*Biochimica et Biophysica Acta*, 459 (1977) 225–240


BBA 47231

# ENERGY TRANSDUCTION IN *ESCHERICHIA COLI*

## THE EFFECT OF CHAOTROPIC AGENTS ON ENERGY COUPLING IN EVERTED MEMBRANE VESICLES FROM AEROBIC AND ANAEROBIC CULTURES

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(Received July 5th, 1976)

SUMMARY

1. The transduction of energy from the oxidation of substrates by the electron transport chain or from the hydrolysis of ATP by the $Mg^{2+}$-ATPase was measured in everted membrane vesicles of *Escherichia coli* using the energy-dependent quenching of quinacrine fluorescence and the active transport of calcium.

2. Treatment of everted membranes derived from a wild-type strain with the chaotropic agents guanidine-HCl and urea caused a loss of energy-linked functions and an increase in the permeability of the membrane to protons, as measured by the loss of respiratory-linked proton uptake.

3. The coupling of energy to the quenching of quinacrine fluorescence and calcium transport could be restored by treatment of the membranes with *N,N'*-dicyclohyexylcarbodiimide.

4. Chaotrope-treated membranes were found to lack $Mg^{2+}$-ATPase activity. Binding of crude soluble $Mg^{2+}$-ATPase to treated membranes restored energy-linked functions.

5. Membranes prepared from a wild-type strain grown under anaerobic conditions in the presence of nitrate retained respiration-linked quenching of quinacrine fluorescence and active transport of calcium after treatment with chaotropic agents.

6. Everted membrane vesicles prepared from an $Mg^{2+}$-ATPase deficient strain lacked respiratory-driven functions when the cells were grown aerobically but were not distinguishable from membranes of the wild-type when both were grown under anaerobic conditions in the presence of nitrate.

7. It is concluded (a) that chaotropic agents solubilize a portion of the $Mg^{2+}$-ATPase, causing an increase in the permeability of the membrane to protons and (b)

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Abbreviation: DCCD, *N,N'*-dicyclohexyl-carbodiimide; $F_0F_1$, proton-translocating and DCCD-sensitive $Mg^{2+}$-ATPase; $F_0$, intrinsic membrane protein portion of the $F_0F_1$; $F_1$, extrinsic membrane protein portion of the $F_0F_1$. When prefixed with B, the terms $F_0F_1$, $F_0$ and $F_1$ refer to those isolated from a bacterial source, *Escherichia coli* in this case.

that growth under anaerobic conditions in the presence of nitrate prevents the increase in proton permeability caused by genetic or chemical removal of the catalytic portion of the $Mg^{2+}$-ATPase.

## INTRODUCTION

According to the hypothesis of Mitchell, the $Mg^{2+}$-ATPase or $F_0F_1$ ($BF_0F_1$ from bacterial sources) acts as a primary active transport system for protons [1, 2]. When an electrochemical proton gradient is established by action of the electron transport chain, the $F_0F_1$ uses that energy to catalyze the phosphorylation of ADP. When ATP is hydrolyzed by the $F_0F_1$, an electrochemical gradient of protons is established.

Studies of $F_0F_1$ complexes from a variety of organisms have led to the suggestion that the rate of flow of protons through the $F_0$ is governed by the presence of the $F_1$ [3–8]. One class of mutants of the $BF_1$ are defective in the coupling of respiratory energy to processes such as active transport of some sugars, amino acids and ions [3, 6–9], and the energy-dependent quenching of the fluorescence of quinacrine [6–8]. Removal of the $BF_1$ from the membrane of vesicles prepared from wild-type *Escherichia coli* (stripped vesicles) similarly causes a loss of these functions [3, 6–8]. Treatment of stripped or mutant membranes with *N,N'*-dicyclohexylcarbodiimide (DCCD), an inhibitor of the $BF_0F_1$ which binds to a polypeptide of the $BF_0$ [10, 11], restores respiratory-driven functions [3, 6–9], presumably by inactivating the proton channel function of the $BF_0$. Binding of $BF_1$ to stripped membranes or to the membrane of a mutant lacking the $BF_1$ likewise restores respiratory-driven functions [3, 6–9].

A recent report by Patel et al. [12] showed that treatment of right-side-out membrane vesicles of *E. coli* with chaotropic agents caused an increase in the permeability of the membrane to protons, with a concomitant loss of ability to couple respiration to active transport of some amino acids. Boonstra et al. [13] have recently shown that $BF_1$ mutants which normally lack coupling of respiratory energy to active transport accumulate substrates as well as the parental strains when both are grown under anaerobic conditions in the presence of nitrate.

This report extends the studies of Patel et al. [12] on the effect of chaotropic agents on energy transduction in *E. coli* membranes. Using the energy-dependent quenching of quinacrine fluorescence and the active transport of calcium as probes of energy coupling in everted membrane vesicles we confirm that treatment with chaotropic agents uncouple respiration from other energy-linked functions. Concomitantly there is a loss of proton uptake associated with respiration. Treatment with chaotropic agents also results in the loss of $Mg^{2+}$-ATPase activity. Binding of soluble $BF_1$ restored respiration-driven activities. These results suggest that a primary effect of chaotropic agents is the extraction of the $BF_1$, which results in an increase in the permeability of the membrane to protons, causing dissipation of the electronchemical gradient of protons established by the electron transport chain, leading, finally, to uncoupling of respiratory-linked functions.

In conjunction with our investigations into the role of the $BF_1$ in maintaining the electrochemical gradient of protons [6–9], we have studied the coupling of respiration to energy-linked functions in everted membranes of a wild-type and $BF_1$-deficient

mutant after growth under anaerobic conditions in the presence of nitrate. While the quenching of quinacrine fluorescence and active transport of calcium linked to respiration were defective in membranes of the mutant when grown under aerobic conditions, both were comparable to the activitites found in membrane of the parent when the mutant was grown under anaerobic conditions with nitrate. Moreover, respiratory-linked functions in wild-type membranes were resistant to extraction with chaotropic agents when the wild-type was grown under anaerobic conditions, even though the $BF_1$ was still extracted. These results confirm those of Boonstra et al. [13] and suggest that the $BF_0$ portion of the ATP synthetase complex is altered during growth under anaerobic conditions with nitrate such that removal by genetic alteration or chemical extraction no longer causes an increase in the passive flow of protons through the $BF_0$.

