EFFECT OF HYDROGEN–DEUTERIUM EXCHANGE ON ENERGY-COUPLED PROCESSES IN THYLAKOIDS

A new illustration of the hypothesis of local proton gradients with the energy-transducing biomembranes

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1. Introduction

The redox chain control of envelope-free chloroplasts suspended in a deuterated medium is enhanced despite a diminished proton gradient [1,2]. This observation, and others [2,3], led us to hypothesize that, superimposed to the classical 'chemiosmotic' mean transmembrane $\Delta\overline{pH_T}$, a lateral pH difference, ΔpH_L, of some tenths of 'units' (negative inside, positive outside), may exist between the sites of H^+ 'pumping' (plastoquinone, PQ) and leakage (coupling factor, CF), due to their spatial separation and to the existence of a significant lateral resistance to protons [3]. Such a resistance would be increased in ^2H_2O, owing to the lower mobility of deuterons along the membrane. Thus, the back-pressure at the plastoquinone level would be exerted by an internal pH locally lower than the average value measured in the inner compartment, this difference, $\overline{pH_i} - pH_i^{PQ}$, being enhanced in ^2H_2O. A corollary of our hypothesis is that for a similar $\Delta\overline{pH_T}$ with both isotopes, the local ΔpH_T at the CF level should be smaller in ^2H_2O than in ^1H_2O, which should reduce the rate of ATP synthesis. We have therefore investigated the effect of heavy water on the photophosphorylation, measured correlatively with the $\Delta\overline{pH_T}$ and the electron flow.

Abbreviations: CF, coupling factor; chl, chlorophyll, FeCy, (potassium) ferricyanide; PQ, plastoquinone; 9AA, 9-aminoacridine; top bar denotes a mean value; $_e$ and $_i$ refer to the external and internal compartments of a vesicle [e.g., $\Delta\overline{pH_T} = \overline{pH_e} - \overline{pH_i}$, the index T being used to distinguish this (mean) transversal pH difference from the lateral ΔpH_L discussed here]

Earlier works on mitochondria gave inconsistent results: the 'P/O' ratio was found unchanged [4,5], decreased [6] or increased [7,8] in deuterated media, but no attention was then paid to ΔpH, the existence and role of which were not known at that time.

Our results indicate that the isotopic substitution slows down the ATP synthesis even more than the electron flow, resulting in a decrease of the 'P/e' ratio, (number of ATP molecules formed per electron transferred). That is, the P/e *vs* $\Delta\overline{pH_T}$ plot is translated in ^2H_2O towards the positive $\Delta\overline{pH_T}$ values, which is, as expected, a shift opposite to that seen for the redox chain control [1,2]. These results are therefore consistent with our concept of a steady-state lateral pH heterogeneity along the membrane, and the increase of the lateral resistance to protons (i.e., deuterons) in ^2H_2O should enhance the difference between local and mean transmembrane $\Delta\overline{pH_T}$.

2. Methodology

Envelope-free chloroplasts were prepared from lettuce as in [9] and suspended at final conc. 15 μM chl in 1.5 ml of the following medium prepared in pure ^1H_2O or ^2H_2O: sorbitol 0.2 M, Hepes 0.01 M, tricine 0.01 M, KCl 0.01 M, $MgCl_2$ 6 mM, K_2HPO_4 2 mM, ADP 0.1 mM. The equivalent pH was set at 7.8, the p^2H value being corrected as in [1]. The sample was contained in a 1 × 1 cm spectroscopic cuvette, stirred and thermostatted at 20°C. 9-Aminoacridine 4 μM and ferricyanide 0.8 mM (no side effects of ^2H_2O on their spectroscopic properties) were added for the simulta-

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neous measurements of the ΔpH [10]; i.e., $\overline{\Delta pH_T}$ in our symbolism, and of the electron flow, respectively (sometimes, methylviologen 50 μM + NaN_3 0.5 mM was used instead of FeCy and the O_2 consumption was then appreciated with a laboratory-mark Clark electrode). The same weak narrow-band blue light (420 nm) was used to excite 9-aminoacridine and to detect FeCy reduction. On the opposite cuvette window, a double fiber guide conducted the fluoresced (9 AA) and transmitted (FeCy) lights to a couple of S-20 photomultipliers, shielded by appropriate sets of filters: one (λ_{max} 420 nm, half-bandwidth 8 nm) allowed us to detect only the transmittance at 420 nm, converted into absorbance with a logarithmic amplifier; the other (λ_{max} 499 nm, half-bandwidth 34 nm) selected the 9-AA fluorescence, whilst rejecting the direct 420 nm beam. Typical recordings are reproduced in fig.1a. The red actinic light (maximum ~0.4 kW/m^2), perpendicular to the analytic, was varied with calibrated neutral filters. The illumination time (1–15 min, depending on the light intensity) was always sufficient to reach the steady state and to form significant amounts of ATP, synthesis of which proceeded at a constant rate (fig.1c).

