot assay as described in Materials and Methods. 10 mM MES and 10 mM Tris were used as the buffers. After radioautography, relevant pieces of the membrane were cut and weighed. The radioactivity was measured by liquid scintillation counting. The background level of radioactivity related to nonspecific Ca^{++} binding with PVDF membrane (membrane pattern without protein) was subtracted. (○) *E. coli* subunit c; (□) thylakoid subunit III.

Ca^{++} Binding to Different Subunit III (c) Proteins

Although all the known subunit c (III) polypeptides of ATP synthases have the common feature of a hydrophobically located, DCCD-reactive Glu (or Asp as in *E. coli*), there is some variation in the N- and C-termini amino acid sequences and in the length of the polypeptides. Sequences for six proteolipids are shown in Fig. 6, all aligned with the highly conserved Glu (or Asp) in the matching position (at position 61 of the *E. coli*, thylakoid, and *Synechocystis 6803* sequences). Assuming that the transmembrane α-helices have similar arrangements, then the plant (potato) and bovine mitochondrial and PS-3 proteolipids have shorter segments outside of the membrane at one or both termini. Moreover, the bovine mitochondrial proteolipid, in contrast to the plant mitochondrial protein, is not formylated at the N-terminus. Figure 7 shows that plant mitochondrial (lanes 9 and 10) and PS-3 (lanes 5 and 7) subunit III proteins do not bind Ca^{++}. The thylakoid protein (lanes 1 and 3) and the quite similar *Synechocystis 6803* protein (lanes 2 and 4) gave Ca^{++} binding. The comparable isolated and purified F_0 subunit from bovine mitochondria was not available, although a purified heart mitochondrial F_0F_1 preparation generously provided by Dr. Y. Hatefi gave good stained bands corresponding to the expected F_0 subunits, but no Ca^{++} binding was observed (data not shown).

Transmembrane H^+ Flux-Driven ATP Formation

Thylakoids (Jagendorf and Uribe, 1966) and intact *E. coli* cells (Maloney *et al.*, 1974) can form

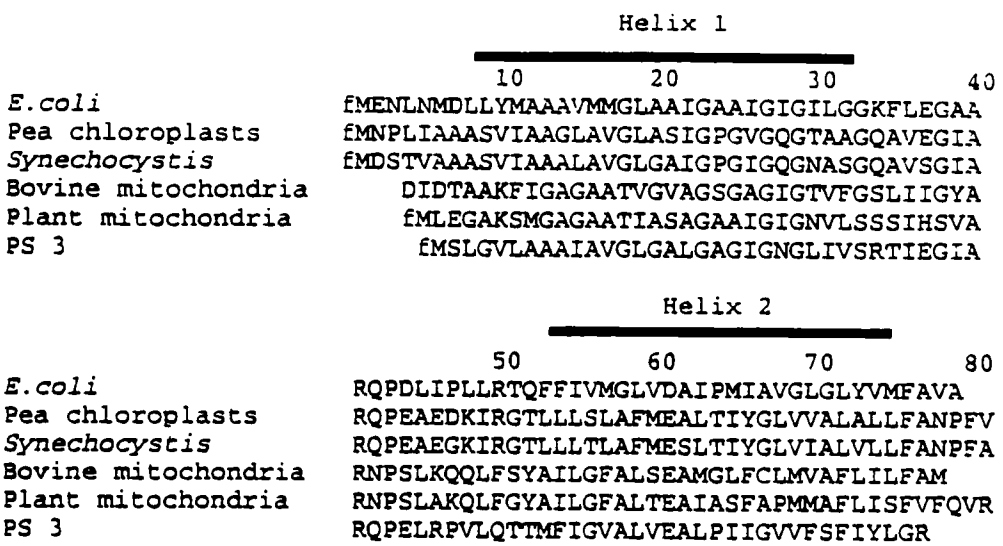

Fig. 6. Amino acid sequences of subunit c (III) and its analogues from different organisms. The sequences were aligned by placing the DCCD-sensitive Asp or Glu of the various proteins directly under position 61 in the *E. coli* and chloroplast sequences. The line above the sequences indicates the portions predicted to be in the membrane (α-helices).