## MATERIALS AND METHODS

### *Growth of cells*

*Escherichia coli* K12 strain 7 [14] and its neomycin-resistant derivative strain NR70 [9] were grown aerobically in a basal salts medium [15] supplemented with 68 mM glycerol as a carbon source. When grown anaerobically, the medium was supplemented with 0.1 % yeast extract (Difco), 50 mM potassium nitrate, 1 $\mu$M sodium selenate and 1 $\mu$M ammonium molybdate. Cultures were harvested in exponential phase of growth, except that stationary phase cultures were used for the preparation of vesicles used to assay fluorescence quenching.

### *Preparation of everted vesicles*

Everted membrane vesicles were prepared as described previously [16]. Extraction with chaotropic agents was performed as follows. Suspensions of membrane vesicles (3–5 mg protein/ml) in a buffer consisting of 10 mM Tris · HCl, pH 7.2, containing 0.14 M KCl, 0.5 mM dithiothreitol, and 10 % glycerol (v/v) (buffer A) were adjusted to either 1.0 M guanidine-HCl or 5M urea by addition of a concentrated solution of chaotrope. Samples were incubated at 0 °C for 5–10 min, followed by centrifugation at 105 000 $\times g$ for 1 h. The pellet was washed once with buffer A and resuspended in the same buffer at a concentration of 3–5 mg of protein per ml. Reconstituted vesicles were prepared by incubating chaotrope-treated vesicles with crude soluble $BF_1$ (12 ATPase units per mg of membrane protein) at 23 °C for 15 min, followed by centrifugation for 1 h at 4 °. The pellet was washed twice with buffer A and resuspended in the same buffer at a protein concentration of 3–5 mg per ml.

### *Transport assays*

Calcium transport was measured under aerobic conditions as described previously [16]. When vesicles prepared from anaerobically grown cells were assayed, the assay buffer was supplemented with 10 mM potassium nitrate.

### *Other methods*

Previously described procedures were used for the measurement of proton translocation [7], quenching of quinacrine fluorescence [8], and ATPase activity [6]. Crude soluble $BF_1$ was prepared as described previously [16]. A previous report has described the preparation of antiserum to the $BF_1$ [16]. Protein concentrations were

estimated by a micromodification of the procedure of Lowry et al. [17] using bovine serum albumin as a standard.

*Chemicals*

$^{45}CaCl_2$ (1.3–1.4 Ci/mmol) was purchased from New England Nuclear Corp. DCCD was obtained from Eastman Organic Chemical Co. Quinacrine dihydrochloride was purchased from Sigma Chemical Co. Urea (special enzyme grade) was obtained from Schwartz/Mann. Guanidine-HCl (ultrapure) was obtained from Heico Chemical Co. All other chemicals were reagent grade and purchased from commercial sources.

## RESULTS

*Effect of anaerobic growth on the energy-dependent quenching of quinacrine fluorescence by everted membrane vesicles*

We have shown previously that the fluorescence of quinacrine becomes quenched in the presence of everted membranes of aerobically grown *E. coli* when either D-lactate, a substrate of the electron transport chain, or ATP, a substrate of the $BF_0F_1$ complex, is added [6, 8]. As shown in Fig. 1, α-glycerophosphate is also an effective substrate for causing quenching in everted membrane vesicles derived from aerobically grown cultures of strain 7, a wild-type K-12 strain. Formate, on the other hand, does not cause quenching, even when the cells are grown in the presence of formate. Everted membranes from aerobically-grown cultures of strain NR70, which lack the $BF_1$, shows little quenching caused by either D-lactate or α-glycerophosphate, unless the membranes are treated with DCCD. This is consistent with our previous results [7, 8], which showed that the $BF_1$ was necessary to maintain the impermeability of the membrane to protons. In the absence of the $BF_1$, the electrochemical gradient of protons established by the electron transport chain cannot be maintained, resulting in uncoupling of energy-linked membrane functions. The uncoupling is prevented by DCCD, which inactivates the proton channel of the $BF_0$ [3], restoring the proton impermeability of the membrane.

In contrast with the results obtained for aerobically grown cultures of strain NR70, membranes prepared from anaerobically grown cells show respiration-dependent quenching which is comparable to that found in strain 7 (Fig. 1). In those experiments formate was used as an energy donor. These results suggest that the membrane of strain NR70 is not proton-permeable when the strain is grown under anaerobic conditions. This effect is not due to the induction of a different $BF_1$, since ATP cannot drive quenching in such membranes (Fig. 1), and no $Mg^{2+}$-ATPase activity is found in everted vesicles prepared from anaerobically-grown NR70 (data not shown).

*Effect of chaotropic agents on energy-linked quenching of quinacrine fluorescence by everted membrane vesicles*

Fig. 2 illustrates the effects of treatment with 1 M guanidine-HCl on membranes derived from aerobically-grown cultures of strains 7 and NR70. It can be seen that guanidine treatment renders membranes from strain 7 similar to those of untreated NR70. In both cases, addition of crude soluble $BF_1$ restores respiratory-driven

