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ELECTRICAL RESPONSE OF BEEF-HEART SUBMITOCHONDRIAL PARTICLES BOUND TO PHOSPHOLIPID-IMPREGNATED MILLIPORE FILTERS DURING ATP HYDROLYSIS

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Summary

1. Beef heart submitochondrial particles bound to asolectin impregnated Millipore filter, according to the method described earlier (Drachev et al. (1974) Nature 249, 321—324), are able to generate, upon addition of ATP, an electrical potential which can be directly measured.

2. The transmembrane electrical potential generated by ATP hydrolysis reaches values up to 80 mV. The half-time required to attain the plateau of potential is paradoxically long (5 to 10 min at room temperature) and is temperature-dependent. Among different phospholipid species which have been used to impregnate the Millipore filter, phosphatidylethanolamine was found to be the most effective for generation of electrical potential.

3. The potential generated by ATP hydrolysis is inhibited by inhibitors of mitochondrial ATPase, by the uncoupler FCCP and by reagents collapsing the membrane potential.

4. Addition of inhibitors of mitochondrial ATPase, when the plateau of potential is attained, results in a decay of potential. This decay of potential is as slow as the generation of potential induced by ATP hydrolysis.

5. The initial rise in electrical potential is proportional to the ATPase activity.

Abbreviations: FCCP, *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone; $\Delta\psi$, membrane potential; ΔpH, pH gradient; ΔU, potential difference measured in the 'thick membrane system'.

Introduction

Drachev et al. [1] have described a convenient means to follow the electrogenic properties of enzymatic systems embedded in lipid vesicles. They bound the vesicles to a planar thick phospholipidic membrane separating two aqueous compartments; the electrical current generated by this system was then measured using standard electrical devices. This method, referred to as the 'thick membrane system', has been extensively studied with reconstituted proteoliposomes, in which the embedded protein has electrogenic properties [2—9], and extended to non-reconstituted systems [10—12]. To be fully valid, this method must lead to an electrical signal quantitatively related to the adhered proteins, i.e. (1) proportional to the amount of protein bound to the filter-this relationship has been clearly demonstrated with bacteriorhodopsin [6], (2) proportional to the enzymic activity of the embedded protein. For this purpose, mitochondrial ATPase is a suitable tool, because the activity of this enzyme can be easily controled by appropriate inhibitors. Proteoliposomes reconstituted from oligomycin-sensitive H^+-ATPase were shown to generate an electrical signal upon ATP hydrolysis [5]. However, the generated potential with the purified enzyme was too low (10—15 mV) to study the correlation between ATPase activity and the electrical signal. Submitochondrial particles from beef heart mitochondria appeared to be a more suitable material. In fact (1) the potential generated by submitochondrial particles in the 'thick membrane system' is 5 to 10 times higher than that generated by proteoliposomes containing the oligomycin-sensitive H^+-ATPase (80—100 mV vs. 10—15 mV) [10,11], (2) the ATPase complex in submitochondrial particles is more stable than the isolated ATPase complex. Furthermore, the submitochondrial particles used are inside-out and ATP has a direct access to the catalytic site of ATPase. The results presented here show that the electrical signal generated by submitochondrial particles upon ATP hydrolysis, and detected by the 'thick membrane system' is inhibited by ATPase inhibitors to the same extent as ATPase activity. The signal is abolished by uncouplers and by reagents collapsing the membrane potential ($\Delta\psi$), but not by reagents collapsing the pH gradient (ΔpH) across the mitochondrial membrane.

Materials and Methods

Reagents. ATP, phospho*enol*pyruvate, pyruvate kinase, NADH and lactate dehydrogenase were obtained from Boehringer; oligomycin from Serva; *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone (FCCP) and 4-chloro-7-nitrobenzofurazan from Pierce; valinomycin from Calbiochem; and asolectin from Associated Concentrates. Phosphatidylcholine was extracted from egg yolk as described in [13], the other phospholipids extracted and purified according to Ref. 14. Nigericin was a generous gift from Dr. R.J. Hosley (Lilly Research Laboratories, Indianapolis, IN). 4-chloro-7-nitrobenzo[^{14}C]-furazan was purchased from Commissariat à l'Energie Atomique (Saclay, France).

Mitochondria and submitochondrial particles. Beef heart mitochondria were prepared according to Smith [15] and stored at −20° C. EDTA-sub-

mitochondrial particles and Mg-ATP-submitochondrial particles were prepared by sonication of the thawed mitochondria according to Löw and Vallin [16] and stored at −70° C. Both types of particles yielded similar results.

Inhibition by 4-chloro-7-nitrobenzofurazan was performed essentially as described by Ferguson et al. [17]: the particles (4 mg/ml) were incubated for 30 min at room temperature in the presence of inhibitor with 10 mM triethanolamine, 0.25 M sucrose, 4 mM EDTA, pH 7.5; they were then centrifuged at 4° C and the pellet resuspended in the same buffer.

Electrical measurements. The apparatus used for electrical measurements was as described before [1,10,11] (cf. Fig. 1). The 'thick membrane' consisted of a 9-mm diameter Millipore filter (type 'Mitex' LSWP, pore size 5 μm), covered on each side with 20 μl of an asolectin solution in *n*-decane (100 mg/ml); the excess was removed by capillarity. The two half-cells were immediately filled with 3.5 ml of appropriate medium. The resistance of the membrane was in the range $0.5–2.10^9$ Ω for currents in the range $10^{-9}–10^{-10}$ A. The sign of the potential difference, ΔU, refers to the particle-free compartment ('out'), the 'in' compartment (to which particles are added) being connected to ground (Fig. 1). The electrical time-constant of the system was approximately 20 s.

