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Biochemistry

Evidence for the involvement of coupling factor B in the H^+ channel of the mitochondrial H^+-ATPase

(dithiol/cadmium/H^+ conduction/membrane H^+ permeability)

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ABSTRACT **Membrane energization by ATP has been measured in vesicles containing purified bovine heart mitochondrial H^+-ATPase (ATP synthase) with the voltage-sensitive dye oxonol VI. The dithiol chelator, Cd^{2+}, and the thiol oxidant, copper *o*-phenanthroline, produced discharge of the membrane potential when added at the steady state and inhibited its establishment when added prior to energization by ATP. These effects, which were reversed by dithiothreitol, were not accompanied by an increase in the nonspecific H^+ permeability of the membrane. Passive H^+ conduction in proteoliposomes containing F_0 (hydrophobic segment of ATP synthase) was assayed by the quenching of 9-aminoacridine fluorescence after establishing a K^+ diffusion potential. This conductance was blocked by Cd^{2+}, an inhibitor of coupling factor B (F_B). Labeling of F_0 with $^{115}Cd^{2+}$ at the concentrations that inhibited the F_0 conductance followed by gel electrophoresis yielded a single radioactive band with a molecular weight corresponding to F_B, the presence of which in the F_0 preparation was confirmed by immunoblot staining. The data offer strong evidence that F_B is an essential component of the H^+ channel of F_0, because H^+ conduction through the channel is inhibited by chemical modification of F_B.**

It is well established that the mitochondrial ATP synthase or H^+-ATPase is an inner mitochondrial membrane complex consisting of a hydrophobic segment (F_0) anchored in the bilayer region and an extrinsic water-soluble multiprotein complex (F_1) with ATPase activity (1). F_1 has been characterized extensively, but knowledge of the structure of F_0 and the function of its subunits is far from complete. Bovine heart F_0 has four principal subunits with identifiable function; they are oligomycin-sensitivity-conferring protein, coupling factor B (F_B), coupling factor 6, which, together with oligomycin-sensitivity-conferring protein, may have a role in linking F_1 to F_0, and the oligomycin and dicyclohexylcarbodiimide binding proteolipid. The proteolipid appears central to the process of H^+ translocation (2). Recent work has also implicated F_B in H^+ translocation (3), and we now provide stronger evidence for its involvement. In addition to these four proteins, our best F_0 preparations show at least two other prominent bands in gel electrophoresis, whose role, if any, is unknown, and there may also be traces of other unknown components.

The properties of F_B, which may be Racker's F_2, have been reviewed recently (3). Its coupling activity is inhibited by Cd^{2+} and phenylarsine oxide under conditions that render these reagents specific to vicinal dithiols—namely, in the presence of excess monothiol (4). We now report that inactivation of H^+-ATPase by Cd^{2+} decreases the electrochemical H^+ gradient ($\Delta\mu_{H^+}$) generated by ATP without significant alteration of the nonspecific H^+ permeability of the membrane. Cd^{2+} also inhibits the F_0-mediated H^+ conduction measured by quenching of the fluorescence of 9-aminoacridine.

EXPERIMENTAL

The preparations of H^+-ATPase and F_0 from it by dissociation of F_1 in 3.5 M NaBr have been described (5, 6). Membrane potential was monitored in this preparation with the voltage-sensitive dye, bis(3-propyl-5-oxoisoxazol-4-yl) pentamethine oxonol (oxonol VI), essentially as described (7). Briefly, 100 μg of H^+-ATPase and 2 μl of 1.5 mM oxonol (dissolved in ethanol) were added to 2 ml of 40 mM Tris acetate, pH 7.5/0.25 M sucrose/2 mM $MgCl_2$, equilibrated for 2 min at 25°C, and the reaction was started by adding a solution containing ATP and ADP as shown in the figures. The absorbance change caused by the redistribution of oxonol VI in response to membrane energization was measured with a Perkin–Elmer model 557 dual wavelength spectrophotometer using 594 nm and 630 nm as reference and measuring wavelengths.