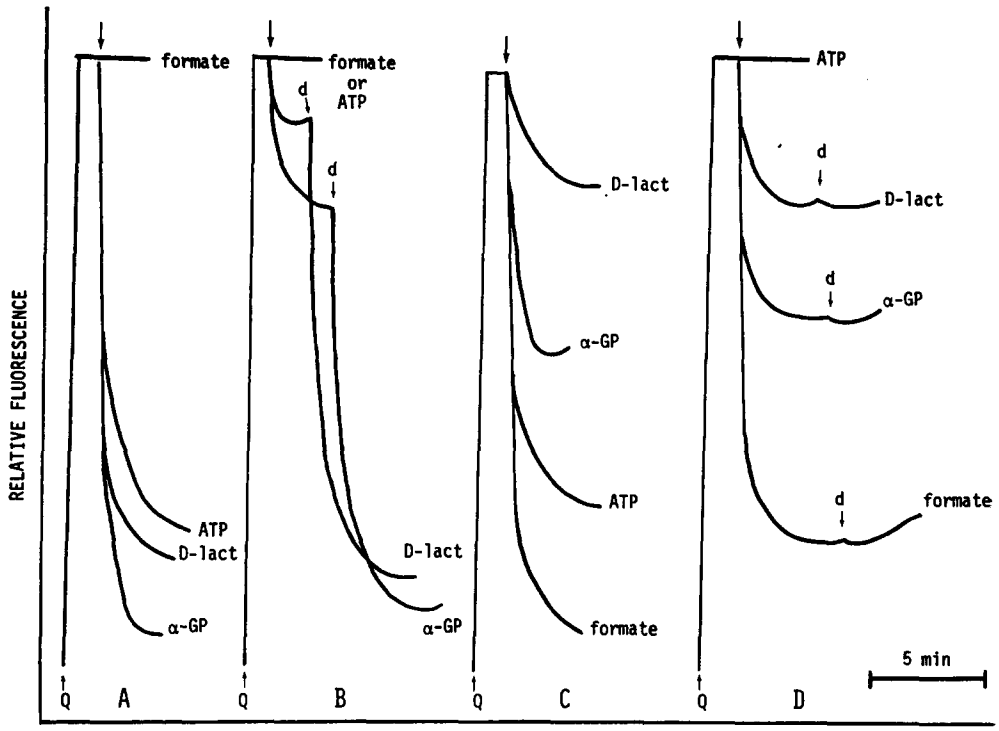


Fig. 1. Effect of aerobic and anaerobic growth on quenching of quinacrine fluorescence in membrane vesicles of strains 7 and NR70. Assays were performed at 23 °C in 2.5 ml of a buffer consisting of 10 mM Tris · HCl, pH 8.0, containing 5 mM $MgCl_2$ and 0.14 M KCl. Fluorescence was measured using an Aminco-Bowman spectrofluorometer with an excitation wavelength of 420 nm and an emission wavelength of 500 nm. At the lower arrow quinacrine (Q) was added to 0.2 $\mu$M. At the time indicated by the upper arrow energy sources were added as indicated at final concentrations of 20 mM formate, 4 mM ATP, 4 mM D-lactate, or 20 mM α-glycerophosphate. At the times indicated with d, DCCD was added to 30 $\mu$M. A, 237 $\mu$g of membrane vesicles prepared from aerobically-grown cells of strain 7. B, 280 $\mu$g of aerobic vesicles from strain NR70. C, 252 $\mu$g of membrane vesicles prepared from anaerobically-grown cells of strain 7. D, 240 $\mu$g of anaerobic vesicles from strain NR70.

quenching. The restoration observed by the addition of crude soluble $BF_1$ was prevented by the addition of anti-$BF_1$ serum but not by control serum (data not shown). Thus, even though a crude soluble fraction was used, it appears that the necessary component is the $BF_1$. This is similar to what occurs when membranes are stripped with an EDTA-containing buffer at low ionic strength (stripping buffer). Treatment with guanidine-HCl has been shown to remove enzymes, specifically the D-lactate dehydrogenase [18]. We have found that guanidine-treated membranes lack D-lactate dehydrogenase activity and show no D-lactate-dependent quenching even after reconstitution with soluble $BF_1$.

Our results also indicate that guanidine treatment removes the $BF_1$. Guanidine-treated membranes have little ATPase activity and bind soluble $BF_1$ in a saturable manner, with saturation occurring at about the same level as found in untreated membranes (Fig. 3). Moreover, immune diffusion of the guanidine extract demon-

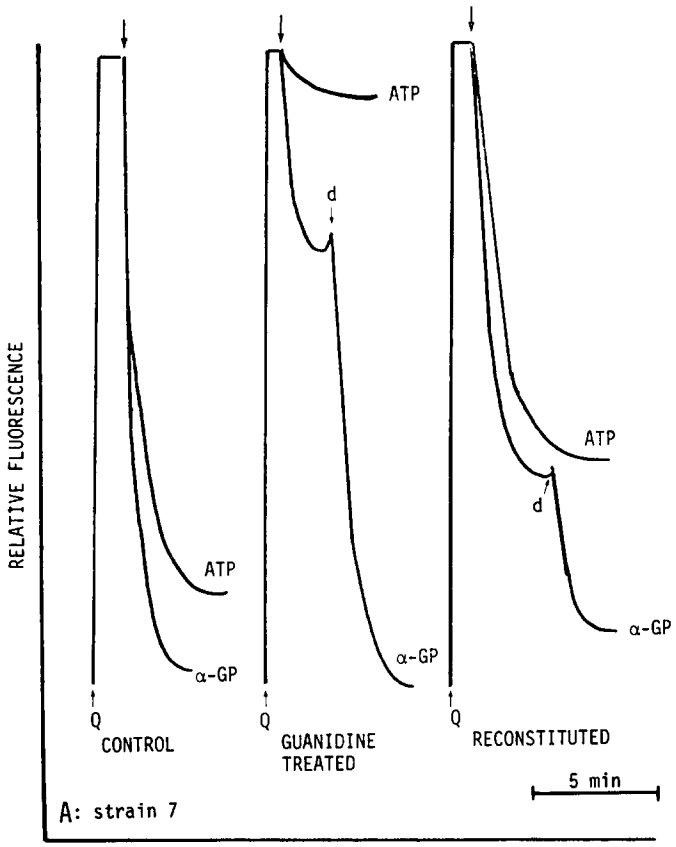


Fig. 2. For legend see next page.

strated a small amount of material which reacted with anti-$BF_1$ antiserum (data not shown). However, no ATPase activity was found in the guanidine extract, and the precipitin arc found upon immune diffusion was smaller than would have been expected if all of the $BF_1$ had been extracted in an active form. It appears that guanidine treatment inactiviates the $BF_1$, with at least some of it becoming solubilized. That treatment results in a loss of respiratory-driven fluorescence quenching, which is restored by rebinding of soluble $BF_1$ (Fig. 2). Since the amount of soluble $BF_1$ necessary to saturate the membrane is about the same as the amount on the membrane prior to extraction, (Fig. 3), it seems reasonable to conclude that guanidine treatment extracts the $BF_1$ from the membrane.

