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pH-Dependent Ca^{2+} Binding to the F_0 *c*-Subunit Affects Proton Translocation of the ATP Synthase From *Synechocystis* 6803

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It was shown before (Wooten, D. C., and Dilley, R. A. (1993) *J. Bioenerg. Biomembr*. **25**, 557–567; Zakharov, S. D., Li, X., Red'ko, T. P., and Dilley, R. A. (1996) *J. Bioenerg. Biomembr*. **28**, 483–493) that pH dependent reversible Ca^{2+} binding near the N- and C-terminal end of the 8 kDa subunit *c* modulates ATP synthesis driven by an applied pH jump in chloroplast and *E. coli* ATP synthase due to closing a "proton gate" proposed to exist in the F_0 H^+ channel of the F_0F_1 ATP synthase. This mechanism has further been investigated with the use of membrane vesicles from mutants of the cyanobacterium *Synechocystis* 6803. Vesicles from a mutant with serine at position 37 in the hydrophilic loop of the *c*-subunit replaced by the charged glutamic acid (strain plc 37) has a higher H^+/ATP ratio than the wild type and therefore shows ATP synthesis at low values of $\Delta\mu_H{}^+$. The presence of 1 mM $CaCl_2$ during the preparation and storage of these vesicles blocked acid–base jump ATP formation when the pH of the acid side (inside) was between pH 5.6 and 7.1, even though the ΔpH of the acid–base jump was thermodynamically in excess of the necessary energy to drive ATP formation at an external pH above 8.28. That is, in the absence of added $CaCl_2$, ATP formation did occur under those conditions. However, when the base stage pH was 7.16 and the acid stage below pH 5.2, ATP was formed when Ca^{2+} was present. This is consistent with Ca^{2+} being displaced by H^+ ions from the F_0 on the inside of the thylakoid membrane at pH values below about 5.5. Vesicles from a mutant with the serine of position 3 replaced by a cysteine apparently already contain some bound Ca^{2+} to F_0. Addition of 1 mM EGTA during preparation and storage of those vesicles shifted the otherwise already low internal pH needed for onset of ATP synthesis to higher values when the external pH was above 8. With both strains it was shown that the Ca^{2+} binding effect on acid–base induced ATP synthesis occurs above an internal pH of about 5.5. These results were corroborated by $^{45}Ca^{2+}$- ligand blot assays on organic solvent soluble preparations containing the 8 kDa F_0 subunit *c* from the S-3-C mutant ATP synthase, which showed $^{45}Ca^{2+}$ binding as occurs with the pea chloroplast subunit III. The phosphorylation efficiency (P/*2e*), at strong light intensity, of Ca^{2+} and EGTA treated vesicles from both strains were almost equal showing that Ca^{2+} or EGTA have no other effect on the ATP synthase such as a change in the proton to ATP ratio. The results indicate that the Ca^{2+} binding to the F_0 H^+ channel can block H^+ flux through the channel at pH values above about 5.5, but below that pH protons apparently displace the bound Ca^{2+}, opening the CF_0 H^+ channel between the thylakoid lumen and H^+ conductive channel.

KEY WORDS: ATP synthase; H^+; energy conservation; proton translocation; Ca^{2+} binding; CF_0 subunit *c*; enzyme regulation; cyanobacteria.

Key to abbreviations: Chl, chlorophyll; CPZ, chlorpromazine; ΔG_p, phosphate potential; $\Delta\mu_H{}^+$, proton electrochemical potential difference; F_1, peripheral hydrophilic part of the ATP synthase complex; F_0, membrane-spanning hydrophobic part of the ATP synthase complex; P/*2e*, phosphorylation efficiency; PMS, phenazine methosulfate; PMSF, phenylmethylsulfonyl fluoride; S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide.

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INTRODUCTION

Proton gradient-driven ATP formation in cell organelles and microorganisms involves the well-characterized multisubunit energy-transducing complex, the F_0F_1(or CF_0CF_1 in chloroplasts). The F_0 part is the transmembrane H^+ channel connecting the high proton potential side to the F_1 part where the ADP and Pi are converted to ATP coupled to the dissipation of the proton gradient (Weber and Senior, 1997). Understanding this important bioenergetic process involves gaining insight into both the structural–catalytic mechanistic aspects and the regulatory features of the F_0F_1. Despite impressive recent advances-particularly in the structural aspects-there is not yet a good understanding of the way H^+ ions interact with the F_0F_1 in the energy transducing mechanism.

A factor with potentially great significance for understanding H^+–F_0 interactions is that Ca^{2+} ions bind to the 8 kDa subunit of chloroplast CF_0 and *Synechocystis* 6803 and *E. coli* F_0 subunit *c*, as was shown by a $^{45}Ca^{2+}$ binding assay using the purified proteins after transfer from SDS-PAGE gels to polyvinyl difluoride membranes (Zakharov *et al.*, 1993, 1995, 1996). There appears to be an organelle- and/or a species-dependent factor that determines the Ca^{2+} binding capacity of the 8 kDa F_0 subunit, because the mitochondrial subunit *c* from bovine heart and the potato tuber did not bind Ca^{2+} (Zakharov *et al.*, 1996). NMR studies of Moody *et al.*, using La^{3+} ions as Ca^{2+} analogues (Moody *et al.*, 1987) and $^{45}Ca^{2+}$ binding studies (Zakharov *et al.*, 1993, 1995, 1996) suggest that the binding site is at the side where the N- and C-termini protrude from the membrane, i.e., on the high H^+ electrochemical potential side of the F_0 H^+ channel.