For the assay of passive H^+ conduction, 20 mg of asolectin that had been dried from $CHCl_3$ solution in a thin layer under N_2 was suspended in 1 ml of 200 mM KCl/10 mM Tricine buffer, pH 7.5, shaken in a Vortex mixer, and finally exposed to sonic dispersion until clarity. One milligram of F_0 vesicles in 10 ml of 10 mM Tricine at pH 7.5 and 1 mM dithiothreitol was spun down and the resulting membrane fragments were resuspended with the liposomes. The mixture was frozen in liquid N_2, then thawed, sonicated for a few seconds, and passed through a Biogel P-6 DG column that had been swollen in 200 mM NaCl/10 mM Tricine buffer to remove external K^+. Small aliquots of K^+-loaded liposomes were assayed in 2 ml of 200 mM NaCl/10 mM Tricine buffer/5 μM 9-aminoacridine. The fluorescence was monitored with a Perkin–Elmer MPF-44A spectrofluorometer. The excitation and emission wavelengths were 365 nm and 451 nm, respectively. The reaction was initiated by the addition of 50 ng of valinomycin in 5 μl of ethanolic solution, which promotes K^+ efflux and a consequent flux of protons inward through the H^+ channel.

RESULTS

On the addition of ATP/ADP to the H^+-ATPase, there is an immediate increase in the bound oxonol, shown by the decrease in $A_{594-630\ nm}$ (Fig. 1, *Inset*). This change is indicative of the establishment of a membrane potential associated with influx of H^+ into the inverted vesicles. When oligomycin or dicyclohexylcarbodiimide is added at the steady state, the H^+ pump is inhibited and the absorbance returns to the

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Abbreviations: F_0, hydrophobic segment of ATP synthase; F_1, water-soluble segment of ATP synthase; F_B, coupling factor B, $\Delta\mu_{H^+}$, electrochemical H^+ gradient.

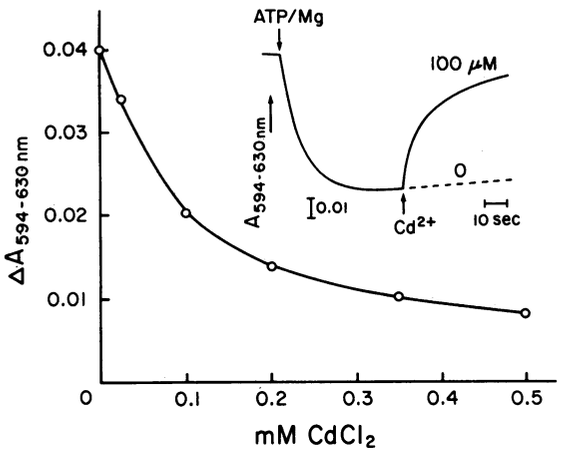

FIG. 1. Response of ATP-energized oxonol VI absorbance change in H^+-ATPase to Cd^{2+}. Cd^{2+} was added to the reaction mixture containing 100 μg of H^+-ATPase and oxonol VI, and the reaction was started with the addition of 20 nmol of ATP and 2 nmol of ADP dissolved in the same medium. The resulting maximal absorbance change is shown on the ordinate. (*Inset*) The absorbance trace on adding ATP/ADP and the reversal of the absorbance change brought about by 100 μM Cd^{2+}.

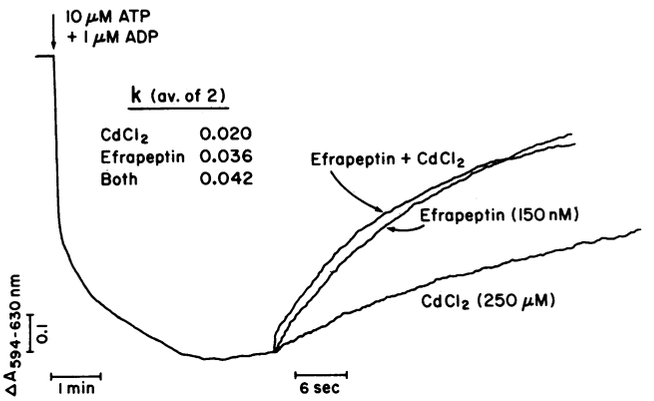

FIG. 2. Effects of Cd^{2+} and efrapeptin on the rate of oxonol release. The experiment was carried out as in Fig. 1 (*Inset*). When the steady state was reached, the discharge of bound oxonol was effected by the addition of 1.25 μg of efrapeptin in 25 μl or by 500 nmol of $CdCl_2$ in 25 μl (or both in 30 μl) of buffer. Note the change in chart speed. At the end, the membrane potential was completely discharged by the addition of 5 μl of 0.5 mM carbonylcyanide *m*-chlorophenylhydrazone in ethanol and the resulting reading served as the reference point for measuring the decay rate. The first order rate constants (k) were determined from a log absorbance vs. time plot.