In the case of membranes prepared from anaerobically grown cultures, however, guanidine treatment has no effect on the quenching of quinacrine fluorescence caused by respiration with formate (Fig. 4). ATP-driven quenching in membranes of anaerobically-grown strain 7 is eliminated by guanidine treatment and is restored by binding of soluble $BF_1$ (Fig. 4). Thus guanidine treatment would appear to remove the $BF_1$ from membranes whether the cells are grown aerobically or anaerobically, resulting in a loss of ATP-driven quenching in both cases and restorable by rebinding

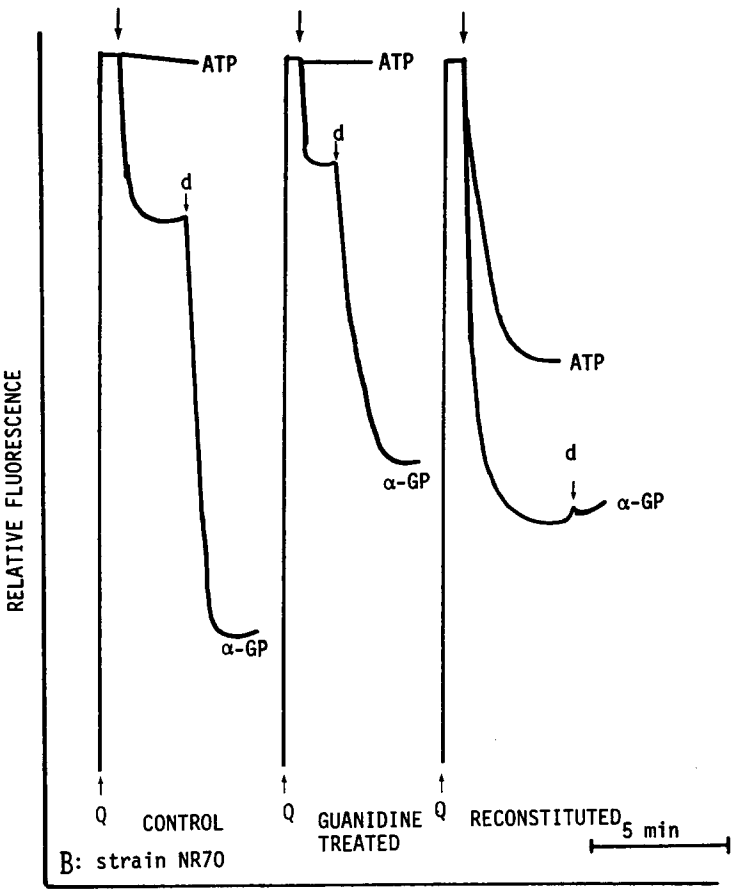


Fig. 2. Effect of guanidine-HCl treatment on quenching of quinacrine fluorescence in membrane vesicles of aerobically grown cells of strains 7 and NR70. All conditions were as given in the legend to Fig. 1. Guanidine-HCl treatment and reconstitution with crude $BF_1$ were performed as described under Methods. A, membrane vesicles from strain 7 with 265 $\mu$g of control membranes, 250 $\mu$g of guanidine-HCl treated membranes or 245 $\mu$g of reconstituted membranes. B, membrane vesicles from strain NR70 with 224 $\mu$g of control membranes, 200 $\mu$g guanidine-HCl treated membranes or 200 $\mu$g of reconstituted membranes.

of the $BF_1$ in both cases. But respiration-driven quenching in the case of membranes from anaerobically grown cells is not affected by guanidine extraction of the $BF_1$ or by genetic loss of the $BF_1$ in the case of strain NR70.

It should be mentioned that urea treatment gave essentially the same results as guanidine treatment, in all experiments except that urea treatment had no effect on the D-lactate dehydrogenase but decreased α-glycerophosphate dehydrogenase activity and α-glycerophosphate-driven fluorescence quenching. The stripping procedure, previously described for removing the $BF_1$ [8], eliminated formate dehydrogenase activity in membranes prepared from anaerobic cells, so that it was not possible to determine the effect of removal of the $BF_1$ by stripping on such membranes.

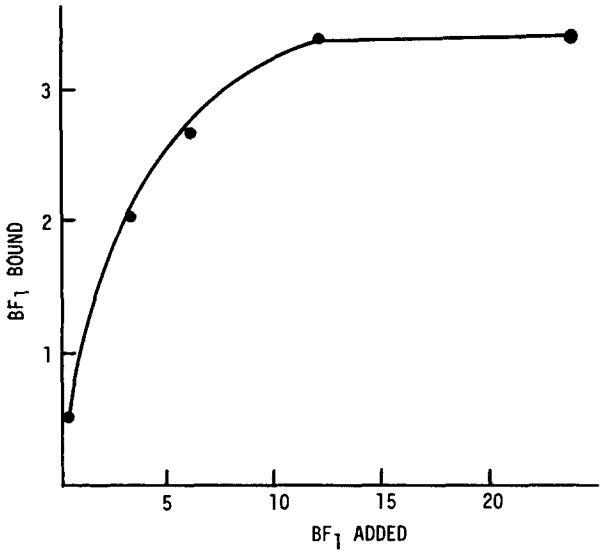


Fig. 3. Binding of crude $BF_1$ to guanidine-HCl treated membrane vesicles prepared from aerobically-grown cells of strain 7. Reconstitution was performed as described under Methods by incubating a suspension of guanidine-treated vesicles of strain 7 (1.8 mg of membrane protein per ml) with varying amounts of crude $BF_1$, followed by removal of unbound $BF_1$ by centrifugation and washing of the vesicles. Abscissa, units of $Mg^{2+}$-ATPase activity added per ml of membrane suspension. Ordinate, units of $Mg^{2+}$-ATPase activity bound per mg of membrane protein.

