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CORRELATIONS BETWEEN ATP HYDROLYSIS, ATP SYNTHESIS, GENERATION AND UTILIZATION OF ΔpH IN MITOCHONDRIAL ATPase-ATP SYNTHASE

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Using fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine induced either by ATP hydrolysis in the ATPase-ATP synthase complex or by succinate oxidation in inverted submitochondrial particles, correlations have been established between ATP hydrolysis, ATP synthesis and the generation and utilization of ΔpH. The results obtained are best explained in terms of local circuits of protons.

Introduction

According to the chemiosmotic hypothesis, substrate oxidation in mitochondria creates a proton gradient between the two sides of the inner membrane [1]. The ATPase-ATP synthase complex can make use of this gradient to synthesize ATP through a reverse flow of protons. Although proton translocation has been investigated in submitochondrial particles or in reconstituted systems, precise kinetic measurements of H^+ flow coupled with the catalytic turnover of the complex are still lacking (see the review of Fillingame [2]). In addition, the localization of the efficient protons either in bulk phases or in specific circuits is still a matter of controversy (see reviews Refs. 3 and 4). According to R.J.P. Williams [5], 'the difference between local circuits and chemiosmosis requires studies of the rates of ATP synthesis, of proton flow, and of the rates of rise of bulk ΔpH and $\Delta\Psi$'. So far, the experiments of Venturoli and Melandri [6] with chromatophores, in the presence of inhibitors limiting the proton flux or the rate of phosphorylation, suggest that there are local circuits in the case of photophosphorylation. In the present work we have established a direct correlation between proton translocation, ATP hydrolysis and ATP synthesis with pig heart inverted submitochondrial particles and with a vesicular preparation of ATPase-ATP synthase complex by controlling the generation of ΔpH with limited amounts of substrates. The results are difficult to explain if local proton circuits are not taken into account. The proton translocation was estimated by fluorescence quenching of ACMA [7,8], the membrane potential $\Delta\Psi$ having been collapsed with the addition of K^+ plus valinomycin [9].

Materials and Methods

Pig heart mitochondria [10], inverted submitochondrial particles prepared by sonication of mitoplasts and vesicular ATPase-ATP synthase complex were obtained as previously described [11]. Inverted submitochondrial particles and

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

ATPase-ATP synthase complex were suspended in 0.25 M sucrose/10 mM Tris-H_2SO_4/10 mM $MgSO_4$ (pH 7.5), frozen in liquid nitrogen and kept at −80°C until used. The particles or the complex were rapidly thawed before each experiment and then kept on ice. This ensured a stable activity of the preparations for at least 1 h. All experiments were performed in the same conditions in parallel assays in the following basic medium: 30 mM Tris/10 mM Mes/10 mM $MgSO_4$/1 mM dithiothreitol/10% glycerol, the pH being adjusted to 7.4 with HCl. To 1.75 ml of this medium were added 10 μl 0.15 mM valinomycin and 10 μl 0.25 mM ACMA (1.25 μM final concentration) and the final volume was adjusted to 2 ml with distilled water, taking into account protein and effectors addition as indicated in the legends of figures. Proteins (submitochondrial particles or ATPase-ATP synthase complex) were preincubated 1 min in this medium at room temperature. For proton translocation the fluorescence of ACMA in the assay medium was taken as 100% for a full scale of the recorder. Then the reaction was initiated by adding ATP or succinate (adjusted at pH 7.4 with Tris) as indicated in the legends. The initial rate of the fluorescence quenching of ACMA was estimated from the slope of the trace recorder in less than 30 s. A Farrand manual spectrofluorimeter was used and the excitation and emission wavelengths were 420 and 510 nm, respectively. The estimation of ΔpH induced in inverted submitochondrial particles by succinate oxidation was performed as described by Dufour et al. [9]. The internal pH of the energized vesicles was estimated by acidification of the external medium by addition of small amounts of 1 M HCl until the initial fluorescence intensity was restored. The final pH was checked with a pH meter. The ΔpH was calculated as the difference between the initial value of 7.4 and the final pH value.