Further significance of the Ca^{2+} CF_0 (F_0) interactions is indicated by the observation that when Ca^{2+} was bound to the CF_0 site in thylakoids it inhibited most of the acid–base driven ATP formation by a pH 5.5–8.5 pH jump, but did not inhibit ATP formation by a pH 4.0–7.5 jump, i.e., suggesting that the pH 4.0 conditions were sufficiently acidic for H^+ ions to displace the Ca^{2+} (Wooten and Dilley, 1993). With intact *E. coli* cells, tight Ca^{2+} binding blocked base-to-acid jump ATP formation (Zakharov *et al.*, 1996). The term "tight Ca^{2+} binding" in this context refers to the enhanced binding affinity induced by the pharmacological agent chlorpromazine, a widely used drug in studies of Ca^{2+}/calmodulin function (Prozialeck *et al.*, 1981). It is known that chlorpromazine (CPZ) and other phenthiazine drugs tighten the Ca^{2+} binding to Ca^{2+}-specific protein binding sites (Massom *et al.*, 1990). Earlier studies (Chiang *et al.*, 1992) found that photoactivated [^{3}H] chlorpromazine covalently labeled the 8 kDa CF_0 subunit III of thylakoids in a Ca^{2+}-enhanced way, and it increased the binding affinity for Ca^{2+} ions. The conditions of maximal photoaffinity labeling of the subunit III were precisely those that had been found to favor a localized $\Delta\mu_{H}{}^{+}$ energy coupling response, i.e., when Ca^{2+} was not depleted from thylakoids by high KC1 concentrations or EGTA (Beard and Dilley, 1988; Chiang *et al.*, 1992). Of importance for understanding bioenergetics in relation to regulatory processes is the finding that when the Ca^{2+}-chlorpromazine adduct blocked the CF_0 H^+ channel, it caused nearly complete inhibition of acid–base jump ATP formation (Wooten and Dilley, 1993) but it did not inhibit the light-driven ATP formation (Chiang *et al.*, 1992). The residual ATP formation, about 20 nmol ATP $(mg\ Chl)^{-1}$ is believed to be driven by protons from the localized domains. The domains contain up to 150 nmol H^+ $(mg\ Chl)^{-1}$, mostly endogenous carboxyl, with some low pKa amine buffering groups in the sequestered membrane regions (Dilley *et al.*, 1987) more than sufficient to account for 20 nmol ATP $(mg\ Chl)^{-1}$ in an acid–base jump experiment (Wooten and Dilley, 1993). Those studies resulted in formulating a hypothesis for Ca^{2+} control of the expression of localized or delocalized proton gradient energy coupling modes (Dilley, 1991).

Further testing is necessary of the hypothesis that the Ca^{2+}—CF_0 binding state can gate the H^+ flux through the CF_0 H^+ channel. One aspect, reported here, is to determine the pH at which the Ca^{2+}—CF_0 blocked channel is opened, i.e., the acidic pH needed to displace the putative bound Ca^{2+} from the CF_0 channel opening to the lumen. This was tested herein using the acid–base jump ATP formation protocol in thylakoid vesicles isolated from *Synechocystis* 6803 cells (Scholts *et al.*, 1996). The protocol included careful control of the acid stage pH and the overall pH jump to the base stage to allow a pH titration of the proposed reaction:

$$Ca^{2+}\text{—}F_{0\,(closed)} \Leftrightarrow Ca^{2+}{}_{(free)} + F_{0\,(open)}.$$

The work includes the use of mutants with certain helpful changes in the amino acid sequence of the 8 kDa subunit *c*, one of which increases the H^+/ATP stoichiometry and hence decreases the magnitude of the ΔpH threshold needed for energizing ATP formation (see accompanying paper (Van Walraven *et al.*, 2002)).

MATERIALS AND METHODS

Chemicals

ADP, valinomycin, and all enzymes were purchased from Boehringer (Mannheim, Germany). PMS, PMSF, and CPZ (chlorpromazine) were from Sigma

(St. Louis, MO), kanamycin sulphate from Duchefa (Haarlem, The Netherlands). The luciferin/luciferase monitoring kit was from LKB/Pharmacia (Uppsala, Sweden). S-13 was kindly donated by Dr P. C. Hamm (Monsanto Co., St. Louis, MO). All other chemicals were of analytical grade.

Culture Conditions, Description of Strains and Determination of Growth Parameters

The cyanobacterial *Synechocystis* PCC 6803 strains were semicontinuously cultured at 34–35°C in 2-L airlift fermentors in BG-11 medium (Lubberding and Bot, 1984) supplied with 25 mg/L kanamycin. The cells were diluted to an optical density (at 798 nm) of 0.2. Cells from about a 1-week-old culture were harvested. In the mutant strain, plc 37, S (serine) at position 37 of the ATP synthase c-subunit was replaced by E (glutamic acid). In another mutant strain S-3-C, serine on position 3 of the *c*-subunit was replaced by C (cysteine). The control strain contained a kanamycin resistance as did the mutants but was otherwise identical to the wild-type *Synechocystis* 6803. Mutants were created as described in the accompanying paper (Van Walraven *et al.*, 2002).

Growth was determined by the optical density at 798 nm.

Preparation of Membrane Vesicles

Membrane vesicles from the *Synechocystis* 6803 strains were prepared according to Scholts *et al.* (1996). A total of 400 mL of cyanobacteria at an optical density (at 798 nm) of 1.2–1.6 were harvested and incubated in 30 mL of Mannitol medium (500 mM Mannitol, 10 mM Tricine-KOH, 10 mM $MgCl_2$, 5 mM NaH_2PO_4, 2.5 mM K_2HPO_4, pH 7.8) supplied with 0.2% lysozyme, and 100 μM PMSF for ca. 2 h at 35°C. Samples were tested for phycocyanin release upon addition of water. As soon as phycocyanin release could be observed, the spheroplasts were centrifuged (2 min, 10,000g) and resuspended in Mannitol medium plus 100 μM PMSF at a chlorophyll *a* concentration of 0.1–0.3 mg/mL. The spheroplasts were kept at 0°C and every 2 h fresh PMSF (100 μM) was added. Spheroplasts were prepared fresh for each day's work and could be used for 5–10 h.

Chlorophyll a concentration was measured according to Arnon *et al.* (1974).

Calcium and EGTA Treatments

In the case of Ca^{2+}-treated vesicles spheroplasts were resuspended in Mannitol medium supplied with 1 mM $CaCl_2$. No $CaCl_2$ was added to other media since Ca^{2+} at high concentrations has been reported to inhibit spinach thylakoid ATP formation as a competitive inhibitor with Mg^{2+} (Jagendorf and Avron, 1959). Furthermore, during actual ATP formation the external Ca^{2+} concentration was diluted out to ca. 15–30 μM (see procedures below), which is more in the physiological range for Ca^{2+} effects and far below the 2–5 mM Mg^{2+} present in the assay medium.

In the case of EGTA-treated vesicles spheroplasts were resuspended in Mannitol medium supplied with 1 mM EGTA. For those treatments osmotic shock prior to acid–base transition (see below) was carried out in acid stage medium at pH 5.8 instead of pH 7.5 for 5 min to allow EGTA to penetrate the thylakoid membrane, and all acid stage media were supplied with 1 mM EGTA.