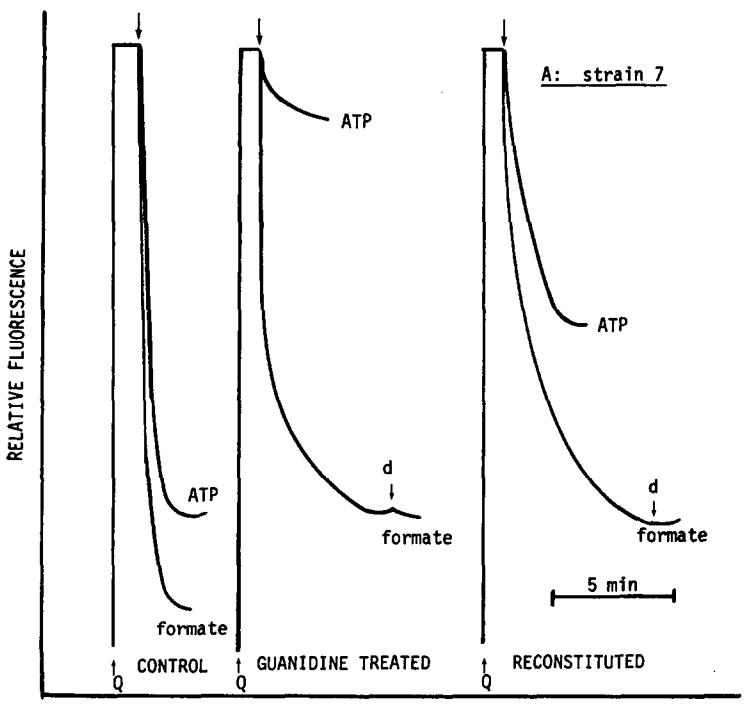


Fig. 4. For legend see next page.

*Effect of anaerobic growth and chaotropic agents on the active transport of calcium in everted membrane vesicles*

The active transport of calcium by everted membrane vesicles can be supported by respiration or ATP hydrolysis [16, 19]. As seen in Fig. 5, membranes prepared from anaerobically grown strain 7 cells loose most ability to accumulate calcium driven by ATP hydrolysis after treatment with guanidine. Transport activity is restored by rebinding soluble $BF_1$, again supporting the idea that guanidine extracts the $BF_1$. Similarly, calcium transport driven by oxidation of NADH is reduced by guanidine treatment, but can be restored either by rebinding of the $BF_1$ or by treatment with DCCD (Fig. 6). Formate-driven calcium transport in membranes from anaerobically grown strain 7, on the other hand, is only slightly reduced by guanidine treatment, with little effect of DCCD or soluble $BF_1$ (Fig. 6). ATP-driven transport is eliminated by guanidine treatment in either type of vesicle but is restored by rebinding of the $BF_1$ (date not shown). Again, those results are consistent with a removal of the $BF_1$ by guanidine treatment. Vesicles prepared from strain NR70 exhibit little respiratory-driven transport when the cells are grown aerobically, but have transport

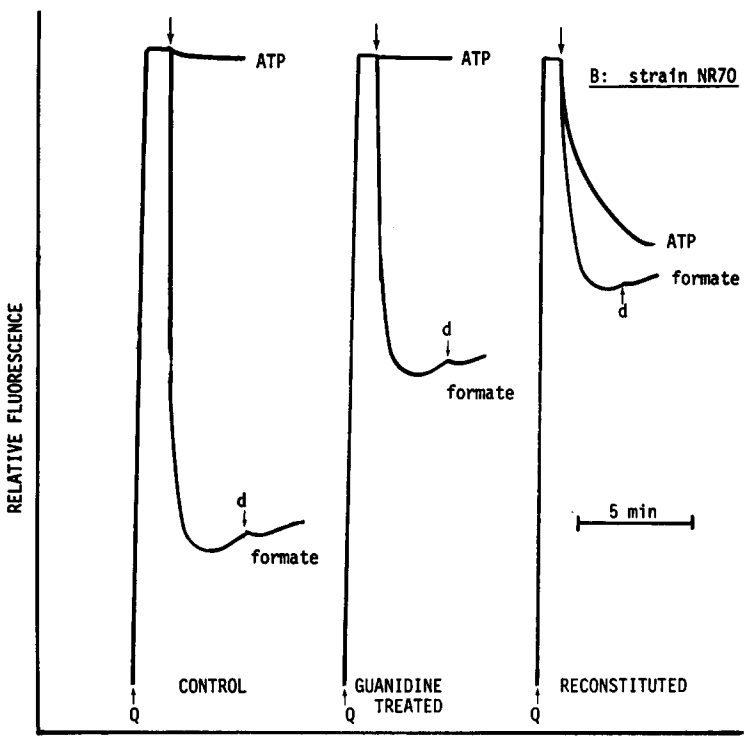


Fig. 4. Effect of guanidine-HCl treatment on quenching of quinacrine fluorescence in membrane vesicles of anaerobically grown cells of strains 7 and NR70. All conditions were as given in the legend to Fig. 1. A, membrane vesicles from strain 7 with 224 $\mu$g of control membranes, 200 $\mu$g of guanidine-HCl treated membranes, or 200 $\mu$g of reconstituted membranes. B, membrane vesicles from strain NR70 with 200 $\mu$g of control membranes, 150 $\mu$g of guanidine-HCl treated membranes of 150 $\mu$g of reconstituted membranes.

