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KINETIC FACTORS LIMITING THE SYNTHESIS OF ATP BY CHROMATOPHORES EXPOSED TO SHORT FLASH EXCITATION

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Summary

ATP synthesis was measured after chromatophores from *Rhodopseudomonas capsulata* had been subjected to illumination by single turnover flashes fired at variable frequencies. Three processes were examined, which under different conditions can limit the net yield of ATP.

(1) A process with an apparent relaxation time of 10—20 ms. This reaction probably limits the rate of ATP synthesis in continuous illumination. It has a similar time dependence to the stimulation of the carotenoid shift decay by ADP after a single flash.

(2) An active state of the ATPase only persists when the chromatophores are excited more often than once in 10 s. This state decays with similar kinetics to the entire carotenoid shift decay. Full activation is achieved after two flashes.

(1) and (2) are not significantly affected by concentrations of antimycin A sufficient to block electron flow through the cytochrome b/c_2 oxidoreductase and abolish phase III in the generation of the carotenoid shift.

(3) In the presence of antimycin A, after the third, fourth and subsequent flashes ATP synthesis is limited by the quantity of reducing equivalents transported through the reaction centre rather than by the level of the electrochemical proton gradient.

Introduction

The excitation of photosynthetic tissues with flashes fired at different frequencies has revealed several factors which limit the reactions that give rise

Abbreviation: BChl, bacteriochlorophyll.

to ATP synthesis. Under some circumstances the rate of ATP synthesis may be limited by the rate of turnover of the electron transport chain. For instance in *Rhodospirillum rubrum* chromatophores the ATP yield is dependent on the integrity of the cytochrome *c* reaction centre complex [1]. In chloroplasts [2,3] and in chromatophores from *Rhodopseudomonas capsulata* treated with valinomycin [4] a lag in the onset of ATP synthesis indicates that there may be a delay in the generation of sufficient high energy state to drive the ATPase. In fact it has been shown that under short flash activation of chloroplasts, the amount of ATP synthesised may be limited by the level of either the electrical or chemical components of the electrochemical gradient of H^+ across the membrane [5]. Interaction between the ATPase and its endogenous inhibitor may also limit ATP synthesis. In chloroplasts the displacement of the inhibitor is dependent on the number and frequency of flashes in a train [6]. An apparent activation of the ATPase after two flashes has also been reported for *Rhs. rubrum* chromatophores [7].

Even when chromatophores are excited by flashes that are so short that the photosynthetic reaction centres expel and accept single electrons there is a significant amount of ensuing ATP synthesis [8,9]. The yield is low, about one ATP/5—15 reaction centres [9]. Under conditions of single and multiple flash activation the fate of the single electrons entering and leaving the reaction centre is now fairly well defined [10]. Similarly the kinetics of H^+ binding on the outside of the chromatophore membrane have been examined in detail [11]. Data on the rate of H^+ release inside the vesicles are still unavailable [12] but there is a good reason to believe that the chromatophore carotenoid shift faithfully reflects the membrane electrical potential generated by combined electron and proton translocations [13,14]. In the experiments reported below, our aim was to relate the yield of ATP synthesis after single and multiple flashes with electron transfer events and carotenoid shift measurements in order to determine which processes are rate determining.

Methods

The growth of *Rps. capsulata*, the preparation of chromatophores and the spectroscopic methods are outlined and referenced in the accompanying paper [9]. 20 μs half-peak width flashes were provided with a single xenon flash tube, triggered with a pulse generator. Flash repetition at consistent intensity was limited to frequencies less than 50 Hz. Where signal averaging was necessary, for example in carotenoid shift determinations, the high frequency pulse trains were repeated at intervals of not less than 50 s [9].

Results

When chromatophores from *Rps. capsulata* are illuminated with a sufficiently short flash of light, each reaction centre delivers a single electron to a low potential ubiquinone pool and accepts an electron from cytochrome c_2 [10]. This situation is closely realised with a 20 μs flash and, in our experiments, provided the bacteriochlorophyll concentration is below 20 μM, more than 85% of the reaction centres within the cross-section of the measuring beam

are excited. After the flash, electrons proceed through the ubiquinone-cytochrome b/c_2 complex at a rate which is dependent on the ambient redox potential of the chromatophore suspension [10]. In the aerobic suspensions employed in the present series of experiments, carotenoid band shift phase III amounts to 20—30% of phases I and II and takes place wiht a half-time of about 1 ms [14]. This means that this proportion of the electrons arriving in the low potential ubiquinone pool proceed through the b/c_2 complex at this rate. The remaining electrons cycle with a half-time of the order of hundreds of milliseconds or, in those reaction centres deficient in cytochrome c_2, return in a back reaction with a half-time close to 1 s [15].