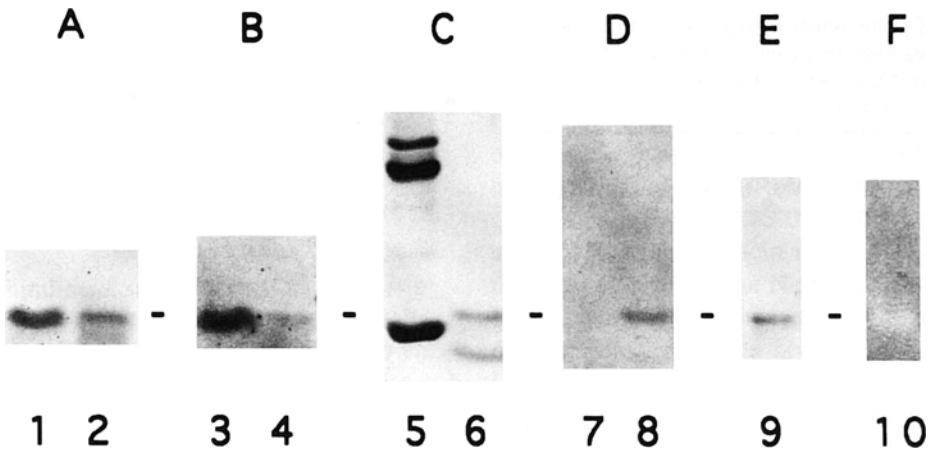

Fig. 7. Calcium binding to the ATP-synthase subunit c (III) of higher plant chloroplasts, the cyanobacterium *Synechocystis* 6803, a gram-positive thermophilic bacterium PS-3, and plant mitochondria. The protein samples were run in SDS-PAGE, electroblotted onto PVDF membranes, and assayed by the Ca^{++}-ligand blot as in Materials and Methods. A, C, E—Coomassie stained gel; B, D, F—radioautographs. Lanes 1, 3—HPLC-purified chloroplast subunit III; lanes 2, 4—subunit III from *Synechocystis* 6803; lanes 5, 7—PS-3 TF_0 preparation; lanes 6, 8—butanol extract of thylakoids; lanes 9, 10—chloroform/methanol extract of potato tuber mitochondria isolated as described earlier (Neuburger *et al.*, 1982).

ATP following appropriate pH jumps in the absence of electron transport-driven H^+ pumping. For thylakoids, an acid-to-base jump is used and for intact *E. coli* a base-to-acid jump. Maloney *et al.* (1974) showed that starved *E. coli* cells subjected to a pH 8 → pH 5 jump form ATP via the F_0F_1 mechanism. It was shown by Chiang *et al.* (1992) that [^{3}H]chlorpromazine can be photoaffinity-attached to the subunit III in thylakoids, and that the covalent attachment is enhanced by having adequate Ca^{++} present. Wooten and Dilley (1993) showed that chlorpromazine photoaffinity treatment of thylakoids blocks greater than 50% of the acid-base jump ATP formation, but only under conditions of adequate Ca^{++}; i.e., the same conditions that maximize the photoaffinity binding of chlorpromazine, maximize the inhibition of ATP formation. The inhibition was observed only when the acid stage was kept above pH 5.0, in keeping with the notion that chlorpromazine bound to Ca^{++} occupied protein sites increases the Ca^{++} binding affinity (Massom *et al.*, 1990), but sufficiently acidic conditions result in eventual loss of Ca^{++} from the binding site.

With that background in mind, intact *E. coli* cells were used for base-acid jump ATP formation experiments (Maloney *et al.*, 1974) under conditions of normal Ca^{++} availability or with Ca^{++} removed by EGTA and with or without UV photoaffinity activation of chlorpromazine. The cells were starved to lower the cytosolic ATP content, allowing better detection of the base-acid jump ATP formation. Table IA shows that the combination of chlorpromazine plus UV light inhibited the pH jump ATP yield by about 50%. The UV treatment without chlorpromazine gave a separate slight inhibitory effect on the pH jump ATP yield (compare lines 2 and 4, Table IB). In four experiments (different days), the inhibitory effect ranged from 3% to 10%, averaging 6% inhibition. The chlorpromazine treatment without UV exposure (Table IA) gave a slight but statistically not significant decrease in the ATP yield.