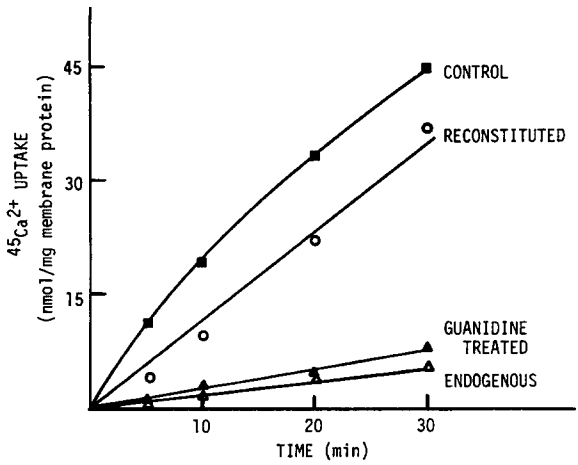


Fig. 5. Effect of guanidine-HCl treatment on ATP-driven calcium transport in membrane vesicles prepared from anaerobically grown cells of strain 7. Calcium transport was performed as described under Methods using 5 mM ATP+5 mM $MgCl_2$, ■–■, control membranes; ▲–▲, guanidine-HCl treated membranes; ○–○, reconstituted membranes; △–△, control membranes with no added energy source.

comparable with the wild-type when grown anaerobically (Fig. 7). DCCD has a large effect on membranes from aerobically grown NR70, but only a small effect on those from anaerobically-grown cultures (Fig. 7). Guanidine treatment has no significant effect on calcium transport driven by formate respiration in membranes prepared from anaerobically grown cells.

As mentioned above for the quenching of quinacrine fluorescence, urea treatment affected calcium transport in vesicles in much the same manner as did guanidine.

*Proton translocation driven by respiration in guanidine-treated everted membrane vesicles*

The $BF_1$ has previously been shown to be necessary in aerobically grown cells not only for its catalytic role in ATP synthesis, but also in maintaining the impermeability of the membrane to protons [3, 6–8]. Such an increase in proton permeability results in uncoupling, much in the same way that artificial proton conductors do. If the effect of chaotropic agents on energy coupling were the removal of the $BF_1$, then it should be reflected in the permeability of the membrane to protons. When everted membranes are incubated with D-lactate in the absence of oxygen, the pH of the solution remains constant. Addition of oxygen results in an increase in the pH of the medium, reflecting electrogenic translocation of protons by the electron transport (Fig. 8). After the oxygen is used up, the proton gradient collapses, resulting in an efflux of protons. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, a potent proton conductor, prevents alkalinization of the medium, presumably by discharging the proton gradient as fast as the electron transport chain can establish it. Urea treatment prevents alkalinization of the medium as well (Fig. 8). Urea treatment was used in these experiments because it does not affect oxidation of D-lactate. Since DCCD, which is known to decrease the flow of protons through the $BF_0$, restores

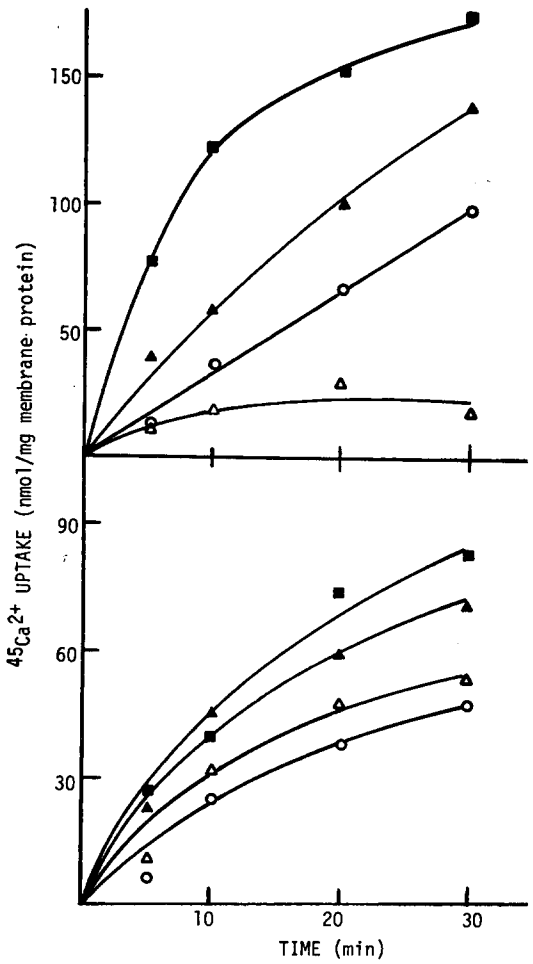


Fig. 6. Effect of guanidine-HCl treatment on respiratory-driven calcium transport in membrane vesicles prepared from aerobically- and anaerobically-grown cells of strain 7. Top: calcium transport in vesicles of aerobically-grown cells using 5 mM NADH as an energy source. Bottom: calcium transport in vesicles of anaerobically-grown cells using 50 mM formate as an energy source. ■–■, control vesicles; △–△, guanidine-HCl treated vesicles; ▲–▲, guanidine-HCl treated vesicles+30 $\mu$M DCCD; ○–○, reconstituted vesicles.

respiratory-driven proton translocation, it is reasonable to assume that the electron transport chain was still capable of proton transportation even after urea treatment, but that the proton gradient could not be maintained. In confirmation of that idea, rebinding of the $BF_1$ to the urea-treated membranes restored D-lactate-driven proton uptake (Fig. 8).

Respiration-driven proton movements in membranes prepared from anaerobically grown cells were not measured, since the amounts of membranes required for such assays would have required larger volumes of anaerobic cultures than were possible in our laboratory.

