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^2H_2O EFFECT ON THE ELECTRON AND PROTON FLOW IN ISOLATED CHLOROPLASTS
An indication for lateral heterogeneity of membrane pH

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SUMMARY : To determine how the actual pH on the membrane surfaces regulate the redox reactions, a study of the electron and proton flow in thylakoids was made in heavy water, in which $^2H^+$ has a smaller mobility than $^1H^+$. The decrease of the redox rates by ^2H_2O is stronger in coupled than in uncoupled conditions, although the bulk ΔpH is slightly diminished; therefore changing $^1H^+$ by $^2H^+$ enhances the electron transfer control. This is particularly so when the two systems are linearly connected : i.e. the plastoquinone pool, not the water-splitting complex, is the main point of the redox chain regulation by ΔpH and of the deuterium action. The role of the proton diffusion barriers is emphasized and the concept of a heterogeneity – accentuated in ^2H_2O – of the pH along the membrane is proposed. The corresponding local pH , different at the points of H^+ active translocation and passive leakage, would be the real factors controlling the membrane-bound processes.

INTRODUCTION

An important feature of biological membranes is that they are electrically charged. In consequence, the protons may be repelled by the inner side of illuminated thylakoids, if it becomes positive, and attracted by their negative outer side (vice versa for energized mitochondria). This makes the membrane-surface pH different from those measured in their adjacent solutions (1). However, only these bulk pH were considered until now in the control of the electron flow, through a mechanism still unraveled : see refs. 2-6 for chloroplasts. Taking advantage of the remarkable deuterium isotopic effects (7-10), we have attempted to determine how a restricted motion of $^2H^+$, compared to that of $^1H^+$, could affect the surface pH and, thence, the role of the latter in the regulation of the – photosynthetic, here – redox reactions.

Few and old experiments have been made in photosynthesis with deuterated water, mostly with unicellular algae (11), though one study on the O_2-evolution oscillations pattern under flashes of light (12) and another on the trans-membrane electrical field (13) have been recently published. From most of these investigations it follows that mainly the dark limiting step of overall photo-

*Abbreviations : DAD, diaminodurene; DBMIB, dibromothymoquinone; DCMU, dichlorophenyl-dimethylurea; DCPIP, dichlorophenol-indophenol; DMQ, 2,5 dimethylquinone; FeCy, potassium ferricyanide; MV, methylviologen.

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synthesis is inhibited by ^2H_2O, the "primary" reactions being almost insensitive to it. However, the most relevant information in the present context is given by mitochondria. Their respiration rate is reduced by ^2H_2O (14-18), less in the presence of dinitrophenol (14), but their phosphorylating activity is variably affected : the "P/O" ratio is unchanged (15,16), decreased (14), or even increased (17,18). No observations have been reported on the proton gradient, and therefore on its correlation to the coupling process, and photosynthetic membranes have not been examined.

We have thus investigated some of the differential effects which ^2H_2O has on the maximum (i.e. under strong light) steady-state electron and proton flow in thylakoids (isolated chloroplasts). The results lead us to hypothesize a heterogeneity of the pH along the membrane surface, which may place the various membrane components in quite different environments.

METHODOLOGY

Envelope-free chloroplasts from Lettuce (*Lactuva sativa* L.) were prepared in the classical way (20) and resuspended before the experiment at 10 µM of chlorophyll in the buffer sorbitol 0.2 M + Tricine 0.01 M + KCl 0.01 M prepared in ^1H_2O or in ^2H_2O. The pH was adjusted to 7.8 in both cases, that with ^2H_2O being corrected according to (21) : true pH in pure ^2H_2O = pH read on the pH-meter + 0.4. The final ^2H_2O concentration was $\sim$ 97 %. The ΔpH, estimated with 4 µM 9-amino-acridine (no special ^2H_2O effect), and the O_2 exchange-rate, followed with a laboratory-made Clark-type electrode, were determined in a stirred cuvette, under air at 20°C; a strong red light (> 0.4 kW m^{-2}) was used for actinic illumination (20). The redox chain was also measured with a recording spectrophotometer, under similar condition of stirring, temperature, and actinic light (provided by an optical guide). The coupled and uncoupled activities were obtained with the same sample, the ionophore (generally 1 µM nigericin) being injected in the middle of the 1-min dark interval between two 1-min illuminations, long enough to reach the steady-state. The other added substances* — when needed — were : ascorbate, 1 mM; DAD or DCPIP : 50 (or 30) µM; DBMIB : 2 µM; DMQ : 400 µM; FeCy : 800 µM; MV : 50 µM, with 500 µM NaN_3; DCMU : 5 µM.