Assay procedure for potential measurements. Assays were performed at room temperature, unless otherwise specified. The particles were added to one side of the cell ('in') (0.05 mg protein/ml in routine experiments). Preliminary assays showed that adhesion of particles to the thick membrane required neither the addition of $CaCl_2$, nor a long preincubation period, in contrast to experiments reported earlier [1—9,11,18]. In fact, when particles and ATP were added simultaneously in the cell, without $CaCl_2$, the increase in potential began with only a small time-lag (1 to 2 min), but the rate of appearance and the extent of the electrical signal were the same as in the absence of $CaCl_2$. In all the experiments described here, particles were left in contact with the thick membrane for 15 min, in the absence of $CaCl_2$, before addition of ATP. In some experiments, the unbound particles were removed by rinsing. This consisted of pouring out the content of the 'in' compartment after adhesion of the

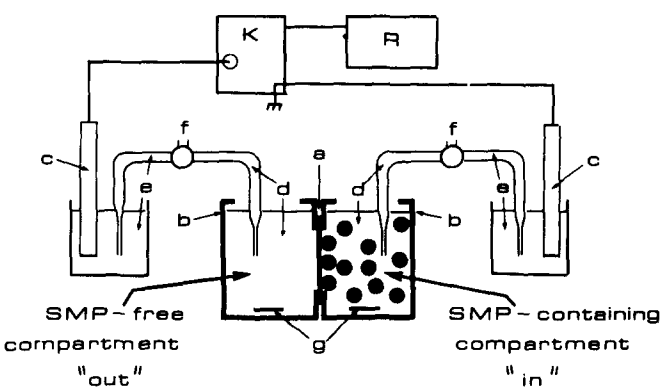

Fig. 1. Diagram of the cell and of the electrical circuit. a, thick membrane; b, teflon cell; c, calomel electrodes; d, reaction mixture; e, saturated KCl; f, salt bridges (three-branches-taps with teflon plugs); g, magnetic bars; K, Keithley electrometer (602); R, register Sefram Servotrace; SMP, submitochondrial particles.

particles and refilling it with fresh medium. During the course of the experiments, additions were made simultaneously to both compartments (unless otherwise specified). ATP was added after the potential had equilibrated. Control experiments showed that no artifacts were introduced by addition of 1% ethanol or 1% methanol; little or no potential difference could be detected when either methylamine or KSCN were added to both compartments. A small shift of potential (about 5 mV) was observed after addition of valinomycin (in the presence of KCl) (cf. also Ref. 7). The resistance of the thick membrane was unchanged by adhesion of particles or by addition of the reagents used in this work: ATP, phospho*enol*pyruvate, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, dicyclohexylcarbodiimide, 4-chloro-7-nitrobenzofurazan. Only valinomycin (0.4 μg/ml) and FCCP (10^{-6} M) were found to lower the resistance of the membrane by a factor of 10 and 50 respectively.

ATPase assay. Test-tube assay: ATPase activity was measured at 30° C in a total volume of 0.5 ml. The reaction medium contained 40 mM Tris-HCl, 10 mM ATP, 5 mM $MgCl_2$, 2 mM phospho*enol*pyruvate, 20 μg pyruvate kinase, pH 8.0. The reaction was started by addition of particles (about 0.05 mg protein), and stopped after 5 min by 0.2 ml of trichloroacetic acid (50% w/v). After cooling to 0° C, denatured proteins were centrifuged for 5 min at 12 000 × *g* and the amount of inorganic phosphate released was determined by the method of Fiske and SubbaRow [19].

Electrical cell assay: ATPase activity was assayed by the inorganic phosphate released into the electrical cell. In experiments with unrinsed cells, aliquots of 50—200 μl were taken simultaneously from both compartments. The reaction was stopped by addition of 20-50 μl of trichloroacetic acid (50% w/v) and the inorganic phosphate in the supernatant assayed according to Fiske and SubbaRow [19]. In experiments with rinsed cells, where the amount of released phosphate was very low, the ATPase activity was determined via the coupled reaction of ATPase, pyruvate kinase and lactate dehydrogenase [20], the reaction medium (containing 1 mM phospho*enol*pyruvate and 14 μg/ml pyruvate kinase) was supplemented with 0.2 mM NADH and 15 μg/ml lactate dehydrogenase. NADH consumption was followed spectrophotometrically at 340 nm on aliquots taken from the electrical cell at time intervals of 5 min.

Protein determination. The biuret method of Gornall et al. [21] or the method of Zak and Cohen [22] with Folin-Ciocalteu reagent were used with bovine serum albumin as a standard.

To determine the amount of proteins in vesicles bound to the thick membrane, we took advantage of the covalent labeling of particles by 4-chloro-7-nitrobenzo[^{14}C]furazan. We first determined the specific radioactivity of the labeled particles. Then the particles were added to the 'thick membrane system'. After a 15 min contact with the membrane, the unbound particles were removed by the rinsing procedure, as described above. The radioactivity of the filter was counted and its value was used for calculating the amount of bound particles.

Results

1. *Generation of electrical potential during ATP hydrolysis*

When the particles were added in the 'in' compartment to a final concentra-

tion higher than 0.02 mg/ml, addition of ATP resulted in the generation of an electrical potential ΔU, positive in the particle-free compartment ('out' compartment), that reached a plateau (ΔU_{max}) of 80—100 mV (Fig. 2a). This plateau could last for 1 hour. The half time of potential generation was 5—10 min at room temperature, corresponding to an initial rate of increase of the potential, $(\Delta U/\Delta t)_0$, of 5—10 mV/min.