ATP was titrated, by luminescence of the luciferine–luciferase complex [11], in a 15 μl aliquot taken out from the cuvette before (sample D) and after (sample L) the illumination. These samples were first diluted at room temperature in 0.6 ml 20 mM tricine + 10 mM Na_2HAsO_4 + 1 mM EDTA + 5 mM $MgSO_4$ + 0.1% (w/w) BSA at pH 7.8, and 30 μl from this new suspension were then injected into a 1 × 1 cm spectroscopic cuvette containing 1.5 ml of this same medium with luciferine 30 μM plus luciferase 70 nM, and placed in front of an S-20 photomultiplier. Thus, the enzyme was always functioning in normal water, in optimum conditions. The calibration was made by injections of 6 pmol ATP (fig.1b). In some cases, the fully uncoupled electron flow was measured in a second illumination after addition of 1 μM nigericin.

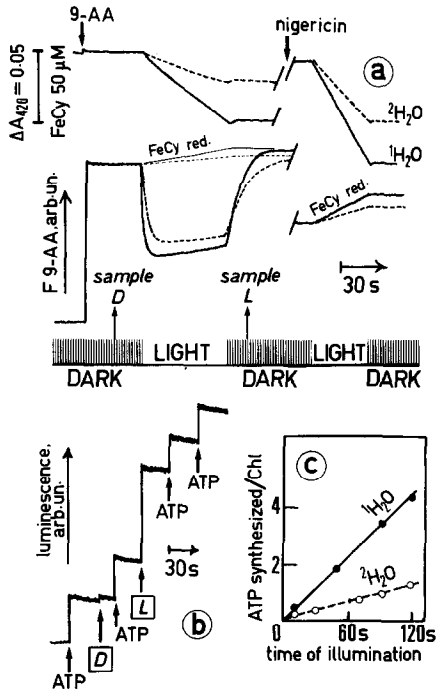

Fig.1. Electron flow, ΔpH and ATP synthesis measured on a same sample. (a) Simultaneous recording of the FeCy absorbance (top) and 9-AA fluorescence, F 9AA (bottom); (——) ^1H_2O; (– – –) ^2H_2O. The aliquots D (dark) and L (light) were taken out at the time indicated; when needed, nigericin 1 μM was injected before a second illumination. For the ΔpH computation [10], the 9-AA fluorescence was corrected for the reference drift, due to the decreased 420 nm light absorption in the time-course of the FeCy reduction (see especially the F 9AA traces with nigericin, where no ΔpH occurs). (b) Luminescence of the luciferine–luciferase complex. The samples D and L after dilution (see section 2) are calibrated by 6 pmol ATP injections. (c) The rate of ATP synthesis (=$[ATP]_{L-D}$/time) is constant during the illumination, which indicates that no back-reactions occur and that the steady-state ATP synthesis is maintained a time long enough to neglect the dark–light and light–dark transients in the computation of the phosphorylation rate.

3. Results and discussion

Fig.2 shows the light-dependency of the electron flow (a), proton gradient (b) and rate of ATP synthesis (c) measured on a same sample (chain $H_2O \rightarrow$ PS II $\rightarrow$ PS I $\rightarrow$ ferricyanide). The phosphorylating conditions do not change qualitatively the ^2H_2O effects which we had reported for the 'basal' electron flow and the associated $\overline{\Delta pH_T}$. The redox chain is severely inhibited (~65%) in strong light, but much less, or not at all, in low light, confirming that only the thermal steps are slowed down [2,3]. The $\overline{\Delta pH_T}$ at the same time is diminished in strong light, as generally observed at external pH 7.0–8.5 [1–3]; it is slightly increased in low light, as noticed only at alkaline external pH [2,3]. The new fact is the sharp drop of the photophosphorylation in deuterated water, by

