

Temperature Dependence of the O₂-Oscillation Pattern in the Filamentous Cyanobacterium *Oscillatoria chalybea* and in *Chlorella kessleri*

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Five characteristic discontinuities of the pattern of oxygen evolution have been detected for the filamentous cyanobacterium *Oscillatoria chalybea* in the temperature range of 0°C to 30°C. The temperatures at which these discontinuities occur are: ≈ 5°C, ≈ 11°C, ≈ 15°C, ≈ 21°C and ≈ 25°C. The calculated initial 5-S state distribution, the miss parameter and the fraction of the fast transition S₃ → S₀ + O₂ are affected. The discontinuities are observed at the same transition temperature also for *Chlorella kessleri* hence are not specific for the cyanobacterium. Based on these studies it is concluded that the not vanishing oxygen signal under the first flash of a flash train in *Oscillatoria* cannot have its origin in interactions between oxygen-evolving complexes. A decrease of temperature should slow down the expected charge exchanges, improve the oscillations, thus reduce or lower the first two oxygen amplitudes of the oscillatory pattern. Lowering of the temperatures improves the oscillations but does not lower the first O₂ signal of the pattern.

Introduction

Since Joliot *et al.* (1969) have investigated in dark adapted algal cells and chloroplasts oxygen yields under sequences of short saturating flashes, the analysis of the oxygen pattern, which shows the pattern of a damped oscillation with the periodicity of four, has become a powerful method to study redox changes and electron transfer in photosystem II (PS II). The quantitative description of the kinetic properties of O₂ release is provided by the Kok model (Kok *et al.*, 1970) which is based on the storage mechanism of four positive charges leading finally to the oxidation of two molecules of water after five redox states S_i in PS II have been gone through. The amount of oxygen evolved under the n-th flash is a measure of the concentration of the centers which were in the S₃

state. In order to explain the observed sequences of O₂ yield, Kok *et al.* (1970) assumed that only the S₀ and S₁ states are stable in darkness with a typical initial distribution being 25%–S₀ and 75%–S₁. The Kok model describes very well the oxygen patterns observed with chloroplasts or thylakoids from higher plants. A different kind of oxygen pattern is observed in the cyanobacteria *Oscillatoria chalybea* (Bader *et al.*, 1984) and *Synechococcus leopoliensis* (Mauzerall and Dubinsky, 1993), where oxygen is already evolved under the first flash, where the fourth flash gives the maximal amplitude and where the oscillations are particularly strongly damped. There are two principal alternative explanations of this behaviour of the oxygen pattern. The first one, proposed by Bader *et al.* (1983), follows the original Kok idea and assumes a non-standard initial S-state distribution after dark adaptation. The authors consider the possibility of population of S₂ and S₃ states in darkness, i.e. longer living S-states S₂ and S₃. Alternatively, Mauzerall and Dubinsky (1993) think that the spectra of flash induced oxygen evolution in cyanobacterium can be understood in the frame of a model of partial pair-wise interactions of the O₂ evolving complexes in their organism. Given a distribution of dark relaxed S-states, maximal for S₁ but containing some S₂, charge exchange

Abbreviations: PS II, photosystem II; OEC, oxygen-evolving-complex.

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between pairs of centres could form O_2 under the first flash. The interactions must be limited, otherwise no oscillations of the O_2 yield would be observed. Mauzerall and Dubinsky have observed a difference in the effective optical cross-sections derived from the saturation curves for the individual flashes in the case of the cyanobacterium *Synechococcus leopoliensis* but not in the chlorophyte *Chlorella vulgaris*. This observation leads them to the conclusion that the oxygen evolving complexes (OEC) in cyanobacterium are not independent in contrast to the independent OEC in chlorella. On the other hand, the difference in the effective optical cross-sections for the different S-states of the cyanobacterium can be explained by a non-standard initial distribution of S states which would affect the flash induced oxygen evolution (manuscript in preparation). Additionally, the model proposed by Mauzerall and Dubinsky requires a non-standard initial S-state distribution, i.e. the most stable state should be S_0 (62%) with S_3 (13%) being almost as stable as S_1 (18%).

The understanding of the mechanisms which lead to the oxygen pattern is important to get a better inside into the electron transport in PS II. From this point of view it is very important to reconsider the two models (Bader *et al.*, 1983; Mauzerall and Dubinsky, 1993) and consequences which follow from them.

