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STUDIES ON THE RATE-LIMITING REACTION OF PHOTOSYNTHETIC OXYGEN EVOLUTION IN SPINACH CHLOROPLASTS

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SUMMARY

The modulated oxygen polarograph has been used to study the rate-determining steps of photosynthetic oxygen evolution in spinach chloroplasts. The rate constant, k, of the reaction has a value of 218 ± 10 (S.E.) s^{-1} at 23 °C and an activation energy of 7 ± 2 (S.E.) kcal · mol^{-1}. A kinetic isotope experiment indicated that this step is probably not the water-splitting reaction. These findings resemble previous results with the unicellular alga *Chlorella* (Sinclair, J. and Arnason, T. (1974) Biochim. Biophys. Acta 368, 393–400). In other experiments we changed the pH, O_2 concentration and osmolarity of the medium, and treated the chloroplasts with 1 mM NH_4Cl without detecting any significant change in k. These results suggest that the step is irreversible. However, a significantly lower value of k, 110 ± 20 (S.E.) s^{-1} was obtained when all salts except 1 mM $MgCl_2$ were removed from the medium bathing the chloroplasts.

INTRODUCTION

In a previous paper [1] we studied the rate-limiting thermal reaction of photosynthetic oxygen evolution in the unicellular alga *Chlorella*. Using the modulated oxygen electrode developed by Joliot [2], we found that the rate constant for this reaction was about 305 s^{-1} at 20 °C, had an activation energy of 5.9 kcal · mol^{-1} and probably did not involve the splitting of an O-H Bond. In the present study, we report on similar experiments performed with spinach chloroplasts. Since isolated chloroplasts might be expected to be more responsive to changes in their suspension medium than whole cells, we have also examined these organelles under a variety of conditions to see if any of these would have an effect on the rate constant. The conditions studied included changes in osmolarity, pH, ion content and oxygen concentration in the suspending medium, as well as addition of the uncoupler, NH_4Cl, to the medium.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

MATERIALS AND METHODS

Intact chloroplasts were prepared according to the method of Cockburn et al. [3] from four-week-old spinach grown in a greenhouse. Some of the basic experiments were repeated with chloroplasts prepared by the method of Schwartz [4] which gave identical phase results although the signal size was smaller. The chlorophyll content of the suspension medium was assayed by Vernon's procedure [5] and the suspension was then diluted to a chlorophyll concentration of 0.5 mg/ml and kept on ice until needed.

In our previous paper [1] we described in detail the modulated oxygen polarograph used in the present experiments. The rate constant, k, of the rate-limiting step of oxygen evolution was determined by measuring the phase lag, ϕ, of the oxygen signal at different frequencies, ν, of modulation. Errors in the phase measurements were somewhat higher ($\pm 2°$) in our present study than in the previous experiments with *Chlorella*. This can be attributed to the greater lability of chloroplasts and the smaller signal size obtained with them. Joliot's theoretical equation [2] for the frequency dependence of the phase:

$$\phi = \phi_0 + p\sqrt{\pi/D} \cdot \nu^{\frac{1}{2}} + \tan^{-1}(2\pi\nu/k)$$

where ϕ_0 is the phase at zero frequency, p is the distance from the platinum electrode to the oxygen sources and D is the diffusion coefficient of oxygen in the medium, was fitted by a computer procedure to the phase results and the optimum values of k, ϕ_0 and $p\sqrt{\pi/D}$ determined. More details of this method can be found in our previous paper [1].

The chloroplasts were perfused with a buffered medium at the rate of a few ml/min during the experiment. Chloroplasts doing CO_2 fixation were perfused with a medium containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)/NaOH buffer (pH 7.6), 0.33 M sorbitol, 1 mM $MgCl_2$, 5 mM $NaHCO_3$ and 100 mM KCl (medium A). In experiments where the signal was maximized by providing NADP as an electron acceptor, the medium contained 20 mM HEPES/NaOH buffer (pH 7.6), 100 mM KCl, 1 mM $MgCl_2$ and 1 mM NADP (medium B). In the latter case the chloroplasts were enriched with ferredoxin (0.4 mg/mg of chlorophyll) before injection into the apparatus. The ferredoxin (type III) and HEPES were obtained from the Sigma Chemical Corporation. The effect of NH_4Cl, KCl, $MgCl_2$, pH and O_2 concentration were monitored by perfusing one sample of chloroplasts first with one of the standard media, and then with the altered medium. Special compressed O_2/N_2 gas mixtures were obtained from Linde for the O_2 experiments.

