THE PROTON-TRANSLOCATING ATPase OF *ESCHERICHIA COLI*

Ian C. WEST * and Peter MITCHELL
Glynn Research Laboratories, Bodmin, Cornwall PL30 4AU, UK

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1. Introduction

It was postulated by Mitchell [1, 2] that the ATPase systems of mitochondrial cristae membranes, chloroplast grana membranes and bacterial plasma membranes perform an essential bioenergetic function by coupling the reversible hydrolysis of ATP to the translocation of protons across the membrane. Direct observation of proton translocation by appropriate pH measurements during ATP hydrolysis was subsequently reported for mitochondria [3–5], chloroplast grana [6] and chromatophores from photosynthetic bacteria [7]. However, in spite of the important and suggestive work of Harold [8], the proton-translocating property of the ATPases of non-photosynthetic bacteria does not yet seem to have been directly demonstrated. In the absence of this crucial information, students of bacterial ATPase systems have unfortunately tended to regard an easily-dissociable water-soluble fragment of these systems (corresponding to the F_1 component of mitochondrial and chloroplast ATPases [9]) as the 'pure ATPase' or simply as 'the ATPase'.

The present paper attempts to encourage more functionally relevant studies of bacterial ATPase systems by showing, with direct pH measurements, that protons are translocated during the DCCD-sensitive hydrolysis of ATP by membrane vesicles prepared from *Escherichia coli*.

* Present address: Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

Abbreviations: DCCD: dicyclohexylcarbodi-imide; FCCP: carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; pH_0 $-\log_{10}$ (chemical activity of H^+ ions in the outer aqueous phase); $\rightarrow H^+/P$: proton translocation quotient giving number of protons translocated per ATP molecule hydrolysed.

2. Materials and methods

E. Coli ML308-225 was grown at 37°C on a mineral medium with succinate as carbon source containing (in 1 litre): 7.0 g of KH_2PO_4, 3.2 g of Na_2HPO_4, 2.0 g $(NH_4)_2SO_4$, 0.41 g of $MgSO_4 \cdot 7H_2O$, 1 mg of $CaCl_2$ and 2.5 g of succinic acid. The medium was adjusted to pH 7.0 with KOH. The organisms were harvested one hour after growth had become limited by exhaustion of succinate, when the cell density was 0.5–0.7 mg cell dry weight/ml.

Membrane vesicles were prepared by the procedure of Kaback [10] and were stored overnight at 4°C. For most experiments the stock suspension of membrane vesicles (7–10 mg of protein/ml) was made 5 mM with respect to $MgCl_2$ and sonicated for 30 sec using an MSE 60 W sonicator at full power, with the suspension and the probe initially cooled to 0°C.

Techniques for measuring the outer pH (pH_0) of vesicle suspensions under anaerobic conditions, and for adding anaerobic solutions, have been described previously [11]. The hydrolysis of ATP was followed essentially by the method of Nishimura, Ito and Chance [12]. At pH_0 6.25 in the presence of 5 mM Mg^{2+} about 0.15 moles of H^+ are produced per mole of ATP hydrolysed, while in the absence of Mg^{2+} about 0.3 moles H^+ are produced. At pH_0 7.05 the quantities of H^+ produced per ATP hydrolysed are 0.72 with Mg^{2+} and 0.80 without Mg^{2+} (J. Moyle, personal communication; and see Alberty [13]).

Proton translocation was estimated from the pH_0 changes, making allowance for net acidification due to ATP hydrolysis. The net acidification was observed in the presence of FCCP which equilibrates the electrochemical of H^+ across the membrane. Proton

translocation plus net acidification was observed in the presence of valinomycin, which makes the membrane permeable to K^+ ions and prevents H^+ ions being drawn back by the membrane potential which would otherwise develop. Most experiments were done at pH_0 6.25 to minimise the net pH_0 changes directly due to ATP hydrolysis compared with the pH_0 changes due to proton translocation across the vesicle membrane. The anaerobic ATP solution was titrated to pH_0 6.250 so that there was no initial displacement of the pH_0 trace on adding ATP.

Stock solutions of valinomycin (2 mg/ml), DCCD (100 mM) and FCCP (1 mM) were prepared in ethanol. Standard acid (50 mM HCL) and alkali (50 mM KOH) were made up in 100 mM KCl and freed of O_2 by evacuating, and flushing with O_2-free N_2. Carbonic anhydrase (25 μg/ml, final concentration) was routinely added to catalyse the equilibration of the CO_2–H_2CO_3–HCO_3^- system.