In this paper we report on temperature measurements of oxygen evolution under short saturating flashes for *Chlorella kessleri* and *Oscillatoria chalybea*. The connectivity of oxygen evolving complexes in cyanobacterium should give a significantly different temperature dependence of the oxygen pattern than the one observed for independent centers of chlorella. If charge transfer between OEC (Oxygen Evolving Complexes) is expected, one has to remember that changes of electron delocalization accompany the structural and chemical changes caused by temperature. It means that this kind of charge transfer proposed in Mauzerall and Dubinsky, 1993 should follow the Arrhenius law and should be very sensitive to temperature changes. The temperature transitions would be strongly influenced by an electron transfer between oxygen evolving complexes.

Materials and Methods.

Plant material

Oscillatoria chalybea was cultured in the medium D of Kratz and Myers (1955). The cultures were grown in $26^\circ\text{C} \pm 1^\circ\text{C}$ in 12 h light / 12 h dark cycle under a light intensity of 1000 lux. The preparation of protoplasts and thylakoids from cells of *Oscillatoria chalybea* was carried out as given by Bader *et al.* (1983).

Chlorella kessleri was cultured in the nutrition solution given by Kowallik (1963) and grown at 30°C under a light intensity of 10 000 lux. The atmosphere contained about 1.5% CO_2 .

Oxygen measurements

The measurements of oxygen evolution under short saturating flashes have been carried out by polarography with the *Three Electrode System* described by Schmid and Thibault (1979). The electrode system was connected with a thermostat in order to permit measurements within a temperature range of 0°C to 30°C . The temperature was stabilized within $\pm 0.1^\circ\text{C}$.

Thylakoids of *Oscillatoria chalybea* (25 μg of chlorophyll) were suspended in 0.6 ml of reaction buffer (0.05 M Tricine and 0.12 M KCl, pH 7.5).

Chlorella cells (5 days old) corresponding to 195 μg chlorophyll were suspended in their growth medium (pH 8.15) of 0.6 ml volume.

Before measurement the samples have been kept for 20 min in ice in darkness. Then the samples have been put on the electrode (handling was performed in dim green light) and were then incubated for 25 min. in darkness at the respective temperature. Immediately after each measurement the temperature of the sample on electrode was measured with an accuracy of $\pm 0.1^\circ\text{C}$.

Fluorescence measurements

The fluorometer PAM-2000 WALTZ (Germany) was used for fluorescence measurements. Two signals have been measured: a) the relative fluorescence yield induced by red light (650 nm) of the intensity of $30 \mu\text{E m}^{-2}\text{s}^{-1}$, F_0 ; b) the maximum level of the relative fluorescence yield induced by white light (8V/20W halogen lamp) passing through a Balzers DT Cyan short-pass filter

($\lambda < 670$ nm), F_m , with the intensity of this light being $730 \mu\text{E m}^{-2}\text{s}^{-1}$.

The holder of the sample was cooled via connection with the thermostat. The temperature treatment of the samples was exactly the same as in the case of oxygen measurements.

Results and Discussion

The ratios of oxygen yield due to the first and second flash (a) and third and fourth flash (b) for the cyanobacterium *Oscillatoria chalybea* in dependence on the temperature are shown in Fig. 1. For *Chlorella kessleri* the ratio of oxygen evolution under the third and fourth flash is depicted in

Fig. 1c. In this case the first flash gives no O_2 yield and the second amplitude is very low.

Two characteristic features of the temperature dependence of the pattern of oxygen evolution in these two species are observed: 1) decreasing temperatures improve the oscillations; 2) the oscillations exhibit in both species the same temperature transitions. There are brakes and discontinuities of the ratios of oxygen yield under certain flashes (Fig. 1) which appear at about 25°C , 21°C , 15°C , 11°C and 5°C . These transitions can have a complex origin and might be due for example to conformational changes of photosystem II (proteins and lipids) or to small modifications on the donor or/and acceptor side of PS II.

