PROTON TRANSLOCATION CATALYZED BY THE PURIFIED YEAST PLASMA MEMBRANE ATPase RECONSTITUTED IN LIPOSOMES

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1. Introduction

The plasma membrane of animal cells contains a sodium- and potassium-pumping ATPase involved in such important physiological functions as regulation of cellular volume, generation of membrane potentials and driving of the cotransport of nutrients with sodium [1]. This enzyme does not seem to be present in plants, algae and fungi, probably because the rigid cell wall of these cells obviates the need for osmotic regulation [2]. On the other hand, on the basis of physiological evidence it has been postulated that the plasma membrane of the eukaryotic cells contain a proton-pumping ATPase. This enzyme would explain membrane potentials, proton secretion and the cotransport of nutrients with protons observed in these cells [3].

Although there is circumstantial evidence that the fungal [4–7] and plant [8] plasma membrane ATPases operate as proton pumps, this important point should be established by demonstrating ATP-dependent proton transport in proteoliposomes reconstituted with the purified enzyme. We have reported that the purified yeast plasma membrane ATPase reconstituted in liposomes catalysed a $^{32}P_i$–ATP exchange inhibited by proton ionophores [10]. Here, we present more direct evidence for the proton-transport activity of the enzyme by demonstrating an ATP-induced quenching of acridine dye fluorescence.

Abbreviations: CCPP, carbonylcyanide-*m*-chlorophenylhydrazone; 1799, 2,2'bis (hexafluoroacetonyl) acetone; ACMA, 9-amino-6-chloro-2-methoxyacridine; Mes,2-(*N*-morpholino) ethanesulfonic acid; Tricine, *N*-tris-(hydroxymethyl) methylglycine

2. Experimental

Yeast plasma membrane ATPase was purified [9] and reconstituted in soybean phospholipid vesicles [10] as described. $^{32}P_i$–ATP exchange [9] and ATPase activity [11] were measured as indicated. Quenching of acridine dye fluorescence was measured at 30°C in 2 ml medium containing 10 mM Mes and 2 mM $MgSO_4$ adjusted to pH 6.0 with NaOH. After addition of 50 μl reconstituted vesicles (10 μg protein, 1 mg phospholipid) and 2 μl 1 mM ACMA, the reaction was started with 25 μl of a solution containing 0.1 M NaATP and 0.1 M $MgSO_4$ adjusted to pH 6.0 with NaOH. Fluorescence was measured with a Perkin Elmer model 204 Fluorescence Spectrophotometer, with exciter and analyzer wavelengths of 415 and 485 nm, respectively, and registered in a Perkin Elmer model 56 recorder. Separation of protein-free liposomes from reconstituted proteoliposomes was achieved by layering 2.5 ml reconstitution mixtures over 1.5 ml 35% (v/v) glycerol, 10 mM tricine, 3 mM 2-mercaptoethanol and 1 mM EDTA, pH 7.5 (NaOH). After centrifugation for 2 h at 0°C at 40 000 rev./min in a Beckman SW 56 rotor, the protein-free liposomes remained at the interphase and the proteoliposomes were recovered in the 35% glycerol layer. Protein and phospholipid were 0.2 and 0.7 mg/ml, respectively. The calibration of fluorescence changes with known pH gradients was performed as in [17] and is described in fig.2.

The acridine dye ACMA was a gift of Dr R. Kraayenhof (Free University, Amsterdam). The uncoupler 1799 was obtained from Dr P. G. Heytler (Du Pont de Nemours, Wilmington DE) and nigericin from Dr W. E. Scott (Hoffmann-La Roche, Nutley NJ).

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Monensin was purchased from Calbiochem and CCCP, valinomycin and gramicidin D from Sigma. All these compounds were dissolved in methanol and this solvent was ≤0.5% in the assays.

3. Results and discussion

Although there is some controversy about the exact mechanism of the phenomenon [12], the quenching of the fluorescence of acridine dyes has been widely employed to monitor pH gradients (acidic inside) in submitochondrial particles [13,14], chloroplasts [15], liposomes [16], gastric microsomes [17] and proteoliposomes reconstituted with energy-transducing enzymes [18–20]. This method is more sensitive than direct pH measurements [18,20] and the dye 9-amino-6-chloro-2-methoxiacridine (ACMA) proved to be the most suitable [20].

When ATP was added to a suspension of proteoliposomes reconstituted with the purified yeast plasma membrane ATPase, the fluorescence of ACMA was quenched (fig.1A). After depletion of ATP by hexokinase and glucose, the fluorescence slowly recovered to the original value (fig.1A). That this response was caused by an ATP-dependent internal acidification of the vesicles was indicated by the complete inhibition caused by the weak base imidazole (25 mM, adjusted to pH 6.0 with H_2SO_4) and by the proton ionophores gramicidin D (2 μg), monensin (0.5 μg) and nigericin (0.2 μg, in the presence of 10 mM potassium). At the pH 6 of the assay medium imidazole exist >10% as the unprotonated form. This uncharged molecule is freely permeable and therefore it would prevent the development of pH gradients by crossing the membrane and combining with the pumped protons [15]. Gramicidin creates a non-specific permeability for monovalent cations, including protons, and monesin and nigericin catalyze the exchange of protons for sodium and potassium, respectively [21]. None of these substances inhibited the ATPase activity (not shown).