The electrical signal arose from the particles that adhered to the filter: rinsing the compartments after particle adhesion, prior to ATP addition, did not alter the initial increase in voltage, nor the amplitude of the signal. The time constant of the electrical response (5—10 min) did not reflect the electrical time-constant of the apparatus (20 s) nor the rate of adhesion of the particles: signals generated 1 hour after addition of particles, or after rinsing, had the same 5—10 min time constant. The electrical signal cannot be due to the ADP and inorganic phosphate (P_i) gradients generated by ATP hydrolysis:

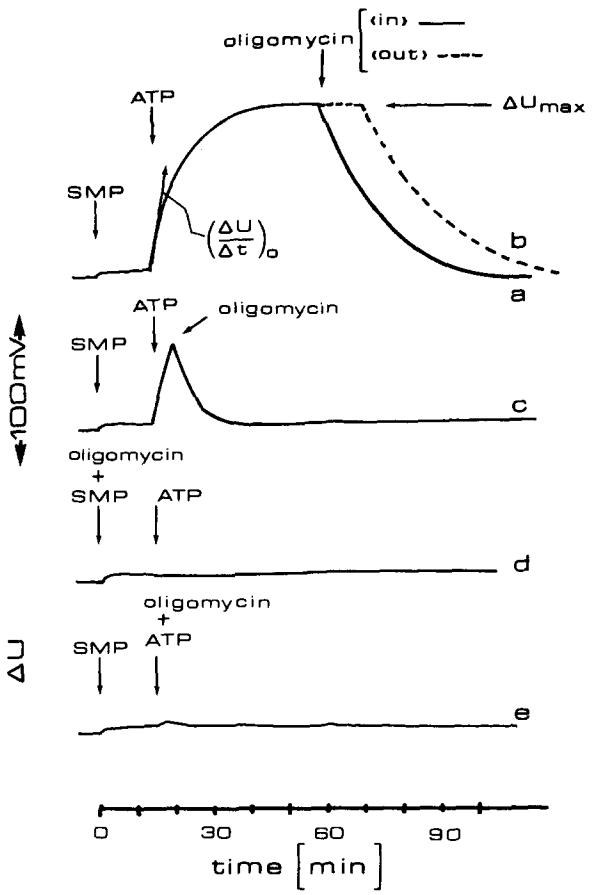

Fig. 2. Effect of oligomycin on the electrical signal generated by ATP hydrolysis. The reaction mixture contained 250 mM sucrose, 50 mM Tris-HCl pH 7.5, 2.5 mM $MgCl_2$, 1.5 mM phospho*enol*pyruvate, 14 μg/ml pyruvate kinase. Additions were beef heart submitochondrial particles (SMP) (0.5 mg/ml), 1 mM ATP and 15 μg/ml oligomycin.

neither addition of those products, nor replacement of particles by purified soluble F_1-ATPase [23] led to a detectable signal.

None of the following parameters had an influence on the generation of the signal: the size of the aqueous 'in' and 'out' compartments (down to 0.7 ml), the pH of the medium in both compartments (from 6.5 to 8.5), the buffering power of the medium (from 5 to 50 mM Tris-HCl at pH 7.5), or the existence of a pH gradient through the thick membrane (pH 6.5 'out', 8.5 'in').

Temperature strongly influenced the initial rate of increase of the signal $(\Delta U/\Delta t)_0$, but not the amplitude of the signal (Fig. 3). From the Arrhenius plot of $(\Delta U/\Delta t)_0$ vs. $1/T$ we calculated the activation energy of the process to be 18 kcal/mol.

Soybean asolectin is a mixture of phospholipids including 40% phosphatidylcholine (PC), 29% phosphatidylethanolamine (PE), 14% monophosphoinositide (PI), 4% phosphatidylserine (PS) [24] and 8% cardiolipin (CL) [25]. To assay the phospholipid requirement for generation of the electrical signal, thick membranes were impregnated with solutions of the different phospholipids in *n*-decane. The typical signal obtained with asolectin ($\Delta U_{max} \simeq 80$ mV, $(\Delta U/\Delta t)_0 \simeq 5$ mV/min) could not be recovered with pure phosphatidylcholine or with mixtures of phosphatidylcholine with charged lipids (90% PC + 10% PI or 90% PC + 10% CL); in all these cases, the observed ΔU_{max} and $(\Delta U/\Delta t)_0$ were nearly zero. On the contrary, with pure phosphatidylethanolamine, we could generate a signal in the thick membrane system, with an amplitude of 80 mV and an initial rate of increase of 5 mV/min, as for asolectin. A mixture of 90% phosphatidylcholine and 10% phosphatidylethanolamine lead to a signal of lowered amplitude ($\Delta U_{max} \simeq 5$ mV, $(\Delta U/\Delta t)_0 \simeq 2$ mV/min). Obviously, phosphatidylethanolamine appears as the active component of asolectin for the generation of the electrical potential in this system.

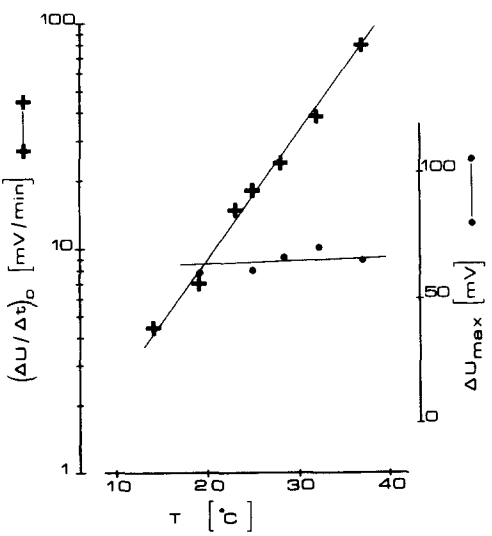

Fig. 3. Effect of temperature on the initial rate of increase, $(\Delta U/\Delta t)_0$ (+———+) and maximal value (ΔU_{max}) (•———•) of the potential generated in the thick membrane system upon ATP hydrolysis. Same conditions as in Fig. 2.