Protein was determined by the method of Lowry et al. [14], standardizing with bovine serum albumin.

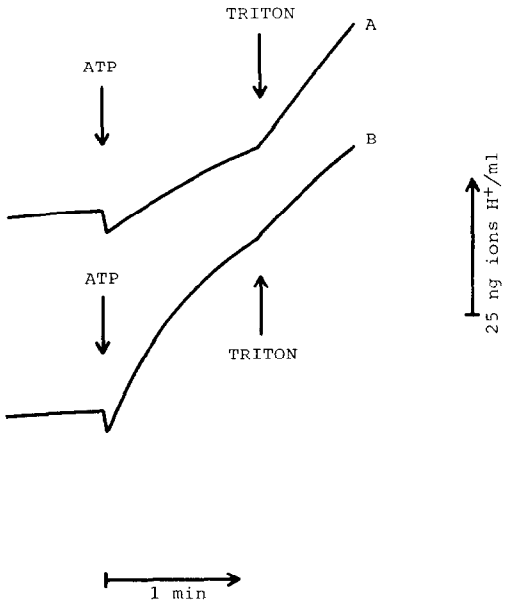

Fig. 1. Strip-chart recordings of pH_0 on adding ATP to aerobic suspensions of membrane vesicles. The suspension contained (in 4 ml): 2.44 mg of vesicle protein, 150 mM KCl, 5 mM $MgSO_4$, 2.86 mM glycylglycine. The pH_0 was adjusted to 7.05 and the vesicles were equilibrated at 25° C for 20 min. Where indicated, 20 μl of ATP solution (50 mM Na_2H_2ATP containing 55 mM $MgCl_2$ adjusted to about pH 7.1 with KOH), or 50 μl of Triton x-100 solution (20 g/l) were added. The pH_0 displacement (0.031 pH units) caused by the addition of 100 nmoles of HCl was determined after the addition of ATP. (A) Unsonicated vesicle suspension. (B) Sonicated vesicle suspension.

3. Results and discussion

There was virtually no hydrolysis of extracellular ATP by intact cells of *E. Coli,* presumably because the active site of the ATPase is accessible only from inside the intact plasma membrane, and no specific ATP/ADP antiporter, such as that of mitochondria, is present in the membrane. Membrane vesicles prepared by Kaback's method hydrolysed externally added ATP rather slowly, but the addition of Triton x-100 to a final concentration of 0.25 g/l caused considerable stimulation as shown in the typical experiment of fig. 1, curve A. Brief sonication (30 sec) of the Kaback vesicle preparations also caused considerable stimulation of ATPase activity, whereas the addition of Triton x-100 to these briefly sonicated suspensions caused only a relatively small further stimulation (fig. 1, curve B). It is concluded that most of the membrane vesicles of the Kaback preparations are topologically closed and retain the outside-out orientation of the original bacteria, but that some broken or inverted membrane material is also present.

By analogy with work on mitochondria (see [15]) and *Mycobacterium phlei* [16], it appeared possible that the increase in ATPase activity observed on sonication of Kaback vesicles might be partly caused by the formation of topologically closed inside-out vesicles rather than by mere fragmentation. We therefore used sonicated preparations in order to examine the possibility of H^+ translocation during ATP hydrolysis.

When the anaerobic suspensions of sonicated vesicles contained FCCP but not valinomycin, the production of net acid, corresponding to ATP hydrolysis, began immediately after adding anaerobic ATP solution (fig. 2, curve A). However, when the FCCP was replaced by valinomycin, there was a period of about 20 sec during which the suspension medium went alkaline (fig. 2, curve B). Fig. 2, curve C, shows the pH_0 changes corrected for the net acid produced during ATP hydrolysis. It can be seen that there was