[γ-^{32}P]ATP hydrolysis and ATP-$^{32}P_i$ exchange activities were measured as previously described [11]. The measurements of net ATP synthesis induced by succinate oxidation in the presence of inverted submitochondrial particles were performed by phosphorylation of ADP with $^{32}P_i$ in the above medium except for the addition of 10 mM glucose plus 30 hexokinase units. The glucose 6-[^{32}P]phosphate formed was extracted and counted as above for the [γ-^{32}P]ATP.

$^{32}P_i$ was obtained from the CEA, France, [γ-^{32}P]ATP from Amersham, hexokinase from Sigma and nucleotides from Boehringer. ACMA was a generous gift of Dr. Blasco and Dr. Kraayenhof.

Results and Discussion

Preliminary experiments

It has been shown that the energization of energy transducing membranes induces a quenching of ACMA fluorescence [12,13]. We see in Fig. 1 that the quenching obtained after addition of ATP (Fig. 1A) or of succinate (Fig. 1B) to inverted pig heart submitochondrial particles proceeds at a rapid initial rate to reach a steady state. With ATP as the substrate, oligomycin fully restores the initial fluorescence, since ATP hydrolysis is blocked and therefore the supply of protons also. In contrast, oligomycin increases the quenching of fluorescence due to succinate oxidation, suggesting that protons can no longer get through the proton channels of the F_0F_1 complex. In both cases, the

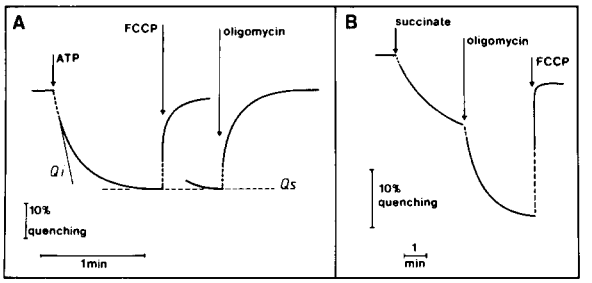


Fig. 1. Fluorescence quenching of ACMA induced by energization of inverted submitochondrial particles. (A) ATP-induced fluorescence quenching of ACMA. Inverted submitochondrial particles (0.26 mg) were preincubated for 1 min in the medium described in Materials and Methods, except that 50 mM KCl was present. The reaction was initiated with 0.05 mM ATP. FCCP (2.5 μM) or oligomycin (5 μg) were added when indicated. Q_i: initial rate of fluorescence quenching; Q_s: quenching of fluorescence at steady state. (B) Succinate-induced fluorescence quenching of ACMA. Inverted submitochondrial particles (0.26 mg) were preincubated for 1 min in the medium described in Material and Methods, except that 50 mM KCl/10 mM glucose/5 mM ADP/20 mM potassium phosphate/30 units hexokinase was present in each assay. The reaction was initiated by 0.3 mM succinate. Oligomycin (0.5 μg) and FCCP (2 μM) were added when indicated by the arrows.

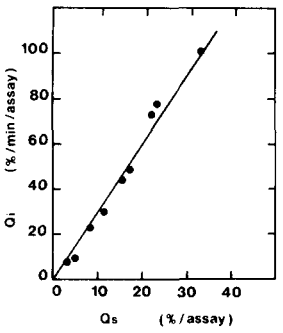


Fig. 2. Correlation between initial rate of ACMA fluorescence quenching (Q_i) and ACMA fluorescence quenching at steady state (Q_s) when energization was induced by various ATP concentrations. Conditions as in Fig. 1A except that the ATP concentration varied from 0.02–0.24 mM.

addition of FCCP restores the initial fluorescence, but if it is added before ATP or succinate, it prevents any quenching. Similar results were obtained when ATP was hydrolyzed by the F_0F_1 complex preparation.