2. Effect of ATPase inhibitors on the potential generated by ATP hydrolysis

ATPase inhibitors including oligomycin, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, 4-chloro-7-nitrobenzofurazan, azide, dicyclohexylcarbodiimide have been found to reverse or prevent the generation of the electrical signal, indicating that particles bound to the thick membrane responded to ATPase inhibitors in the same way as unbound particles.

Oligomycin reversed the signal generated by ATP hydrolysis when added either 'in' (Fig. 2a, 2c) or 'out' (Fig. 2b). In that latter case, decay occurred after a time-lag of about 10 min. It must be stressed that upon addition of oligomycin, ATPase activity was stopped instantaneously [26], but the decay of electrical potential was slow, the time constant being 5—10 min. Oligomycin totally inhibited the potential increase when added simultaneously with the particles before ATP (Fig. 2d) or simultaneously with ATP after the particles (Fig. 2e).

N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline inactivates both soluble and membrane bound ATPase [27], the inactivation being more pronounced at acidic pH. As shown in Fig. 4 the decay of the potential due to this inhibitor was faster when the pH was lowered from pH 8.5 to pH 6.5 irrespective of the fact that ATP hydrolysis has taken place at pH 7.5 (curve c) or 6.5 (curve d).

Addition of azide and dicyclohexylcarbodiimide [29,30] after generation of the electrical potential also resulted in a slow decay of the electrical potential.

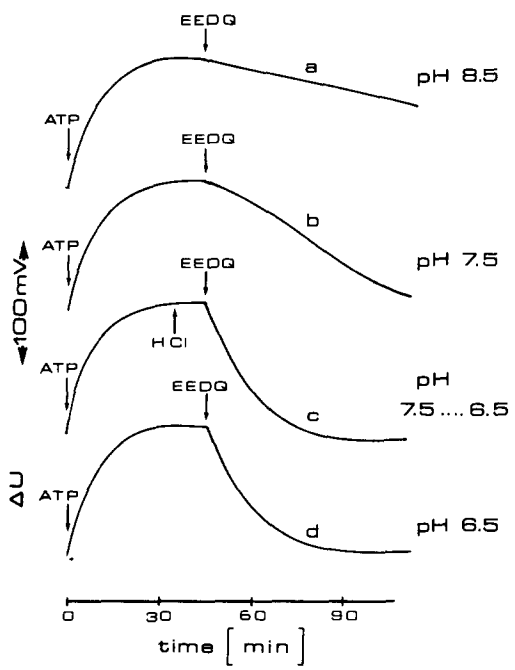

Fig. 4. Effect of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) on the electrical signal generated by ATP hydrolysis. Effect of pH. The reaction mixture contained: 250 mM sucrose, 25 mM Tris, 25 mM Mops, 5 mM $MgCl_2$, 1.5 mM phospho*enol*pyruvate, 14 μg/ml pyruvate kinase and bovine heart submitochondrial particles (0.05 mg/ml). Additions were 6 mM ATP and 3 mM EEDQ. HCl was used to shift the pH.

4-Chloro-7-nitrobenzofurazan inhibits ATPase activity by reacting at neutral pH with a tyrosine group of the hydrophilic F_1 factor of the ATPase complex; this inhibition is reversed by thiols [17]. As shown in Fig. 5a, the electrical signal was completely abolished in about 40 min by 1 mM of this inhibitor. No reversion was obtained by adding 5 mM dithioerythreitol. When, however, particles were first treated by 4-chloro-7-nitrobenzofurazan (see Methods) and then added to the cell, the corresponding inhibition could then be reversed with 2 mM dithioerythreitol. Fig. 5b illustrates an experiment where the electrical potential was generated upon ATP hydrolysis by particles pretreated with 4-chloro-7-nitrobenzofurazan. The ATPase activity in these particles was inhibited to an extent of 80%. The potential generated reached a plateau of only 20 mV. Addition of 2 mM dithioerythritol increased the plateau to a value of 80 mV, similar to that of the control experiment (Fig. 5a). Among these different ATPase inhibitors, 4-chloro-7-nitrobenzofurazan offered two major advantages: (1) the ATPase activity of particles pretreated with this inhibitor can be adjusted accurately to a given value (in this respect, dicyclohexylcarbodiimide was unsuitable since, even after the washing step, inactivation still increased), (2) inhibition can be reversed by dithioerythritol.