Samples of the different vesicle preparations were tested for phenazine methosulfate (PMS) mediated light-induced proton uptake and ATP synthesis, and ATP hydrolysis, all by determination of proton uptake or release (see accompanying paper (Van Walraven *et al.*, 2002).

ATP Synthesis by Acid-Base Transition

ATP synthesis was driven by an acid–base transition, as described by Krenn *et al.* (1993) (cf. Van Walraven *et al.*, 2002, for composition of acid and base stages, etc.), at 37°C and ATP production was measured with the luciferin/luciferase assay. All media were supplied with 100 μM PMSF. First, 0.1 mL of spheroplasts (50 μL for strain S-3-C) in mannitol medium (composition listed under "Preparation of Membrane Vesicles") with chlorophyll concentration of 0.1–0.3 mg/mL were osmotically shocked for 5 min in 0.4 mL of acid stage medium at pH 7.5 (in the case of EGTA treatment, pH 5.8, see above). Then 0.2 mL of this suspension was added to 1 mL of acid stage medium with variable pH for 1 min and finally acid–base transition was carried out by addition of 0.5 mL of this suspension to 0.5 mL of base stage medium. After 1 min, 5 μL samples were tested for ATP content by addition to the luciferin/luciferase assay medium according to the manufacturers' instructions. For those experiments with an acid stage medium below pH 5, incubation was shortened to 30 s to diminish harmful effects of acid to the vesicles. The pH of the acid- and base-stage were measured afterwards to determine the exact value of the ΔpH (and $\Delta\mu_H{}^+$). All ATP productions were corrected for myokinase activity and ATP already present by uncoupling with 5 μM S-13.

ΔG_p values were calculated according to Krab and Van Wezel (1992).

Phosphorylation Efficiency (P/*2e*)

Light-induced linear electron transport and ATP synthesis rates were measured respectively by the reduction of $K_3Fe(CN)_6$ and the reduction of $NADP^+$ in a coupled enzymatic reaction. First, 1 mL of spheroplasts in Mannitol medium with a concentration of ca. 0.2 mg Chl/mL were osmotically shocked in the dark at 35°C for 10 min in 27 mL of reaction medium (15 mM Tricine–NaOH (pH 8.0), 50 mM KCl, 50 mM NaCl, 5 mM $MgCl_2$, 2.5 mM K_2HPO_4, 1 mM glucose, and 100 μM PMSF). Then the suspension was placed on ice and 1.2 mM $K_3Fe(CN)_6$, 1 mM ADP (final concentrations) and the above reaction medium were added to bring the total volume to 32 mL. Three milliliter aliquots of that suspension at 35°C were given 5 min of high intensity white light. The reaction was stopped by cooling on ice and addition of 0.3 mL $HCLO_4$ (6 M), and the suspension was neutralized by addition of 6 M KOH–1 M Tris until the final pH was 7.5 ± 0.5. The $KCLO_4$ precipitate was removed by centrifugation and reduction of $K_3Fe(CN)_6$ of the supernatant was measured at 420 nm. Subsequently the ATP formation was measured in a coupled enzymatic reaction (Bergmeyer, 1970) by the reduction of $NADP^+$. Samples kept dark by wrapping in an aluminum foil provided a control for dark ATP formation. Samples given the uncoupler S-13 (1 μM final concentration) and illuminated provided an indication of the coupling quality of the spheroplasts. Each data point was the result of triplicate determinations and P/*2e* determinations on both Ca^{2+} and EGTA treated vesicles from both strains have been carried out eight times over a period of 3 months.

Calcium Binding to Proteins

The hydrophobic subunit III from the *Synechocystis* 6803 S-3-C mutant strain membranes was isolated by the butanol extraction/ether precipitation method, and SDS-PAGE electrophoresis (Zakharov *et al.*, 1993). Electroblotting of the proteins onto polyvinyl difluoride membranes were as described in Zakharov *et al.* (1993). The $^{45}Ca^{2+}$ ligand blotting method to detect Ca^{2+} binding to the protein (Maruyama *et al.*, 1984) was as described in Zakharov *et al.* (1993).

RESULTS

The rationale for this work is twofold: (A) to test other organisms for the occurrence of Ca^{2+} gating of H^+ flux through the $F_0\,H^+$ channel as found for CF_0 in higher plant (pea) thylakoids (Chiang *et al.*, 1992; Wooten and Dilley, 1993) and for *E. coli* cells (Zakharov *et al.*, 1996) and (B) to determine the pH dependence of the Ca^{2+}—$F_0\,H^+$ flux gate for the open or closed states. The critical pH for influencing the Ca^{2+}—CF_0 structure is the thylakoid lumen (inside) pH. The expectation, based on the previous work mentioned above (Chiang *et al.*, 1992; Wooten and Dilley, 1993; Zakharov *et al.*, 1996), is that at high internal pH, Ca^{2+} would be bound to the F_0 *c*-subunit and the H^+ gate is closed to "bulk phase" internal protons. At some acidic pH, the H^+ ions should protonate the putative Ca^{2+} binding carboxyl groups of the *c*-subunit and displace Ca^{2+}, opening the H^+ channel to the bulk phase. Our approach is to use acid–base transition to generate the $\Delta\mu_H{}^+$ because the internal and external pH values can be controlled and there is no local proton gradient that can be higher than the bulk ΔpH across the thylakoid membrane. In the accompanying paper, it is shown that membrane vesicles from the control strain (wild-type) *Synechocystis* 6803 can synthesize ATP upon acid–base transition with a minimum of about 2 ΔpH units (Van Walraven *et al.*, 2002). In this paper, we will use different states of Ca^{2+} availability and test whether such conditions influence the pH dependence of acid–base jump ATP formation. It was advantageous to use two mutant strains of *Synechocystis* 6803, one having serine 37 replaced by glutamate (plc 37) and the other having serine 3 replaced by cysteine (S-3-C). The plc 37 strain is useful due to a higher H^+/ATP (near or slightly above 4; see, Van Walraven *et al.* (2002)), which allows the vesicles to start making ATP at a lower pH jump compared to the wild-type. This allows an easier and clearer determination of the Ca^{2+} effect in closing the $F_0\,H^+$ channel using a titration of the internal pH. The S-3-C mutant is useful owing to an apparent tighter Ca^{2+} binding at the $F_0\,H^+$ gate improving the resolution of the data on the acid–base jump-induced ATP formation with Ca^{2+} present compared to the EGTA treatment used to remove bound Ca^{2+}. This strain already shows some Ca^{2+} induced closing of the H^+ gate without having to add extra $CaCl_2$, showing this to be a phenomenon that very likely occurs naturally.