RESULTS

Seven types of essentially linear redox chains were studied, which involve System I, System II, or Systems I+II (cf.(22)). With DCPIP, $DCPIPH_2$ or $DADH_2$, however, a cyclic electron flow around System I, not detected in the redox activity measurements, adds its contribution to the ΔpH built up; also, whereas the reduction of DBMIB seems to exclude the plastoquinone pool (23), that of the lipophilic "class III" acceptor DMQ (24) apparently occurs within it (we have observed that neither DMQ nor $DMQH_2$ react with System I).

Table I and Fig. 1 describe the effect of the ^1H_2O replacement by ^2H_2O on these reactions. Although variable, depending on the complexity of the redox chain tested and the chloroplast preparation, the slowing down of the coupled,

TABLE I

Comparison of the coupled and uncoupled electron flow, and of ΔpH, in ^1H_2O (first line) and in ^2H_2O (second line). The rates (in milliequivalent s^{-1} mol^{-1} chlorophyll) were measured under strong illumination by O_2 evolution or uptake, or — spectrophotometrically — by the dye reduction or oxidation; ΔpH was estimated with 9-aminoacridine. $DCPIPH_2$ was obtained by DCPIP reduction with 500 μM ascorbate or by a long enough pre-illumination. With $DADH_2$ and $DCPIPH_2$, 5 μM DCMU was present. See Methodology for details.

Type of chain	Coupled rate	Uncoupled rate	ΔpH †
System I :			
$DADH_2$ ⟶ MV	107	115	3.53
	91	77	3.86
$DCPIPH_2$ ⟶ MV	58 ± 15	98 ± 19	2.91 ± 0.04
	32 ± 9	54 ± 18	2.74 ± 0.31
System II :			
H_2O ⟶ DCPIP	43 ± 17	165 ± 55	3.26
	38 ± 10	102 ± 32	3.38
H_2O ⟶ DMQ	79 ± 16	114 ± 22	3.53 ± 0.02
	44 ± 18	70 ± 13	3.42 ± 0.31
H_2O ⟶ DBMIB+FeCy	40 ± 19	37 ± 12	—
	21 ± 1	18 ± 1	—
Systems I + II :			
H_2O ⟶ FeCy	68 ± 21	192 ± 89	3.60 ± 0.36
	25 ± 11	113 ± 91	3.48 ± 0.39
H_2O ⟶ MV	31 ± 7	95 ± 25	3.65 ± 0.24
	15 ± 7	63 ± 15	3.44 ± 0.28

† The optical artifacts created in 9-aminoacridine measurements, mainly by DCPIP (ox or red) but also by FeCy or DAD, were eliminated with baselines corrections or, for $DCPIPH_2$ in the presence of ascorbate, by using as reference the fluorescence of the non-permeating dye fluorescein.

"basal", electron flow in saturating light is universal. It is nevertheless higher when the two Systems are linearly connected : ∿ 50-55 % on the average, than when only one of them is concerned : ∿ 15-45 % for System I, ∿ 25-45 % for System II. In the uncoupled conditions and under the same strong illumination, this differential action apparently vanishes, and the inhibition is around 40 % as a mean in all cases. The uncoupling (= ratio of the rates with and without saturating nigericin) varies with the chains, being absent for the System I reaction $DADH_2$ ⟶ MV or the System II reaction H_2O ⟶ DBMIB, moderate for the System I reaction $DCPIPH_2$ ⟶ MV or the System II reaction H_2O ⟶ DMQ, and high for the others, which involve the two Systems participation. Moreover, the Fig. 1 insert points out that the uncoupling in ^2H_2O increases in an enhanced manner with that obtained in ^1H_2O. The main odd points concern the chain H_2O ⟶ DCPIP, probably because this dye, which also behaves as a weak uncoupler (24), has a dual site of action (see above and ref. 25).