3. ATPase activity of adhered particles

From the determination of the amount of protein bound to the filter and the rate of ATP hydrolysis in a rinsed cell (see Methods), we calculated the specific ATPase activity in bound particles: in a series of assays where 1.8 mg of particles protein in 3.5 ml of aqueous medium was left in the presence of a 2.5-cm^2 thick membrane during 15 min, 2.5 ± 1 μg protein were found to adhere to the filter. The rate of ATP hydrolysis was 6 ± 2 nmol/min, corresponding to a mean specific activity of 2.5 ± 2 μmol P_i/min/mg, which was of

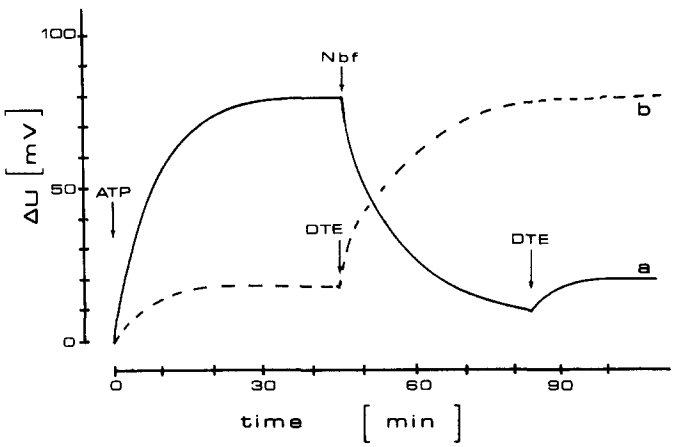

Fig. 5. Effect of 4-chloro-7-nitrobenzofurazan (Nbf) and dithioerythritol (DTE) on the electrical signal generated by ATP hydrolysis. The reaction mixture and the concentration of particles were as in Fig. 2. ATP was used at the final concentration of 1 mM to generate the electrical signal. In trace (a), 1 mM Nbf was added after the plateau of potential was reached, followed by addition of 5 mM DTE. In trace (b), the particles were previously 80% inhibited by incubation with Nbf (see Methods). 2 mM DTE was sufficient to regenerate the potential.

the same order as the specific ATPase activity in free particles (1.7—2 μmol P_i/min/mg).

4. Relation between ΔU and ATPase activity. Influence of protein concentration

Since ATPase activity is the same in bound and free particles, this study was carried out on unrinsed cell. We essentially used particles partially inhibited by 4-chloro-7-nitrobenzofurazan (see above section).

At a protein concentration of 0.05 mg/ml, both the initial rate of generation of the electrical signal $(\Delta U/\Delta t)_0$ and the plateau value (ΔU_{max}) were dependent of the activity of the preparation: in the experiment of Fig. 6, ATPase activity was halved by preincubation with 4-chloro-7-nitrobenzofurazan, and the initial rate of generation of the electrical signal $(\Delta U/\Delta t)_0$ was also about half that observed in the control experiment. The plateau was lower in inhibited particles than in control particles. Addition of dithioerythritol to control particles after the plateau was reached changed neither the value of the electrical signal nor the rate of ATP hydrolysis. When added to inhibited particles, the thiol induced a new electrical signal and a marked

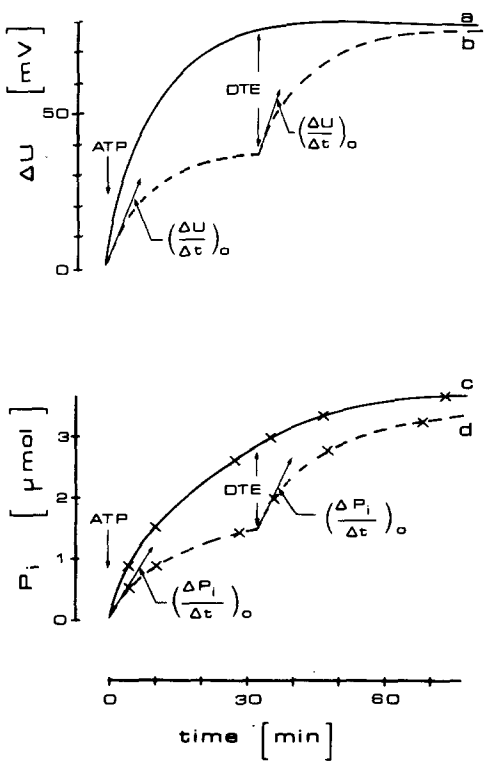

Fig. 6. Effect of 4-chloro-7-nitrobenzofurazan (Nbf) and dithioerythritol (DTE) on the ATPase activity of submitochondrial particles and the generation of the electrical signal upon ATP hydrolysis. The reaction mixture contained: 250 mM sucrose, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, particles (0.05 mg/ml), 1.5 mM phospho*enol*pyruvate, 14 μg/ml pyruvate kinase. Additions were 12 mM ATP and 4 mM DTE. Traces (a) and (c): control particles. Traces (b) and (d): particles preincubated with Nbf (see Methods) to give a roughly 50% inhibition of the ATPase activity.

stimulation of the rate of ATP hydrolysis. The plateau obtained after addition of thiol was equal to that found in the control (80 mV) (Fig. 6). In assays with 4-chloro-7-nitrobenzofurazan-treated particles in which ATPase was inhibited to different extents, there was a linear relationship between the ATPase activity and $(\Delta U/\Delta t)_0$, with a slope of 0.15 mV · nmol^{-1} P_i liberated · mg^{-1} protein (Fig. 7). The potential plateau (ΔU_{max}) was also related to ATPase activity (Fig. 8, open circles). After dithioerythritol addition, the increment in potential and in ATPase activity (cf. Fig. 6) also followed the same linear relationship (Fig. 8, triangles).

Another way to modify the rates of potential generation and ATP hydrolysis was to vary the pH of the medium. Assays were carried out at pH 6.5, 7.5, 8.5. Here again, correlation between $(\Delta U/\Delta t)_0$ and ATP hydrolysis holds (Fig. 7).

