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Adjustable microchemiosmotic character of the proton gradient generated by Systems I and II for photosynthetic phosphorylation in thylakoids

Claude Sigalat, Francis Haraux, Françoise de Kouchkovsky, Suong Phung Nhu Hung and Yaroslav de Kouchkovsky

Laboratoire de Photosynthèse, C.N.R.S., B.P. 1, 91190 Gif-sur-Yvette (France)

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To clarify the debate on the localized character of proton gradients in energy-transducing membrane systems, the way in which the osmolarity and ionicity of the medium, which should affect the thylakoid lumen properties, may modulate the relative efficiency of $\Delta\tilde{\mu}_{H^+}$ generated by PS I or PS II (restricted here to ΔpH: valinomycin present) was examined. Although the results depended on the preparations and the conditions, a trend towards proton delocalization, especially in 50 mM KCl vs. the usual 5–10 mM, was observed when thylakoids were suspended in a sorbitol-free buffer for only a short time before the experiment. It was also verified that the better efficiency of PS I vs. PS II protons was not due to the 9-aminoacridine method used to quantify ΔpH. One main argument is that similar results were found when the proton gradient was estimated by total H^+ translocation, measured with a glass electrode, and by probe partitioning, followed in parallel. Lastly, it was observed that, even when protons are emitted by water-splitting enzymes, i.e., far from coupling factors, the rate of ATP synthesis is less sensitive to nigericin than expected from the ΔpH decrease. This suggests that protons are flowing, from redox to phosphorylating pumps, in an anisotropic medium. The role of vesicular configuration and topological organization of energy-transducing membranes in the microchemiosmotic behaviour of organelles is stressed. It is suggested that besides water, polypeptide chains, rather than lipid heads (owing to the limited effectiveness of lipophilic nigericin), may ensure the lateral H^+ transport between their points of influx and efflux.

Introduction

The chemiosmotic theory [1] is widely considered as the most adequate description of the energy-transducing processes in biomembranes. However, the precise nature of the high- and low-potential compartments for protons remains controversial. Although a purely intramembraneous coupling [2,3] seems now improbable, many data reported these last years suggest that hydrogen ions are not fully delocalized in the aqueous phases, as proposed by Mitchell. Whereas classical chemiosmosis requires univocal relationships between thermodynamic fluxes (electron flow, rate of ATP synthesis) and forces (transmembrane $\Delta\tilde{\mu}_{H^+}$, Gibbs free enthalpy change of phosphorylation ΔG_P), variable correlations were obtained with bacterial chromatophores [4,5], mitochondria [6–13], and – see below – chloroplasts. These anomalous results were generally interpreted according to a scheme

Abbreviations: $\Delta\tilde{\mu}_{H^+}$ and ΔpH, difference of proton electrochemical potential and of pH between two phases (absolute values here), respectively; PS I and PS II, Photosystems I and II; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

intermediate between chemiosmosis and direct coupling: a microscopic $\Delta\tilde{\mu}_{H^+}$, different from that averaged across the whole membrane, drives and regulates the energy-dependent processes [14–16]. This is called localized chemiosmosis or microchemiosmosis.

Local events were also invoked to explain flash-induced ATP synthesis by chloroplasts, before any formation of significant delocalized $\Delta\tilde{\mu}_{H^+}$ [17], and its relative insensitivity to permeant buffers [18], although opposite results were also claimed [19]. Some other facts, such as the variation of $\Delta G_P/\Delta\tilde{\mu}_{H^+}$ ratio independently of the way $\Delta\tilde{\mu}_{H^+}$ is modulated, were better interpreted by a slightly different model, mosaic chemiosmosis [20], according to which each redox pump exchanges its protons with one given ATP-synthetase, in separate 'coupling units'. In mitochondria and chromatophores, 'titration' of redox and phosphorylating pumps with appropriate inhibitors [21], used together [22,23] or with uncouplers [24], were rather consistent with this latter model, but some of these experiments were criticized [25], and their theoretical basis is disputable [26]. With chloroplasts, our group has shown that replacement of protons by deuterons increases the redox control, despite a diminished H^+ gradient, whereas phosphorylation falls more than expected from this $\Delta\tilde{\mu}_{H^+}$ decrease [27–29]. We gave to these data a microchemiosmotic interpretation [16], but partly different from the already published models. According to our results, a significant resistance for protons would separate the sites of H^+ input (redox carriers) and output (coupling factors). This means that a lateral pH drop may occur not only between coupling units and other points of the membrane, as with natural or artificial leaks, but also between redox proton pumps and ATP-synthetases. We strengthened this view by showing that protons emitted in granal regions of the thylakoid lumen are less able to drive ATP synthesis than protons coming from stromal areas, where coupling factors are located [30]. This latter result contradicts reports from McCarty's group [31,32], which concluded that there was an equal efficiency of $\Delta\tilde{\mu}_{H^+}$ generated by PS I and PS II in thylakoids. We present here new facts, confirming our previous data, and showing that the osmolarity and ionicity of the medium may influence the localized character of electrochemical proton gradients. However, some discrepancies between our results and others remain to be solved.

Materials and Methods

Chloroplasts were prepared by chopping in a blender, for a few seconds, 50–200 g of washed lettuce leaves with twice as high a number of ml of the following buffer: 0.4 M sorbitol, 10 mM Tricine, 10 mM NaCl, 20 μM bovine serum albumin (M_r 68 000), 40 mM sodium isoascorbate (pH 7.8). The extract was filtered through ten layers of cheesecloth and one layer of nylon (mesh size: 25 μm), then centrifuged at $1000 \times g$ for 5 min. The pellet was washed with 10 mM Tricine, 10 mM NaCl (pH 7.8) and centrifuged at $2000 \times g$ for 8–10 mn. In some cases (see Results), chloroplast hypotonic disruption was avoided by adding 0.4 M sorbitol to this washing medium. The new pellet was resuspended in a medium containing 0.2 M sorbitol, 10 mM Tricine, 10 mM Hepes, 10 mM KCl (pH 7.8), and diluted to a chlorophyll concentration of 900 μM (as determined by Mackinney's method [33]); the suspension was kept on ice and maintained in darkness. All steps were carried out under dim light and below 5°C.