Since we wanted to make a correlation between the quenching of fluorescence and constant rates of ATP hydrolysis or synthesis at various substrate concentrations, it appeared suitable to measure not the extent of quenching at steady state, but the initial rate of quenching as described by Dufour et al. [9] for yeast plasma membrane ATPase. However, Fig. 2 shows that under our conditions for various ATP concentrations, a linear relationship was obtained between the initial rates of fluorescence quenching and the extent of quenching at steady state.

Correlation between the ATPase activity of the ATPase-ATP synthase complex and the quenching of fluorescence of 9-amino-6-chloro-2-methoxyacridine due to proton movements

Fig. 3A shows that a significant increase in the initial rate of ATP-dependent fluorescence quenching is observed in the presence of KCl plus valinomycin and that no ATP-dependent fluorescence quenching of ACMA occurs in the presence of oligomycin. The maximal increase was observed with 50 mM KCl (data not shown). Under the latter condition, the $\Delta\Psi$ becomes negligible [14] and the fluorescence quenching of ACMA is related only to ΔpH generation [9]. The initial rate of fluorescence quenching is dependent on the ATP concentration. Double-reciprocal plots (Fig. 3B and C) are linear and give a K_m-ATP of about 0.1 mM for both fluorescence quenching and ATP hydrolysis with or without KCl. KCl increases the V_m by 3.6-fold only for the fluorescence quenching (Fig. 3B). A linear plot of the relative initial rate of fluorescence quenching versus the initial rate of ATP hydrolysis is obtained (Fig. 3D).). It passes through the origin, indicating that a direct correlation exists between the two phenomena. This proves the absence of any other quench-inducing process under our conditions. The presence of KCl plus valinomycin does not modify these characteristics

Therefore, since the K_m-ATP is the same for both the rates of fluorescence quenching and ATP hydrolysis and since there is a linear correlation between the two phenomena, the quenching of ACMA fluorescence observed appears essentially related to the proton movements due to ATP hydrolysis.

Correlation between $^{32}P_i$ incorporation into ATP and fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine in the presence of the ATPase-ATP synthase complex

As previously shown [11], our preparation of vesicular ATPase-ATP synthase complex is a tightly coupled system. In this kind of system, which is no longer linked to the respiratory chain, the energy necessary for ATP synthesis is supplied by ATP hydrolysis and the synthesis of ATP is estimated by incorporation of $^{32}P_i$ into the γ position of ATP (ATP-$^{32}P_i$ exchange). Therefore the rates of ATP hydrolysis, $^{32}P_i$ incorporation into ATP in addition to the rate of fluorescence quenching of ACMA have been studied in parallel assays. The ATP concentration was low (0.2 mM) to permit precise measurement of the initial rates of fluorescence quenching. ADP was present in the medium to obtain an immediate linear rate of $^{32}P_i$ incorporation into ATP as previously described [11]. The effects of P_i concentration on the reaction rates were studied. In Fig. 4A, ATP hydrolysis activity (closed squares) and P_i incorporation into ATP (open squares) increase with P_i concentration. In the latter case, Michaelis-Menten kinetics was observed and in reciprocal plots (not