The Effect of Addition or Depletion of Ca^{2+} on ATP Synthesis Driven by Acid-Base Transition

Without Ca^{2+} added to the plc 37 mutant spheroplasts the $\Delta\mu_H{}^+$ needed to reach the threshold level for ATP formation was about 8 kJ/mol (less than 1.5 pH units, Fig. 1(A)). With a base stage pH of 8.4, such vesicles gave acid–base jump ATP with internal pH of about 7.1 or less (Fig. 1(B)). The ΔpH needed to reach the threshold was lower than reported for wild type spheroplasts (in that work the base stage pH was 8.34) due to the higher

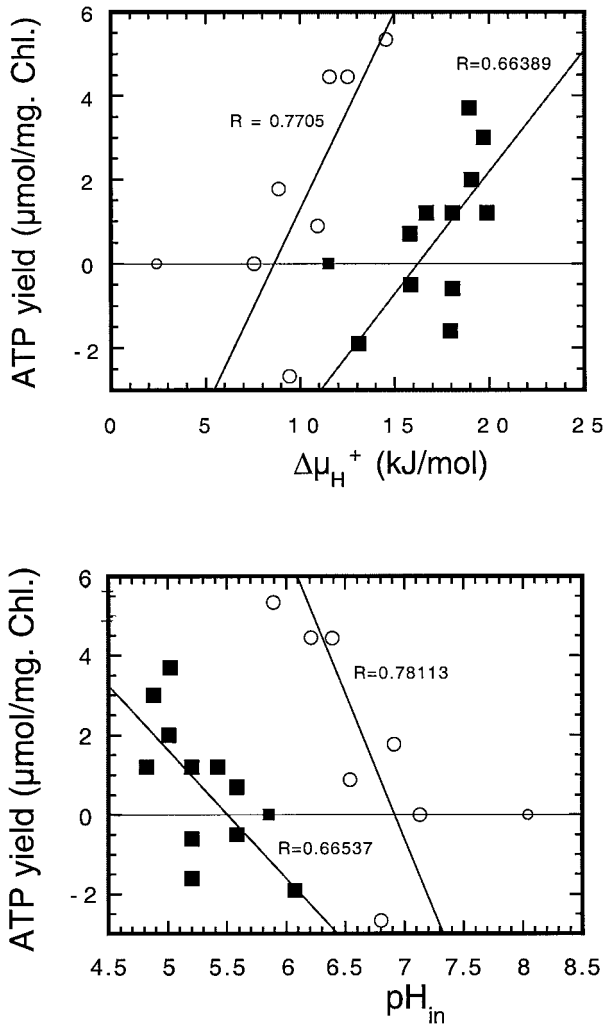


Fig. 1. Representative experiments of ATP production (within 1 min) as a function of $\Delta\mu_H{}^+$ (A) and as a function of internal pH (B) applied by acid–base transition of vesicles from *Synechocystis* 6803 strain plc 37 with (■) and without (○) added Ca^{2+} at high pH_{out}. (The experiments were carried out at 37°C. For the $-Ca^{2+}$ vesicles (○) [Chl] during phosphorylation was 1.9 μg/mL and pH_{out} was 8.40. Initial ΔG_p was 36.31 kJ/mol. For the $+Ca^{2+}$ vesicles (■) [Chl] was 2.5 μg/mL and pH_{out} was 8.28. $[K^+]_{in}$ and $[K^+]_{out}$ were 10 mM in both experiments. The ATP produced was corrected for myokinase activity and ATP already present, running a control with uncoupler (cf. Methods section). Data within 5 kJ/mol of the equilibrium $\Delta\mu_H{}^+$ for ATP synthesis/hydrolysis were fitted with the linear fit option in the program KaleidaGraph. The *R* values of the regression lines are given in the graph.)

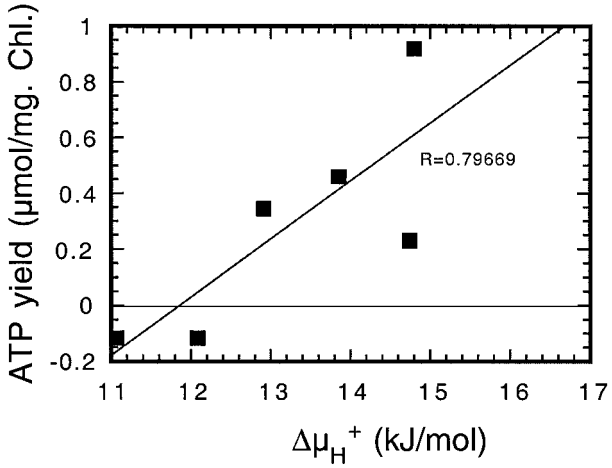
H^+/ATP of this mutant strain (see, Van Walraven *et al.*, 2002)). Using the $\Delta G_p = n_H{}^+\Delta\mu_H{}^+$ relationship, this computes to an H^+/ATP ratio near 4.3, consistent with the high H^+/ATP reported for the plc 37 strain.

With 1 mM $CaCl_2$ added to the spheroplast storage suspension (but not in the assay buffers, cf Methods) a much greater ATP formation threshold $\Delta\mu_H{}^+$ was observed (about 16 kJ/mol, a ΔpH of more than 2.5 units, Fig. 1(A)) and ATP formation did not occur until the acid pH reached about 5.6 (the base stage was pH 8.28, about the same as for the $-Ca^{2+}$ experiment). In this case the calculated $n_H{}^+$ is 2.3 H^+/ATP, an unrealistically low value, especially in view of the demonstrated much higher $n_H{}^+$ value found for the $-Ca^{2+}$ conditions. Clearly, a 2.5 or greater ΔpH (translating to a $\Delta\mu_H{}^+$ of about 16 kJ/mol) needed to obtain acid–base jump ATP formation in the $+Ca^{2+}$ case suggests something other than energetic considerations controls the onset of ATP formation at pH values above pH 5.8. We suggest that Ca^{2+} closure of the CF_0 H^+ gate is the factor involved.