For measurements, chloroplasts (15 μM chlorophyll) were suspended in one of the following media: (1) 0.2 M sorbitol, 10 mM Tricine, 10 mM Hepes, 10 mM KCl, 6 mM $MgCl_2$, 2 mM K_2HPO_4 (pH 7.8) = isotonic medium; or (2) 5 mM Tricine, 2 mM Hepes, 5 mM KCl, 4 mM $MgCl_2$, 1 mM K_2HPO_4 (pH 7.8) = hypotonic medium; in some experiments, KCl was 50 mM instead of 5. 500 μM ADP, 50 nM valinomycin and 4 μM 9-aminoacridine were always present. The desired electron acceptor, 50 μM methyl viologen, 800 μM potassium ferricyanide, 500 μM dimethylquinone or 50 μM pyocyanin, was added according to the electron chain tested. It was checked that the probe fluorescence yield was the same with these different substances, and especially unaffected by their redox state. To obtain (apparent) ΔpH from 9-aminoacridine fluorescence quenching [34], the internal volume of thylakoids was computed as in [35], using an osmolarity of 275 mM for isotonic and 35 mM for hypotonic media (on rare occasions, hypertonic buffer at 355 mosM/l was used,

with essentially the same effect as the isotonic one); ionic strength was between 30 and 105 mM (65 mM in isotonic buffer). ATP was measured by luminescence of the luciferase-luciferine complex [36], and the phosphorylation rate was corrected for a slight kinase activity, which never exceeded 1 or 2 mmol ATP/mol Chl per s and could be cancelled out by 5 μM diadenosine pentaphosphate [37]; the photophosphorylation rate was unaffected by this inhibitor. Each chloroplast sample was incubated 2 min before the experiment, which was run under air in a magnetically stirred spectrophotometric cuvette, thermostatted at 20°C, with a specially designed set-up [29]. Actinic 620–750 nm red light (max: $\approx 700\ W \cdot m^{-2}$) was varied in intensity with neutral filters; no mutual screening effect of chloroplasts was detected at twice the chlorophyll concentration used. Time of illumination (1–5 min) was always sufficient to reach steady-state ΔpH and to obtain a constant rate of ATP synthesis. In some experiments, Hepes and Tricine were omitted, and the light-induced pH shift of the suspension was measured with a glass electrode, in order to estimate the total proton translocation and the rate of ATP synthesis according to Nishimura's method [38].

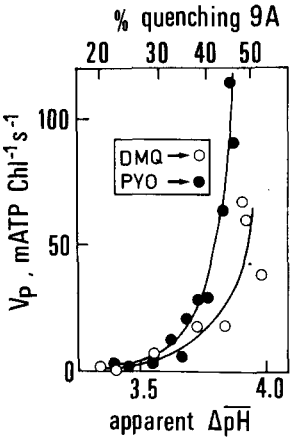

Fig. 1. Rate of ATP synthesis as a function of ΔpH measured by 9-aminoacridine fluorescence quenching. Medium with sorbitol 0.2 M; ●, pyocyanin loop (PS I); ○, $H_2O \rightarrow$ dimethylquinone chain (PS II). An experiment similar to that in Ref. 30, but here with valinomycin. Curves traced by varying light intensity; other conditions: see Materials and Methods.

Results

Effect of osmolarity on PS II and PS I protons efficiency

Fig. 1 illustrates a typical experiment realized according to our standard procedure [30]; that is, chloroplasts were disrupted during their preparation in a sorbitol-free buffer, then stored and used in the presence of 0.2 M sorbitol. To collapse the electrical component of $\Delta\tilde{\mu}_{H^+}$, valinomycin and K^+ were always present in the reacting medium, which was not the case in the corresponding figure of Ref. 30. Our previous data were confirmed, which incidently shows the limited weight of the electric component of proton gradient at steady-state in thylakoids: a given ΔpH, estimated with 9-aminoacridine, allows more ATP synthesis when protons are transported by the pyocyanine PS I loop, i.e., in the vicinity of coupling factors, than when PS II is involved. This was in fact observed independently of the osmolarity during washing and preincubation stages (not shown). If now the buffer used for storage and measurement was rendered hypotonic by the absence of sorbitol, the PS I-generated ΔpH was still the most efficient for phosphorylation. We supposed that, during the long storage of chloroplasts in a low-osmolarity medium, slow membrane rearrangements might have followed the initial swelling of thylakoids. To avoid such possible effects, all media needed for the different stages preceding the experiment itself contained thereafter sorbitol, and the hypotonic shock occurred only a few minutes before running it. This was done by diluting the sample to its final concentration in the sorbitol-free buffer. Fig. 2 shows two extreme situations, obtained with different chloroplast preparations, but following a rigorously identical experimental protocol. No such fluctuations in the results were observed in isotonic conditions, which always gave a clear discrimination between the two systems. The important point, nevertheless, is that in a hypotonic medium, proton gradients induced by either photosystem could have similar, and even equal, phosphorylating efficiencies. Thus, medium osmolarity seems indeed to play a role in the proton localization, as detected in the PS II/PS I comparison.

To obtain a more complete picture, we further compared the phosphorylating capacities of PS I (pyocyanin loop) and of PS II + PS I ($H_2O \rightarrow$

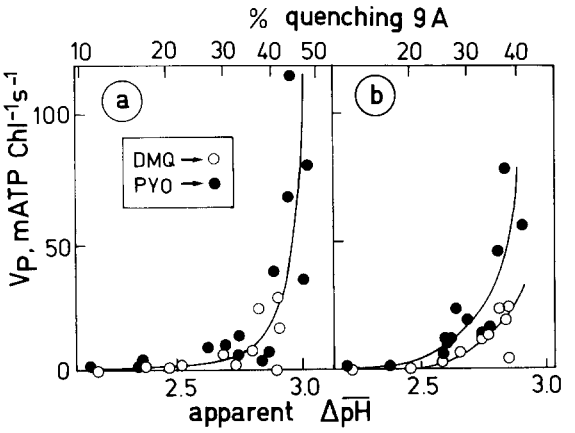

Fig. 2. Experiments as in Fig. 1, but with a low osmolarity medium. (a) and (b) correspond to observed extreme situations. ●, pyocyanin loop (PS I); ○, $H_2O \rightarrow$ dimethylquinone chain (PS II). See text for experimental details and statistical considerations (case b is less frequent than case a).