Equipment for controlling and monitoring the temperature have been described previously [1]. Experiments to determine the influence of temperature on k were performed on a single sample of chloroplasts within the temperature range of 19–32 °C. Below 19 °C the oxygen signal was too small for reliable measurements while above 32 °C the chloroplasts were rapidly denatured.

For the kinetic isotope experiments, a deuterated perfusion medium was prepared by freeze-drying the basic water based medium (medium A). The dried chemicals were then dissolved in ^2H_2O and freeze-dried a second time, before a final dissolution in ^2H_2O. Chloroplasts were perfused 45 min in this medium before

measurements of the oxygen signal were made to determine k. The chloroplasts were then perfused with an H_2O based medium for 45 min before a determination of k in H_2O was made. The ^2H_2O contained 99.8 % of the heavy isotope and was obtained from Stohler Isotope Corporation.

RESULTS

In Fig. 1, the dependence of the phase and amplitude of the oxygen signal on the square root of modulation frequency is shown, from which the rate constant is determined. This experiment was performed at 23 °C using basic medium B, with a bilayer of chloroplasts, which approximates to the "thin layer" of cells of Joliot's theory. The solid lines were generated using the theoretical equations and show that values of k, ϕ_0 and $p\sqrt{\pi/D}$ could be obtained which gave excellent agreement between theory and experiment. The equations assume that the rate-limiting reaction is first order. In fact, we found that the phase was independent of the intensity within the linear range, a

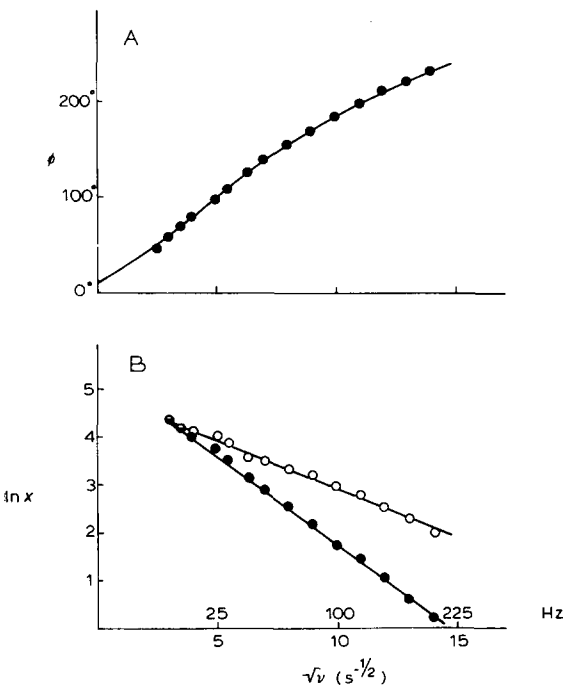


Fig. 1. (A) The phase lag between the oxygen signal and the light modulation plotted against the square root of frequency (●) obtained with the modulated polarograph using a sample of spinach chloroplasts. The curve is generated by the best fit of the equation:

$$\phi = \phi_0 + p\sqrt{\pi/D} \cdot \nu^{\frac{1}{2}} + \tan^{-1}(2\pi\nu/k)$$

Best fit values were 200 s^{-1} for k, 9.82° $s^{-\frac{1}{2}}$ for $p\sqrt{\pi/D}$ and 13° for ϕ_0. The standard deviation between the experimental and calculated points was 2.2°. (B) The amplitude results of the O_2 signal for the same sample as in Fig. 1A. For the closed symbols $x = \ln A$ and for the open symbols $x = \ln A\,(k^2+4\pi^2\nu^2)^{\frac{1}{4}}/k$, where A is the amplitude of the O_2 signal. The open circles show only the influence of diffusion, while the closed circles also reflect the effect of the rate-limiting reaction.

