The efficiency of energized protons for ATP synthesis depends on the membrane topography in thylakoids

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In thylakoids system II water-splitting proton generation is mainly localized in grana stacks, whereas system I plastoquinol reoxidation, is essentially restricted to non-appressed regions, such as stromal lamellae; the same is true for the coupling factor. For a given mean proton gradient, a system II chain was found to be less able to drive phosphorylation than a system I or a system I + II chain. These results support our microchemiosmotic hypothesis, based on the existence of lateral resistances to H^+ movements. They confirm that the proton gradients at the redox chain and at the coupling factor are unequal and that both are different from their mean experimentally measured value.

Electron transfer *Proton gradient* *Phosphorylation* *Membrane topography* *Microchemiosmosis* *Thylakoid*

1. INTRODUCTION

Several results have cast doubt on Mitchell's concept [1] of a fully delocalized $\Delta\mu_{H+}$, without necessarily supporting William's view [2] of a purely intra-membraneous proton loop connecting the redox chain to the phosphorylating coupling factor: mitochondria [3–5]; bacterial chromatophores [6–7]; chloroplasts [8–12]; discussion [13–17]. We have shown by replacing $^1H^+$ with the slower $^2H^+$ that a lateral protonic resistance exists along the membrane interface between the proton generator site of PQH_2 oxidation and the different H^+ leaks [10,11], as well as between the points of H^+ input, PQH_2 oxidation by SI and H_2O splitting by SII, and the coupling factors, CF [12].

Abbreviations: $\Delta\mu_{H+}$, ΔpH, $\Delta\Psi$, transmembrane differences of the proton electrochemical potential, of pH, and of electric potential, respectively; a top bar indicates mean, as experimentally measured, values; ^{C}, the local situation at the coupling factor level; SI, SII, (photo)systems I, II; CF, coupling factor; PQ, PQH_2, plastoquinone, plastoquinol; DBMIB, dibromothymoquinone

Here, we chose an approach based on the main location of SII in the grana regions and of SI and CF in the stromal lamellae [18]. We have inferred that the lateral resistance to H^+ should be higher between SII and CF than between SI and CF and therefore that the real $\Delta\mu_{H+}$ at the CF level should be lower in the former case than in the latter for a given mean $\Delta\overline{\mu_{H+}}$. We had proposed that the proton gradients at the sites of H^+ generation in the redox chain and of H^+ backflow across the coupling factors are different from the measured $\Delta\overline{\mu_{H+}}$, which is only an average value: this is a direct consequence of the lateral resistances to H^+ movements [10–12,17].

Since in chloroplasts the electric potential difference $\Delta\Psi$ is negligible in the steady-state [19,20], ΔpH could reasonably account for the whole $\Delta\mu_{H+}$ and was therefore favoured here.

2. EXPERIMENTAL

Envelope-free chloroplasts were prepared from lettuce [21] and suspended at 15 μM chl in 1.5 ml sorbitol (0.2 M) + Hepes (0.01 M) + Tricine (0.01 M) + KCl (0.01 M) + $MgCl_2$ (6 mM) +

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K_2HPO_4 (2 mM) + ADP (0.1 or 0.5 mM) + 9-aminoacridine (4 μM) (to estimate $\Delta\overline{pH}$). Ferricyanide reduction and 9-aminoacridine fluorescence were simultaneously measured in a stirred aerobic cuvette, thermostated at 20°C and illuminated with red light (max ~0.5 $kW.m^{-2}$) [12,21]. Aliquots of the suspension were diluted to determine immediately the ATP formed with the luminescent complex luciferin–luciferase [12].

The complete chain tested was $H_2O \longrightarrow$ SII $\longrightarrow$ SI $\longrightarrow$ ferricyanide: the system II chain was $H_2O \longrightarrow$ dimethylquinone or, via DBMIB, ferricyanide; the system I chain was the cyclic flow with pyocyanine.

3. RESULTS

In a first set of experiments, electron transfer, $\Delta\overline{pH}$, and phosphorylation were modulated by decreasing the light intensity (as in [12]: see top curves fig.2) or by adding increasing amounts of DBMIB, an inhibitor between SII and SI [22]. Fig.1 shows the corresponding crude results for the chain $H_2O \longrightarrow$ ferricyanide. Such curves presenting a minimum have been previously interpreted [23] as due, for their descending part, to a progressive disconnection of plastoquinone molecules from SII and, for their ascending part, to their replacement by DBMIB; in this case, electrons are directly transferred from SII to ferricyanide. That is, when the SII + SI chain is replaced by a SII chain, only one site of proton generation, water-splitting, operates instead of two. For middle DBMIB concentrations, the relative contribution