methyl viologen chain). Fig. 3 displays extreme cases obtained in the presence of 0.2 M sorbitol. Depending again on chloroplast preparations, ΔpH generated by PS I + PS II was found to be less (a) or similarly (b) powerful – but never more – compared to that produced by PS I alone. This variability must be related to the fact that PS I and PS I + PS II situations are close, because both involve efficient PS I H^+-translocation and the weight of PS II protons is reduced in the full chain. Indeed, for ATP synthesis, a PS II-dependent $\Delta\tilde{\mu}_{H^+}$ is, according to our views, less active than a PS I one, owing to a pH lateral drop between the points of proton emission (water oxidation) and utilization (ATP synthesis). When pyocyanin and methyl viologen chains were compared after an osmotic shock, similar to the last treatment described above, the phosphorylation vs. ΔpH curves were always identical (Fig. 4). This result agrees with our hypothesis of a trend towards a proton delocalization in a hypotonic medium (Figs. 2a and 4), and of a generally limited discrimination between PS I and PS I + II reactions (Fig. 3b).

We tried to determine the parameters which may have some connection with the more or less great variability of observations made with dimethyl quinone in hypotonic buffers or methyl viologen in isotonic ones, compared to pyocyanin taken as a reference. Species (lettuce or, rarely, spinach), season of the year, external leaf state, or slight changes in preparation of thylakoids did not play a crucial role. Pigment content (chlorophylls *a* and *b*, carotenoids), maximum turn-over of the redox chain or rate of phosphorylation, magnitude and relaxation-time of the proton gradient – all

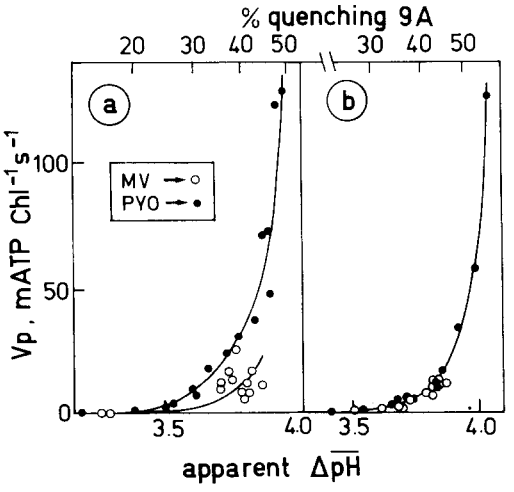

Fig. 3. Experiments as in Fig. 1 (sorbitol 0.2 M present), but with methyl viologen (○) and pyocyanin (●) as redox carriers. (a) and (b) correspond to extreme cases obtained (see text for comments).

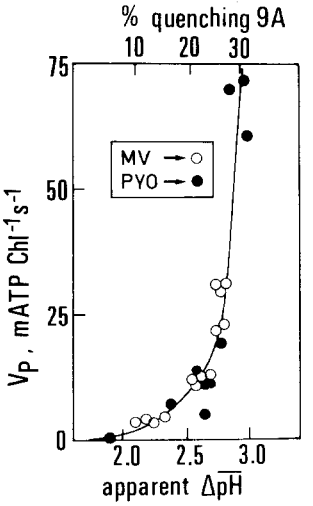

Fig. 4. Experiments as in Figs. 1–3, with low osmolarity medium and methyl viologen (○) or pyocyanin (●).

these activities measured in strong light – seem not to be correlated with the reported changing behaviour. On the whole, for about 30 experiments, we found: (1) for dimethyl quinone vs. pyocyanin, 100% discrimination in sorbitol-containing media, approx. 10% similarly distinct and approx. 30% merging behaviours in sorbitol-free ones, most of the results in these conditions giving only a partial approach of the two curves; (2) for methyl viologen vs. pyocyanin, slightly more distinct than similar behaviour in the presence of sorbitol, but always identical in its absence.

Comparison of PS I and PS II efficiency by analysis of the rate and extent of light-induced external pH shift

The use of 9-aminoacridine has been discussed by numerous authors, especially by McCarty's group [32] for PS I vs. PS II comparisons. These investigators, who utilized labeled hexylamine, found that, independently of the electron pathway, ΔpH was univocally correlated to ATP synthesis. We have already recalled that in fact all amines should obey the same basic mechanisms [35,39], even though their specific properties, such as the partition coefficient [39], may be responsible for quantitatively different overestimations of ΔpH. On the other hand, the intermediate centrifugation required to separate organelles from the medium in which the radioactive probe is counted [32] may lead to some underestimation. But the main point is that the comparative studies made by either group do not require knowledge of the absolute values of the proton gradient (restricted, in our case, to ΔpH). Because no probe use is devoid of problems, we tried to confirm our results by another method.

To do that, the light-induced pH shift of the suspension, buffered only slightly, was measured with a glass electrode, the rate of phosphorylation being computed from the disappearance of 'scalar' protons [38]: $(ADP + P)^{a-} + (a - b)\ H^+ \rightarrow ATP^{b-}$, with $(a - b) \approx 0.94$ at pH 7.9. (No scalar H^+ imbalance is expected—and this was checked in uncoupled conditions—with the redox acceptors used, because of the 1/1 ratio of scalar protons to transferred electrons in the redox reaction.) Concerning ΔpH, not only did we obtain it by the conventional 9-aminoacridine method, but also by the transient decay of external pH when light was turned off, a measure of total proton translocation (Fig. 5a). According to delocalized chemiosmosis, in effect only one ΔpH may be obtained for a given proton translocation, in fixed conditions as realized here, and this ΔpH strictly represents the actual one utilized for phosphorylation. In contrast, microchemiosmosis predicts that no unique relationships may exist. A complication could arise from some uptake of scalar protons from the medium accompanying a temporary post-illumination ATP synthesis, perhaps not negligible, and which would be superimposed on the dark-relaxation of the proton gradient (Fig. 5b). We tried to overcome this problem by dissipating ΔpH with a rapid protonophore injection in place of, or in addition to, light extinction, but the effect seemed to be not always instantaneous and complete. Since, for the present aim, only relative values were required, it was in fact sufficient to consider the resulting balance, at light-dark transition, between proton uptake and release. Indeed, if ΔpH is fully delocalized, proton transport and the phosphorylation rate are rigorously correlated at any time. Therefore the number of scalar protons which should be added to the apparent proton

