THE EFFECT OF INTRATHYLAKOID pH* ON THE RATE OF CHLOROPLAST ELECTRON TRANSPORT REACTIONS AT SUBZERO TEMPERATURES

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1. Introduction

The rate of chloroplast electron transport under coupled conditions may depend on the internal pH of the thylakoids [1–3]. We reported [4] that chloroplasts suspended in a fluid medium at subzero temperatures show light-induced proton uptake coupled to electron transport from water to methyl viologen. These and earlier experiments under similar conditions [5] also showed that reduction of cytochrome *f* and *P*-700 in the dark following continuous illumination is stimulated by the addition of uncouplers, suggesting that the rate of electron flow under these conditions is also limited by the internal pH* (pH* is the effective pH in aqueous/organic media [6]). Here we have studied the effect of internal pH* on the rate of reduction of cytochrome *f* and *P*-700 in the dark following illumination and on the rate of reduction of ferricyanide in continuous light, by measuring these in the presence of uncouplers at different pH* values. From a comparison of these experiments and similar measurements in the absence of uncouplers we can estimate that chloroplasts suspended in 1:1 (v/v) ethanediol : water at −17°C can develop a proton gradient in continuous light of ~2.6 pH* units.

2. Experimental

Chloroplasts were prepared from spinach (*Spinacea oleracea*) and stored frozen in liquid N_2 as in [4].

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; *P*-700, reaction centre chlorophyll of photosystem I

Light-induced absorbance changes were measured in a dual-wavelength spectrophotometer (Hitachi Perkin-Elmer 356) adapted for side illumination. The spectrophotometer was linked to a minicomputer by an interface with analog inputs, to which the signal from the spectrophotometer was connected, and logic outputs, which were used to control a shutter permitting actinic illumination. The experiments were controlled by a program which allowed repetitive operation to improve the signal-to-noise ratio. Normally 10–30 experiments were averaged. The apparatus is detailed in [4].

Actinic light from a slide projector was passed through a Wratten 70 filter for illumination with red light or a combination of Wratten 47B or Schott BG 12 (2 mm thick) filters and 2 cm of saturated $CuSO_4$ for illumination with blue light. Cytochrome *f* was measured at 554 minus 543 nm with the photomultiplier protected by a combination of a Schott BG 18 (3 mm) (illumination with red light) or a Wratten 16 filter (illumination with blue light) plus 5 mm of saturated $CuSO_4$. *P*-700 was measured at 703 minus 720 nm with the photomultiplier protected by a 3 mm Schott RG 715 filter. Ferricyanide reduction during illumination with red light was measured at 400 minus 460 nm with the photomultiplier protected by a combination of a 2 mm Schott BG 12 filter and 5 mm saturated $CuSO_4$. Rates were calculated using a measured extinction coefficient for ferricyanide in 1:1 (v/v) ethanediol:water of 800 $l \cdot mol^{-1} \cdot cm^{-1}$ at 400–460 nm.

The usual reaction medium consisted of a 1:1 (v/v) mixture of water:ethanediol containing 0.33 M sorbitol and 5 mM $MgCl_2$ plus different buffers

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(50 mM). Potassium phosphate was used at pH* 6.0–8.0, potassium acetate at pH* < 6.0 and Hepes + 50 mM KCl at pH* > 8.0. The pH* of the acetate and phosphate buffers were calculated from the values in [7]. The pH* of the Hepes buffers were estimated using coloured indicators which had themselves been calibrated with phosphate. Chlorophyll was measured as in [8].

3. Results and discussion

The reduction of *P*-700 and cytochrome *f* in the dark following illumination at −17°C followed first-order kinetics as in [4,5]. Using $\Delta\epsilon_{554-543} = 2.2 \times 10^4$ l .mol^{-1} .cm^{-1} and $\Delta\epsilon_{703-720} = 6.4 \times 10^4$ l .mol^{-1} .cm^{-1} for cytochrome *f* and *P*-700, respectively [9,10] we could estimate a ratio of photoreducible cytochrome *f* to *P*-700 of 1.2 in agreement with the observations in [5]. We found no significant difference either in the rate of cytochrome *f* reduction nor in the amount of photoreducible cytochrome on illumination with either blue or red light.

Figure 1A shows plots of the observed first-order rate constants for the reduction of cytochrome *f* and *P*-700 at different pH* values, in the presence of the uncoupler gramicidin D so that the intrathylakoid pH* was the same as the pH* of the medium. Figure 1A also shows the rate of ferricyanide reduction measured under the same conditions. If these results are expressed as mol ferricyanide reduced .mol *P*-700^{-1} .s^{-1}, there is a good correlation between the rate of ferricyanide reduction in continuous light and the rate of *P*-700 reduction following illumination over the whole pH* range studied. A similar result was obtained [1] for experiments at room temperature. We find that the rate of *P*-700 reduction is ~20% faster than the rate of ferricyanide reduction.

All three reactions show the pH* dependence expected if they depended on the deprotonation of a component with p*K** 6.8 as shown in fig.1B. This is rather higher than the corresponding p*K* 5.5 observed at room temperature [1].

The rate of cytochrome *f* reduction is only 20–30% of the rates of *P*-700 and ferricyanide reduction, as reported previously [5]. This observation is one of several pieces of kinetic evidence [11–13] which are difficult to reconcile with a scheme of electron transport where cytochrome *f* is an obligatory intermediate between plastoquinol and plastocyanin.