The addition of either uncoupler, CCCP (Table IB) or nigericin (Table IC) gave complete inhibition of the base-to-acid jump ATP yield, as expected from the results of Maloney *et. al.* (1974).

Reducing the free Ca^{++} concentration of the cell suspension with addition of 2 mM EGTA prior to giving the UV photoaffinity treatment abolished the inhibitory effect of the chlorpromazine (Table II). This is as expected if the binding of chlorpromazine to the F_0 subunit c requires Ca^{++} being on the binding site, which is the case for chlorpromazine binding to calmodulin and other Ca^{++} binding proteins (Prozialeck *et. al.*, 1981, Roberts *et. al.*, 1986).

The apparent binding affinity of Ca^{++} to the site(s) responsible for the UV plus chlorpromazine inhibition of the base-to-acid jump ATP formation was estimated by using an EGTA-Ca^{++} buffer to control the free

Table I. Effect of Chlorpromazine and Ultraviolet Light and the Uncouplers Nigericin (Nig) and Chlorocarbonyl Cyanide Phenylhydrazone (CCCP) on the Base-to-Acid Jump ATP Formation in *E. coli*[a]

	pH Shift (base pH → acid pH)	CPZ	UV	Uncoupler	Cytosolic ATP (mM)
A	1. 8 → 8	−	−	−	0.10 ± 0.02
	2. 8 → 5	−	−	−	0.66 ± 0.04
	3. 8 → 5	+	−	−	0.64 ± 0.06
	4. 8 → 5	+	+	−	0.30 ± 0.07
B	1. 8 → 8	−	−	−	0.12 ± 0.01
	2. 8 → 5	−	−	−	0.32 ± 0.04
	3. 8 → 5	−	−	10 μm CCCP	0.12 ± 0.02
	4. 8 → 5	−	+	−	0.30 ± 0.02
C	1. 8 → 8	−	−	−	0.16 ± 0.01
	2. 8 → 5	−	−	−	0.44 ± 0.03
	3. 8 → 5	−	−	5 μM Nig	0.17 ± 0.03

[a] *E. coli* cells from mid-log phase growth were starved to lower cytosolic ATP levels, then washed and resuspended in 0.2 M sodium phosphate pH 8.0 (cf. Materials and Methods) according to Maloney *et al.* (1974). The cells (10^{11} cells per ml) were treated, as indicated, with or without uncouplers or 30 μM chlorpromazine and 14 min of UV radiation (366 nm main excitation using a model UVL-21 Blak-Ray lamp (UVP Inc., San Gabriel, California) placed 12 cm above the stirred cell suspension kept at 4°C). For the base-acid jump, the cells in the above medium were diluted 20-fold into 0.2 M sodium phosphate (pH 5.0) in the presence of 10 μM nonactin. The control lines 1 in experiments A to C was a comparable dilution into pH 8.0 medium. The effect of chlorpromazine with no UV treatment is given in A (line 3), and the effect of UV light with no chlorpromazine is given in B (line 4). The inhibitory effect of both factors given together to cause photoaffinity cross-linking of CPZ to the subunit C of the F_0 is given in A (line 4). The effects of either 10 μM CCCP or 5 μM nigericin, present for 2 min in the pH 8.0 medium and at the same concentrations in the pH 5.0 medium, are given in B and C, respectively. Experiments A to C represent data from a different day's experiment using different cell cultures. Each line of data represents one sample of cells treated as indicated and the cytosolic extract assayed three times in the luciferin-luciferase assay. Each type of experiment was repeated on at least three different days (different cell cultures, etc.) with results typical of those shown.