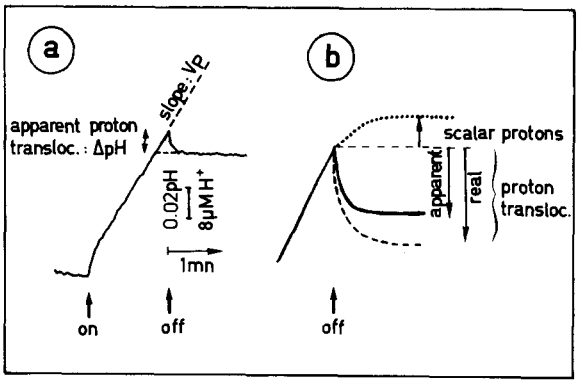

Fig. 5. (a) Typical recording, with a glass electrode, of light-induced shift of external pH (slightly buffered medium), converted into equivalent protons by HCl titration; 40 μM chlorophyll. The arrows show dark-light and light-dark transitions. Conditions: see materials and Methods. (b) Schematic representation of the trace (———) after switching off the light; to obtain the real extent of proton vectorial translocation (- - - - -), one should add to the apparent value (———) an unknown amount of scalar protons (· · · · · ·) consumed by ATP synthesis during H^+ efflux in darkness.

backflow, upon returning to darkness, to obtain the real value of translocated H^+ is solely determined by the amount of ATP then formed. This means that, in a delocalized situation, the deficit of vectorial protons due to the post-illumination scalar H^+ is simply a fraction of these vectorial protons. Consequently, the abscissa scale is uncertain by only a proportional factor and the relation between apparent H^+ uptake and rate of ATP synthesis should not depend on whether PS I or PS II are operating. A possible cause of error would be a change in basal proton conductivity, which should modify the ratio between scalar and vectorial protons upon relaxation. Actually, we verified that the rate of H^+ leakage in darkness, measured both with the glass electrode and by 9-aminoacridine fluorescence, was independent of the redox carrier used, in basal and in phosphorylating conditions. It turned out that practically all these possible sources of distortion were of minor importance. For example, Fig. 6 shows that a unique relationship links the estimated proton translocation to the corresponding apparent mean

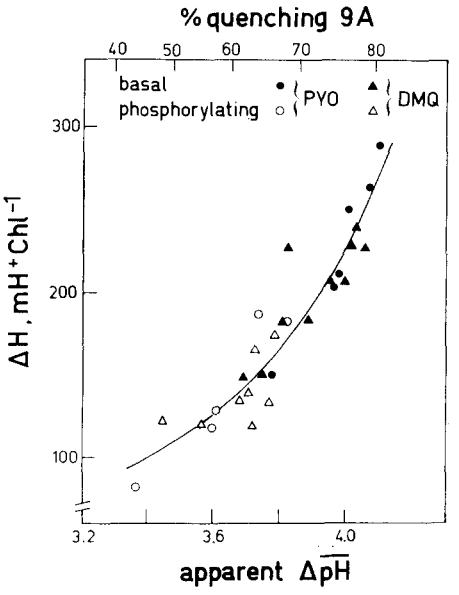

Fig. 6. Unchanged correlation with the type of redox chain and the functional state of thylakoids, between total H^+ translocation, estimated with glass electrode, and the corresponding mean ΔpH, expressed by fluorescence quenching of 9-aminoacridine. Curve traced by varying light intensity; ●, ○, pyocyanin, △, ▲, dimethylquinone; ●, ▲, basal; ○, △, phosphorylating.

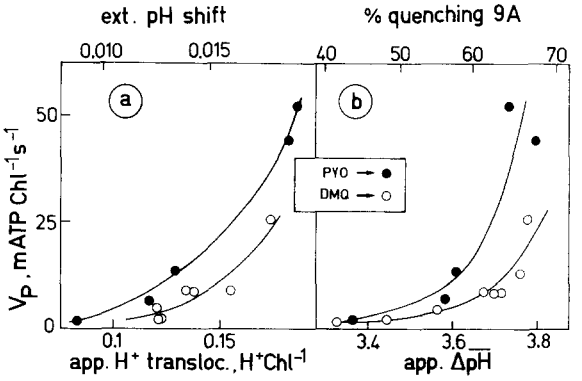

Fig. 7. Rate of ATP synthesis vs. (a) apparent proton translocation, measured as indicated Fig. 5, or (b) apparent ΔpH, measured with 9-aminoacridine. Simultaneous measurements by both methods, with pyocyanin (●) or dimethylquinone (○); variable light. Isotonic conditions: sorbitol 0.2 M present.

ΔpH, regardless of the redox mediator or of the functional state (basal or phosphorylating). Therefore, if a divergent relationship between the rate of ATP synthesis and the apparent (= vectorial minus scalar) proton movement is observed, it strongly suggests a ΔpH heterogeneity. Fig. 7a indeed shows that, in the presence of 0.2 M sorbitol, the pyocyanin curve is above that with dimethylquinone, and Fig. 7b exhibits an identical situation in a 9-aminoacridine plot, both measurements being made in parallel on the same sample. Thus, the latter method does not qualitatively bias our results; besides, in the conditions where identical PS I and PS II curves were obtained according to 9-aminoacridine data, the same was observed using instead total proton translocation, using a glass electrode.