TABLE I

BEST FIT PARAMETERS FOR EXPERIMENTS WITH CHLOROPLASTS AT 23 °C

	k (s^{-1})	$p\sqrt{\pi/D}$ ($^0s^{-\frac{1}{2}}$)	ϕ_0 (degrees)
Medium A*	215± 9 (S.E.)	10.3±0.5 (S.E.)	3±5 (S.E.)
Medium B**	218±10 (S.E.)	9.7±0.2 (S.E.)	13±7 (S.E.)

* Medium A contained 20 mM HEPES/NaOH buffer (pH 7.6), 0.33 M sorbitol, 100 mM KCl, 1 mM $MgCl_2$ and 5 mM $NaHCO_3$.

** Medium B contained 20 mM HEPES/NaOH buffer (pH 7.6), 100 mM KCl, 1 mM $MgCl_2$ and 1 mM NADP.

characteristic which is expected for a first, but not for a second order reaction.

The best fit parameters for chloroplasts in medium A or in medium B are shown in Table I. Each value is an average of seven and all experiments had a standard deviation between experimental and theoretical values of less than 3 °. The differences in the best fit parameters for the two situations shown in Table I probably result from the change in osmolarity of the medium. The chloroplasts, doing CO_2 fixation and suspended in the sorbitol-containing medium (medium A) were smaller than those in the sorbitol-free buffer (medium B). This was reflected in the smaller intercept in the sorbitol medium. Also viscosity measurements allowed us to estimate from the best fit values of $p\sqrt{\pi/D}$, that the average chloroplast volume was about 10 % smaller in medium A.

The value of k was determined at two temperatures with seven different samples of chloroplasts yielding an average value of E_a of 7±2 (S.E.) kcal · mol^{-1}. The average value of E_D was 6.1±9 (S.E.) kcal · mol^{-1}, which is close to an independent physical measurement of 6.0 kcal · mol^{-1} [1].

The results of four kinetic isotope experiments gave a ratio of $k_{H_2O}/k_{^2H_2O}$ of 1.1±0.1 (S.E.). Hence it does not appear likely that the rate-limiting step of oxygen evolution in spinach chloroplasts involves the breaking of an O-H Bond. The ratio of $D_{H_2O}/D_{^2H_2O}$ was 1.2 which compares well with a value of 1.25 calculated from viscosity measurements.

The results of the experiments with different concentrations of inorganic ions is shown in Table II. Each value is an average of four to six determinations. The effect

TABLE II

EFFECT OF INORGANIC IONS

In addition to the salts mentioned, the medium contained 20 mM HEPES/NaOH buffer (pH 7.6), 0.33 M sorbitol, and 5 mM $NaHCO_3$. Values are given ±S.E.

Salts in medium	k (s^{-1})	$p\sqrt{\pi/D}$ ($^0s^{-\frac{1}{2}}$)	ϕ_0 (degrees)
1 mM $MgCl_2$	110±20	10.9±5	11±1
25 mM $MgCl_2$	215±16	10.8±5	6±3
1 mM $MgCl_2$ + 100 mM KCl	215±11	10.3±5	3±5

of the salts on the value of k was reversible and did not depend on whether the chloroplasts were first perfused with the low salt medium (containing only 1 mM $MgCl_2$) or one of the high salt media (with 25 mM $MgCl_2$ or 100 mM KCl). In addition, some other salts were investigated. When we perfused the chloroplasts with a medium containing 100 mM sodium acetate or 100 mM $NaNO_3$, results similar to those with 100 mM KCl were obtained. Hence, it does not appear that the presence of any one ion is responsible for the stimulation of k.

Many of the other situations that we investigated had little effect on the rate constant. In the pH range 5.8–8.2, where the signal size was large enough to make reliable phase measurements, there was no significant change in the value of k within the resolution of our technique. Similarly, when 1 mM NH_4Cl was added to the medium or when the medium was bubbled with 10 %, 20 % or 30 % oxygen mixtures, no significant change in k was observed.