That Ca^{2+} addition was not, say, changing the H^+/ATP ratio or otherwise having an unexpected influence on the intrinsic bioenergetics of the vesicles is shown by the data of Fig. 2, where the base stage was lowered to pH 7.16 (and Ca^{2+} was present in the spheroplast storage buffer). In that case the plc 37 strain spheroplasts allowed ATP formation to be initiated with a ΔpH of about 2, and that required an acid side (inside) near pH 5.2. That is, it can be understood, based on previous results with higher plant thylakoids which showed Ca^{2+} gating (Wooten and Dilley, 1993), that such an acidic pH would cause loss of Ca^{2+} binding to the CF_0 gating site.

Fig. 3(A) shows a similar Ca^{2+} effect on the $\Delta\mu_H{}^+$ threshold values for the S-3-C strain as for the plc 37 strain presented in Fig. 1(A). However, to investigate ATP synthesis in the absence of Ca^{2+} in this strain it was necessary to add EGTA to spheroplasts because apparently, the F_0 subunit of this strain naturally binds some Ca^{2+}. In the Fig. 3(A) and (B) data the base stage pH was 8.33 and Ca^{2+} present caused the threshold ΔpH to be about 3.3 units (pH storage near 5.0), but when Ca^{2+} was depleted with EGTA, ATP formation began when the acid stage reached about 6.2. Again, the lower acid stage pH threshold was not because of an energetic threshold requirement, as shown by Fig. 4(A) and (B) where the same Ca^{2+}-supplemented spheroplasts were used, but the base stage was brought to pH 7.3. Our interpretation is that with the lower base stage pH, sufficient energy for ATP formation in the pH jump is only realized when the acid stage is acidic enough to displace Ca^{2+} from the F_0, and that requires only a ΔpH of 2.5 to reach the pH to displace Ca^{2+}, much less than the 3.3 ΔpH seen for the $+Ca^{2+}$ data of Fig. 3. For the S-3-C mutant strain, even though it shows an H^+/ATP ratio near 3 and the acid–base jump needs to be larger than for the plc 37 strain, with its H^+/ATP near 4, the same argument can be made as to Ca^{2+} effects blocking the F_0 above pH values near 5.0–5.5.

These experiments are consistent with the Ca^{2+}-gating hypothesis we are testing. When Ca^{2+} is available



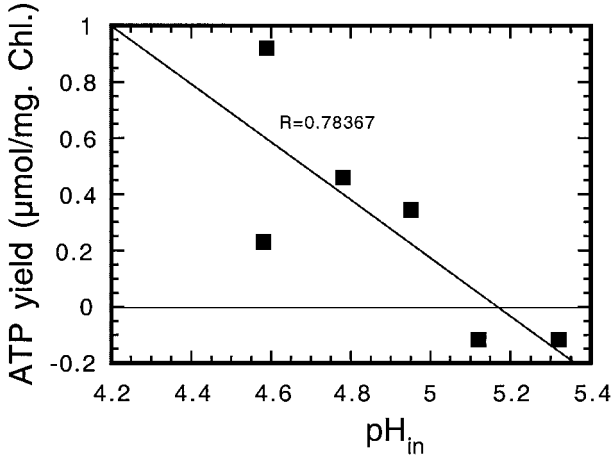
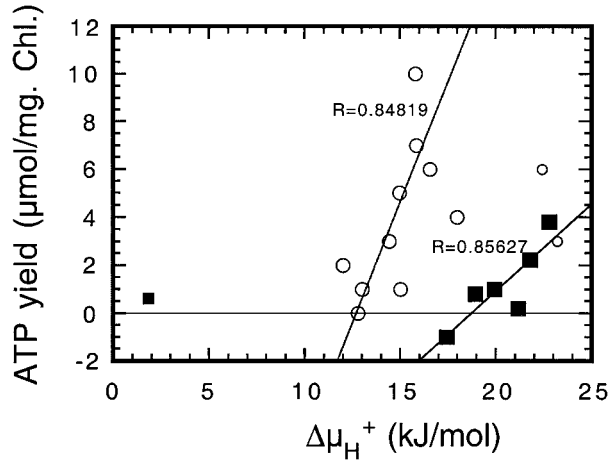


Fig. 2. ATP production by acid–base transition (within 0.5 min) as a function of $\Delta\mu_H^+$ (A) and as a function of internal pH (B) of vesicles from *Synechocystis* 6803 strain plc 37 with added Ca^{2+} at lower pH_{out}. (The experiments were carried out at 37°C. [Chl] during phosphorylation was 3.05 μg/mL and pH_{out} was 7.16. $[K^+]_{in}$ and $[K^+]_{out}$ were 10 mM in both experiments. The ATP produced was corrected for myokinase activity and ATP, already present running a control with uncoupler as mentioned in Fig. 1. The regression line is obtained as in Fig. 1.)



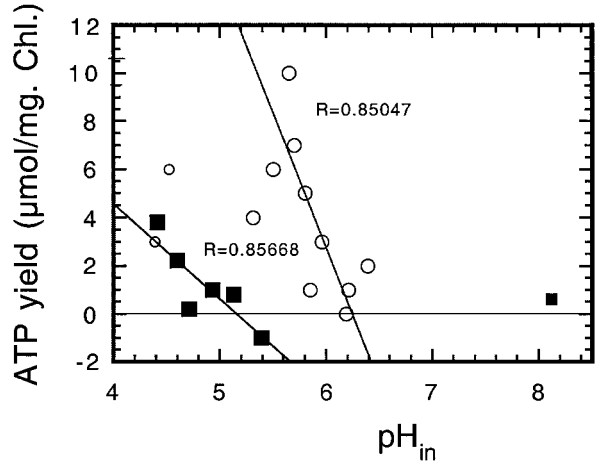


Fig. 3. Representative experiments of ATP production (within 1 min) as a function of $\Delta\mu_H^+$ (A) and as a function of internal pH (B) applied by acid–base transition of vesicles from *Synechocystis* 6803 strain S-3-C in the presence of added Ca^{2+} (■) or EGTA (○) at high pH_{out}. (The experiments were carried out at 37°C. For the +EGTA vesicles (○) [Chl] during phosphorylation was 0.93 μg/mL and pH_{out} was 8.35. Initial ΔG_p was 36.11 kJ/mol. For the $+Ca^{2+}$ vesicles (■) [Chl] was 2.45 μg/mL and pH_{out} was 8.33. $[K^+]_{in}$ and $[K^+]_{out}$ were 10 mM in both experiments. The ATP produced was corrected for myokinase activity and ATP already present, as mentioned in Fig. 1. The regression lines are obtained as in Fig. 1.)

it appears that above pH 5.5 the proton gradient, though energetically capable of driving ATP formation, cannot do so. If Ca^{2+} was not added to the plc 37 mutant, or if EGTA pulls Ca^{2+} away from the S-3-C mutant membranes, the system can make ATP at acid stage pH values above 5.5, if the base stage has a sufficiently high pH to generate the threshold energy required.