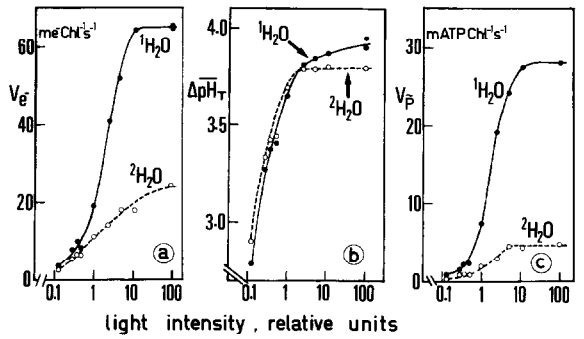

Fig.2. Light-dependency of electron flow (a), proton gradient (b) and phosphorylation rate (c), measured as indicated in section 2 and in fig.1: (–•–) ^1H_2O; (–○–) ^2H_2O; chain $H_2O \rightarrow$ ferricyanide.

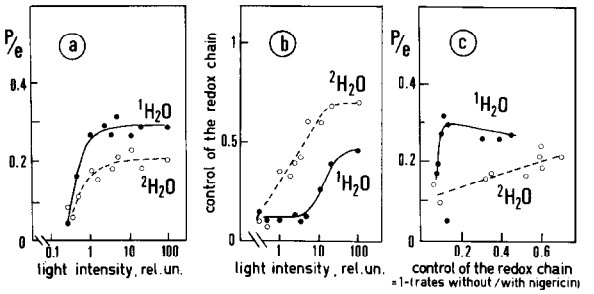

Fig.3. Light-dependency of the P/e ratio (a) and of the ΔpH-control of the electron flow (b) measured on the same sample in ^1H_2O or ^2H_2O, and their correlation (c). Conditions as in section 2, chain $H_2O \rightarrow$ ferricyanide: (–•–) ^1H_2O; (–○–) ^2H_2O. P/e = ratio of the rate of ATP synthesis to that of the electron flow: control of the redox chain = 1 – ratio of phosphorylating to nigericin-uncoupled electron flow. (Due to the phosphorylating conditions, the redox rates without nigericin are already above the basal one, hence the limited uncoupling by this ionophore.)

a value (~85%) much above that affecting the electron flow, and which is too high for being simply explained by a trivial solvent/substrate effect on the H^+-involving steps of the ATP synthesis. Consequently, the P/e ratio is strongly decreased by the isotopic substitution (a similar behaviour is obtained with the chain $H_2O \rightarrow$ methylviologen). Insofar as the H^+/e and the H^+/ATP ratios are absolute stoichiometries, P/e in the steady state is related to the proportion of the proton efflux crossing the membrane through the CF_0–CF_1 complex allowing ATP synthesis. For $H^+/e = 2$ (full PS II + PS I chain) and $H^+/ATP = 1/3$, a phosphorylating yield of 1 for the proton output should give a theoretical P/e ratio = 0.67 maximum. Depending on the chloroplast preparations, we found in ^1H_2O and strong light a P/e ratio from 0.3–0.45 (the basal rate is not subtracted here): 45–65% of the protons escaped through a phosphorylating pathway.

Fig.3a shows that the P/e ratio, thence the yield of photophosphorylation, increases with the light intensity. This should occur via a membrane 'energization': the coupling factor CF_1 would be activated by the transmembrane 'electric field' [12,13] and by the chemical component ΔpH_T of the overall transmembrane proton gradient $\Delta\tilde{\mu}_{H^+}$ [13–15]. Additionally a possibly related energy-dependent increase of the proton permeability has been attributed to the opening of the CF_0 channels [16,17]. Thus, the 'energization' diminishes the relative amount of the wasteful-leaking of protons. In accordance with thermodynamics, $\Delta\tilde{\mu}_{H^+}$ regulates also the redox chain, which is verified at least for the chemical component ΔpH_T: its suppression by nigericin increases the rate of the electron flow, which allows therefore to measure the slowing down of the redox chain by this ΔpH_T. We have plotted the 'control' (=1 – rates without/with nigericin) *vs* the light intensity in fig.3b. The control is always enhanced in ^2H_2O, except in very limiting light, in a way similar to that reported for basal rates [1,2]. If the redox chain and the CF_0–CF_1 complex activities were regulated by the same parameter, whichever it is, changing a common factor should preserve the relation between the P/e ratio and the control of the electron flow. Fig.3c shows that it is not the case: for an identical control of the redox-chain, P/e is much lower in ^2H_2O than in ^1H_2O. The isotope substitution cannot alter the intimate nature of the mechanisms which determine, for a given magnitude of the membrane 'energization', the P/e and control values: therefore, only the actual size of this parameter has been unequally changed at the CF_0–CF_1 and at the redox-chain regulation sites.