pre-energized level owing to the nonspecific leakage of H^+ from the vesicle (data not shown). Cd^{2+} addition produces a similar effect on the oxonol absorbance. If Cd^{2+} is added before ATP, there is little or no absorbance change, and the effect of increasing Cd^{2+} on the steady oxonol absorbance change is shown in Fig. 1. The Cd^{2+} block is seen even in the presence of 2-mercaptoethanol but it is inhibited if dithiothreitol is present, as in the experiments on the effect of Cd^{2+} on P_i–ATP exchange (4). Earlier experiments have shown that binding of Cd^{2+} does not affect the oligomycin sensitivity of the H^+-ATPase and that the site of Cd^{2+} binding is probably F_B dithiol (4). Because Cd^{2+} is bound with high affinity to vicinal dithiols, it seemed feasible to show the binding directly. In fact, when H^+-ATPase was treated with $^{115}Cd^{2+}$ in the presence of excess 2-mercaptoethanol and $NaDodSO_4$/PAGE was carried out, the radioactivity was recovered in a peak located at the position of F_B (see figure 11 in ref. 3).

The discharge of the bound oxonol on addition of Cd^{2+} to the energized H^+-ATPase could occur either because of an increase in H^+ permeability of the membrane or because of an inhibition of the low level of continuing H^+ pumping activity maintained by the residual ATP, with subsequent nonspecific slow discharge of the H^+. One test to distinguish between these two explanations is to inhibit the pump completely without affecting the H^+ channel and to measure the effect of Cd^{2+} on the efflux rate. This was accomplished with efrapeptin, an inhibitor of ATPase (8). In our experiments, in the presence of 0.5 μg of efrapeptin, the ATP-hydrolyzing activity of H^+-ATPase was not detectable. The same amount of efrapeptin reduced the ATP-induced oxonol binding by at least 98%. Fig. 2 shows that when efrapeptin is added to H^+-ATPase after establishing the membrane potential with ATP, the oxonol VI absorbance reverted to its pre-energized level considerably faster. This decay, expressed as the first-order rate constant, is probably close to the true rate of nonspecific H^+ leakage through the membrane. Although Cd^{2+} by itself also promoted a similar absorbance change, any additional effect of this ion on the decay rate in the presence of efrapeptin was barely noticeable.

We have also recently studied the inactivation of purified F_B by the relatively specific thiol-oxidizing reagents, copper *o*-phenanthroline and iodosobenzoate. The inactivation followed pseudo-first-order kinetics with respect to F_B protein, which suggests intramolecular disulfide formation (data not shown), and is consistent with the presence of a vicinal dithiol in F_B. These reagents also inhibited the P_i–ATP exchange activity of H^+-ATPase and blocked the membrane potential-dependent binding of oxonol VI with IC_{50} values of 25–40 μm for the copper chelate. The ATPase activity, however, was unaffected, an effect similar to that of Cd^{2+}. Experiments similar to those shown in Fig. 2 using copper *o*-phenanthroline instead of Cd^{2+} also showed that thiol oxidation produced a slow discharge of bound oxonol. The decay rates for efrapeptin and efrapeptin together with copper *o*-phenanthroline, however, were practically the same, again indicating that disulfide formation in F_B did not produce any significant increase in the H^+ permeability of the membrane.

The above results, however, differ from the observations with intact mitochondria. It is known that Cd^{2+} uncouples mitochondrial oxidative phosphorylation, inhibits P_i–ATP exchange, and stimulates ATPase activity (3). All these effects can be explained on the basis that Cd^{2+} produces an increase in the proton permeability of the mitochondrial membrane. The data in Fig. 3 show that Cd^{2+} does in fact produce rapid discharge of the pH across the membrane of respiring rat liver mitochondria. Oligomycin does not affect this discharge rate; the $t_{1/2}$ values for Cd^{2+}-induced decay with and without oligomycin were 16.2 and 16.4 sec, respectively. It may be noted that Cd^{2+} is not a protonophore in liposomes (3). Because the experiments with the purified H^+-ATPase show that Cd^{2+} does not alter membrane permeability by interaction with F_B, it would seem that Cd^{2+} must react with some other unknown membrane protein in the mitochondria and produce a rapid H^+ leak. $^{115}Cd^{2+}$-binding experiments in the presence of a 10-fold excess of 2-mercaptoethanol show that rat liver mitochondria bind ≈2.6 ng equivalents of Cd^{2+} per mg of protein or about 8 times the cytochrome *a* content (unpublished data). There are clearly several unidentified Cd^{2+} binding sites that could account for the increase in the H^+ permeability.