Under these conditions but with the addition of ADP and P_i, ATP is synthesised with a yield equivalent to one molecule/5—15 reaction centres, depending on the preparation [9]. Antimycin, at a concentration sufficient to inhibit electron flow through the ubiquinone-cytochrome b/c_2 oxidoreductase completely and eliminate carotenoid band shift phase III inhibits the single flash yield of ATP synthesis by 30—50%. Baltscheffsky has also reported minimal inhibition of single flash ATP synthesis in chromatophores from *Rhs. rubrum* [7].

By direct assay of the ATP yield with luciferin and luciferase it is not possible to resolve the kinetics of the synthesis, since the time course of light emission itself is fairly slow [16]. One estimate for the turnover time of the ATP-synthesising apparatus (including electron transport) can, however, be estimated by giving two activating flashes with varying times between them. In *Rps. capsulata* chromatophores the rate of hydrolysis of the newly synthesised ATP is extremely slow (half-time about 20 s) so that the double flash yield may be determined from the level of light emission 500—1000 ms later. Fig. 1 shows the ATP synthesis due to the second flash (the double flash minus the single flash yields) as a function of the dark time between the flashes from 20 to 256 ms and Fig. 2, with longer periods between the flashes.

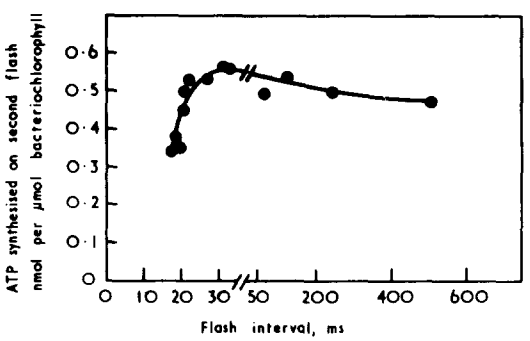

Fig. 1. Second flash yield of ATP as a function of time (18—512 ms) between two flashes. Chromatophores from *Rps. capsulata* strain N22 were suspended to a bacteriochlorophyll concentration of 20 μM in 2.5 ml of a medium containing 100 mM glycylglycine, 10 mM magnesium acetate, 100 μM sodium succinate, 2 mM potassium phosphate, 50 mM KCl, 30 μM ADP, 5 μM antimycin, 150 μM luciferin, 0.2 mg luciferase at a final pH of 7.75. Light emission was recorded as described [9] and the second flash yield estimated as in the text. The single flash yield was 0.35 nmol ATP/μmol bacteriochlorophyll.

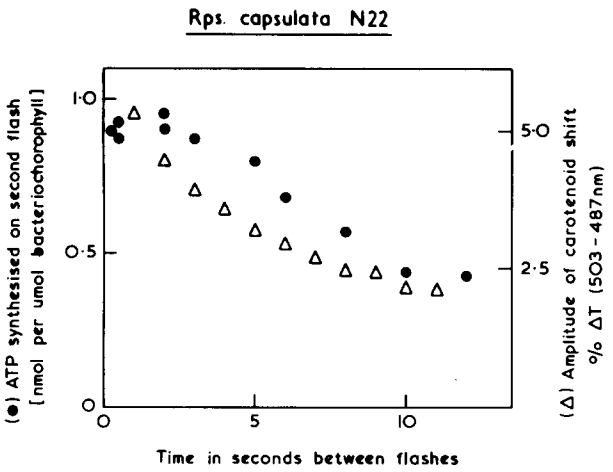

Fig. 2. Second flash yield of ATP as a function of time (0.25—12 s) between two flashes. Conditions as Fig. 1. The single flash yield in this chromatophore preparation was 0.5 nmol ATP/μmol bacteriochlorophyll. The carotenoid decay kinetics after a single flash were measured at 503 nm in a similar medium, lacking luciferin and luciferase, and as described in the previous paper [9].