Similar studies have been carried out at a higher protein concentration (0.5 mg/ml); Fig. 9 shows two representative experiments where the rates of ATP hydrolysis differed markedly, due to different initial concentrations of ATP and different ratios of ATP to $MgCl_2$. At high ATP concentration (Fig. 9a, c) the rates of potential generation and ATP hydrolysis were higher than at low ATP concentration (Fig. 8b, d). However, this relationship obviously does not hold any more when the plateau of potential is attained: the same plateau (90 mV) was obtained after 75 min (curves a, b) and yet at this time

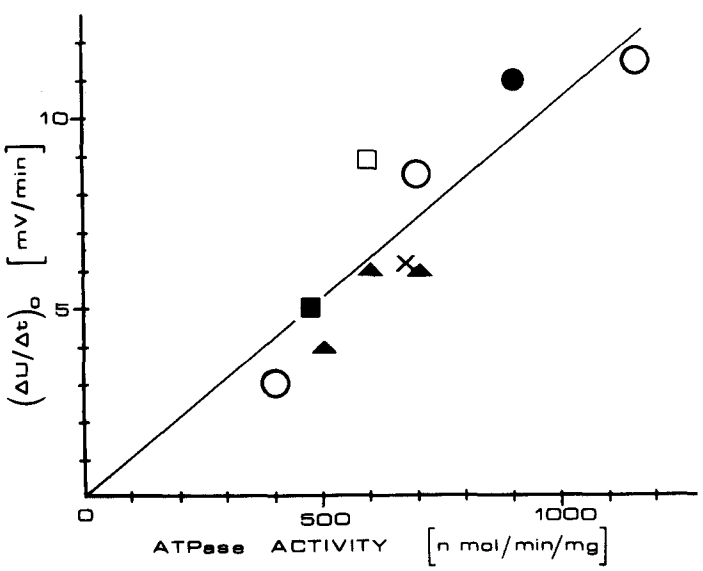

Fig. 7. Relationship between the initial increase of the electrical signal and the ATPase activity. In all cases the protein concentration was 0.05 mg/ml and the ATPase activity measured without the rinsing step. In a first set of assays carried out at pH 7.5, the reaction mixture and the additions were: 250 mM sucrose, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, 1.5 mM phospho*enol*pyruvate 14 μg/ml pyruvate kinase and 12 mM ATP: ●, control; ○, particles pretreated with 4-chloro-7-nitrobenzofurazan (cf. Fig. 6); ▲, same as ○ after treatment by 4 mM dithioerythritol. In a second set of assays carried out at different pH values, the medium was made of 250 mM sucrose, 25 mM Tris, 25 mM Mops, 5 mM $MgCl_2$, 1.5 mM phospho*enol*pyruvate, 14 μg/ml pyruvate kinase, 6 mM ATP: ■, final pH 6.5; □, final pH 7.5; X, final pH 8.5.

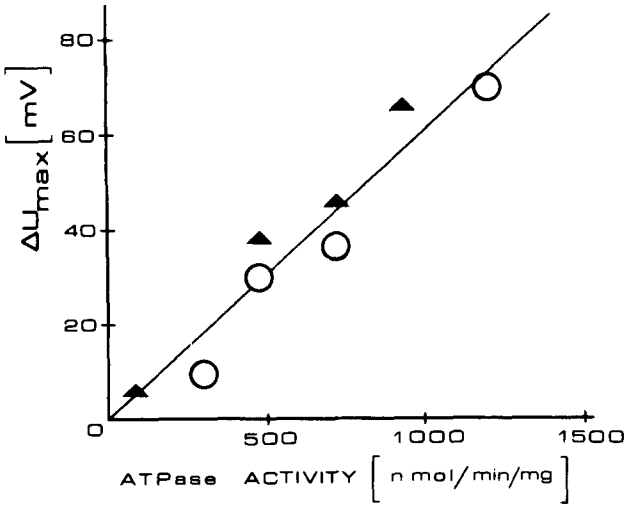

Fig. 8. Relationship between the plateau of potential and the ATPase activity. Same conditions as in Fig. 6. ○, particles pretreated with 4-chloro-7-nitrobenzofurazan; ▲, same as ○ after treatment by 4 mM dithioerythritol.

the amounts of P_i released were markedly different (9 μmol (curve c) and 4 μmol (curve d)). Furthermore, despite the fact that the plateau of potential was attained, ATP was still hydrolyzed quite rapidly. However the rate of ATP hydrolysis (curve c) (120 nmol · min^{-1}) was double that in curve d. From

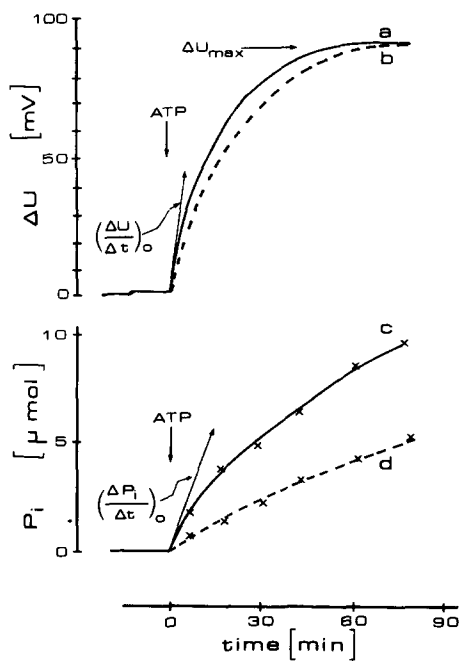

Fig. 9. Electrical signal (ΔU) and ATPase activity (P_i) at high protein concentration (0.5 mg/ml). The reaction mixture contained: 250 mM sucrose, 50 mM Tris-HCl, 2.5 mM $MgCl_2$, pH 7.5. Additions were: 0.5 mg/ml submitochondrial particle, 1.5 mM phospho*enol*pyruvate, 14 μg/ml pyruvate kinase and (a, c), 3.5 mM $MgCl_2$, 6 mM ATP; (b, d), 1.2 mM ATP.

a series of experiments carried out with 4-chloro-7-nitrobenzofurazan-treated particles or with different concentrations of ATP or different ATP/Mg ratios, we found a linear relationship between $(\Delta U/\Delta t)_0$ and ATPase activity (slope of 0.5 mV · nmol^{-1} P_i · mg^{-1} protein), whereas a variation of the ΔU_{max} value began to appear only when the ATPase inhibition was higher than 60—70%.