A more direct method for testing the role of F_B in the H^+ pump involves the use of F_0 for the study of passive H^+ conduction driven by a K^+ diffusion potential (9). On adding valinomycin to K^+-loaded liposomes containing F_0, there is K^+ efflux and a counter movement of H^+, which is measured by the quenching of the fluorescence of 9-aminoacridine. Both oligomycin and dicyclohexylcarbodiimide blocked this

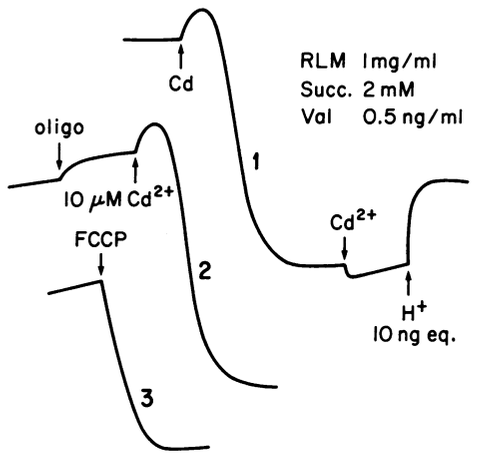

FIG. 3. Discharge of the H^+ gradient in respiring mitochondria by Cd^{2+}. Rat liver mitochondria (RLM) were in 0.25 M sucrose/0.02 M KCl/3 mM Hepes buffer, pH 7.1. Succinate (Succ.) and valinomycin (Val) and then the reagents indicated in the figure were added. Oligomycin was 2.5 μg and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was 2 μM. The pH was monitored with a glass electrode. The $t_{1/2}$ for the discharge was determined from a log $[H^+]$ vs. time plot, which was linear for >75% of the response.

H^+ translocation almost completely. Similar results have been reported by Glaser *et al.* (10), but these workers only obtained partial block with the inhibitors. If Cd^{2+} exerts its uncoupling effect by producing a H^+ leak, in this system the fluorescence quenching would be expected to occur faster and the extent of quenching to remain unaltered in the presence of the ion. On the contrary, the results show that Cd^{2+} blocked the quenching of valinomycin-induced fluorescence (Fig. 4). Furthermore, the average $t_{1/2}$ for the signal establishment in the presence of different concentrations of Cd^{2+} was 23.7 ± 4.4 (n = 10) sec, which was not significantly different from the value of 22.3 ± 2.4 (n = 6) sec in the absence of Cd^{2+}. No difference in $t_{1/2}$ would be expected if each proteoliposome contained a single F_0 complex.

F_B has been detected by immunoblot staining in the F_0 preparations used for these experiments. Moreover, when ^{115}Cd was added to F_0 proteoliposomes under conditions that inhibit quenching of aminoacridine fluorescence, the radioactivity was recovered on $NaDodSO_4$/PAGE exclusively at a position corresponding to F_B (Fig. 5).

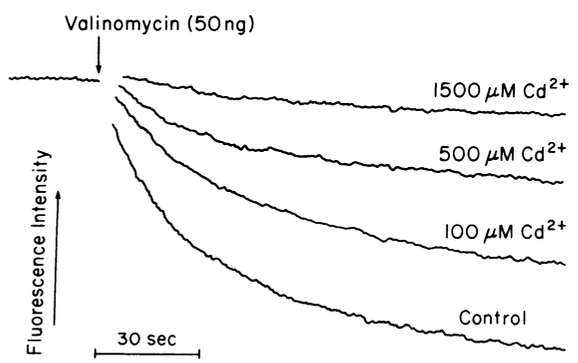

FIG. 4. Effect of Cd^{2+} on passive H^+ conduction through F_0. Ten microliters of K^+-loaded proteoliposomes, containing ≈10 μg of mitochondrial F_0 protein, was added to 2 ml of K^+-free buffer that contained 5 μm 9-aminoacridine. Fluorescence quenching occurred on addition of 50 ng of valinomycin. Total quenching for control samples was 9.3%. Small aliquots (2–20 μl) of $CdCl_2$ were added 2 min prior to the addition of valinomycin.