Table II. Effect of the Ca^{++} Chelator EGTA on the Chlorpromazine Plus UV Light Inhibition of Base-to-Acid Jump ATP Formation in *E. coli*[a]

	pH Shift (base pH → acid pH)	CPZ	UV	EGTA	Cytosolic ATP (mM)
A.	1. 8 → 8	−	−	−	0.15 ± 0.01
	2. 8 → 5	−	−	−	0.59 ± 0.04
	3. 8 → 5	+	+	−	0.32 ± 0.08
	4. 8 → 5	+	+	+	0.56 ± 0.05
	5. 8 → 5	+	−	+	0.54 ± 0.04
	6. 8 → 5	−	+	+	0.56 ± 0.02
	7. 8 → 5	−	−	+	0.57 ± 0.03

[a] Conditions were as in Table I part A except that the cells were washed and resuspended with medium supplemented with 2 mM EGTA (see Materials and Methods) prior to CPZ and UV light treatment, and EGTA was present at 0.5 mM in the pH 5.0 stage. The experiments were repeated three or more different days with results consistent with the data shown.

Ca^{++} concentration (Bers *et. al.*, 1994) during the UV treatment. Figure 8 shows a progressive inhibition of the ATP yield as the free [Ca^{++}] increased from zero to about 200 nM. The half inhibition concentration appears to be near 80–100 nM, which can be taken as an approximate binding affinity of the inhibitory site (assumed to be the subunits of the F_0 H^+ channel).

The free Ca^{++} concentration was checked with the Fura-2 fluorescence assay system (Grynkiewicz *et. al.*, 1985) for the EGTA-Ca^{++} buffers used, both without and with the usual amount of starved *E. coli* cells present. In the latter case, the starved cells were allowed to equilibrate with the buffer system for 3 min prior to centrifuging the cells down and taking the

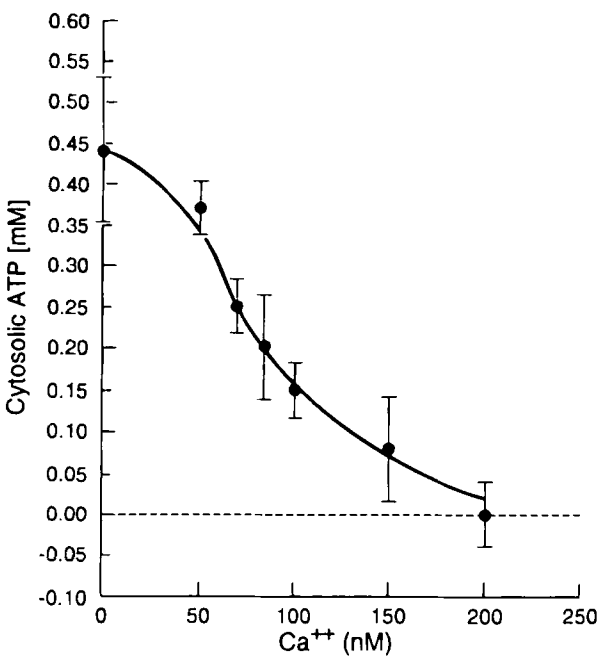

Fig. 8. The effect of free calcium concentration on the UV plus chlorpromazine inhibition of base-to-acid jump ATP formation in intact *E. coli* cells. Intact, starved *E. coli* cells were suspended in various EGTA-Ca^{++} buffers to give the specified free [Ca^{++}] (Bers *et al.*, 1994) as described in Materials and Methods. Other conditions were as described in Table I. The Y-axis represents the difference between the control (no pH jump) ATP level (0.20 mM ATP) and the ATP level resulting from the pH jump.

supernatant for assaying the free Ca^{++} concentrations. The free Ca^{++} concentration was essentially the same with and without cells present (data not shown) as expected for the EGTA-buffered system.