In the absence of uncoupler the reduction of cyto-

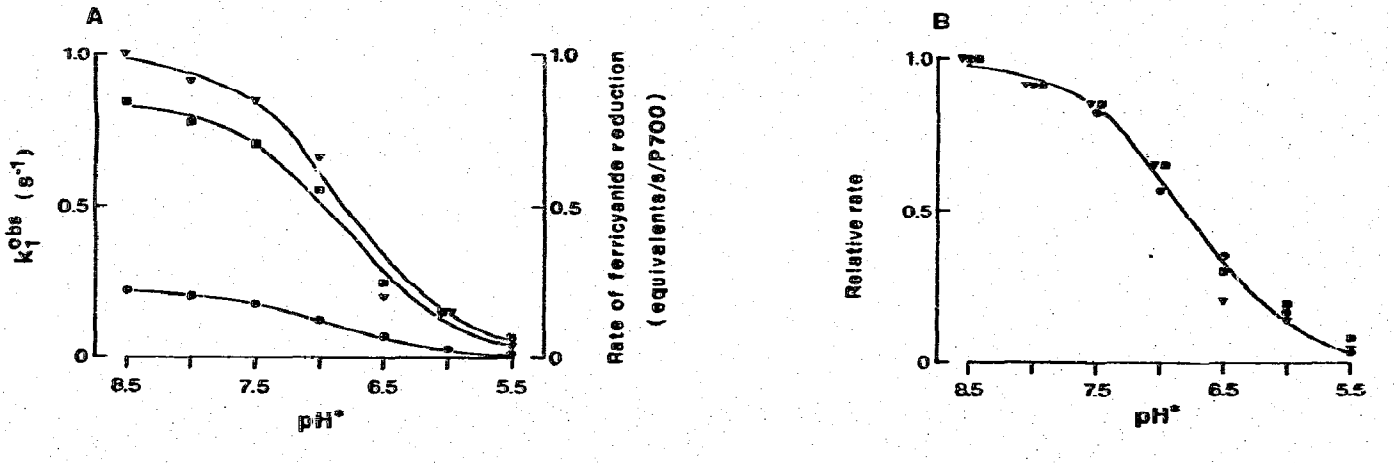

Fig.1. (A) Effect of internal pH* on the apparent first-order rate constant for the dark reduction of cytochrome *f* and P-700 following illumination and on the rate of ferricyanide reduction in continuous light. (•) cytochrome *f* reduction; (▾) *P*-700 reduction; (▪) ferricyanide reduction. (B) The same results following normalisation to the rate at pH* 8.5. The curve is the theoretical titration curve for a compound with pK* 6.8. The reaction medium contained 0.2 mM ferricyanide when its reduction was being measured and otherwise 40 μM methyl viologen, 4 μM gramicidin D and chloroplasts (corresponding to a final chlorophyll concentration of 40 μg/ml for cytochrome *f* measurements and 20 μg/ml otherwise). The temperature was −17°C. Other details are given in section 2.

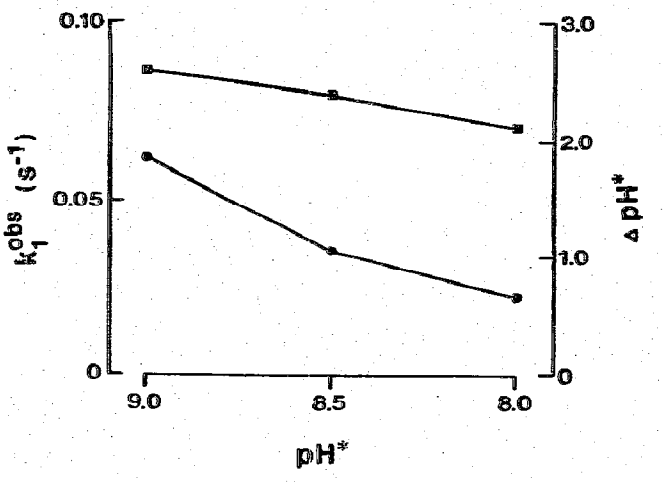

Fig.2. Estimation of the transmembrane pH* gradient generated in the absence of uncoupler. (•) Variation of the apparent first-order rate constant for cytochrome *f* reduction in the dark with the external pH*. (▪) ΔpH* calculated using these results and those from fig.1. The experimental conditions were as in the legend to fig.1 except that gramicidin was omitted.

chrome *f* and *P*-700 still showed first-order kinetics. Both reactions were stimulated to the same extent by gramicidin D or NH_4Cl. Figure 2 shows a plot of the observed first-order rate constant for cytochrome *f* reduction in the absence of uncoupler and the corresponding pH* gradient across the thylakoid membrane, calculated by using the data from fig.1A, as a function of the pH* in the external medium. It is noteworthy that the value of 2.6 found for the ΔpH* under these conditions is close to the values measured at room temperature [14].

The method used here for measuring the transmembrane pH gradient, introduced [1], has been criticised by the suggestion [3] that in addition to the internal pH the rate of electron transport is also dependent on the ΔpH and the external pH. However, some of the data in [3] was replotted [5] and shown to be compatible with the idea that at internal pH < 8.2, the rate of chloroplast electron transport is mainly determined by the internal pH. An analogous argument should hold for our experiments.

The results presented here confirm the impression from [4,5,16] that electron and proton transport in chloroplasts suspended in mixtures of water and ethanediol at subzero temperatures have remarkably similar properties to those of chloroplasts suspended in aqueous media at room temperature.

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