Both the initial rate and the extent of quenching were increased by the permeant anion nitrate, while the less permeable chloride was much less effective (fig.1B,C). Similar results were obtained with the sodium, potassium and Tris salts of nitrate. This stimulation suggest an electrogenic character of the proton pump so that the development of a membrane potential would inhibit proton transport. In the presence of nitrate the potential would be discharged and protons together with nitrate would accumulate inside the vesicles. In accordance with this interpretation, in vesicles prepared with potassium inside valinomycin produced the same stimulation as nitrate (fig.1D–F). In this case the pumping of protons inside the vesicles would be electrically compensated by the exit of

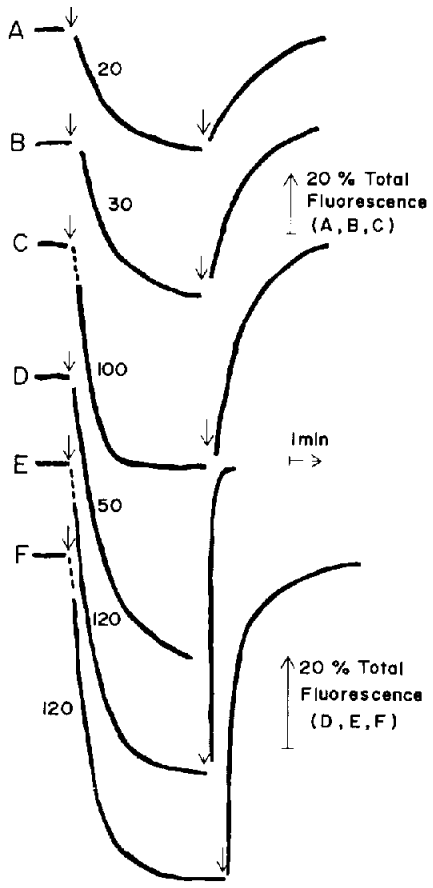

Fig.1. ATP-driven intravesicular acidification. Proteoliposomes were reconstituted with 0.2 mg purified yeast plasma membrane ATPase/ml and 20 mg of sonicated soybean phospholipids/ml by 2 cycles of freezing–thawing–sonication as in [10]. The reconstitution medium contained 10 mM tricine–NaOH (pH 7.5), 1 mM EDTA, 3 mM 2-mercaptoethanol, 4% glycerol and either 5 mM $MgSO_4$ (A–C) or 25 mM K_2SO_4 (D–F). Quenching of ACMA fluorescence was measured as in section 2, except that the assay medium included 50 mM KCl (B), 50 mM KNO_3 (C,F), 25 mM K_2SO_4 (D,E) and 1 μg valinomycin (E). The first arrow indicates the time of ATP addition and an instantaneous quenching of ~25% has been corrected in all the traces. The second arrow corresponds to the addition of either hexokinase and glucose (0.1 mg and 20 μmol, respectively (A–C)) or 30 nmol 1799 (E,F). The initial rate of quenching expressed as % total fluorescence/min is indicated.

potassium mediated by valinomycin. The proton transport observed in the absence of permeant ions (fig.1A,D) can be ascribed to the passive permeability of the vesicles towards the ions present in the assay medium.

The identity between the plasma membrane ATPase and the proton pump was indicated by the following similar properties exhibited by the ATPase activity and by the ATP-dependent quenching of ACMA fluorescence:

(i) Dicyclohexylcarbodiimide (45 μg) and diethylstilbestrol (45 μg) inhibited >90% but the mitochondrial ATPase inhibitor oligomycin (40 μg) was without effect.
(ii) pH optimum was ~6, with <10% activity at pH 4.5 and 7.5.
(iii) Nucleotides other than ATP such as ITP, GTP, UTP, ADP and adenosine 5′-(α,β-methylene) triphosphate were ineffective.
(iv) The K_m for ATP was >1 mM.

Energy transduction by an electrogenic proton pump should be completely blocked by uncouplers [22] and, accordingly, the quenching of ACMA fluorescence was abolished by 1799 (fig.1E,F). However, in [10] it was shown that the uncoupler CCCP produced only partial inhibition of the $^{32}P_i$–ATP exchange unless valinomycin and potassium were present. These results are confirmed in table 1, where both the $^{32}P_i$–ATP exchange and the quenching of ACMA fluorescence are only partially inhibited by CCCP but completely blocked by the same uncoupler in the presence of valinomycin. Our interpretation in [10] was that the CCCP-resistant activity corresponded to an electroneutral proton–potassium exchange. Uncouplers would only dissipate the pH gradient generated by this pump when the proton efflux mediated by the uncoupler could be electrically compensated by potassium influx mediated by valinomycin.