DISCUSSION

The $^{45}Ca^{++}$-binding assay used for this work is a well-accepted, widely used method for detecting high-affinity Ca^{++}-binding sites in most of the well-known Ca^{++}-binding proteins, such as calmodulin, etc. (Charuk *et. al.*, 1990), and in other less well-known Ca^{++}-binding proteins, for example, spinach ferredoxin (Surek *et al.*, 1987). An important fact concerning high-affinity Ca^{++}-binding sites in proteins is that the coordinating oxygen ligands—seven being the most common coordination number (Strynadka and James, 1989)—often come from widely separated segments of the polypeptide chain (McPhalen *et al.*, 1991) rather than from the familiar calmodulin-type helix-loop-helix motif where a contiguous 12-residue segment provides most of the coordinating oxygens (water can supply one or more coordinating oxygen atoms). This point has relevance for the present study of the subunit c (III) of ATP-synthases inasmuch as the subunit C (III) proteins do not have a calmodulin-type E–F hand (Roberts *et al.*, 1986), Ca^{++} binding motif.

The use of this Ca^{++}-binding assay in concert with other data discussed below leaves little doubt that the subunit c (III) part of various $F_0(CF_0)$ H^+ channel complexes bind Ca^{++}. Moreover, there is no reason to suspect that a contaminating protein, inadvertently running with the subunits c and III on SDS-PAGE gels, could be the actual $^{45}Ca^{++}$-binding moiety. The evidence supporting this point is that the subunit c preparation, as purified by the Fillingame group, contained only one protein component as deduced from sequencing the first six residues. The thylakoid subunit III was purified in this laboratory by HPLC and SDS-PAGE (Chiang *et al.*, 1992; Zakharov *et al.*, 1993), and after deformylation of the protein and transfer to PVDF paper from the SDS gels it also gave sequence data indicating that a single protein component was present. In this work, the HPLC-purified subunit III was used for the data shown in Fig. 7 [as in Fig. 2 of Zakharov *et al.* (1993)]. The other three CF_0 subunits present in the complete CF_0CF_1 complex (Fig. 2) showed no Ca^{++} binding, suggesting that the 8-kDa subunit is the sole agent responsible for the Ca^{++} binding capacity.

Using thylakoid membranes, [^{3}H]chlorpromazine and UV light treatment was shown to photoaffinity-label the subunit III, further evidence for a high-affinity Ca^{++}-binding site being present [chlorpromazine is a well-known photoaffinity probe for protein high-affinity Ca^{++}-binding sites (Roberts *et al.*, 1986)].

Calcium binding to the subunits c and III was abolished after mild acid hydrolysis which removes the formyl group on the N-terminal Met (Fig. 3), suggesting that the formyl oxygen group may provide one of the critical oxygens needed for coordinating the Ca^{++}. A question to consider is whether the dilute acid treatment caused denaturation which may have altered the conformation in a way that altered the Ca^{++} binding capacity. We have no data on protein conformation, but the buffer used to solubilize the protein (having 2% SDA and 6 M urea) is itself a denaturing agent. The HCl treatment does not significantly alter the mobility on the SDS-PAGE gels, so it seems likely, but we have no other evidence in hand to support the point, that the deformylation and not acid denaturation caused the loss of Ca^{++} binding.

The C-terminal carboxyl group is probably also necessary for Ca^{++} binding, first suggested by the NMR experiments of Moody *et al.* (1987). The failure of the plant mitochondrial and PS-3 subunit c to bind Ca^{++} (Fig. 7), even though they have the formyl Met at the N-terminus, may be because both have an Arg^+ at the C-terminus. The Arg cation could perturb the Ca^{++} coordination capability of the terminal COOH group. The beef heart mitochondrial F_0 subunit corresponding to subunit c did not bind Ca^{++} (data not shown) and in common with some other mammalian systems it lacks the f-Met at the N-terminus. However, the mitochondrial and the PS-3 proteins are shorter by four and five residues, respectively, on the N-terminal end, and the PS3 is shorter by four residues on the C-terminal end (cf. Fig. 6); and their shorter length may preclude forming the Ca^{++} binding site.