The first point of interest, emerging from these data is that in either the presence or absence of antimycin A the second flash yield of ATP is about 50% greater than that after a single flash when the double flash dark time is of the order of 40 ms. A similar effect has been observed with chromatophores from *Rhs. rubrum* [7]. When the flashes are moved closer together the second flash yield decreases. For technical reasons the flashes cannot be fired at less than 18-ms intervals but, nevertheless some of the kinetics of the recovery may be observed between 20 and 100 ms. The half-time of recovery appears to be about 5—10 ms. This appears to correspond neither with rapid electron flow through the ubiquinone-cytochrome b/c_2 complex ($t_{1/2}$ approximately 1 ms) nor with slow electron return ($t_{1/2}$ values approximately 200—1000 ms). Moreover, since similar recovery kinetics could be observed in the presence of antimycin A, the rate-limiting step in the turnover does not appear to be one associated with electron transport through the cytochrome b/c_2 complex. There may be a correlation between the recovery of ATP synthesis on the second flash and that component of the carotenoid shift decay which is accelerated by ADP and P_i and which has a $t_{1/2}$ = 5—10 ms [9].

When the time between the two flashes is increased beyond about 500 ms the second flash yield decreases. It subsides to the same value as the single flash yield at dark times greater than about 5—10 s. Fig. 2 compares the decrease in second flash yield with the decay kinetics of the carotenoid shift. Under these conditions the carotenoid shift decay kinetics resemble those of the decay of the pH-indicating absorption change of added cresol red [16]. It therefore appears that the ability of the chromatophores to synthesise extra ATP on the second flash is related to the level of $\Delta\bar{\mu}_{H^+}$ before that flash.

When a chromatophore suspension, in the absence of antimycin A is excited with a train of flashes, 256 ms apart, the ATP yield varies with the number of

pulses in the train as shown in Fig. 3. The increased yield of ATP synthesis is only observed from the first to the second flash; thereafter the yield/flash remains constant. If the flash frequency is either increased or decreased significantly from once every 256 ms then the ATP yield decreases for the reasons given above, i.e. when the flashes are fired at 20 s apart, the 'activation' phenomenon decays between the flashes and when they are fired 18 ms apart the ATP-synthesising machinery has insufficient time to recover (Fig. 3).

In the absence of antimycin A, ATP synthesis is ultimately limited at high excitation frequencies by that process with a half-time of the order of 10—20 ms. In saturating, continuous light, we should therefore expect between 37 and 70 turnovers/s. After the first flash, the yield for a single flash in a train is approximately 1.4 nmol ATP/μmol bacteriochlorophyll. In steady-state light we would therefore predict a rate of ATP synthesis equal to 3—6 mol ATP/mol bacteriochlorophyll per min, in excellent agreement with our routine rates under continuous illumination (1—3 mol ATP/mol bacteriochlorophyll per min).

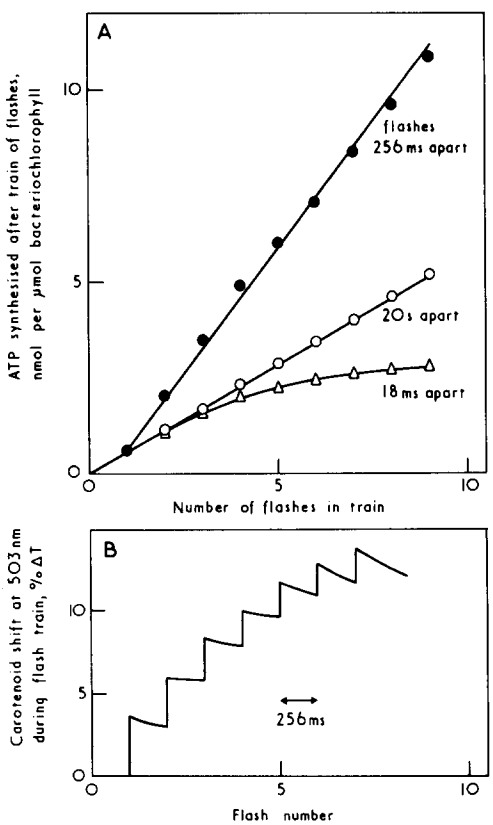

Fig. 3. ATP synthesis in a flash train in the absence of antimycin. (A) Conditions as Fig. 1 but in the absence of antimycin A. (B) Experiments were carried out in the same suspension medium (minus luciferin and luciferase) with the carotenoid shift measured at 503 nm. The scales of (A) and (B) have been normalised but note that (B) illustrates the kinetics of the carotenoid shift during flash activation on a real time scale whereas (A) only indicates the amount of ATP synthesised at the end of a train of flashes.