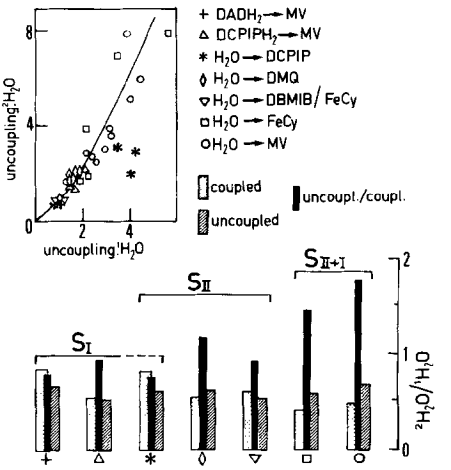

Fig. 1. Bottom : diagram of the mean ^2H_2O effect on the coupled and uncoupled electron transfer rates with various redox chains. The black vertical bars represent the ratios : uncoupling in ^2H_2O/uncoupling in ^1H_2O ; they are means of the individual ratios, not ratios of the mean values : the numbers may thus noticeably differ, as in the $H_2O \longrightarrow MV$ case, but this does not change the nature of the observed effect. Top insert : correlation between uncoupling in ^2H_2O and ^1H_2O for individual values (different experiments). Conditions : see Methodology.

Since the uncoupling depends on the internal pH (2,6), or in addition on the magnitude of the transmembrane ΔpH (3,5), the ΔpH (and hence, internal pH_i) was measured in ^1H_2O and ^2H_2O. Table I indicates that this isotope interchange has no strong influence on the steady-state ΔpH in saturating light : indeed, we have measured that both the $^2H^+$ influx and efflux are slower than their $^1H^+$ counterpart. With a few exceptions in the opposite direction, Δp^2H is less than Δp^1H by a mean < 0.2. In Fig. 2, the individual data, obtained with various chloroplast preparations, indicate that no special discrimination between the different chains exists and that, grossly, Δp^2H and Δp^1H vary in a parallel way (the dashed diagonal is for $\Delta p^2H = \Delta p^1H$).

From these two observations (Figs. 1 and 2), it results that the redox-chain control by ΔpH — or by pH_i — is more pronounced in ^2H_2O than in ^1H_2O. This is illustrated in Fig. 3, which shows also some additional features. No specific conclusion may be drawn for System I, because of its relatively small ΔpH, but for System II and Systems I + II chains, the uncoupling at a given ΔpH, already greater in the latter case, is even more so when the electrochemical gradient concerns $^2H^+$ instead of $^1H^+$. Because of the scattering of the points due to the blending of different experiments made on various chloroplast preparations, this is indicated by enclosing the different groups of data into their proper domains, which obviously are not superimposable. To put it more clear, a