Calcium binding to the CF_0 H^+ channel in thylakoids has been suggested to regulate H^+ flux across the inner opening of the H^+ channel (Chiang and Dilley, 1987; Chiang *et al.*, 1992; Wooten and Dilley, 1993). This function has particular relevance in thylakoids owing to the considerable body of evidence suggesting that thylakoid energy coupling can occur with either localized or delocalized H^+ gradients as the driving force for ATP formation (for reviews cf. Ferguson, 1985; Dilley, 1991). These two points, Ca^{++} binding to the CF_0 and there being two modes of H^+ gradients that energize ATP formation, are joined in the concep-

tual scheme that suggests that calcium binding to the 8-kDa subunit III can close the H^+ channel at the lumenal side, allowing the protonmotive force in localized membrane domains to drive ATP formation. Displacement of the Ca^{++} ions by (1) excessive H^+ accumulation in the domains, as occurs under basal electron transport conditions, (2) Ca^{++} chelator treatment, or (3) exposure of thylakoids to high KCl or NaCl concentrations, leads to increased lumenal H^+ accumulation and to the expression of delocalized $\Delta\tilde{\mu}_H{}^+$ energy coupling (cf. Dilley, 1991, for details). Those data and other data discussed below give good support for the proposed regulatory role of the Ca^{++}-CF_0 H^+ gating hypothesis.

In the review just mentioned above, it was suggested that the biological relevance for thylakoids having the putative dual proton gradient energy coupling modes appears to be as a stress sensor for a thylakoid system of protection against excess energization. We postulate that the switchover from localized $\Delta\tilde{\mu}_H{}^+$ coupling to the delocalized mode results in an increased acidification of the lumen (Renganathan *et al.*, 1993), and that signal then triggers a protective mechanism in the thylakoid involving the conversion of violaxanthin to zeaxanthin. The formation of zeaxanthin is triggered by light intensities too high to be utilized in photosynthesis (Demmig *et al.*, 1987). It is believed that zeaxanthin (and antheraxanthin), which only form under acidic pH conditions (over-acidification of the lumen relates to the excess energization), interacts with excited-state chlorophyll, resulting in the dissipation of the excitation energy into infrared radiation (Demmig-Adams, 1990; Noctor *et al.*, 1991; Gilmore and Yamamoto, 1991; Pfundel *et. al.*, 1994; Owens, 1994). Thus, the demonstrable occurrence of Ca^{++} regulation of localized and delocalized proton gradient energy coupling in chloroplasts may be more than a laboratory curiosity, perhaps having important physiological consequences.

The situation in *E. coli* cells is not so clear, and there is not the broad experimental base on which to test hypotheses concerning localized versus delocalized $\Delta\tilde{\mu}_H{}^+$ gradient energy coupling. Nonetheless, the *E. coli* F_0 subunit c binds Ca^{++} just as effectively as the thylakoid subunit III, which led us to test for Ca^{++} regulation of H^+ flux into the H^+ channel using intact *E. coli* cells in the base-to-acid pH jump ATP formation assay of Maloney *et al.* (1974). The photoaffinity Ca^{++}-binding site probe, chlorpromazine (Prozialeck *et al.*, 1981), had the effect—similar to that found in thylakoids (Wooten and Dilley, 1993)—of blocking a portion of the ATP formation yield energized by an imposed ΔpH (Table I). Table IA shows about a 50% inhibition of this base-to-acid jump ATP yield by the UV plus 30 μM chlorpromazine treatment. It should be noted that the UV light source used had a far-UV emission (366 nm), not the lethal 260 nm emission. This wavelength is not expected to cause cell death or widespread damage to proteins. In the calmodulin system chlorpromazine binding to the Ca^{++}-protein complex causes an increase in Ca^{++} binding affinity for its site (Massom *et al.* 1990). Our previous results with chloroplasts also are consistent with such an effect (Wooten and Dilley, 1993). The observed inhibition of the ΔpH-driven ATP formation by chlorpromazine could be explained by a similar increased affinity for Ca^{++} binding to the F_0 site owing to the photoaffinity-attached drug. In the absence of the UV photoaffinity treatment the chlorpromazine had no inhibitory effect on the pH jump ATP formation (compare lines 2 and 3, Table IA) even though at high concentrations, chlorpromazine can inhibit ATP formation in thylakoids (Good *et al.*, 1966), and it is an inhibitor of F_1 ATPase activity in mitochondria (Chazotte *et. al.*, 1982). The likely explanation for the lack of chlorpromazine inhibitor when UV was not given could be that cells were washed free of the chlorpromazine-containing medium and resuspended in fresh pH 8.0 medium without chlorpromazine, followed by the pH jump. The washing step would dilute the chlorpromazine to levels too low to be inhibitory.