Relative resistance of phosphorylation to nigericin

Localized proton circuits in bacterial chromatophores [4,5] and in mitochondria [8] were also suggested on the basis of limited protonophore action on ATP synthesis, or on ΔG_P, despite a severely diminished $\Delta\tilde{\mu}_{H^+}$. We have repeated this type of experiment with thylakoids. Fig. 8 discloses how the rate of phosphorylation varies with the overall proton gradient, when the latter is depressed by increasing the membrane leaks (nigericin addition) or by decreasing the H^+ influx (light lowering). It confirms that ATP synthesis is less

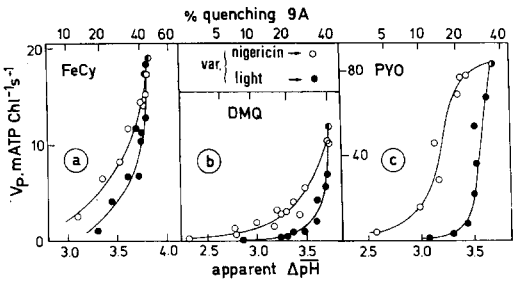

Fig. 8. Rate of ATP synthesis as a function of apparent ΔpH, varied by light intensity (●), 100 to 0.26%, or by nigericin (○), 10 to 100 nM. (a): $H_2O \rightarrow$ ferricyanide chain; (b): $H_2O \rightarrow$ dimethylquinone chain; (c): pyocyanin loop. Isotonic conditions: sorbitol 0.2 M present; different experiments.

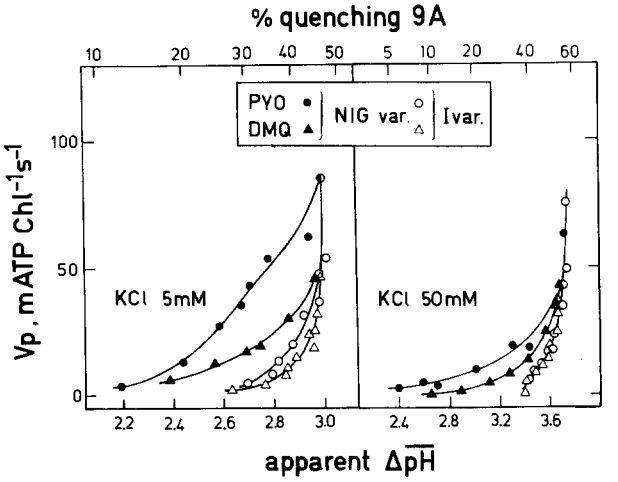

Fig. 9. Effect of ionicity on the phosphorylation-rate dependency upon ΔpH in a low-osmolarity buffer. (a): low salt concentration (KCl 5 mM); (b): identical medium, but with 10-times higher KCl concentration (50 mM). ΔpH decreased by light attenuation (○, △) or by nigericin addition (●, ▲); ▲, △, dimethylquinone; ●, ○, pyocyanin. Two different chloroplast preparations.

sensitive to a ΔpH reduction by nigericin than classical chemiosmosis would predict. This could not result from a heterogeneous population [40], that is, in the present case, only a fraction of the vesicle population would have fixed the uncoupler. If this were true, the dark-decay of the proton gradient would have been found at least biphasic: actually, though accelerated, it stayed monophasic, with a linear increase of its rate constant with protonophore concentration. Therefore a microchemiosmotic interpretation seems to us the most reasonable interpretation and, in agreement with the above authors, we conclude that the local $\Delta\tilde{\mu}_{H^+}$ driving ATP synthesis resists more a membrane leak increase than the mean (= measured) one. This was true for a PS II + PS I chain ($H_2O \rightarrow$ ferricyanide: Fig. 8a), or for separate PS II ($H_2O \rightarrow$ dimethylquinone: Fig. 8b) and PS I segments (pyocyanin: Fig. 8c). In the latter case, one may notice that the divergence between nigericin and light curves is the highest, as if the spatial proximity of PS I and coupling factors favoured some kind of privileged proton pathway between them. But an unexpected finding was for the opposite situation, of a PS II remote from coupling factors: even there, nigericin molecules seem partly unable to intercept hydrogen ions between their sites of production and utilization.

We tried again to shift the partly localized coupling to a more delocalized situation, through changes in osmolarity and ionicity of the medium. The latter case, studied in the absence of sorbitol, is illustrated in Fig. 9. In low KCl (Fig. 9a), PS I and PS II curves, traced by illumination decrease, were still divergent, though less than in the presence of sorbitol, as already noticed (see above). If now, at constant strong light, nigericin is added to decrease the proton gradient and hence the phosphorylation rate, new curves are obtained for PS I and even PS II. With a 10-fold higher KCl concentration, pyocyanin and dimethyl quinone curves obtained by light-modulation of H^+ input became closer to each other and could even be superimposed, as in Fig. 9b, but modulation of H^+ output by nigericin reintroduces a differential behaviour, less pronounced, however, than in the standard medium. Thus, to increase the internal volume of bulk water—at least in principle, since it cannot be easily measured—by an osmolarity decrease and to raise its conductivity by an ionic strength increase help $\Delta\tilde{\mu}_{H^+}$ to equalize in the lumen. Conversely, that some coupling flexibility may thus be obtained shows that no strictly isolated pathways exist for protons, at variance with what is proposed by mosaic chemiosmosis [20].

Discussion

Phosphorylating efficiency of $\Delta\tilde{\mu}_{H^+}$ generated by PS I or PS II