5. Effects of reagents that collapse either the membrane potential or the pH gradient

ATP hydrolysis promotes an uptake of protons in particles. The proton motive force ($\widetilde{\Delta\mu}_H$) which describes the proton movement is made of two components: an electrical potential difference, $\Delta\psi$, and a proton concentration gradient, ΔpH, where ΔpH is the pH difference between the two compartments separated by the membrane, so that $\widetilde{\Delta\mu}_H = \Delta\psi - 2.3\,(RT/F)\,(\Delta pH)$ [31,32].

As shown in Table I the proton carrier FCCP collapsed the electrical signal, indicating that this signal is related to transport of protons. We assayed a number of other reagents known to collapse either $\Delta\psi$ (valinomycin in the presence of KCl, or KSCN) or ΔpH (nigericin in the presence of KCl, or methylamine) (Table I). There was no effect of ΔpH-collapsing agents. By contrast, the electrical signal was decreased by 80—90% upon addition of 10—15 mM KSCN, and by 50% upon addition of 0.7 μg valinomycin per mg protein.

6. Response of the thick membrane to a pH gradient

We carried out experiments where a proton gradient across the thick membrane was generated, in the absence of submitochondrial particles, by simply adding HCl to one of the compartments. Different medium were used without significant changes in the observed results (Tris-Mops, Tris-Mes or Tris buffers, containing or not sucrose and $MgCl_2$).

For most of their characteristics, the signals observed here mimicked those reported in section 1: (i) The 'HCl-free' side became positive with respect to the other. Moreover the effect could be reversed by adding HCl in the second compartment, the sign of the overall resulting signal depending only on the final pH gradient. (ii) The half-time required to attain the plateau was long

TABLE I

EFFECT OF ΔpH AND $\Delta\psi$ COLLAPSING AGENTS ON THE SIGNAL GENERATED BY ATP HYDROLYSIS

The medium contained 0.5 mg/ml submitochondrial particle, 1.5 mM phospho*enol*pyruvate, 14 μg/ml pyruvate kinase, 1 mM ATP, 1 μM FCCP, nigericin (4 μg/mg protein, added 'in' only), valinomycin (0.7 μg/mg protein, added 'in' only), 10 mM methylamine, 20 mM KSCN, pH 7.5.

Conditions	Additions	ΔU_{max} (mV)
125 mM sucrose, 60 mM KCl,	ATP	100—110
50 mM Tris-HCl, 2.5 mM $MgCl_2$	ATP + FCCP	0
	ATP + nigericin	90—100
	ATP + valinomycin	50— 65
250 mM sucrose, 50 mM Tris-HCl	ATP	100—100
2.5 mM $MgCl_2$	ATP + FCCP	0
	ATP + methylamine	90—100
	ATP + KSCN	<10

(3—6 min at 20° C). The temperature dependence of the initial rate of increase of the potential corresponded to an activation energy of about 20 kcal/mol.

The amplitude of the signal observed in these experiments was proportional to the pH gradient in a range from pH 2 to 10, with a slope of 40—50 mV/pH-unit i.e. less than what would be predicted by the Nernst law for protons. When FCCP was added to the cell, the potential reached, within 1 min, a new higher plateau corresponding to the value predicted by the Nernst law for protons; this important difference with the experiments described in section 1 will be discussed below.

Discussion

Adhesion of 'inside out' submitochondrial particles to a thick membrane does not introduce any major artifact in the ATPase function: the enzymatic properties of ATPase in bound particles and its response to inhibitors are essentially similar to those of free particles. By inhibiting the ATPase activity of the bound particles by 4-chloro-7-nitrobenzofurazan, a clear correlation could be established between the electrical signal generated upon ATP hydrolysis in the thick membrane system and the enzymatic activity. Most important is the effect of the concentration of particles on the electrical signal depending on ATP hydrolysis. Both the rate of appearance of the electrical potential and the plateau of potential are related to the specific ATPase activity only in a narrow range of protein concentration (0.02—0.5 mg/ml in experiments described here).

A peculiarity of the electrical signal generated upon ATP hydrolysis by submitochondrial particles in the thick membrane system is the length of time (15 to 20 min) necessary to attain the plateau of potential. This time is much longer than that required for generation of electrical potential ($\Delta\psi$) or pH gradient (ΔpH) in free particles, as studied by fluorescent probes [33] or by uptake of phenyldicarbaundecaborane anion [11,34]. Moreover, the decay of the signal, initiated by addition of oligomycin, 4-chloro-7-nitrobenzofurazan, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, or azide (i.e., reagents which inhibit ATPase activity) is as slow as the generation of the signal by ATP hydrolysis.

Another characteristic of the electrical signal generated in this system is that it corresponds essentially to a difference of electric potential, $\Delta\psi$. Moreover, no pH gradient could be detected between the 'in' and 'out' compartments with conventionnal pH electrodes; if present, it should be less than 0.01 pH unit.