Table 1
Effect of uncouplers and valinomycin on the energy transfer activities of the reconstituted proteoliposomes

Ionophores	$^{32}P_i$–ATP exchange	Extent of quenching of ACMA fluorescence
Expt 1		
CCCP (5 nmol)	75	65
CCCP (20 nmol)	55	40
CCCP (5 nmol) + valinomycin	10	< 5
1799 (5 nmol)	55	60
1799 (20 nmol)	< 5	10
1799 (5 nmol) + valinomycin	25	35
Expt 2		
CCCP (20 nmol)	55	
CCCP (20 nmol) + valinomycin	< 5	
CCCP (0.5 nmol)		85
CCCP (0.5 nmol) + valinomycin		10

The reconstitution medium contained either 25 mM K_2SO_4 (expt 1) or 50 mM KNO_3 (expt 2) and 50 mM KNO_3 was included in the assay medium. Results are expressed as % of the values obtained in the absence of ionophores, corresponding to 19 nmol . min^{-1} . mg $^{32}P_i$–ATP exchange^{-1} and 70% of quenching. The amount of valinomycin added was 1 μg and in the absence of uncouplers it was without effect

Two novel experimental findings have prompted a modification of this view:

(1) As mentioned above, another uncoupler like 1799 produces complete inhibition of energy transfer activities in the absence of valinomycin and the action of sub-optimal doses is much less potentiated by valinomycin than in the case of CCCP (table 1).
(2) Even in vesicles containing nitrate the inhibition produced by CCCP was enormously potentiated by valinomycin (table 1). In these vesicles the electrical balance during proton efflux mediated by the uncoupler should be achieved by nitrate efflux and therefore the effect of valinomycin must have some other explanation.

One possibility is that the anionic form of CCCP and the positively charged valinomycin–potassium complex form a neutral complex inside the membrane which facilitates the carrier activity of the uncoupler. The existence of such ternary complexes has been demonstrated [23] and the low solubility in the membrane of the anionic form of the uncouplers is known to be limiting for its proton transport activity [24]. A similar potentiation by charge neutralization inside the membrane has been postulated to explain the increase in dibenzyldimethyl ammonium permeability produced by tetraphenyl boron [25].

An approximate determination of the pH gradient generated by the ATPase was obtained by calibrating the fluorescence changes with vesicles of known imposed pH gradients [17]. For these experiments it was essential to remove the non-reconstituted liposomes by gradient centrifugation because they would

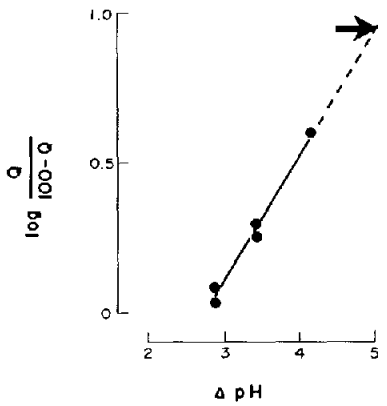

Fig.2. Experimental relationship between the extent of fluorescence quenching and ΔpH values generated artificially. Proteoliposomes purified by glycerol gradient centrifugation were incubated with 25 mM succinic acid at pH 4.25 (NaOH) for 1 min and then 100 μl were diluted in 1.9 ml medium containing 10 mM tricine, 2 mM $MgSO_4$, 50 mM KNO_3 and 1 μM ACMA. The pH was varied from 7–8.5 with NaOH. The % quenching of fluorescence (Q) was calculated taking as 100% the value obtained after addition of 2 μg gramicidin. The arrow indicate the quenching obtained with the same amount of vesicles energized with ATP at pH 6.

respond to artificially created pH gradients but not to ATP. As indicated in fig.2, the results of such calibration extrapolate a pH gradient of ~5 units for the ATP-energized proteoliposomes. This value may be an underestimation because of the possibility that the gradient-purified proteoliposomes still contained vesicles unresponsive to ATP. On the other hand, this calibration curve as well as similar curves described for other systems [17] must be taken with caution because they deviate from the theoretical slope of 1 calculated for an ideal fluorescent amine [16]. With these reservations in mind, the magnitude of the estimated pH gradient suggests a stoichiometry for the proton pump of 1 H^+/ATP. If the energy of ATP hydrolysis were employed to pump 2 H^+, the pH gradient could not exceed 4 pH units. In *Neurospora crassa* hyphae, electrophysiological studies have indicated a reversal membrane potential for the proton pump of ~–400 mV [26]. This value also requires a stoichiometry of 1 H^+/ATP.

The enormous proton gradients needed to reverse a proton pump with such stoichiometry may explain the failure of attempts to synthesize ATP driving back the enzyme with pH gradients either artificially imposed or generated by light in bacteriorhodopsin containing liposomes.

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