We have previously interpreted [16,30,39] in

microchemiosmotic terms the limited ability of PS II protons to drive ATP synthesis: the water-splitting enzymes, which liberate protons in granal parts of the lumen, are remote from coupling factors, essentially located in non-appressed areas, where PS I also predominates. Due to some diffusion barriers, a lateral pH drop should occur in the steady state between redox pumps and ATP-synthetases. This would cause the local $\Delta\tilde{\mu}_{H^+}$ at the coupling factor to be lower than the overall $\Delta\tilde{\mu}_{H^+}$, which is an average for the entire vesicle. The situation is quite different when protons are transported across different regions of the membrane (chains $H_2O \rightarrow$ ferricyanide or methyl viologen) or mainly in its stromal fractions (pyocyanin loop): in these cases, the lateral diffusion barriers may be inefficient enough to make the local phosphorylating proton gradient closer, or even superior, to the mean $\Delta\tilde{\mu}_{H^+}$. Contrary to what was claimed by Davenport and McCarty [32], these results cannot be due to the way $\Delta\tilde{\mu}_{H^+}$ is estimated. (1) In this type of work, one needs only to compare the rates of ATP synthesis at equal ΔpH, whatever their true values, and diffusible amines, such as those used by our two groups, are adequate for such a purpose [34,35,39]. (2) Even though some interference between redox carriers and probes may exist, as visualized by a slight fluorescence quenching of 9-aminoacridine, it was found here the same with pyocyanin and dimethylquinone. Moreover, in the latter case, this quenching was found not only to be quite small, but also to be unchanged, in coupled or uncoupled states, before and after thylakoid illumination, i.e, with fully oxidized or partly reduced quinone. Besides, if some fluorescence change had occurred, it would have been cancelled out in the dark/light fluorescence ratio used for ΔpH computation and measured at the same point of the time-course curve. (3) These side-effects have indeed no influence on our results, as seen when, in the same medium, similar curves were obtained with dimethyl quinone and pyocyanin (Figs. 2a and 9b), but distinct curves with methyl viologen and pyocyanin (Fig. 3a). (4) A completely different method, the glass-electrode measurement of the external-pH shift, confirms the 9-aminoacridine data (Figs. 6 and 7). In addition, one must mention the recent report of Flores and Ort [4]], who extended to flashing-light conditions our previous experiments made in steady state, and again noticed the poor efficiency of protons generated by water-splitting enzymes.

The discrepancies between our results and those of McCarty's group remain to be explained. The differences in the media used led us to investigate the role of osmolarity and ionicity in energy coupling. We found indeed that ΔpH tends to be delocalized in experiments driven in sorbitol-free buffers, and even more so if the ionic strength is high, provided that thylakoids undergo the hypotonic shock only a short time prior to measurement. Otherwise, it may be that when vesicles are maintained a longer time in these swelling conditions, slow solutes and water flows, together with partial rearrangement of membrane components, reestablish the situation initially prevailing in isotonic media. Since the protocols used by the two groups are partly different, somewhat different results could be expected. Nevertheless, because we could obtain a delocalized situation only in some cases, whereas McCarty's group observed it regularly, we feel that other hidden reasons exist, but they are presently out of reach of the experiment.

Relation between proton path, proton metastable pools, and membrane topography

That low osmolarity would favour some equalization of $\Delta\tilde{\mu}_{H^+}$ generated by PS I and by PS II, i.e., delocalize protons, could be related to the proposed dissipation, during thylakoid swelling, of special intramembraneous proton gradients [43,43]. However, these 'metastable proton pools', which would be carried by buried groups and would connect redox and phosphorylating pumps [37,44,45], exhibit properties hardly compatible with some of our data. Thus, whereas nigericin seems to open these microspaces, it does not very efficiently intercept protons between redox carriers and coupling factors. It is therefore difficult to identify such microdomains as the structure where we believe part of the fast lateral H^+ flow does occur.

Even without particular microcompartmentation, the osmolarity effect on coupling efficiency of organelles may be easily understood by attributing a significant H^+ resistivity to the proton conducting media, as illustrated in Fig. 10 for the

high-potential phases of mitochondria (intra-cristae and inter-membrane spaces) and of chloroplasts (lumen). Van Dam et al. [46] have proposed that shrinkage of mitochondrial cristae could facilitate some kind of direct coupling, by shortening the statistical distance between redox carriers and coupling factors, located on opposite membranes (Fig. 10a). This indeed may be realized in mitochondria, where the coupling factors seem randomly distributed (see Fig. 2.14 in Ref. 47). The chloroplast situation (Fig. 10b), however, is quite different, because there coupling factors and

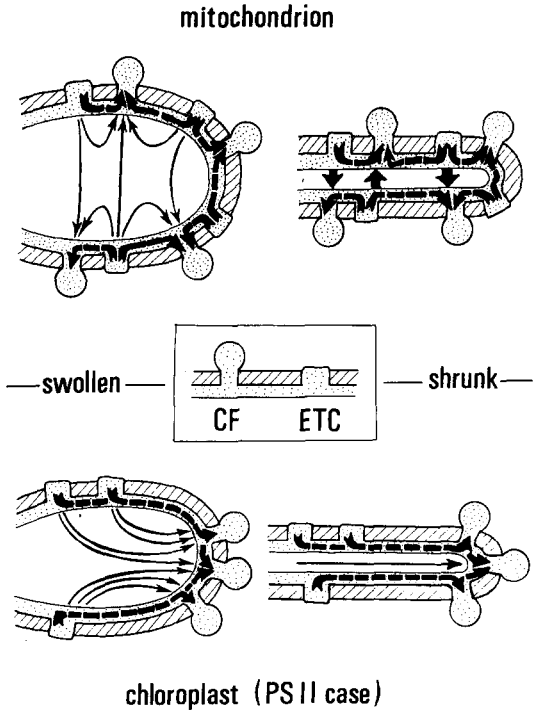

Fig. 10. Sketch of possible osmotic effects on localized coupling in mitochondria cristae and in thylakoids. Due to their differences in the topographical distribution of electron transfer chains (ETC) and coupling factors (CF), shrinkage statistically decreases protonic resistances in mitochondrial cristae (which raises phosphorylation yield), but increases them in thylakoids (which lowers phosphorylation yield). In both cases swelling favours H^+ delocalization. Dotted area: membraneous and/or interfacial structure, including ETC and CF, where the lateral proton-transport (dashed arrows) is supposed to occur; hatched area: rest of the membrane; unmarked area: bulk medium, where there are suggested to be delocalized H^+ internal pathways (plain arrows); only water-splitting enzymes are represented for chloroplast redox H^+ pumps. For the sake of simplicity, only the situation inside is depicted, but the equivalent should or may exist outside.

PS I are mainly located in stromal regions [48,49], cytochromes b_6-f are distributed over the whole membrane [50], and PS II, i.e., oxygen-evolving complexes, are essentially in granal domains [49]. Thus, by creating new H^+ pathways in a larger bulk phase, swelling lowers the overall H^+ resistance and favours their randomization. As a consequence, the proton gradient at coupling factors comes closer to the mean $\Delta\tilde{\mu}_{H^+}$, which raises the phosphorylation rate and reduces the discrimination between PS II and PS I. It is interesting to note that osmotic shrinkage has been reported to increase lumenal viscosity [51], perhaps by changing the bound/free water ratio.