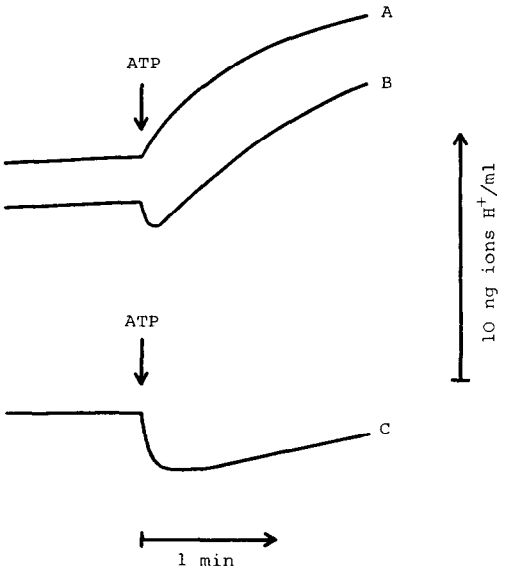

Fig. 2. Strip-chart recordings showing changes in pH_0 on adding anaerobic ATP solution to anaerobic suspensions of sonicated membrane vesicles. The suspension contained (in 4 ml): 1.49 mg of membrane protein, 150 mM KCl, 5 mM $MgCl_2$, 3 mM glycylglycine, 0.1 mg of carbonic anhydrase, and valinomycin or FCCP as indicated. The suspension was adjusted to pH_0 6.25 and incubated anaerobically for 30 min. Where indicated, 10 $\mu\ell$ of ATP solution (50 mM Na_2H_2ATP containing 55 mM $MgCl_2$ adjusted to pH 6.250 with KOH) was added. (A) Contained 2.5 μM FCCP; (B) contained 4 μg of valinomycin; (C) is obtained by subtracting pH_0 in curve A from that in curve B, and represents inward proton translocation.

a rapid inward translocation of protons, presumably until a partial steady state was established between inward translocation and outward leakage across the vesicle membrane. The subsequent slow return of pH_0 towards the new pH_0 baseline occurred as ATP hydrolysis approached completion and the rate of hydrolysis decreased, as shown by curve A of fig. 2. When the Mg^{2+} concentration was lowered to 140 μM by omitting the $MgCl_2$ normally added, the ATPase activity was depressed to 8% of normal, and the H^+ translocation was barely significant. In the normal medium, the presence of 25 μM DCCD virtually abolished the ATPase activity, and 2 mM NaN_3 inhibited the ATPase activity by 88%.

In several experiments like that of fig. 2, by comparing the initial rate of inward proton translocation (curve C) with the initial rate of ATP hydrolysis (curve A), we obtained an $\rightarrow H^+/P$ quotient of 0.58 $\pm$0.18 (S.D., n = 6) ng ions H^+ translocated/nmoles ATP hydrolysed. As it was not expedient in this work to allow for the leakage of H^+ back across the vesicles or to compensate for the hydrolysis of ATP by membranes which had not resealed with the inside-out orientation, the actual proton-translocation stoicheiometry of the ATPase system can safely be assumed to be considerably higher than the $\rightarrow H^+/P$ quotient observed here.

Further work will be required to decide whether the ATPase system of *E. Coli* may exhibit the same $\rightarrow H^+/P$ stoicheiometry (near 2.0) as mitochondrial and chloroplast ATPase systems [3–6]. Meanwhile, it may be helpful in the planning of future research to note that the membrane-located ATPase of *E. coli*, which has been the object of considerable biochemical [17–21] and genetic [22] research, resembles the proton-translocating ATPases of mitochondria and chloroplasts in many respects including the following. Part of this ATPase system corresponds to mitochondrial or chloroplast F_1 and can be isolated from the membrane in soluble form by washing with media of low ionic strength or with detergent. The complete ATPase is activated by Mg^{2+}, and Mg^{2+} also plays a part in attaching the F_1 component to the rest of the ATPase (F_0) which is integral with the membrane. The specificity of the ATPase towards nucleoside triphosphates is low. Activity of the complete F_0F_1 ATPase, but not of the soluble component F_1, is inhibited by DCCD. The ATPase activity of the component F_1 is released from the membrane component, but not when F_1 is cold-labile when F_1 is in the complete F_0F_1 complex. The complete ATPase is inhibited by azide. Under certain conditions, ATPase activity is increased by treatment with trypsin, and this effect may be attributable to the destruction by trypsin of a specific polypeptide inhibitor component of the ATPase. Dissociation of F_1 from the membrane causes energetic uncoupling that appears to arise from the conduction of protons through the F_0 component in the membrane, and this uncoupling effect can be specifically reversed by the ATPase inhibitor DCCD. The ATPases of bacteria other than *E. coli* have also been shown to exhibit similar characteristics [8].

The facts listed above, together with our finding that the complete ATPase system of *E. coli* couples

the translocation of protons to ATP hydrolysis, suggests that the ATPase system of *E. coli* is a member of a large class of reversible proton-translocating ATPases that is widely distributed in the animal, plant and microbial kingdoms.

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