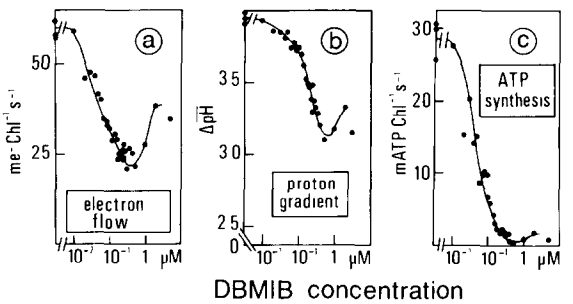

Fig.1. Steady-state electron flow (a), transmembrane mean pH difference (b), and ATP synthesis (c) as a function of DBMIB concentration. Conditions as in section 2: ferricyanide, 0.8 mM; ADP, 0.1 mM; saturating light.

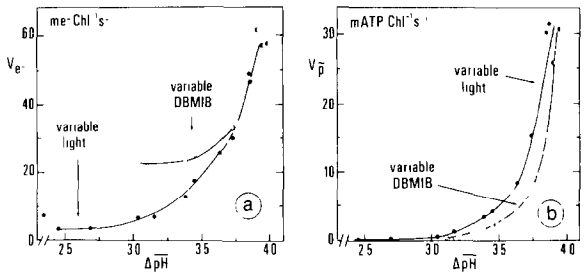

Fig.2. Rate of electron transfer (a) and of ATP synthesis (b) as a function of measured $\Delta\overline{pH}$. Conditions: fig.1. $\Delta\overline{pH}$ was varied by DBMIB addition or light intensity decrease.

of the uninhibited complete chain to the total ferricyanide reduction may be estimated from fig.2a. It shows how the electron-transfer rate varies with $\Delta\overline{pH}$ when the latter is changed by light attenuation (which preserves the two photosystems contribution) or by DBMIB addition. The predominance of the SII chain appears as soon as the two curves start to diverge, which happens for rather small amounts of DBMIB.

In theory, to generate a given ΔpH, twice as many electrons must be flowing through SII alone than through the full chain. Actually, below $\Delta\overline{pH} \sim 3.5$, the DBMIB curve stays quite high. Two non-exclusive reasons may be proposed: either $H^+/e^- > 2$ for the full chain, due for instance to a protonmotive Q-cycle [24]; or $H^+/e^- < 1$ for the SII chain, because some of the protons generated by the water splitting are ejected outside [25]. Another cause of H^+/e^- lowering would be that DBMIB acts as a protonophore, although no significant permeability change was noticed with this inhibitor [26].

According to Mitchell's delocalized chemiosmosis, the phosphorylation rate should be independent of the origin of the proton gradient. Fig.2b proves that this is definitely not so. A $\Delta\overline{pH}$ is less active if it is formed by SII alone than if it is generated by both photosystems. This is easily understandable, considering that what controls the ATP synthesis is the actual $\Delta\mu_H^C$ (i.e., ΔpH^C here) at the coupling factor level. For a given mean $\Delta\overline{pH}$, this ΔpH^C is smaller if the only source of protons for CF is SII than if other H^+ come from SI, as a result of PQH_2 oxidation. We thus confirm our initial hypothesis of localized proton gradients.

Another representation may be used, namely the ΔpH dependence of P/e, ratio of ATP synthesized per electron transferred. P/e is a function of the maximum theoretical $(P/e)_{th}$, which in the chemiosmotic interpretation is equal to the product of the two stoichiometries H^+/e^- and P/H^+, and of a yield factor ϱ, which is the ratio of the phosphorylating H^+ flow over total H^+ efflux: $P/e = \varrho\ (P/e)_{th}$. The coupling factor would be 'closed' in the relaxed state and would be 'opened' when $\Delta\mu_{H^+}$ (i.e., ΔpH here) is applied across it [27]: ϱ will rise from a minimum (~0?), to a maximum. Hence the type of curve displayed in fig.3. One should expect that cutting by two, the H^+/e^- ratio when SII + SI chain (variable intensity) is replaced by SII chain (DBMIB), moves the DBMIB curve below the light-intensity curve by this same factor. Although the observed displacement is mainly due to this cause, the shift is stronger than expected. For instance, whereas at $\Delta\overline{pH}$ ~3.5 the ratio of these two curves is <2 for the electron flow