Test for Side-Effects of the Addition of Ca^{2+} or EGTA

Some important considerations which could contradict the above model required that certain additional controls be carried out, including measuring the ATP/*2e* ratios for the various conditions. It was found that the ATP/*2e* ratios were the same, within experimental error, for the presence and the absence of Ca^{2+}. For example, with the plc 37 membranes typical data were $+Ca^{2+}$ in the storage, 0.85 and for the EGTA-treated sample, 0.87. In a series of measurements with S-3-C membranes, the values were 1.16 with Ca^{2+} and 1.13 with EGTA. Although we cannot be sure about the $H^+/2e$ ratio in these vesicles, the P/*2e* values found correspond with a H^+/ATP value of 4.6 for the plc 37 and 3.5 for the S-3-C strains if we adopt

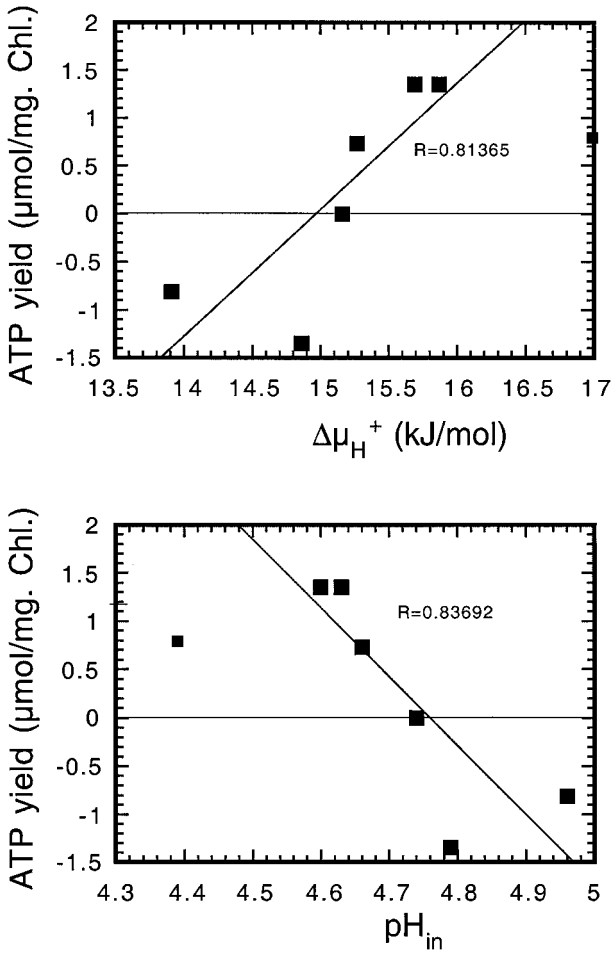


Fig. 4. ATP production by acid–base transition (within 0.5 min) as a function of $\Delta\mu_H{}^+$ (A) and as a function of internal pH (B) of vesicles from *Synechocystis* 6803 strain S-3-C with added Ca^{2+} at lower pH_{out}. (The experiments were carried out at 37°C. [Chl] during phosphorylation was 2.28 μg/mL and pH_{out} was 7.3. $[K^+]_{in}$ and $[K^+]_{out}$ were 10 mM in both experiments. The ATP produced was corrected for myokinase activity and ATP already present, as mentioned in Fig. 1. The regression line is obtained as in Fig. 1.)

the data of Berry and Rumberg (1999) for the $H^+/2e$ of spinach thylakoids at high light intensity. The difference in H^+/ATP between strains is described in the accompanying paper (Van Walraven *et al.*, 2002).

The presence of Ca^{2+} or EGTA had no effect on coupling quality of ATP synthesis activity because light-induced proton uptake and ATP synthesis were equal using vesicles with or without the addition. It was also shown, as in the accompanying paper (Van Walraven *et al.*, 2002), that the ATP synthase of the different cyanobacterial vesicles from both strains remain active during preparation since ATP hydrolysis activity was retained (results not shown).

Binding of $^{45}Ca^{2+}$ to Subunit *c* of the ATP Synthase From *Synechocystis* 6803 ATP Synthase

Because the $^{45}Ca^{2+}$ binding experiments of Zakharov *et al.* (1993, 1995, 1996) indicated that both the N-terminal formyl-Met and the C-terminal Ala carboxyl groups were necessary for Ca^{2+} binding (cf. Moody *et al.* (1987) for NMR evidence for the C-terminal COO-interaction with trivalent lanthanide ion analogues of Ca^{2+}), we thought it worthwhile to check the S-3-C mutant for Ca^{2+} binding activity. The $^{45}Ca^{2+}$ ligand blot assay was applied to the subunit III protein purified from this mutant by the butanol extraction-ether precipitation technique (Zakharov *et al.*, 1993). Figure 5 shows that the S-3-C mutant subunit *c* binds Ca^{2+} as does the pea thylakoid subunit III and the wild type reported earlier (Zakharov *et al.*, 1996). However, since the amount of protein applied to the gel was much less in the case of the mutant sample compared to the pea subunit III (see legend to Fig. 5) less $^{45}Ca^{2+}$ binding was visible. It was not possible to determine a $^{45}Ca^{2+}$/*c*-subunit ration from such an experiment because