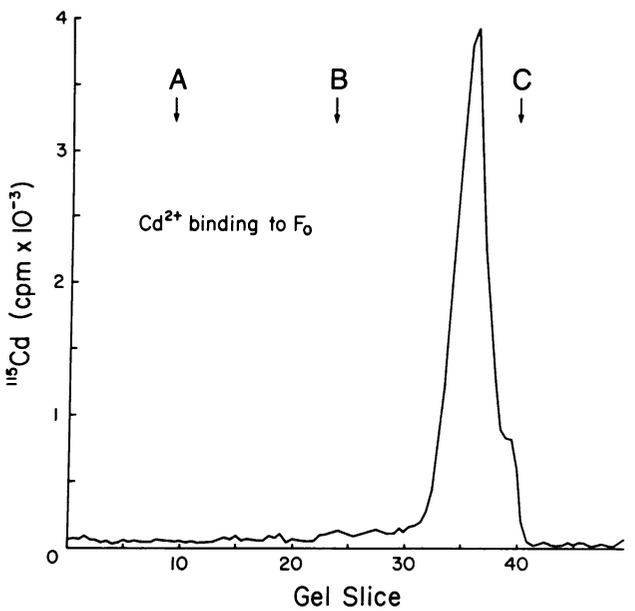

FIG. 5. Radiolabeling of F_0 with ^{115}Cd. A 100-μl aliquot of F_0 (10 mg/ml) in Tris acetate, pH 7.5/250 mM sucrose, was incubated at room temperature for 5 min after addition of 2 μl of 50 mM 2-mercaptoethanol. A 20-μl aliquot of 500 μM $CdCl_2$ (0.5 μCi/nmol; 1 Ci = 37 GBq) was added and the incubation continued for an additional 5 min. An aliquot (24 μl) of 5% $NaDodSO_4$/20% glycerol/1 mM 2-mercaptoethanol was added. Protein samples (50 μg) were immediately transferred to 10% polyacrylamide gels (10 × 0.5 cm) and electrophoresed using a 50 mM phosphate running buffer (pH 7.0). After electrophoresis, gels were sliced (1 mm) and extracted for 24 hr at 70°C with 1.0 ml of 20 mM EDTA (pH 7.5) before addition of scintillation fluid. In the phosphate-gel system, the migration of F_B is somewhat behind that of cytochrome *c* and corresponds to that of the $^{115}Cd^{2+}$ label. The total incorporation of ^{115}Cd was 4.9 nmol per mg of F_0. Assuming a molecular weight of 150,000 for F_0, the incorporation of ^{115}Cd into the peak comigrating with F_B is 0.73 nmol per nmol of F_0. A, bovine serum albumin; B, carbonic anhydrase; C, cytochrome *c*.

DISCUSSION

Several reports have recognized the dicyclohexylcarbodiimide-binding proteolipid as the principal component of the F_0 proton channel (for a recent review, see ref. 11). Experiments in liposomes (12, 13), lipid-impregnated millipore membranes (14), and recently, planar bilayers (15) using partially and extensively purified proteolipid from several different H^+-ATPases have indicated that this hydrophobic protein complex is capable of conducting H^+ through lipid bilayers. The kinetic competence of the incorporated proteolipid to account for the rates observed in intact mitochondria, however, needs to be established. Schindler and Nelson (15) have shown discrete conductance channels with a highly purified preparation of the proteolipid from yeast mitochondria. The significance of these experiments, implicating only the proteolipid for H^+ channel activity, however, may need to be reassessed in light of the results described in this paper, showing blockage of H^+ translocation through the H^+-ATPase as well as F_0 by perturbation of F_B dithiol. Furthermore, there is evidence that the F_0 prepared from *Escherichia coli unc* mutants lacking the a or b subunits have negligible H^+ conducting activity (16–19). Our present evidence that the mitochondrial F_B is also a functional part of the proton channel is thus consistent with recent data that the H^+ channel may consist of other proteins in addition to the proteolipid. The attractive speculation that perturbation of F_B by inhibitors (3) may produce a controlled H^+ leak is not substantiated, and it seems unlikely. Our data, however,

clearly support the conclusion (3) that F_B is a component of the H^+ channel of the bovine heart H^+-ATPase, functional in the reversible synthesis of ATP from the $\Delta\mu_{H^+}$.

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