Microchemiosmotic schemes

The above example illustrates how different topographic configurations may account for various microchemiosmotic situations. For this reason, we do not believe that 'mosaic chemiosmosis' [20], proposed for mitochondria [23] and chromatophores [22,24], necessarily applies as such to chloroplasts. The one-to-one functional relationship between redox and phosphorylating pumps proposed by this model implies their tight association within independent 'coupling units' or through separate connectors, almost perfectly isolated. Such conditions are not warranted in general and seem even improbable for water-splitting complexes and ATP-synthetases. A recent report [54] has suggested that some privileged H^+ exchange may occur between oxygen-evolving complexes and CF_0, but this was observed only after a mild extraction of CF_1, which would allow some polypeptides of the intramembraneous proton channels of coupling factors to migrate in the appressed regions of the membrane. Moreover, although it is still disputed [52], the PS II/PS I stoichiometry – and therefore their ratio to coupling factors – may be far from 1 [53].

One must remark that the microchemiosmotic schemes proposed before us implicitly consider that no resistance separates redox and phosphorylating pumps. This is an intermediate situation between direct coupling and delocalized chemiosmosis. Such models may explain many data, such as the limited inhibition of ATP synthesis by protonophores (see Refs. 4, 5, and Fig. 8), but fail to account for others, such as H-isotope effects

[27–29] or the differential effectiveness of PS I and PS II for phosphorylation [30,41]. These are easily integrated in the model we have proposed [16,27,39], where diffusion barriers exist between the primary and secondary pumps and between these pumps and the rest of the membrane.

Some observations are still difficult to integrate into the different proposed models without calling on addition hypotheses. An example is the enhancement of phosphorylation in chloroplasts by low amounts of uncouplers [56]. The microchemiosmotic interpretation can explain a relative insensitivity of ATP synthesis, rather than a stimulation, except if: (1) protonophores shuttle protons not only transversely, but also laterally, thereby lowering the diffusion barriers which we suggest may separate redox carriers from coupling factors; and/or (2) redox proton carriers coupled to ATP-synthetases are different from those which are regulated by ΔpH, an ad hoc and doubtful hypothesis. However, it should be noticed that this uncoupler-stimulated phosphorylation seems dependent upon the type of chloroplast used, since we never could observe it, although we did explore the same concentration range of protonophore.

Nature of the domains where protons may migrate

In principle, microchemiosmosis does not require special structures or microdomains: if high enough, the resistivity of the osmotic compartment can by itself explain an incomplete proton delocalization between separate input and output points. However, the idea of some microcompartmentation for protons came from different sides. One may cite 'metastable proton pools', sheltered by membrane [37,42–45], or a special 'interface', accessible to protons and lipophilic probes but not to buffers and salts [60,61]. The latter medium would be sensitive to thylakoid swelling and membrane integrity, since it is absent in freeze-thawed chloroplasts [54]. This last fact can be paralleled with the delay which separates light-triggering of H^+ uptake and pH-change of the medium, a delay which vanishes when chloroplasts are vigorously ground [62].

The necessity to reconcile a significant lateral kinetic barrier for protons and a partial inaccessibility of this path to protonophores lead us to the proposal that hydrogen ions migrate between primary and secondary pumps in an anisotropic medium and to imagine several types of lateral protonic conductors besides 'bulk' water, which does not rule out the possibility that some H^+ may always escape into this phase. We firstly suggested that a few layers of 'structured' water, bound on the membranes, could play this role [28], but this must now be questioned [59]. Lipid polar heads would be good candidates [60,61], as illustrated by the high lateral proton conductivity of phospholipid monolayers [62], but this is not very compatible with the limited effectiveness of lipophilic protonophores on phosphorylation. The most probable remains hydrogen bonds of polypeptides chains [63], shielded enough to reduce equilibration of protons so channelled with the adjacent aqueous phase. It is noteworthy that, according to recent electron micrographs, the internal space of mitochondria [64] and the lumen of thylakoids [65] are to a great extent filled with proteins: in isotonic conditions, bulk water could well be scarce there.

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References

1 Mitchell, P. (1961) Nature 191, 144–148
2 Williams, R.J.P. (1961) J. Theor. Biol. 1, 1–17
3 Williams, R.J.P. (1978) Biochim. Biophys. Acta 505, 1–44
4 Baccarini-Melandri, A., Casadio, R. and Melandri, B.A. (1977) Eur. J. Biochem. 78, 389–402
5 Casadio, R., Baccarini-Melandri, A. and Melandri, B.A. (1978) FEBS Lett. 87, 323–328
6 Padan, E. and Rottenberg, H. (1973) Eur. J. Biochem. 40, 431–437
7 Azzone, G.F., Pozzan, T., Massari, S. and Bragadin, M. (1978) Biochim. Biophys. Acta 501, 296–306
8 Azzone, G.F., Pozzan, T. and Massari, S. (1978) Biochim. Biophys. Acta 501, 307–316
9 Holian, A. and Wilson, D.F. (1980) Biochemistry 9, 4213–4221
10 Wilson, D.F. and Forman, N.G. (1982) Biochemistry 21, 1438–1444
11 Rottenberg, H. (1983) Proc. Natl. Acad. Sci. USA 80, 3313–3317
12 Mandolino, A., De Santis, A. and Melandri, B.A. (1983) Biochim. Biophys. Acta 723, 428–439
13 Deléage, G., Penin, F., Godinot, C. and Gautheron, D.C. (1983) Biochim. Biophys. Acta 725, 464–471