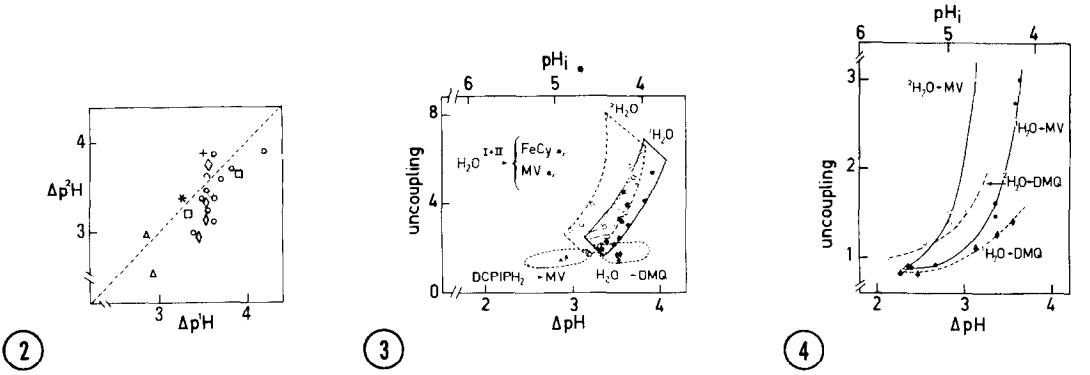

Fig. 2. Correlation between the pH transmembrane differences in ^2H_2O (Δp^2H) and in ^1H_2O (Δp^1H) ; separate experiments in general for the different data (see Fig. 1 for symbols). Conditions : see Methodology.

Fig. 3. Correlation between the uncoupling and the ΔpH (Δp^2H in ^2H_2O, Δp^1H in ^1H_2O) measured on the same samples for each given set of experiment, i.e. couple of open (in ^2H_2O) and closed (in ^1H_2O) symbols. Conditions : see Methodology.

Fig. 4. Correlation between uncoupling and ΔpH in ^2H_2O (open symbols) and ^1H_2O (closed symbols). Same chloroplast preparation. The ΔpH was lowered and the redox rate raised by increasing the nigericin concentration, from right to left, from 0 to 40 nM; the fully uncoupled rate was obtained with a subsequent addition of 1 μM nigericin. See text for details and Methodology for general conditions.

direct comparison of the two representative chains $H_2O \longrightarrow$ DMQ and $H_2O \longrightarrow$ MV was made on chloroplasts from the same batch, to which an increased nigericin concentration was added. The resulting curves, displayed on Fig. 4, strengthen the impression given by Fig. 3 and confirm again that the more is coupled the redox chain (Systems II+I vs. System II alone), the more is enhanced the ^2H_2O effect : compare the uncoupling ratios at the different ΔpH by drawing vertical lines in Fig. 4.

DISCUSSION

The unequal effects of ^2H_2O on the redox chains, disclosed in Table I and Fig. 1, proceed from mechanisms which may differ depending on the coupling state. In uncoupled conditions, since all the chains behave statistically in a comparable way, the ^2H_2O action should be similar to that it has in solution, i.e. it may be due to the so-called substrate (9) – ^2H_2O furnishes the $^2H^+$ – and solvent (10) effects. These two effects may also depend on the presence, on the membrane surface, of a layer of "structured" water (26), different when ^2H_2O replaces ^1H_2O (27). In coupled conditions (non-phosphorylating here), these general mechanisms must still operate, but the discrimination between the chains cannot