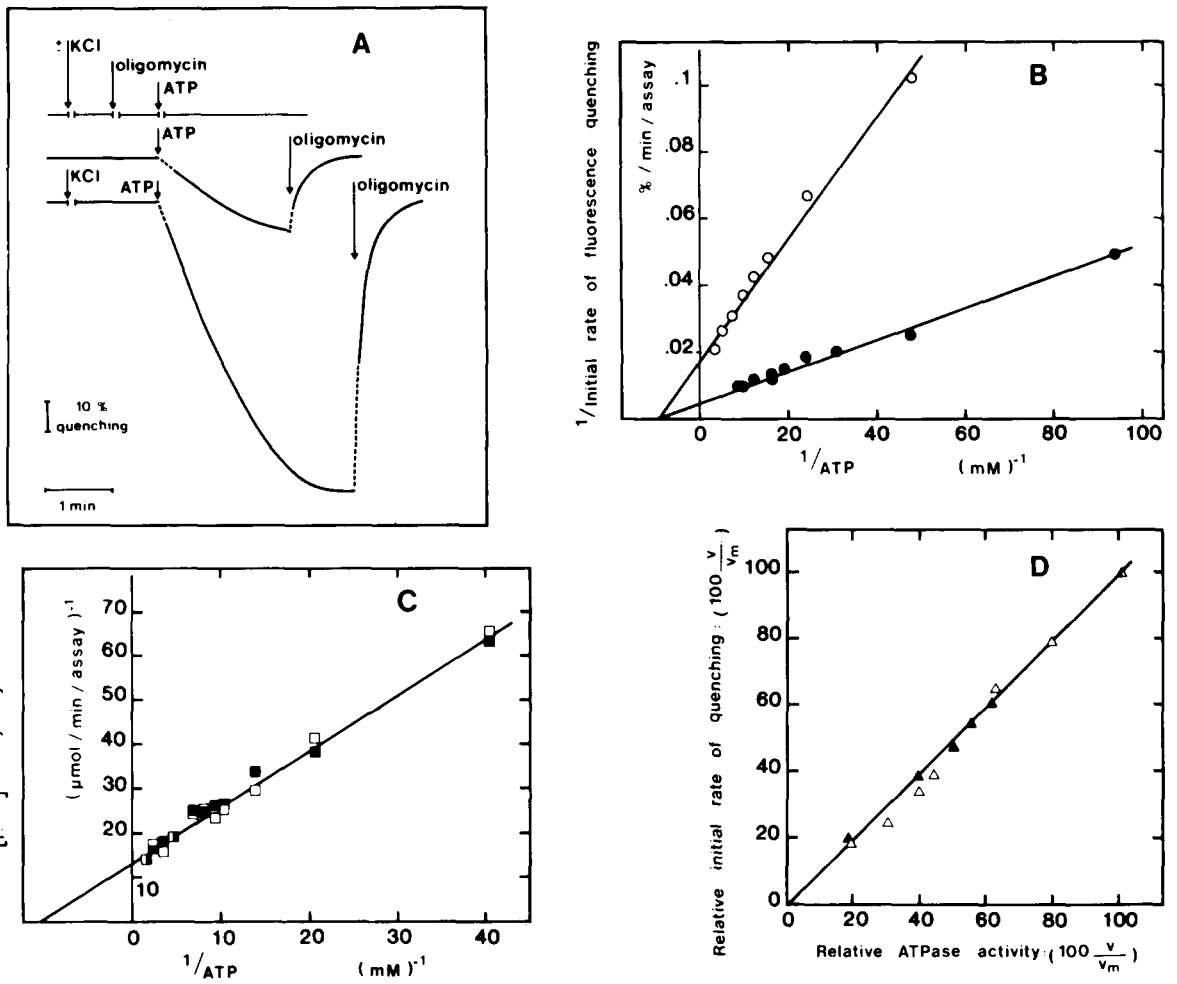


Fig. 3. Correlation between ATP hydrolysis and fluorescence quenching of ACMA in the ATPase-ATP synthase complex. The complex (0.11 mg) was preincubated for 1 min in the medium containing valinomycin as described in Materials and Methods in the presence (●, ■, ▲) or in the absence (○, □, △) of 50 mM KCl. The reaction was initiated by addition of ATP at the indicated concentrations. To measure the rate of [γ-^{32}P]ATP hydrolysis, the reaction was stopped after 15 s by introduction of the sample in the $^{32}P_i$ extraction mixture. The $^{32}P_i$ liberated was counted as previously described [11]. (A) Fluorescence quenching of ACMA induced by 0.04 mM ATP, effect of KCl and oligomycin (5 μg/assay); (B) effect of ATP concentration on the initial rate of the ATP-dependent fluorescence quenching of ACMA in the presence (●——●) or in the absence (○——○) of KCl; (C) effects of [γ-^{32}P]ATP concentration ($7 \cdot 10^3$ cpm/nmol) on the rate of [γ-^{32}P]ATP hydrolysis in the presence (■——■) or in the absence (□——□) of KCl; (D) plot of relative initial rate of fluorescence quenching of ACMA versus initial rate of [γ-^{32}P]ATP hydrolysis in the presence (▲) or in the absence (△) of KCl.