With long dark times between flashes in a train (20 s in Fig. 4), antimycin shows a constant 30% inhibition, independent of the number of flashes in the train (compare Fig. 3). Within the 20 s dark time, electron transport reactions are complete and the activation of the ATPase does not persist. At a flash frequency of about 4 s^{-1} in the presence of antimycin A, the yield of ATP, although only decreased by about 50% after the first and second flashes drops considerably after subsequent flashes. This type of behaviour clearly correlates with the cyclic electron transport reactions during multiple flash activation, which has been documented for example for the photooxidation of cytochrome c_2 or reaction centre bacteriochlorophyll in the presence of antimycin A [18]. Since the majority of the reaction centres in the chromatophore suspension are associated with two cytochrome c_2 haems, extensive photooxidation is observed after each of two closely spaced flashes [19]. If cyclic electron return to cytochrome c_2 is blocked with antimycin A further flashes do not elicit substantial oxidation. Similar behaviour is observed on the acceptor side of the reaction centre for the photoreduction of cytochrome *b*. At the pH and apparent redox potential of the chromatophores in the present experiments we expect H^+ release on the inside of the chromatophore lumen to display a similar pattern [12].

The ATP yield after multiple flashes, in the presence of antimycin A does not correlate with either the level of $\Delta\psi$, indicated by the carotenoid shift, or with the external change in pH indicated by cresol red absorption in unbuffered chromatophore suspensions (see also [17]). After the first flash (plus antimycin, ADP, P_i) the signals from both of these probes are complete within 1 ms. 256 ms after the flash the carotenoid shift has decayed by approximately 20% and the second flash takes the signal amplitudes beyond that achieved by the first flash. During subsequent flashes and dark periods the carotenoid shift and cresol red absorption change remain above the levels achieved after the first flash (Fig. 4B).

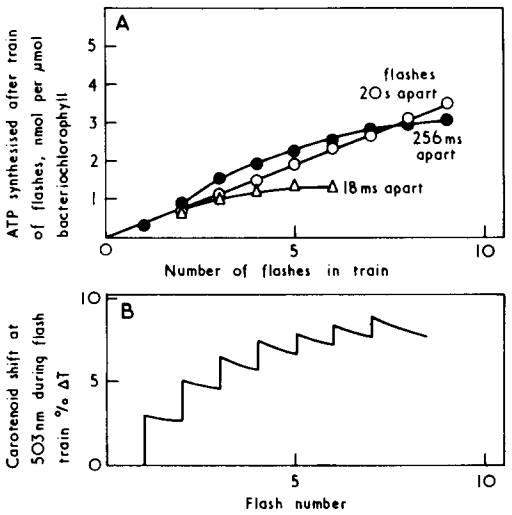

Fig. 4. ATP synthesis in a flash train in the presence of antimycin. As Fig. 3 plus 5 μM antimycin A.

The data of Fig. 4 probably explain why, at the high flash frequencies used by Del Valle-Tascon and coworkers [1], ATP synthesis in *Rhs. rubrum* vesicles is dependent on the integrity of the electron transport chain, i.e. the cytochrome *c* content of the chromatophores. Dislocation of the cytochrome *c* from the reaction centre is equivalent to adding antimycin A to block effective completion of the electron transport cycle. We suspect that the limitation of the cytochrome *c* content would not be serious at very low flash frequencies. Our *Rps. capsulata* preparations routinely possess approximately 0.4—0.6 cytochrome c_2 pair/reaction centre so that in contrast to the results of the Dutch group [1], the ATP yield/flash per functional cytochrome is much less than unity. Baltscheffsky [7], working with single flash excitation of *Rhs. rubrum* observed similar yields to our own. Again we suggest that experiments performed at high flash frequency should be interpreted with caution.

Conventional redox potentiometry under anaerobic conditions is prohibited with luciferase because of the strict oxygen requirement of the reaction. We are able, however to perform aerobic redox titrations with ferri- and ferrocyanide at the high end of the scale, between 300 and 500 mV.