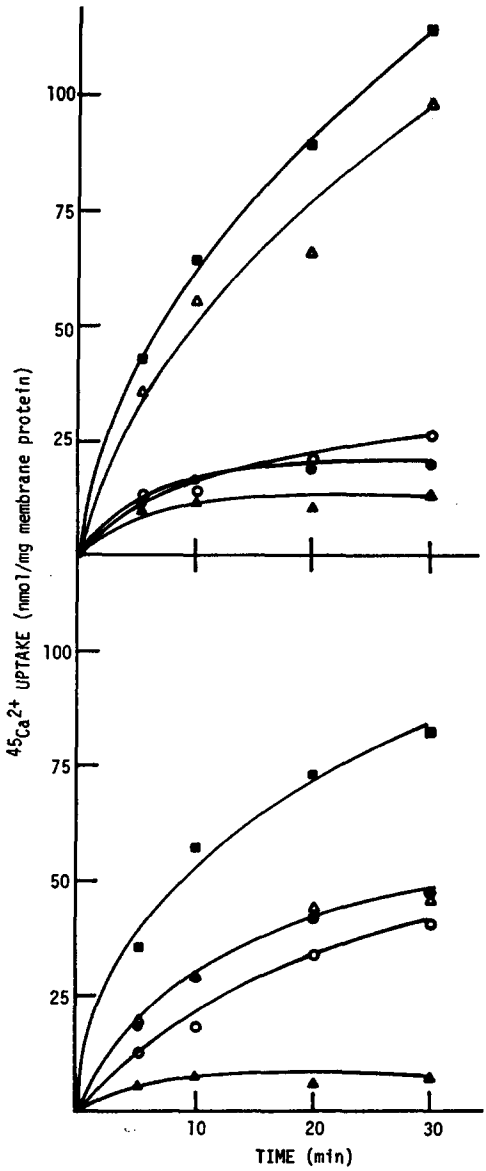


Fig. 7. Effect of guanidine-HCl treatment on respiratory-driven calcium transport in membrane vesicles prepared from aerobically and anaerobically grown cells of strain NR70. Top: calcium transport in vesicles of aerobically grown cells using 5 mM NADH as an energy source. Bottom: calcium transport in vesicles of anaerobically grown cells using 50 mM formate as an energy source. ●–●, control vesicles; ○–○ guanidine-HCl treated vesicles; △–△ guanidine-HCl treated vesicles + 30 $\mu$M DCCD; ■–■, control vesicles + 30 $\mu$M DCCD; ▲–▲, control vesicles with no added energy source.

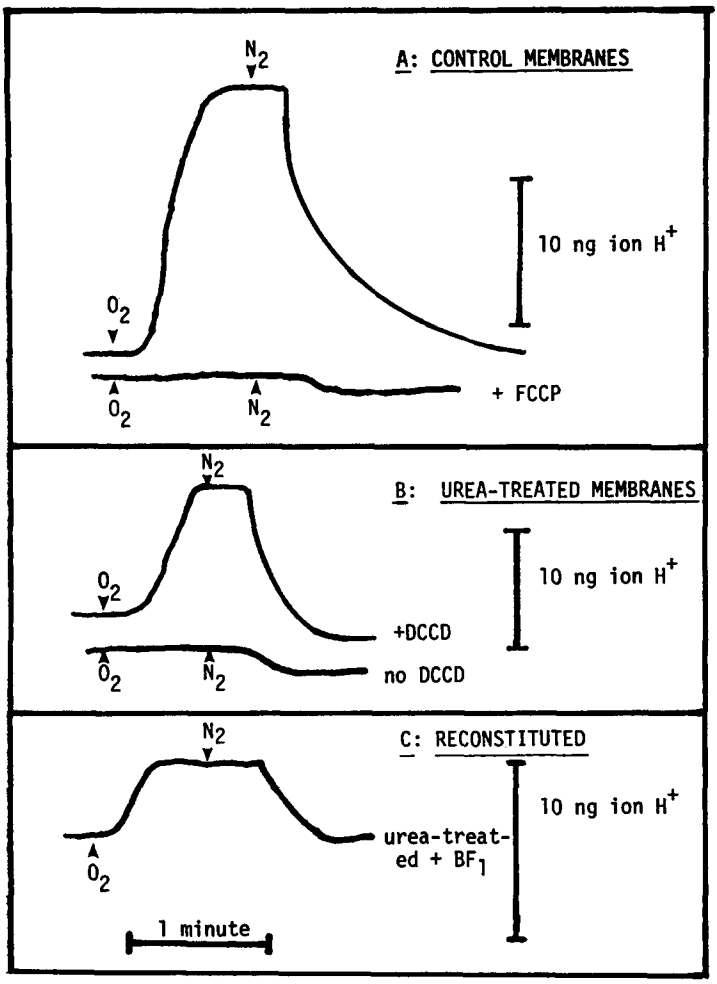


Fig. 8. Effect of urea treatment on proton translocation in membrane vesicles prepared from aerobically grown cells of strain 7. Proton translocation was assayed as described under Methods using 5 mM D-lactate as an oxidizable substrate. Oxygen was introduced by replacing the nitrogen stream with a stream of water-saturated air for 20 s, after which the air was again replaced with nitrogen gas. An upward deflection indicates an increase in pH of the outer medium, reflecting translocation of protons into the vesicles. Standard solutions of HCl were used to calibrate the pH changes. A: 3.5 mg of control vesicles in the absence (upper curve) or presence (lower curve) of 5 $\mu$M FCCP. B: 3 mg of urea-treated vesicles in the presence (upper curve) of 0.15 mM DCCD or absence (lower curve) of DCCD. C: 3 mg of reconstituted vesicles.

## DISCUSSION

Patel et al. [12] have reported that treatment of right-side-out membrane vesicles prepared from aerobically-grown *E. coli* with the chaotropic agents guanidine-HCl or urea causes an increase in the permeability of the vesicles to protons, leading to a reduction in the ability to accumulate amino acids. Moreover, the increase in

proton permeability could be reversed by treatment with carbodiimides, resulting in restoration of active transport. Our results confirm and extend those observations.

Our studies utilized everted membrane vesicles instead of right-side-out ones to allow for the measurement of energy-dependent quenching of quinacrine fluorescence and of calcium transport and to study the effect of binding soluble $BF_1$ to membranes. Considering first vesicles prepared from aerobically grown cells, it is clear from measurements of quinacrine fluorescence that the chaotropic agents guanidine-HCl and urea decrease fluorescence quenching whether energized by energy derived from oxidation of compounds by the electron transport chain or by hydrolysis of ATP by the $BF_0F_1$. Likewise, calcium transport is decreased by treatment with those chaotropes. We have also found that treatment with chaotropic agents increases the permeability of the membrane of everted vesicles to protons, as Patel et al. [12] found in right-side-out vesicles.