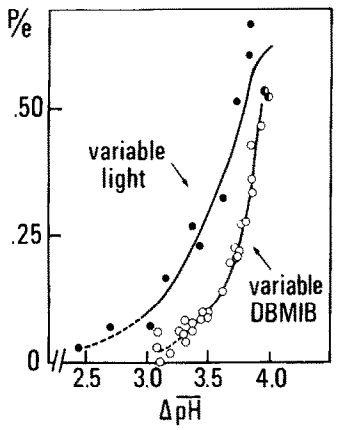

Fig.3. Variation of P/e ratio (mol ATP formed/mol electron transferred in unit time) with measured $\Delta\overline{pH}$. Conditions: as in fig.1. $\Delta\overline{pH}$ was varied by DBMIB addition or light intensity decrease. Owing to an approximate determination of the thylakoid internal volume, $\Delta\overline{pH}$ may be overestimated; identically for DBMIB and light-intensity curves. Also, a constant (dark) signal was subtracted from the total (light) signal in ATP determination with luciferase; the uncertainty of this correction having an increased weight when phosphorylation decreases, P/e drops sharply for low apparent $\Delta\overline{pH}$, hence the dashed lines. However, none of these methodological remarks affects the comparison between the two displayed curves since only their relative position is relevant here.

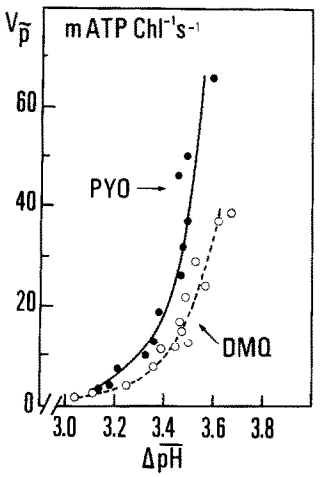

Fig.4. Comparison of ATP-synthesis rate dependence *vs* measured $\Delta\overline{pH}$ for a system II linear chain (DMQ) and a system I cyclic chain (PYO). Conditions as in section 2: 2,5-dimethylquinone, DMQ (0.5 mM); pyocyanine, PYO (50 μM); ADP (0.5 mM). $\Delta\overline{pH}$ was varied by decreasing the light intensity.

(fig.2a), it is >3 there. This additional effect is certainly due to the local ΔpH^C, which activates the coupling factor, being lower in SII situation than if SI also contributes to its formation (fig.2b).

Fig.4 presents a different experiment, where SII and SI manipulation was obtained by making use of their specific pathways, with the lipophilic acceptor dimethylquinone in the first case, or the cyclic cofactor pyocyanine in the second. It substantiates the results in fig.2b.

4. DISCUSSION

The higher efficiency of SI, compared to SII, in driving phosphorylation is simply explained here by assuming that for a given mean $\Delta\overline{pH}$ the local ΔpH^C at the coupling factor is stronger in the first case than in the second. But other factors may intervene, like the reduction of coupling factor sulfur groups by the ferredoxin–thioredoxin reductase–thioredoxin complex, which would divert electrons from SI [28]. It is quite unlikely that this was occurring here, since these carriers are lost during chloroplast breakage and washing. Moreover, such a pathway is significant only if no SI electron acceptor is present [29], which again was not the case here. Therefore, this phenomenon may be disregarded.

Another alternative is that an unsuspected $\Delta\Psi$ variation is at the origin of the lower efficiency of $\Delta\overline{pH}$ generated by system II. This improbable ad hoc argument seems ruled out. Although more precise and systematic measurements are required, the preliminary controls made by measuring the electrochromic band-shift of pigments at 515 nm, confirm that $\Delta\Psi$ is too small to alter the results depicted here. Moreover, if $\Delta\Psi$ changes, it does it in the same direction as $\Delta\overline{pH}$.

Inasmuch as 9-aminoacridine, like any amine, probes also the surface potential [30], the ΔpH comparison may be biased if this potential changes differently with each photosystem. The only significant cause to discuss is the inner charge neutralization by the massive proton influx. This should release a related amount of 'trapped' dye which would then migrate outside. In consequence, the fluorescence quenching would be minimized and the computed $\Delta\overline{pH}$ underestimated. To explain why system I curves are on the lower $\Delta\overline{pH}$ side than the system II curves (figs.2b,3,4), this underestimation should be stronger for system I, that is its activity should liberate more 9-aminoacridine. In other words, more protonizable groups should be present in vicinity of system I. The available data show the contrary: inside, system I carries less negative charges than system II; ~8.5 $mC.m^{-2}$ *vs* 34 [31]. Therefore if it plays a role, such electrostatic effect may only enhance the observed divergence between the two photosystems.

This work supports our microchemiosmotic concept. As discussed in [17], our approach is different from the previous localized hypotheses [5,7–9,13], which propose that fast H^+ currents out-circuit the bulk phases. These interpretations assume, as in Mitchell's and Williams' views, that there is a null resistance between the points of H^+ input (redox chain) and of H^+ output, through the coupling factors. These ideas are at variance with our model of finite H^+ microscopic resistances, topographically determined, which lead to the existence of distinct proton gradients. These local $\Delta\mu_{H^+}$ are the actual driving forces for the different energy-dependent processes.

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