Single flash yields of ATP synthesis are only slightly inhibited when the redox potential of the suspension is raised to between 400 and 420 mV where cytochrome c_2 is more than 90% oxidised before excitation (Fig. 5). Under these conditions, ADP and P_i still give rise to an acceleration of the carotenoid band shift decay after a single flash [15]. The synthesis of ATP is abolished at potentials greater than 500 mV when the primary photochemistry is blocked. When antimycin-treated chromatophores are exposed to a train of closely spaced flashes a significant proportion of the ATP yield is attenuated co incidentally with a single flash cytochrome c_2 photooxidation (Fig. 5). When

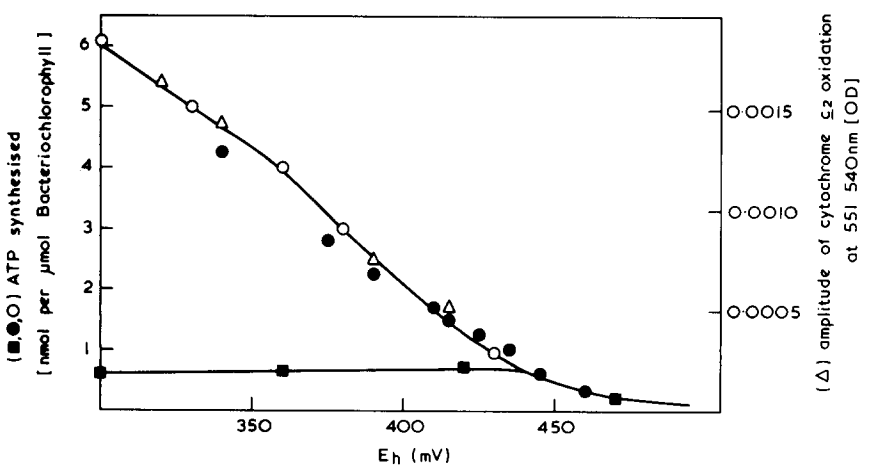

Fig. 5. Redox potential dependence of flash-induced ATP synthesis. Experimental conditions as Fig. 1 except that the redox potential was poised with mixtures of ferri- and ferrocyanide (total concentration 100—400 μM). The redox potential was determined before and after flash excitation with platinum and reference calomel electrodes. ■, single flash yield of ATP; ○, ●, ATP synthesis after a train of ten flashes at 3.9 Hz (○, measurements made on the same sample going from low to high redox potential; ●, measurements on a fresh sample going from high to low redox potential); △, cytochrome c_2 photooxidation at 551—540 nm after single flashes by conventional double-beam spectroscopy.

the cytochrome c_2 is completely oxidised before excitation we observe similar extents of ATP synthesis after single flashes or multiple flashes. The dependence of the ATP yield after multiple flashes upon the redox state of cytochrome c_2 is clearly an electron transport turnover effect: when cytochrome c_2 is chemically oxidised, only the first flash in a train is capable of eliciting charge separation through the reaction centre.

Discussion

Even in the presence of antimycin A, a significant quantity of ATP is synthesised on single turnover excitation of chromatophore suspensions. It may be concluded that energy released in the segment of electron transport:

$$\text{cytochrome } c_2 \rightarrow [\text{BChl}]_2 \rightarrow Q_A(\text{Fe}) \nearrow Q_B \searrow \text{cytochrome } b\text{-}50$$
$$H^+_{OUT} \quad H^+_{IN}$$

may be effectively coupled to ATP synthesis. It seems that the oxidation of cytochrome c_2 by reaction centre $[\text{BChl}]_2$ contributes very little to the single flash yield of ATP since the yield is barely inhibited at redox potentials where cytochrome c_2 is chemically oxidised before the flash. In our aerobic suspensions of *Rps. capsulata* chromatophores antimycin inhibits the extent of the carotenoid band shift by about 30% (i.e. it eliminates phase III) [14] and ATP synthesis by the same degree. It is tempting to suggests that the antimycin-sensitive ubiquinone-cytochrome b/c_2 complex makes an energetic contribution to ATP synthesis equal to that of the electron transport segment detailed above. This interpretation must, however, be qualified by considerations discussed below.