result directly from their intimate nature, otherwise such discrimination would have been preserved in the uncoupled state. One might have thought of a different control due to a Δp^2H different from Δp^1H. Yet, Table I and Fig. 2 show that generally Δp^2H is less than Δp^1H, whereas the Fig. 1 insert indicates an enhanced uncoupling in ^2H_2O as compared to ^1H_2O ; this is especially manifest for the chain $H_2O \longrightarrow$ MV. That is $^2H^+$ (and/or ^2H_2O) acts on the control mechanism itself of the electron flow by the ΔpH. It does not matter here, because this work was done at a fixed external pH_e, whether the key regulating factor is pH_i (2) or the mean of pH_i and pH_e (3), but in any event the pH — and hence ΔpH — to consider are at the very point where the control occurs. More precisely, since at identical bulk ΔpH and,thence, pH_i, the redox-chain regulation is more tight in ^2H_2O than in ^1H_2O, the controlled site should be locally in a more acidic surrounding. This cannot be achieved by a general lowering, in heavy water, of the Gouy's type internal *surface* pH, which is actually above that of the lumen (1), because, for a given bulk pH and equal charge densities, the *transversal pH profile* in the diffuse layer should stay unchanged, as ^2H_2O and ^1H_2O have the same dielectric constant. One must therefore conclude that the pH at the membrane surface — or within hydrophilic regions of the membrane — may differ at different places and that ^2H_2O (i.e. $^2H^+$) accentuates these local differences. Such *lateral heterogeneity* of the membrane pH would result from the remoteness of the locations of protons active transport and passive leakage (cf. the "localized proton currents" concept in ref. 28). Thus, with respect to the mean — surface or bulk — lumen pH, at the steady state, the pH would be higher at the points of H^+ influx (e.g. plastohydroquinone oxidation) and lower at the points of H^+ efflux (e.g. coupling factor and membrane "pores") ; outside, the situation would be opposite : Fig. 5. This wavy form of the pH along the membrane is probably a general feature of the energy transducing membranes (thylakoids here, but certainly also mitochondria and others). It occurs evidently as well in normal water as in heavy, and, due to the slower $^2H^+$ mobility (8), the reported ^2H_2O effect is to increase the "waves" amplitude even though the average pH may be the same : hence the remarkable enhancement of the redox-chain control. A corollary of this hypothesis of *local pH* is that the redox-chain control is also local and should subsist even in presence of nigericin. But then, not only the numerous artificial points of H^+ leakiness so created make them closer to the points of H^+ active transport — which decreases the pH-waves amplitude —, but also no bulk ΔpH is built up : the local pH_i becomes insufficiently acidic (and/or the local ΔpH insufficiently high) to significantly regulate the chain. Indeed, the uncoupling vs. bulk ΔpH curves of Fig. 4 indicate an apparent ΔpH threshold around 2-2.5, and a similar relationship should exist for the local ΔpH.

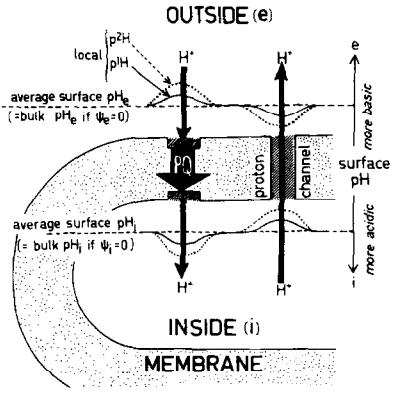

Fig. 5. Heterogeneity of the pH at the membrane level. The existence of diffusion barriers at the points of active H^+ translocation (e.g. the plastoquinone proton channels (19)) and passive H^+ leakage (CF_0 + CF_1 proton channels, and possible other sites) cause a local pH heterogeneity along the membrane interface. Thus, the actual ΔpH at these points are respectively larger or smaller than the average ΔpH value. This heterogeneity is increased in ^2H_2O owing to the slower $^2H^+$ motion (for the sake of simplicity, one has considered here the case where the average Δp^1H = average Δp^2H). Ψ = surface electrostatic potential ; hatched areas : proton channels.

It seems unlikely (4) that the control of the electron flow results from a direct ΔpH effect on the redox-potential differences of protonizable electron carriers. The mechanism(s) must thus be indirect, as through pH-induced "conformational changes", which for instance may close the recently reported "proton channels" (19) and alter the spatial arrangement of the electron transfer chain components in the membrane (cf. (29)). If the control is exerted on the so-called plastoquinone proton channels, it may be expected stronger when these two channels, on the two membrane sides, are concerned (full chain $H_2O \longrightarrow$ methylviologen) than when only the external one is involved (System II chain $H_2O \longrightarrow$ dimethylquinone) and, a fortiori, when both are out-circuited (with DBMIB). Whatever the actual mechanisms are, their effect should be reinforced by the slower diffusion of $^2H^+$ as compared to that of $^1H^+$, which alter the local pH, and the plastoquinone region appears to be the main site not only of the control, but also of the related ^2H_2O effect, specific to the coupled conditions. In contrast, the water-splitting system itself, probably because it is a quasi irreversible process (cf. (30)), does not offer a privileged target to the ^2H_2O action (cf. (12)). But in all circumstances, the pH to be considered for a given membrane bound reaction is that of its local microenvironment, which may be well away from that given by the macroscopic measurements.

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