That the chlorpromazine plus UV inhibition of the ATP yield is related to Ca^{++} being available—presumably for binding to the F_0 subunit c binding site—is supported by the Table II data showing that the UV + chlorpromazine inhibitory effect was not observed when the Ca^{++} chelator EGTA was used to deplete the cells of Ca^{++} prior to the UV exposure. That shows further that the UV + chlorpromazine treatments *per se* were not inhibitory to ATP formation.

An apparent Ca^{++} binding constant for the chlorpromazine photoaffinity inhibition of the ATP yield near 80–100 nM was observed using the Ca^{++}-EGTA buffer system to control the free [Ca^{++}] in the cell suspension (Fig. 8). This is a typical binding affinity for Ca^{++} binding proteins (Roberts *et. al.*, 1986). The cooperativity evident in the Ca^{++} concentration effect on chlorpromazine photoaffinity inhibition of the base-acid jump ATP formation yields a Hill coefficient of 1.4–1.5, rather similar to the Hill coefficient of 1.7 reported for the Ca^{++} dependence of trifluoperazine binding to troponin C (Masson *et al*, 1990). The signifi-

cance of the cooperativity in the chlorpromazine inhibition of the *E. coli* base-acid jump ATP formation is not clear, and further studies would be needed to gain insight into the inhibition pattern. However, one can speculate that a single 10-subunit c F_0 TH^+ channel could have more than one Ca^{++} binding site and that full occupancy of the putative multiple sites may be necessary to allow maximal chlorpromazine binding and hence inhibition of H^+ flux into the F_0 channel.

It is important to note that the base-to-acid pH jump drives many turnovers of the F_0F_1 ATP-synthase complexes, reflecting significant H^+ flux through the H^+ channel. The calculation is as follows: the amount of ΔpH jump ATP formed in our experiments increased the cytosolic concentration by about 0.3–0.6 mM (Table I). Assuming roughly $0.3–1.0 \times 10^{10}$ cells per mg protein, 3–4 μl cell water per mg protein, and about 10^3 F_0F_1 complexes per cell, there could be an estimated turnover of the F_1 mechanism of nearly 1800 ATP per F_1. This gives a calculated turnover time of about 17 msec per ATP formed. This is a reasonable value in that the F_1 is intrinsically capable of a much faster turnover time, easily in the range of 2 msec per turnover (Junesch and Gräber 1987). It follows that the required H^+ flux must be, at minimum, about 5000 H^+ through the F_0 channel (assuming $3H^+$/ATP). The approximately 50% inhibition of pH jump-induced ATP formation caused by chlorpromazine + UV light could be interpreted as resulting from the photo-affinity bound drug blocking the release of Ca^{++} from a portion of the F_0 channel gating sites, thus impeding the entry of external phase (pH 5) protons into the blocked F_0. It is known that chlorpromazine binding to calmodulin causes an increased affinity for Ca^{++} binding (Massom *et al.*, 1990).

It remains for future work to discern in the *E. coli* system what biological role is played by the putative Ca^{++} binding to the F_0 subunit. One possibility, in analogy to the chloroplast system, is that the *E. coli* system uses the Ca^{++} binding to regulate the flux of H^+ ions across the periplasm-exposed part of the F_0 H^+ channel. Alkaliphilic bacillus species have some type of regulation of H^+ flux at the F_0 H^+ channel that permits the bacterium to utilize transmembrane $\Delta\mu_{H^+}$ gradients for energy coupling at pH values below 9.5 and localized $\Delta\mu_{H^+}$ H^+ gradient coupling at pH values above 9.5 (Krulwich, 1995). The thylakoid system is believed to use the Ca^{++} gating of H^+ flux at the CF_0 for control of a stress response signal (Dilley, 1991). It is now an open and interesting question whether the *E. coli* system uses the observed Ca^{++} binding to the F_0 for some kind of physiological regulation.

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