shown), a K_m of 5 mM for phosphate was obtained. In the case of fluorescence quenching (open circles) the initial rates first increase with P_i concentration and then decrease at higher P_i. These results can be interpreted in terms of relative rate of synthesis and hydrolysis. At low P_i, ATP synthesis (open squares) is slow and utilizes a small part of the protons produced by ATP hydrolysis. The remaining protons account for the increase of fluorescence quenching. With higher P_i, ATP synthesis increases quicker than ATP hydrolysis and makes use of an increasing amount of protons; thus, the quenching of fluorescence decreases accordingly.

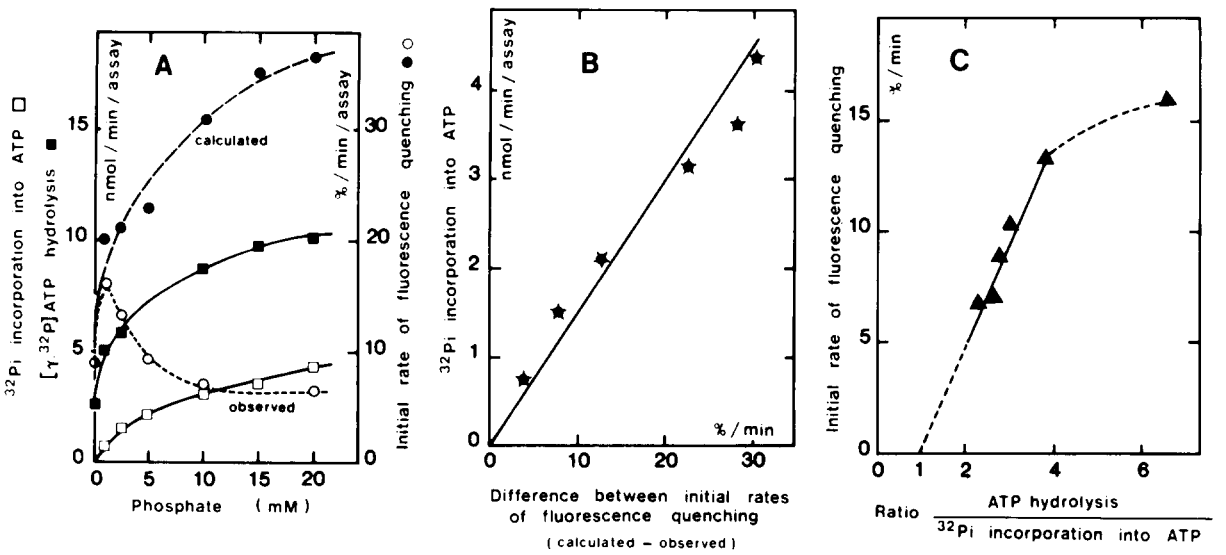
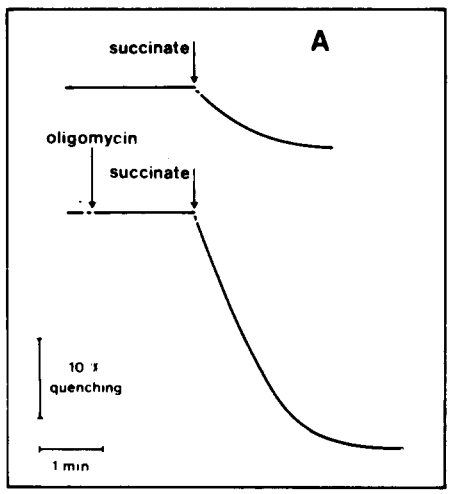
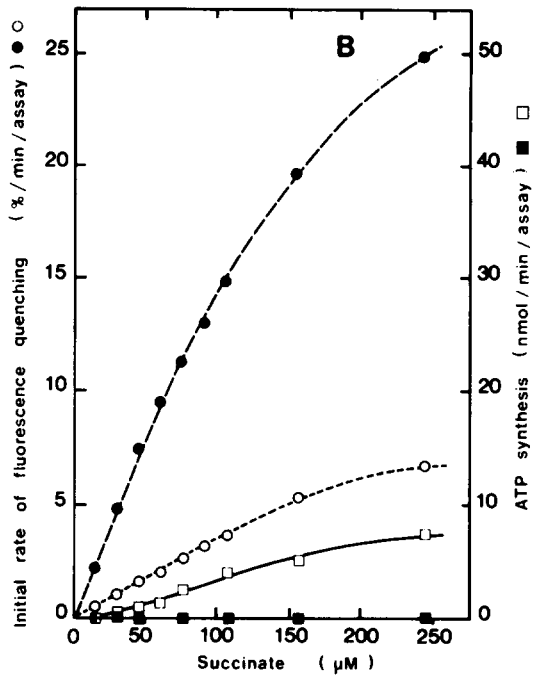