Patel et al. [12] were unable to find a relation of the effect of chaotropic agents with the $BF_0F_1$ complex. A number of laboratories have determined that exposure of the $F_0$ portion of the ATP synthetase complex from a variety of sources by removal of the $F_1$ portion results in an uncoupling of energy-linked reactions [4, 5, 20–22]. Rebinding of the $F_1$ or treatment with carbodiimides could relieve the uncoupling in some cases. Our own results have shown that removal of the $BF_1$ from a wild-type strain of *E. coli* causes a loss of respiratory-driven quenching of quinacrine fluorescence [6, 8], of transport [6, 8] and of proton impermeability [6, 8]. Genetic modification or loss of the $BF_1$ likewise results in those properties and in the loss of active transport of sugars and amino acids in whole cells and right-side-out vesicles as well [3, 6–9]. In all cases we were able to restore active transport by treatment with DCCD [6–9], but only when everted membrane vesicles were used was it possible to demonstrate that binding of soluble $BF_1$ could restore respiratory- and ATP-driven fluorescence quenching and respiratory- and ATP-driven calcium transport [6]. Those observations suggested that the effect of chaotropic agents might be the removal of the $BF_1$. It has been shown, further, that some proteins are extracted from the membrane of *E. coli* by guanidine-HCl [18].

Binding a crude soluble $BF_1$ to chaotrope-treated membranes of wild-type or $BF_1$-deficient strains restored the ability of such vesicles to catalyze the energy-dependent quenching of quinacrine fluorescence and transport of calcium. Thus, the effect seems to be related specifically to a removal of some component in the crude soluble fraction. Since our earlier results with purified $BF_1$ showed that it was that component of the crude soluble fraction which restored energy-linked functions in the $BF_1$-deficient mutant and in the wild-type following removal of the $BF_1$ [6] and, since the effect in this case was prevented with antiserum prepared against purified $BF_1$, it is reasonable to conclude that the $BF_1$ was the necessary component in this case as well. Since we have previously shown that removal of the $BF_1$ results in an increase in the permeability of the membrane to protons [6, 8] and since rebinding of the $BF_1$ decreased the permeability of everted membrane vesicles to protons, as shown in this study, it is again reasonable to conclude that the increase in permeability to protons caused by treatment with chaotropic agents was due specifically to removal of the $BF_1$. Thus, the uncoupling of energy-linked functions was a tertiary effect following an inability to maintain an electrochemical gradient of protons, which in turn followed extraction of the $BF_1$ by the chaotropic agents. However, since chaotropic

agents affect numerous proteins, it is possible that part of the observed effect of those reagents is due to other factors. Quantitation of the electrochemical gradient of protons established by vesicle preparations treated in various ways would be necessary to answer that question.

A more interesting question is the effect of anaerobic growth on the $BF_0F_1$ complex. In membranes of cells grown under aerobic conditions, removal of the $BF_1$, whether by mutation [3, 6–9] or extraction with EDTA in a buffer of low ionic-strength [3, 6, 8] or chaotropic agents results in decrease in activity of reactions which are linked to a protonmotive force, as discussed above. Boonstra et al. [13] recently reported that the aspect of the phenotype of $BF_1$-deficient strains of *E. coli*, including strain NR70, was suppressed when the cells were grown under anaerobic conditions without reversing the primary defect in the $BF_1$. Our results tend to confirm that observation. Membranes prepared from strain NR70 grown under anaerobic conditions in the presence of nitrate to induce nitrate reductase exhibited respiratory-driven calcium transport and quenching of quinacrine fluorescence at nearly the same level as strain 7, the parental strain. Only a slight stimulation was found after treatment with DCCD. Moreover, treatment with guanidine-HCl had little effect on those processes, nor did binding of crude $BF_1$ stimulate further. Boonstra et al. [13] found that membranes from the wild-type strains were still sensitive to guanidine-HCl treatment; however, our findings suggest that the membranes from anaerobically grown strain 7 give essentially the same results as those from strain NR70.

Growth under anaerobic conditions suppressed the defect in respiratory-driven reactions in strain NR70, but had no effect on ATP-driven ones. Binding of $BF_1$ was necessary to restore ATP-driven reactions in membranes from NR70 and in guanidine-HCl-treated membranes from strain 7. Moreover, anaerobically grown NR70 still lacked azide-sensitive $Mg^{2+}$-ATPase activity. Those results confirm the fact that growth under anaerobic conditions does not revert NR70 to a $BF_1$-positive strain.

The major question is, then, the mechanism by which anaerobic growth suppresses the uncoupling found in $BF_1$-deficient strains. One possibility is that the $BF_0$ is modified in some way to decrease the rate at which it translocates protons. In that case the permeability of the membrane of strain NR70 to protons would be decreased. However, the membrane of anaerobically-grown strain 7, the parental strain, appears to be resistant to guanidine-HCl treatment with respect to energy-linked functions, which suggests that the effect of anaerobic growth is not limited to $BF_1$-deficient strains. Also, binding of $BF_1$ to the membrane of mutant or parent restored ATP-driven functions. If the $BF_0$ were much less permeable to protons, that might interfere with phosphorylation of ADP coupled to the anaerobic electron transport chain during growth of the parent. It might also prevent ATP-driven reactions from occurring after binding of $BF_1$ to the mutant. On the other hand, it is difficult to understand how respiratory-driven functions could be suppressed in strain NR70 if the membrane of that strain were unable to maintain an electrochemical gradient of protons. It may be that the interaction between the $F_0$ and $F_1$ portions of the complex is altered such that the $BF_0$ is active in translocating protons only when the $BF_1$ is present. That would still entail a modification of the $BF_0$ during anaerobic growth, since the $BF_1$ used for reconstitution experiments was obtained from aerobically grown strain 7. It would be of great interest to determine the permeability of the membrane of strain NR70 to protons after anaerobic growth. However, we were unable to culture cells in large

enough quantities to perform such measurements. Even if the membrane were less permeable to protons, as might be expected, it would still not explain the mechanism by which that impermeability is maintained. Thus, further investigations into the differences between the $BF_0F_1$ complexes of aerobically and anaerobically grown cells should be performed.

ACKNOWLEDGEMENTS

We thank Dr. T. Tsuchiya for his useful suggestions and Mrs. S. Tucker for technical assistance. This research was supported in part by USPHS Grant No. GM-21648 from the National Institute of General Medical Sciences, Grant No. BMS-75-15986 from the National Sciences Foundation, and was aided by a Basil O'Connor grant from the National Foundation-March of Dimes.

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