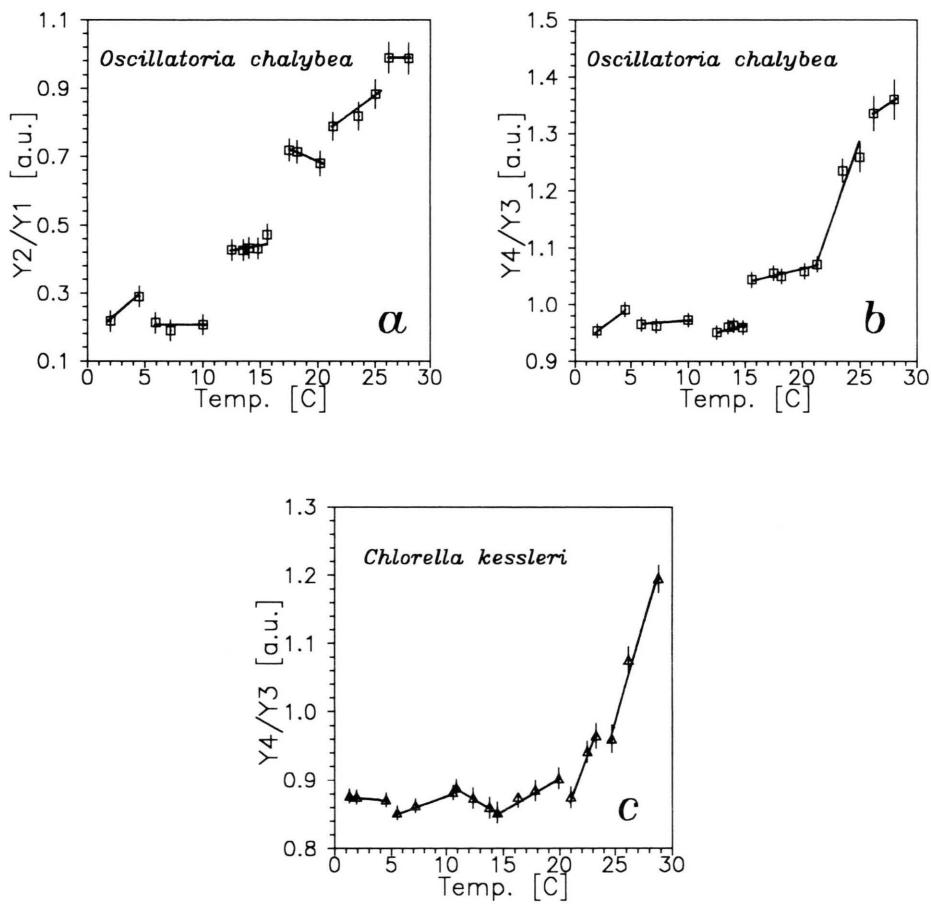


Fig. 1. Temperature dependence of the ratio of oxygen yield (a) of the second and first flash, (b) the fourth and third flash in *Oscillatoria chalybea* and (c) the fourth and third flash in *Chlorella kessleri*. Maximal error bars are given in the figures.

We have also measured F_0 and F_m changes in dependence on the temperature for *Chlorella kessleri* (Fig. 2). Such an experiment was not possible for *Oscillatoria chalybea* because in this case the fluorescence signals are too low in comparison to the noise level. Minimal fluorescence (dark) F_0 represents the fluorescence intensity with all PS II reaction centers open whereas maximal fluorescence (dark) F_m corresponds to the maximal level of fluorescence, when photosystem II reaction centers are closed (Kooten and Snel, 1990; Horton and Ruban, 1993). In Fig. 2a and b the temperature dependences of F_0 and F_m are shown for chlorella. They depend in a comparable way on the temperature changes. In the range of 0–5 °C, 5–11 °C and 11–15 °C they decrease with increasing temperature exhibiting discontinuities at tran-

sition temperatures. When the temperature increases from 15 °C to 25 °C both parameters increase with a brake at about 21 °C. Then they decrease when the temperature increases up to 30 °C.

Looking for the relations of the fluorescence parameters which usually reflect changes on the acceptor side of PS II connected with the donor side we have carried out a mathematical analysis of the pattern of oxygen evolution. We have used a heterogeneous model, considering 5 S-states (S_0 , S_1 , S_2 , S_3 , S_4) assuming two ways of O_2 yield – a fast one passing through the $S_3 \rightarrow S_0$ transition and a slower one including a longer living S_4 -state. The detailed description of this model is given by Burda and Schmid (1996). The calculated initial distribution of S_i states in the case of chlorella