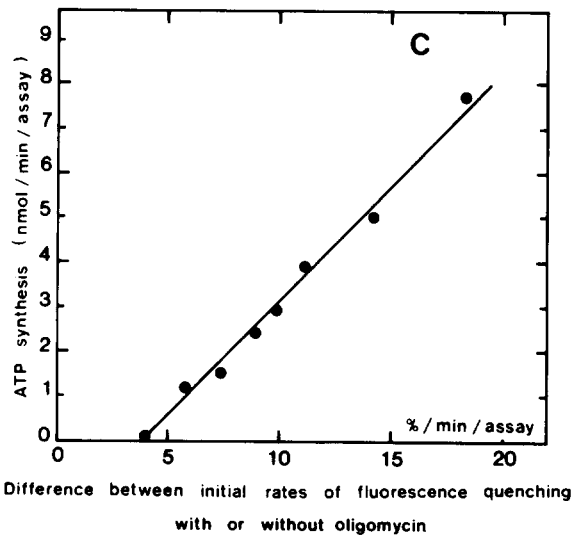
Fig. 4. Correlation between the rate of $^{32}P_i$ incorporation into ATP and relative disappearance of initial rate of fluorescence quenching of ACMA in ATPase-ATP synthase complex. The complex (0.11 mg) was preincubated for 1 min in the medium described in Materials and Methods, except that 0.5 mM ADP and potassium phosphate at the indicated concentration were added. The K^+ concentration was maintained at 50 mM in each assay by an adequate KCl addition. The reaction was initiated with 212 μM ATP and stopped 30 s later to measure the amount of [γ-^{32}P]ATP hydrolyzed or $^{32}P_i$ incorporated into ATP (ATP-^{32}Pi exchange) as previously described [11]. (A) Effect of phosphate concentration on the initial rate of [γ-^{32}P]ATP hydrolysis (■———■), $^{32}P_i$ incorporation into ATP (□———□) and fluorescence quenching of ACMA observed (○———○) or calculated (●———●). Calculated quenching for each P_i concentration: $Q_p = H_p(Q_0/H_0)$, where H_p is the rate of ATP hydrolysis at the indicated P_i concentration, Q_0 and H_0 are quenching and hydrolysis at $|P_i| = 0$. The specific radioactivity used for the [γ-^{32}P]ATP hydrolysis measurements was $22 \cdot 10^3$ cpm/nmol. For ATP-$^{32}P_i$ exchange activity, 50 μCi $^{32}P_i$ was added in each sample. (B) $^{32}P_i$ incorporation into ATP as a function of the difference between the calculated and the observed initial rates of fluorescence quenching of ACMA obtained in (A). (C) Plot of the initial rate of fluorescence quenching of ACMA versus the ratio: ATP hydrolysis over $^{32}P_i$ incorporation into ATP. (The data are obtained from (A).)