In light of these observations, mechanisms of generation of potential with the thick membrane system can be discussed and ruled out. The first hypothesis assumes that bound particles retain their membrane properties, and that the electrical signal directly reflects the accumulation of protons in the internal volume of particles. If this was the case, the time constant of the response would be that required for the establishment of $\Delta\psi$ and ΔpH (<1 min); moreover, addition of reagents collapsing either $\Delta\psi$ or ΔpH would have affected the observed electrical signal. As an alternative, the signal could be related to an efflux of protons from the inside of the adhered particles towards the 'out'

compartment: in thise case, the time constant of the signal would be the electrical time constant of the thick membrane (20 s), and moreover, the generation of the signal would have been highly unfavoured when the 'out' compartment was more acidic (pH 6.5) than the 'in' compartment (pH 8.5).

A way to rationalize the above results is to consider the electrical signal as the effect on the thick membrane of an instantaneous accumulation of protons inside the bound particles during ATP hydrolysis, mimicked by adding HCl to the 'in' compartment (the best fit was obtained for a proton gradient of 1.4 pH unit).

The electrical response of the thick membrane to a proton gradient did not appear to be dependent upon the thickness of the membrane or the solvent used to dissolve the phospholipids; for example, a 5-fold increase in membrane thickness by juxtaposition of 5 Mitex filters or substitution of decane by hexadecane did not change the characteristics of the signal.

Different models for explaining the effect of protons are given in Fig. 10. In Fig. 10a, we emphazise the localization of the proton gradient at the interface of adhesion of the vesicle on the membrane and the possibility that the vesicle somehow penetrates into the thick membrane. This localized proton gradient can be collapsed with FCCP by releasing the protons to the exterior of the vesicles; the resulting pH change in the medium is expected to be much lower because of dilution effects; the final pH gradient across the thick membrane will then be undetectable by our technique. On Fig. 10b and 10c two possible mechanisms of action of protons are proposed: in Fig. 10b, protons are trapped in aqueous compartments, which may exist near the surface of the

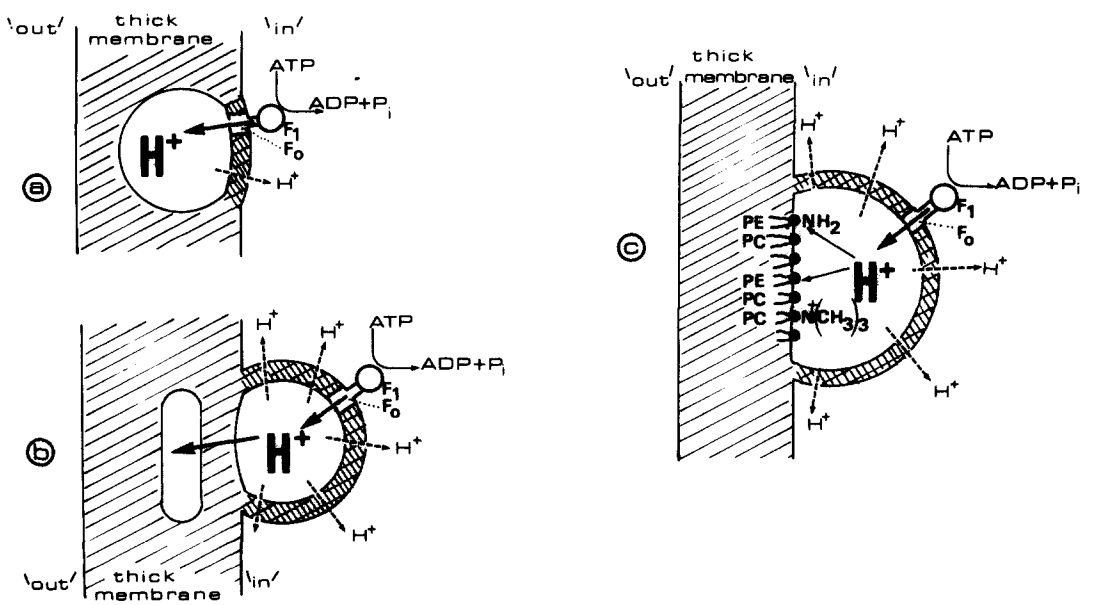

Fig. 10. Hypothetical mechanisms of proton action on the thick membrane during ATP hydrolysis. PE, phosphatidylethanolamine; PC, phosphatidylcholine. F_1, hydrophilic sector of ATPase; F_0, hydrophobic sector of ATPase.

thick membrane [7]. In Fig. 10c, protons react with the phospholipids of the thick membrane. At the pH used in our experiments (pH 7.5), protons could react with the amino group of phosphatidylethanolamine which have a pK of 7.5 but not with the quaternary nitrogen of phosphatidylcholine whose pH is 11.6 [35] and this could be the reason why generation of an electrical signal is observed in this system only when phosphatidylethanolamine is present in the phospholipid mixture (see also Drachev et al. [5]). The consequences of the reaction with protons could be modifications of surface charges of the membrane, possibly followed by reorganizations among phospholipids (for example phase separations [36]), both phenomena being compatible with an activation energy of 18 kcal/mol.

A final comment concerns the percentage of particles bound to the thick membrane. Using a concentration of particles of 0.5 mg protein/ml and a thick membrane with an area of 2.5 cm^2, the amount of bound particles was not higher than 5 μg. Assuming that a submitochondrial particle is a sphere with a radius of 2000—2500 Å, containing 400 to 800 ATPase molecules, and that 10% of the total protein content of a particle consist of ATPase, it can be calculated that 20 to 50 μg of particle protein can cover the whole area of the membrane. Thus, when 10 to 25% of the area of the thick membrane is covered with particles, the electrical signal generated by ATP hydrolysis appears to reach a limit. The reason for this limitation is not apparent.

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