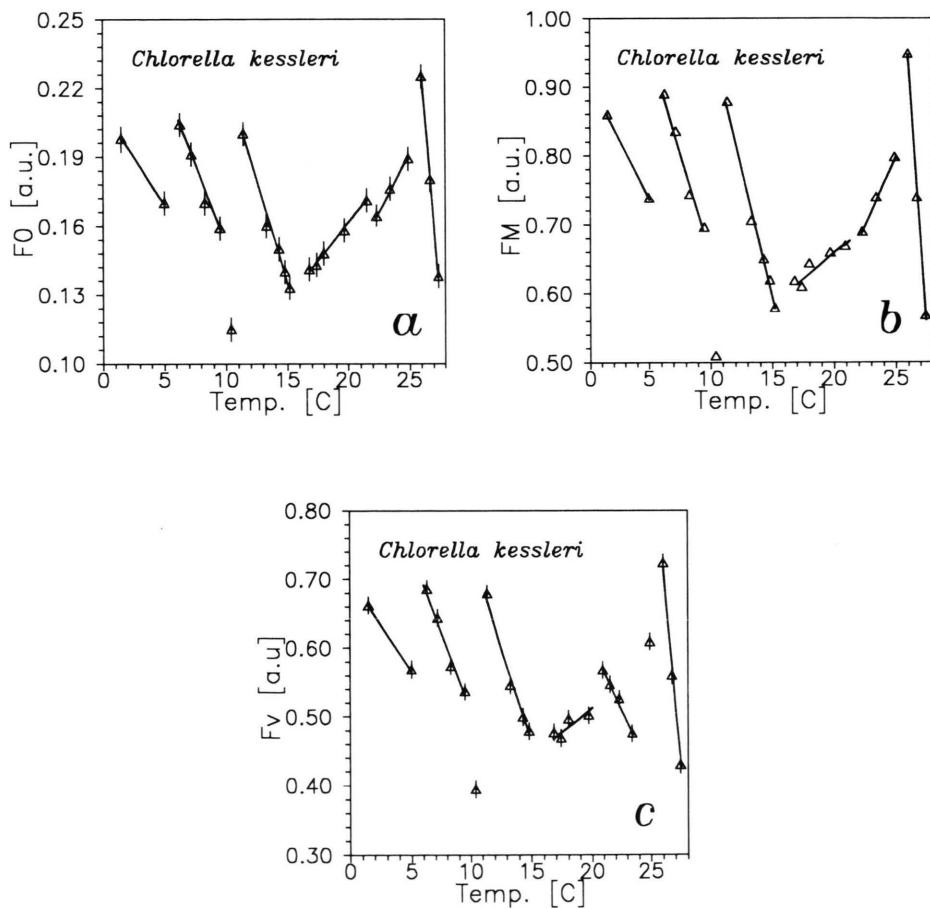


Fig. 2. Fluorescence characteristics (a) F_0 , (b) F_m and (c) F_v as functions of temperature in *Chlorella kessleri*. Maximal error bars are given in (a) and (c). In the case of F_m (b) the error bars are within the sign size of the curve symbols.

shows a main occupation of the S_1 state varying with temperature, a small population of the S_2 state and no occupation of higher states. The temperature dependence of the S_i occupation is depicted in Fig. 3. It is evident that the temperature dependence of S_0 has the same characteristics as the ratio Y_4/Y_3 (Fig. 1c), however without showing the discontinuities at temperatures higher than 15 °C. This correlation is not surprising because the first amplitudes of oxygen evolution are mostly sensitive to the initial S-state distribution. The gradual decrease of the S_0 occupation with temperatures decreasing from 30°C to 15°C and increase of S_1 on the other hand can be explained by the more effective reduction of Tyr_D^{ox} by the S_0 state (Vermaas *et al.*, 1984, 1988; Styring and Rutherford, 1987; Messinger and Renger, 1993)

during the dark adaptation time at lower temperatures. This process seems to be 20% more efficient at 16 °C than at 28°C. Small temperature changes of S_2 cannot significantly influence the oxidation of the S_0 state by Tyr_D^{ox} . Below 15 °C the process of dark stabilization of the donor side of photosystem II seems to be more complicated. It looks as if the modifications on the acceptor side of PS II are more pronounced. The total miss parameter α_t (Fig. 4a) which is the sum of misses for the separate transitions $S_i \rightarrow S_{i+1}$ ($i=0,1,2,3$) comes mainly from the unsuccessful transition between the $S_2 \rightarrow S_3$ states in the range of temperatures from 15 °C to 30 °C. Below 15°C the efficiency of transitions from S_0 to S_1 decreases with decreasing temperature as well.