As shown in Fig. 3D, when only ATP hydrolysis took place, the quenching of fluorescence increased linearly with the hydrolytic rate. We can use this correlation to calculate the expected quenching corresponding to the rates of ATP hydrolysis for each P_i concentration (data given by Fig. 4A closed squares). This enabled us to draw the calculated curve of fluorescence quenching Fig. 4A (closed circles). The differences between the calculated curve and the actually observed curve can be related to the rates of ATP synthesis (Fig. 4B). The linear relationship obtained demonstrates that the difference in the rate of fluorescence quenching is directly correlated with ATP synthesis and reflects the protons used for ATP synthesis.

An additional argument is given in Fig. 4C. As previously defined [11], the ratio between ATP hydrolysis and ATP synthesis (H/S) is a good measure of the efficiency of energy recovery. The smaller and closer the ratio is to 1, the higher the efficiency. The data of Fig. 4A (ATP synthesis, ATP hydrolysis and observed fluorescence quenching) were used to draw Fig. 4C. The latter shows that the plot of initial rate of fluorescence quenching observed versus the ratio H/S extrapolates to 1. This demonstrates that if the energy from ATP hydrolysis were completely recovered for ATP synthesis, no fluorescence quenching would be observed; in other words, no proton movement could be detected by ACMA under these conditions. This could happen if the proton movements in each case could be limited to a single molecule of ATPase-ATP synthase complex. It also means that protons efficient for ATP synthesis are restricted to local circuits not accessible to ACMA fluores-







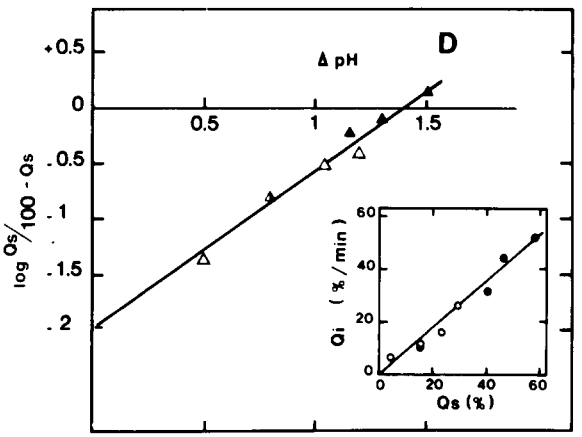


Fig. 5. Correlation between net ATP synthesis and quenching of fluorescence of ACMA in inverted submitochondrial particles oxidizing succinate. Relation between ΔpH and quenching. Inverted submitochondrial particles (0.45 mg) were preincubated for 1 min in the medium described in Materials and Methods, except that glucose (10 mM), ADP (5 mM), potassium phosphate (20 mM) (final concentrations) were added. KCl was also added to match the final K^+ concentration of 50 mM. 30 units hexokinase were present in each assay (2 ml). When indicated, oligomycin (5 μg/assay) was present. The reaction was initiated by addition of succinate at the indicated concentrations, and stopped 30 s later for [γ-^{32}P]ATP synthesis measurements (see Materials and Methods). The specific radioactivity of $^{32}P_i$ was $18 \cdot 10^3$ cpm/nmol. (A) Fluorescence quenching of ACMA induced by 0.15 mM succinate in the presence or in the absence of oligomycin; (B) effect of succinate concentration on the initial rate of [γ-^{32}P]ATP synthesis and of the succinate dependent fluorescence quenching of ACMA in the presence (●, ■) or in the absence (○, □) of oligomycin; (C) [γ-^{32}P]ATP synthesis as a function of the difference between the initial rates of fluorescence quenching of ACMA obtained for each concentration of succinate in the presence of oligomycin and that obtained in the absence of oligomycin (data from (B)); (D) relation between ΔpH and fluorescence quenching of ACMA induced by addition of succinate at concentrations ranging from 0.012–0.15 mM in the presence (▲) or in the absence (△) of oligomycin. The ΔpH was estimated as described in Materials and Methods. Q_s is the quenching at the steady state. Insert: initial rate of fluorescence quenching (Q_i) as a function of quenching at the steady state (Q_s).

cence measurements. To check whether local circuits for protons could also be detected between the respiratory chain and the complex, experiments were performed with inverted phosphorylating submitochondrial electron transport particles using the same technique.