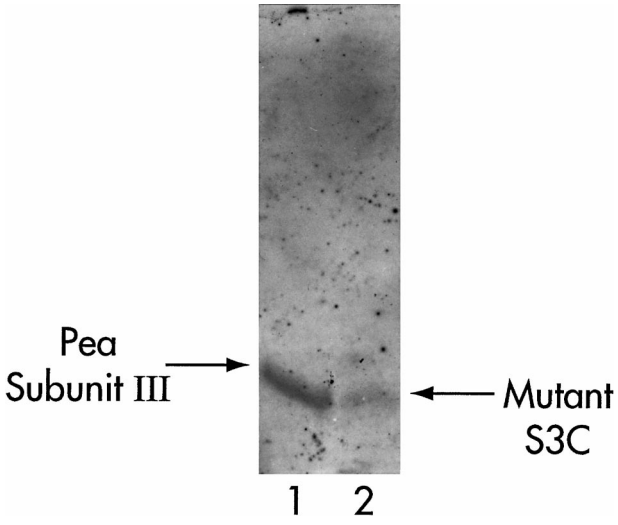


Fig. 5. $^{45}Ca^{2+}$ binding to the 8 kDa *c*-subunit of the S-3-C *Synechocystis* 6803 strain. The *c*-subunit was partially purified as described in the Methods section. After transferring the proteins from the SDS-PAGE gels to polyvinyldifluoride paper by electroblotting, the $^{45}Ca^{2+}$ wash was given and the radioactivity detected by autoradiography (cf. Methods section). For comparison, purified CF_0 subunit *c* of pea thylakoids obtained as described in Zakharov *et al.* (1993), was run side-by-side with the protein extracted from the *Synechocystis* 6803 S-3-C mutant. The amount of *c*-subunit applied to the SDS-PAGE gel lanes was 6 μg for the pea thylakoid and 1–2 μg for the S-3-C mutant. This was estimated from the silver stain intensity necessary to visualize the highly hydrophobic protein. The high SDS concentration (5%) used to solubilize the (partially pure) *c*-subunit makes accurate protein determination difficult).

the amount of *c*-subunits and $^{45}Ca^{2+}$ atoms are difficult to quantify.

DISCUSSION

These results are consistent with the hypothesis that Ca^{2+} ions bound to the 8 kDa F_0 subunit *c* provide a gating action on H^+ flux across the opening of the F_0 H^+ channel. In this work, the acidic pH needed to allow ATP formation by H^+ ion efflux through the F_0F_1 complex with Ca^{2+} present was near pH 5.6 for the plc 37 strain (Fig. 1(B)) and near pH 5.3 for the S-3-C mutant (Fig. 3(B)) despite the fact that with the base stage above 8 the ΔpH was far in excess for providing the thermodynamic driving force needed to energize ATP formation. We interpret this as indicating that above those pH values, Ca^{2+} is bound to the F_0 H^+ channel, blocking protons from entering the channel and hence no bulk phase protons from succinic acid go through the F_0 and no ATP formation occurs. Acidic conditions below pH 5.6 (for the plc 37 strain) or 5.3 (for strain S-3-C) allowed ATP formation and we interpret that as owing to the acidic conditions causing displacement of Ca^{2+} from the F_0 gating site.

A number of control experiments have been carried out to exclude other possible effects of Ca^{2+}:

1. Uncoupling. Since vesicles $+Ca^{2+}$ and $-Ca^{2+}$ (or +EGTA) gave the same light-induced proton uptake and ATP synthesis (data not shown), an uncoupling effect in the $+Ca^{2+}$ case is ruled out. Also uncoupling should have an effect on P/*2e* values and that was not observed.
2. Increase in $\Delta\mu_{H^+}$ needed for activation of the ATP synthase just like the different redox states of CF_0F_1 (see Hangarter *et al.*, 1987; Junesch and Gräber, 1985). If Ca^{2+} addition would lead to an increase in the $\Delta\mu_{H^+}$ needed for activation this effect should also have been obvious at the lower external pH used in the acid–base jump studies. Furthermore, it was shown that a large fraction of the ATP synthases remains active after vesicle preparation, and therefore needs no activation prior to ATP synthesis.
3. Decrease in H^+/ATP. This should also be obvious at the lower external pH used during acid–base jump. Furthermore a decrease in H^+/ATP should logically lead to a higher P/*2e*, but we found similar P/*2e* values with or without Ca^{2+} added.

Higher plant (pea) chloroplast thylakoids were also shown to have a Ca^{2+}-gated CF_0 H^+ channel, and when Ca^{2+} was abundant the thylakoid acid–base jump ATP formation was significantly inhibited when the acid stage was pH 5.5 (base stage 8.5) but not inhibited when the acid stage was pH 4.0 (base pH 7.5) (Wooten and Dilley, 1993). In that work no further testing was done on the pH dependence of the Ca^{2+} blocking effect, but the similarity to these results is noted in that the Ca^{2+} block of lumen-phase H^+ efflux-driven ATP formation was opened somewhere to the acid side of pH 5.5. In the pea thylakoid work, the Ca^{2+} binding was tightened by photoaffinity attaching chlorpromazine (CPZ) to the Ca^{2+}—CF_0 gating site (Chiang *et al.*, 1992; Wooten and Dilley, 1993). It had been shown earlier, that provided Ca^{2+} was present for binding to the F_0, [^{3}H] chlorpromazine was strongly photoaffinity bound to the CF_0 8 kDa subunit III of pea thylakoids with very low labeling of any other thylakoid proteins (Dilley and Chiang, 1989). Chlorpromazine is a well-known pharmacological agent known to target high-affinity Ca^{2+} binding sites in proteins (calmodulin, troponin *c*, etc., cf. Massom *et al.*, 1990; McPhelan *et al.*, 1991; Roberts *et al.*, 1986), With some of the *Synechocystis* 6803 spheroplasts employed in this work CPZ was present in the earlier experiments but proved to be unnecessary and, in fact, it would have interfered with the estimation of the intrinisic H^+—Ca^{2+} competition for the Ca^{2+} binding sites. Hence, we suggest that the data presented herein reveal the intrinsic pH dependence of Ca^{2+} binding to the F_0 H^+ channel.