DISCUSSION

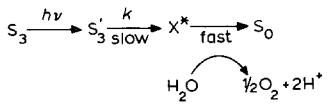
It would appear from this study that the rate-limiting thermal reaction associated with photosynthetic oxygen evolution in spinach chloroplasts is similar to that in *Chlorella*. The rate constant is somewhat similar (218 s^{-1} for chloroplasts vs. 305 s^{-1} for *Chlorella*) but the activation energy is slightly larger (7 kcal · mol^{-1} for chloroplasts compared with 5.9 kcal · mol^{-1} for *Chlorella*). The deuterium isotope effect has a low value in both species, indicating that this step probably does not involve the breaking of an O-H Bond in a water molecule. These similarities indicate that higher and lower plants share a common mechanism in the rate-determining step. This is consistent with the similarities in the flash yield sequences of the two species and the application of the Kok model for oxygen evolution to both [6]. However, it should be noted that our rate constants are somewhat lower than the slowest rate step in the Kok scheme which Bouges Bocquet [7] has estimated to be about 580 s^{-1}.

The effect of inorganic ions on k is not merely an osmotic one, since large changes in the sorbitol concentration of the medium had no effect on it. Although stimulation of oxygen evolution by Cl^- has been observed by many other workers [8–11], our results indicate that the increase in the rate constant cannot be attributed to any one ion, but rather to the effect of inorganic salts in general. Salt induced changes in the membrane structure or protein conformation may be responsible for these effects.

The study of the effect of uncouplers and pH on k was suggested by recent work which has identified two sites of phosphorylation, one of which (coupling site II) appears to be linked to oxygen evolution. Gould and Izawa [12] have suggested that H^+ from the water-splitting reaction accumulate on the inside of the chloroplast lamellar membrane. According to the Mitchell hypothesis [13], the development of an electrochemical potential difference of hydrogen ions across the membrane is an intermediate step leading to the phosphorylation of ADP. Thus, many uncouplers of phosphorylation including NH_4Cl may derive their activity from their ability to inhibit the light-induced movement of hydrogen ions [14]. We investigated the possibility that the rate-limiting reaction of O_2 evolution was directly involved in phosphorylation in two ways. We wished to observe whether a change in the electrochemical potential difference of H^+ across the membrane could affect the value of k. NH_4Cl

was used in our study to see if uncoupling had any effect on k. Neither the pH changes nor the addition of NH_4Cl to the medium appeared to change the value of k. This seems to be consistent with the observations of Gould and Ort [15] that the rate of electron transport through coupling site II is independent of phosphorylation and uncoupling. The results are perhaps best explained if the rate-limiting step of oxygen evolution is irreversible.

Oxygen concentration did not affect the rate constant in these experiments. As oxygen is the product of water splitting, this observation suggests that either the rate-determining step is not the water-splitting step (in agreement with our kinetic isotope experiment), or that the rate-determining step is irreversible, or perhaps both. We feel that the slow build up of an activated compound or charge complex, X^*, followed by its rapid and irreversible reaction with water best explains our results. In terms of the Kok model, the scheme is:



Perhaps the lack of control of photosynthetic oxygen production by the end product can be explained by the observation that oxygen can control other sites in photosynthesis. It has long been known that oxygen inhibits carbon fixation via the Warburg effect [16], and recently Diner and Mauzerall [17] have demonstrated that a co-operative feedback mechanism on the oxidizing side of photosystem II may be controlling oxygen evolution.

In summary, our work suggests the following properties for the rate-limiting reaction of oxygen evolution in spinach chloroplasts:

(1) The reaction is first order and has a rate constant of 215 s^{-1}.

(2) It has an activation energy of 7 kcal · mol^{-1}.

(3) In the deuterium isotope experiment, the ratio of $k_{^2H_2O}/k_{H_2O}$ was estimated to be 1.1. This indicates that this step is probably not the water-splitting reaction in photosynthetic O_2 evolution.

(4) The reaction is sensitive to low salt concentrations with a low rate constant of 110 s^{-1}.

(5) The reaction is unaffected by the addition of 1 mM NH_4Cl to the medium or changes in the oxygen concentration and pH of the medium. This evidence suggests that the reaction is irreversible.

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