When the chromatophores are excited by two closely spaced flashes (dark time 40 ms) the second flash ATP yield exceeds the first by about 50%. This may be an 'activation' of the ATPase as described by Harris and Crofts [6] for chloroplast membranes and by Baltscheffsky for *Rhs. rubrum* chromatophores [7] and may be related to the energised-state dependence of chromatophore ATP hydrolysis [20,21]. Unlike the chloroplast system [6], activation is only observed on the second flash, subsequent flashes in chromatophores elicit the same yield as the second flash. It seems unlikely that further activation is achieved upon continuous illumination since the flash yields may be extrapolated to predict accurately the rates of ATP synthesis which can be measured in steady-state light. Control of the state of activation is perhaps achieved through the level of the electrochemical proton gradient across the membrane (Fig. 3). The activated enzyme does not appear to be stable but decays in parallel to the decay of the carotenoid absorption band shift.

The attenuation of the second flash yield when the two flashes are fired closer together than 40 ms will ultimately limit the net rate of ATP synthesis in steady-state light. The kinetics of this process resemble the acceleration of the carotenoid shift by ADP and may be related to the turnover time of the ATPase. The half-time of the reaction is similar to that described for chloroplast ATPase turnover (Ref. 6, and Graber, P., personal communication). This interpretation would however, be liable to two major problems. First, it is diffi-

cult to see why the energised intermediate driving ATP synthesis does not persist in the 20—50 ms between flashes: at least the carotenoid shift and pH indicator signals of added dyes only fall by about 20% in this time. Second, the efrapeptin and venturicidin titrations suggest that there is one ATPase/100—200 bacteriochlorophyll [9]. Preliminary results with ATPase labelling by 4-chloro-7-nitrobenzofurazan [22] (in collaboration with Dr. S.J. Ferguson) gave similar values. This would suggest that only about 15—20% of the total ATPase turns over after a single flash excitation.

A reaction whose rate has not yet been accerately measured is the release of H^+ on the inside of the chromatophore lumen [12]. This process may be a good candidate for the rate-limiting step in ATP synthesis at high flash frequencies. Such a suggestion may also help to explain why ATP synthesis ceases in antimycin A-treated chromatophores after three or four flashes despite the fact that the carotenoid shift indicates the presence of a substantial membrane potential; release of H^+ inside the chromatophore at external pH values above 7.4 is linked to the reduction of cytochrome *b* by ubiquinone [12] and cytochrome *b* photoreduction ceases after three or four flashes in a train of pulses.

These arguments are leading to a formulation of the coupling mechanism in which the protonic path between the electron transport chain and the ATPase is direct or localised rather than delocalised by way of a transmembrane electrochemical proton gradient. One more observation favouring this view is that from the estimated values of $\Delta\bar{\mu}_{H^+} = 70$ mV after a single turnover flash [23], from a stoichiometry of H^+ transferred to ATP synthesised of 2 [24] and from the standard free energy of hydrolysis of ATP $\Delta G^0 = -8.5$ kcal · mol^{-1} [25], we expect the ATP yield to be far less than that determined experimentally (an observation communicated by Dr. B.A. Melandri). This problem would be circumvented if the protons released in the electron transport reactions proceeded directly to the ATPase [26] without first having to charge up the electrical capacitance of the membrane or the pH-buffering capacitance inside the chromatophore [27].

Against the concept of the local coupling protons we note (i) in the presence of antimycin A, only one proton is bound/electron transferred and yet two protons are required to synthesise one ATP [24], (ii) there is apparently an equivalent number of reaction centres and ATPase complexes and yet only one ATP is synthesised/5—15 reaction centres, (iii) ATP synthesis is accompanied by an acceleration in the decay of the carotenoid band shift which appears to reflect a delocalised membrane potential [9,15].

From the foregoing discussion it is clear that in their present forms neither the chemiosmotic hypothesis [27] nor direct H^+ coupling hypotheses [26] adequately account for our data and compromises may be worth considering. For instance Melandri and coworkers [4] and Jackson et al. [28] have suggested that although $\Delta\mu_{H^+}$ provides the energy for ATP synthesis, a direct interaction between electron transport and the ATPase may also be required. Other investigators have suggested that there may be microscopic domains, for example at the membrane interface which act as proton-conducting paths not in rapid equilibrium with the bulk aqueous phase [29].

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