The k_d for Ca^{2+} dissociation was not determined in this work (but see Zakharov *et al.*, (1996) and it can be questioned why we added such a high Ca^{2+} concentration to the spheroplast storage buffer in these experiments (1 mM $CaCl_2$). The usual Ca^{2+} binding k_d values for biologically relevant tight Ca^{2+} binding sites is in the micromolar range, or less, (McPhelan *et al.*, 1991; Roberts *et al.*, 1986). The high Ca^{2+} concentration added may not have been necessary as Chiang and Dilley (1987) found that even in the presence of 100 mM KCL, thylakoids could bind Ca^{2+} at the F_0 H^+ channel in the 50 μM or lower $CaCl_2$ concentration range. Thylakoids have extremely high levels of Ca^{2+} binding sites (approximately 0.2 Ca^{2+} per Chl (Chiang and Dilley, 1987 and references therein)) so we used 1 mM $CaCl_2$ to assure that from preparation to preparation there was sufficient added Ca^{2+} to saturate all the binding sites. Furthermore, during actual ATP formation the external Ca^{2+} concentration is diluted out to ca. 15 μM (for the acid–base transition experiments) or 30 μM (for the P/*2e* experiments), which is more in the physiological range.

Another question concerns how the proposed H^+ flux gating relates to what is known about the structure of the F_0 H^+ channel. First it should be mentioned that

experiments show that the Ca^{2+} gating works on protons going in both directions, lumen-to-outside and on the flux from the sequestered domains into the lumen. The former effect depends, of course, on acid–base jump ATP formation (this works with similar experiments in pea thylakoids (Wooten and Dilley, 1993)); and the latter effect was shown by measuring net H^+ uptake into the lumen when light-driven H^+ flux was used. In those experiments, CPZ was photoaffinity attached to the CF_0 8 kD subunits with Ca^{2+} present, having the effect of tightening the Ca^{2+} binding affinity (Massom *et al.*, 1990) making the closed-gate configuration more stable. In those conditions (A) light-dependent H^+ accumulation occurred predominantly into the sequestered domains with minimal luminal acidification (cf. Fig. 5 of Chiang *et al.* (1992)) and (B) both flash-induced ATP formation (Table 5 of that ref.) and postillumination ATP formation occurred but there was not the usual lumenal proton pool contribution to the postillumination ATP yield (Fig. 4 of Chiang and Dilley (1992)). We can conclude that the proposed Ca^{2+}—CF_0 H^+ gate acts as a block to H^+ flux in either direction (provided the pH is not so low as to displace Ca^{2+}), an expected feature of a gate site in a two-way H^+ channel.

In preliminary experiments we have observed that Ca^{2+}-treated spinach thylakoids produce ATP at lower light intensity compared to untreated thylakoids with no extra Ca^{2+} added (data not shown). This Ca^{2+} effect was not observed when the thylakoids were reduced first with dithiothreitol nor in vesicles of any mutant *Synechocystis* 6803 strain. It is known (Krab *et al.*, 1993) that the ATP synthase from most cyanobacteria functionally corresponds to the reduced CF_1 of higher plant thylakoids. We interpret these data as consistent with the occurrence of Ca^{2+}-CF_0-stabilized local domains where the local $\Delta\mu_H{}^+$ induced by light-driven electron transport is higher than the lumen bulk phase $\Delta\mu_H{}^+$. Because of the high threshold in proton translocation of the oxidized CF_0F_1 (because of the high energy needed for activation (Junesch and Gräber, 1985; Hangarter *et al.*, 1987)) the Ca^{2+} effect becomes evident. A similar effect was seen as in the studies mentioned before on flash-induced ATP synthesis with (oxidized) pea thylakoids (Fig. 1 of Chiang and Dilley (1987)).

Such a gating function is necessary if, as much evidence suggests, thylakoids can carry out either a localized or a delocalized $\Delta\mu_H{}^+$ coupling mechanism. The physiological function for such a gating action is suggested by recent studies of the stress responses of chloroplasts (Pan and Dilley, 2000). At nonstressful energization levels the energy-coupling proton gradient needed to drive ATP synthesis is hypothesized to be constrained to membrane-localized domains (as yet not defined by rigorous structural information), the thylakoid lumen pH stays above pH 6.0 and the proposed acidic-lumen-dependent stress-response mechanisms are not engaged. It has already been suggested (Dilley, 1991) that when energy-coupled H^+ fluxes linked to ATP formation are not fast enough to keep pace with H^+ production (as in high light conditions) the local domains become overacidified and the excess acidity displaces the Ca^{2+} at the CF_0 gate site, leading to luminal acidification to below pH 6.0. It has been proposed (Dilley, 1991; Krieger and Weiss, 1993) that lumen acidification to below pH 6 permits the operation of at least two stress alleviation responses: (A) an acidic-lumen-dependent Ca^{2+} release from PS II reaction centers with a downregulation of PS II water oxidation rates (Krieger and Weiss, 1993), and (B) an acidic-lumen-dependent stimulation of the violaxanthin deepoxidase activity which results in zeaxanthin (and antheraxanthin) formation leading to dissipation of excess energy in antennae chlorophyll excited states (Demmig-Adams, 1990; Gilmore, 1997).

The H^+ channel pathway into the CF_0 complex from the lumen is not understood, although recent discussions have proposed that somehow subunit *a* is involved along with the ring of 10–14 *c*-subunits (Fillingame *et al.*, 2000 and references therein). One would ask if subunit *a* provides some of the proton-relay sites into the bilayer (to access the Glu 61 of subunit *c*) why or how would Ca^{2+} binding at the N- and C-terminal portions of the *c*-subunit act to gate the H^+ flux across the lumen-to-bilayer-interior H^+ path? There is no evidence for Ca^{2+} binding to subunit *a*. Possible explanations are (A) although there is evidence by mutation that certain residues in *a* are essential in proton translocation the actual inlet might not be in subunit *a*. The proton inlet could well be via the ring of *c*-subunits with the correct orientation of the *c*-subunits requiring some interaction with subunit *a*. (B) the functional H^+ path may require close interaction of subunit *a* with the contact portion of the ring of *c*-subunits; if so, Ca^{2+} bound to the *c*-subunits could alter the conformation, closing the transmembrane pathway but allowing H^+ insertion from the putative local domains into the critical parts of subunit a. How the proposed "localized" protons get into the F_0 domain remains a mystery, in part because the structural nature of the localized domain remains to be determined.

In mitochondrial ATP synthase the *c*-subunit does not bind Ca^{2+}, presumably because of the cationic Arg at the C-terminus (Zakharov *et al.*, 1996) but another subunit might be involved in Ca^{2+}-dependent regulation. A recent paper (Arakaki *et al.*, 2001) describes binding of Ca^{2+} to mammalian F_0 subunit *e* and those authors proposed that subunit *e* may have some Ca^{2+}-dependent regulatory role.

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