Correlation between net ATP synthesis and fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine by the inverted submitochondrial particles oxidizing succinate

Upon addition of succinate the fluorescence quenching of ACMA was recorded until a steady state was reached (Fig. 5A). For a fixed concentration of succinate, the initial rate of quenching is greatly increased in the presence of oligomycin, indicating a proton accumulation somewhere in the vesicles. As shown in Fig. 5B, oligomycin completely inhibits ATP synthesis. This figure also shows the increase, with succinate concentration, of initial rates of ATP synthesis and of fluorescence quenching of ACMA (with or without oligomycin). When protons are used for ATP synthesis, the initial rate of fluorescence quenching is very low when compared with that obtained in the presence of oligomycin. However, oligomycin affects neither the Michaelian kinetics nor the K_m-succinate of 0.6 mM for the initial rate of florescence quenching (plot not shown). For each succinate concentration used in Fig. 5B, the difference in the rates of fluorescence quenching in the presence and in the absence of oligomycin was calculated and plotted versus the initial rate of ATP synthesis (Fig. 5C). A linear correlation is obtained beyond a threshold of 4% difference between initial rates of fluorescence quenching. This means that ATP synthesis cannot take place until this critical value is reached. Using the experimental approach of Dufour et al. [9], described in Materials and Methods, we have measured the pH between the two bulk phases corresponding to the fluorescence quenching of ACMA at the steady state. Fig. 5D shows a linear relationship between the measured ΔpH and log $Q_s/(100 - Q_s)$, Q_s giving the percent of quenching of fluorescence at the steady state. In addition, under these conditions the initial rate of fluorescence quenching is also directly correlated with its extent at the steady state (insert, Fig. 5D). Therefore, these two correlations allow us to conclude that the initial rate of fluorescence quenching reflects the generation of ΔpH. Thus we were able to estimate that, as soon as the ΔpH reached 0.5 units (corresponding to the critical value of 4% difference in quenching of fluorescence in Fig. 5C), the ATP synthesis could be detected.

Conclusions

Without needing to re-evoke the controversies about the mechanism of quenching of ACMA fluorescence in the presence of a membrane, the ΔpH values measured in our experiments by successive additions of HCl appear well correlated with the fluorescence quenching. There is also a linear correlation between the following four processes examined: the initial rate of ΔpH generation, fluorescence quenching, ATP hydrolysis and ATP synthesis (induced either by ATP hydrolysis or succinate oxidation). Under our conditions, in which $\Delta\Psi$ is collapsed as much as possible (K^+ plus valinomycin), the ATP synthesis can be started as soon as ΔpH rises over 0.5 units, which is a very low value. Moreover, when the ATPase-ATP synthase complex catalyzes almost at the same rate both ATP synthesis and ATP hydrolysis, no proton movements could be detected, since the fluorescence quenching of ACMA tends towards zero. This proves that the protons efficient for ATP synthesis are protected against delocalization into bulk phase. Since oligomycin immediately restores the full fluorescence of ACMA when ATP hydrolysis is blocked, it means that as soon as the proton flow of ATP hydrolysis is stopped, the remaining protons diffuse and are no longer accessible to ACMA. On the other hand, addition of oligomycin to inverted submitochondrial particles oxidizing succinate increases the fluorescence quenching, indicating that protons rapidly produced by the respiratory chain can accumulate in a compartment as shown by the increase of ΔpH. Moreover, with the ATPase-ATP synthase complex, the closer the rate of ATP synthesis is to that of ATP hydrolysis, the lower the number of protons detected by ACMA. Therefore, as a general conclusion, the amount of protons efficient for ATP synthesis appears to be the result of a rapid production of protons, either by substrate oxidation or by ATP

hydrolysis and a relatively low diffusion process of protons going towards the bulk phase. Thus efficient protons appear to be localized in a "kinetic compartment".

Acknowledgements

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