14 van Dam, K., Wiechmann, A.H.C.A., Hellingwerf, K.J., Arents, J.C. and Westerhoff, H.V. (1978) in Membrane Proteins (Nicholls, P., et al., eds.), pp. 121–132, Pergamon Press, Oxford
15 Skulachev, V.P. (1982) FEBS Lett. 146, 1–4
16 Haraux, F. and de Kouchkovsky, Y. (1983) Physiol. Vég. 21, 563–576
17 Ort, D.R. and Dilley, R.A. (1976) Biochim. Biophys. Acta 449, 95–107
18 Ort, D.R., Dilley, R.A. and Good, N.E. (1976) Biochim. Biophys. Acta 449, 108–124
19 Westerhoff, H.V., Simonetti, A.L. and Van Dam, K. (1981) Biochem. J. 200, 193–202
20 Westerhoff, H.V., Melandri, B.A., Venturoli, G., Azzone, G.F. and Kell, D.B. (1984) FEBS Lett. 165, 1–5
21 Venturoli, G. and Melandri, B.A. (1982) Biochim. Biophys. Acta 680, 8–16
22 Hitchens, G.D. and Kell, D.B. (1983) Biochim. Biophys. Acta 723, 308–316
23 Westerhoff, H.V., Colen, A.M. and Van Dam, K. (1983) Biochem. Soc. Trans. 11, 81–86
24 Hitchens, G.D. and Kell, D.B. (1983) Biochem. J. 212, 25–30
25 Cotton, N.P.J. and Jackson, J.B. (1983) FEBS Lett. 161, 93–99
26 O'Shea, P.S. and Thelen, M. (1984) FEBS Lett. 176, 79–82
27 de Kouchkovsky, Y. and Haraux, F. (1981) Biochim. Biophys. Res. Commun. 99, 205–212
28 Haraux, F. and de Kouchkovsky, Y. (1982) Biochim. Biophys. Acta 679, 235–247
29 de Kouchkovsky, Y., Haraux, F. and Sigalat, C. (1982) FEBS Lett. 139, 245–249
30 Haraux, F., Sigalat, C., Moreau, A. and de Kouchkovsky, Y. (1983) FEBS Lett. 155, 248–252
31 Portis, A.R. and McCarty, R.E. (1974) J. Biol. Chem. 240, 6250–6254
32 Davenport, J.W. and McCarty, R.E. (1984) Biochim. Biophys. Acta 766, 363–374
33 Mackinney, G. (1941) J. Biol. Chem. 140, 315–322
34 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70
35 Haraux, F. and de Kouchkovsky, Y. (1980) Biochim. Biophys. Acta 592, 153–168
36 Lemasters, J.L. and Hackenbrock, C.R. (1978) Methods Enzymol. 57, 36–50
37 Dilley, R.A. and Schreiber, U. (1984) J. Bioenerg. Biomemb. 16, 173–193
38 Nishimura, M., Ito, T. and Chance, B. (1962) Biochim. Biophys. Acta 59, 177–182
39 de Kouchkovsky, Y., Haraux, F. and Sigalat, C. (1984) Bioelectrochem. Bioenerg. 13, 143–162
40 Duszynski, J. and Wojtczak, L. (1985) FEBS Lett. 182, 243–248
41 Flores, S. and Ort, D.R. (1984) Biochim. Biophys. Acta 766, 289–302
42 Johnson, J.D., Pfister, V.R. and Homann, P.H. (1983) Biochim. Biophys. Acta 723, 256–265
43 Baker, G.M., Bhatnagar, D. and Dilley, R.A. (1981) Biochemistry 20, 2307–2315
44 Theg, S.M. and Homann, P.H. (1982) Biochim. Biophys. Acta 679, 221–234
45 Laszlo, J.A., Baker, G.M. and Dilley, R.A. (1984) J. Bioenerg. Biomemb. 16, 37–51
46 Woelders, H., Colen, A. and Van Dam, K. (1984) EBEC Rep. 3, 279–280
47 Tzagoloff, A. (1982) Mitochondria, p. 36, Plenum Press, New-York
48 Berzborn, R.J., Muller, D., Roos, P. and Andersson, B. (1981) in Photosynthesis (G. Akoyunoglou, ed.), vol. 3, pp. 107–120, Balaban International Science Services, Philadelphia
49 Andersson, B. and Haehnel, W. (1982) FEBS Lett. 146, 13–17
50 Cox, R.P. and Andersson, B. (1981) Biochim. Biophys. Res. Commun. 103, 1336–1342
51 Nesbitt, D.M. and Berg, S.P. (1982) Biochim. Biophys. Acta 679, 169–174
52 Whitmarsh, J. and Ort, D.R. (1984) Arch. Biochem. Biophys. 231, 378–389
53 McCauley, S.W., Taylor, S.E., Dennenberg, R.J. and Melis, A. (1984) Biochim. Biophys. Acta 765, 186–195
54 Junge, W., Hong, Y.Q., Qian, L.P. and Viale, A. (1984) Proc. Natl. Acad. Sci. USA 81, 3078–3082
55 Giersch, C. (1983) Biochim. Biophys. Acta 725, 309–319
56 Junge, W., Ausländer, W., McGeer, A.J. and Runge, T. (1979) Biochim. Biophys. Acta 546, 121–141
57 Hong, Y.Q. and Junge, W. (1983) Biochim. Biophys. Acta 722, 197–208
58 Ausländer, W. and Junge, W. (1974) Biochim. Biophys. Acta 357, 285–298
59 Nachliel, E. and Gutman, M. (1984) Eur. J. Biochem. 143, 83–88
60 Kocherginskii, N.M. (1979) Biofizika 24, 954–959; Engl. Transl. (1980): Biophysics 24, 982–987
61 Haines, T.H. (1983) Proc. Natl. Acad. Sci. USA 80, 160–164
62 Soucaille, P., Prats, M., Tocanne, J.-F. and Teissié, J. (1983) in Premier Colloque National de Bioélectrochimie (Y. de Kouchkovsky, ed.), pp. 155–156, Reprographie CNRS, Gif-sur-Yvette
63 Nagle, J.F. and Morowitz, H.J. (1978) Biophysics 75, 298–302
64 Srere, P.A. (1980) Trends Biochem. Sci. 5, 120–121
65 Weibull, C., Carlemalm, E., Villiger, W., Kellenberger, E., Fakan, J., Gautier, A. and Larsson, C. (1980) J. Ultrastruct. Res. 73, 233–234