One may suspect, nevertheless, that part of the P/e lowering in heavy water was due to some inactivation of the phosphorylating complex (CF) itself. But such 'solvent effects' on enzymes are limited: for instance, the myosin [18] or brain [19] ATPases are inhibited by 20–25% at the utmost. Therefore, (average) $\Delta\tilde{\mu}_{H^+}$ is 'used' differently by the redox chain and the coupling factor. A tempting hypothesis is that, as suggested [1,2], $\Delta\tilde{\mu}_{H^+}$ (and especially ΔpH) is not fully delocalized at the membrane interface. Fig.4 gives the

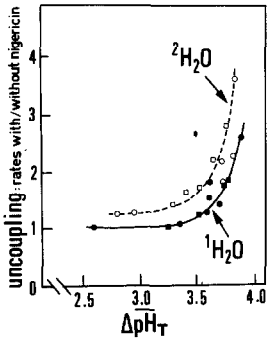

Fig.4. Uncoupling, i.e., ratio of the rates of nigericin-uncoupled to phosphorylating electron flow, as a function of $\Delta\overline{pH_T}$ (=ΔpH measured by 9-AA, see section 1). Conditions as in fig.1; variable parameter, light intensity; chain $H_2O \rightarrow$ ferricyanide.

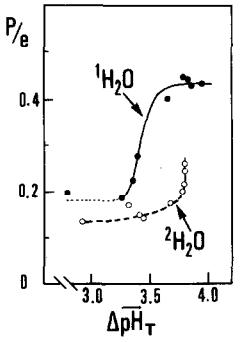

Fig.5. P/e rate-ratio as a function of $\Delta\overline{pH_T}$. Conditions as in fig.1; variable parameter, light intensity; chain $H_2O \rightarrow$ ferricyanide.

ratio of nigericin-uncoupled/phosphorylation-coupled electron flow as a function of $\Delta\overline{pH_T}$ (this fraction, called 'uncoupling', is equal to $1/(1-\alpha)$, α being the 'control' of the redox chain plotted in fig.3b,c). We have reported [1] that the regulation of the 'basal electron flow (as expressed by its uncoupling ratio) is, for a given $\Delta\overline{pH_T}$, more important in ^2H_2O than in ^1H_2O. All is as if the site of H^+ influx, where this feedback effect occurs, is submitted to a more important transmembrane ΔpH_T than the mean $\Delta\overline{pH_T}$, measured by the 9-AA fluorescence. Since the lateral resistance between the points of H^+ input and output is greater for deuterons than for protons [3], a same average $\Delta\overline{pH_T}$ should correspond, at the plastoquinone level, to a local ΔpH_T more important in ^2H_2O than in ^1H_2O, leading to an enhanced control. Consequently, the uncoupling *vs* $\Delta\overline{pH_T}$ is shifted in heavy water on the lower side of the $\Delta\overline{pH_T}$ [1,2]. The situation should be opposite at the points of H^+ efflux, such as the coupling factors. Fig.5 shows that the P/e *vs* $\Delta\overline{pH_T}$ plot is indeed translated towards the higher ΔpH_T-values. The electric-potential may contribute to these phenomena, but its value in the steady state is quite small [20,21], especially with the phosphorylating ferricyanide chain [22].

Although further works are needed on the latter topic, we think that the existence of a lateral difference of up to 0.2–0.5 pH between the points of H^+ translocation and leakage [3] is therefore the simplest explanation of the ^2H_2O effect on the electron flow, ΔpH and ATP synthesis. The kinetic restriction to the proton movement causing this lateral ΔpH_L means that any precise study of the bioenergetics of chloroplasts and other organelles can no longer ignore the existence of local pH, hence local ΔpH, at the reactive points of the membrane interfaces. Finally, we hope that our concept of the coexistence of lateral and transversal proton gradients, which is strengthened by the present study, may offer a common ground to the 'chemiosmotic' theory [22] and to the 'membrane-proton' hypothesis [23].

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