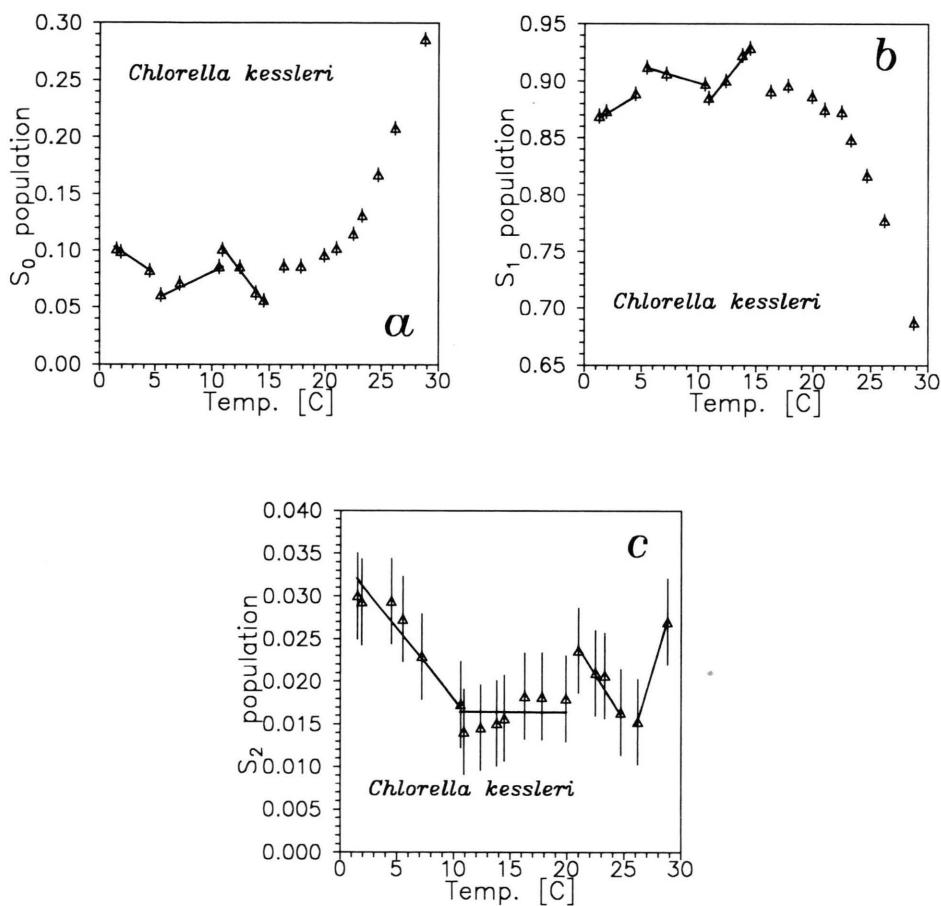


Fig. 3. Temperature dependence of the initial S-states distribution: (a) the S_0 state, (b) the S_1 state and (c) the S_2 state in *Chlorella kessleri*. The S_i states are calculated from a heterogenous 5 S-state model. There is no population of the S_3 and S_4 states. Maximal error bars are given in the figures.

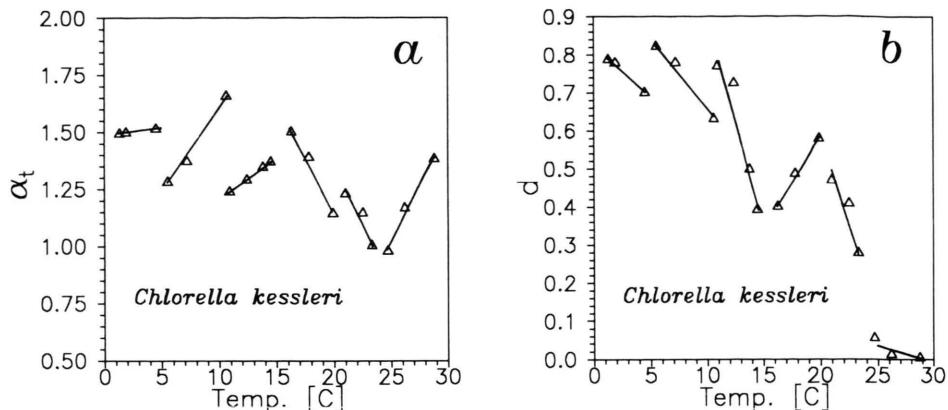


Fig. 4. The total miss parameter α_t , (a) and the probability of a fast transition $S_3 \rightarrow S_0$, d , (b) as a function of temperature in *Chlorella kessleri*. These parameters are calculated from a heterogenous 5 S-state model. The error bars are within the sign size of the curve symbols.

The most interesting correlation exists between the d parameter and F_v . d expresses the fraction of fast transition of S_3 via/through S_4 to the S_0 state leading to O_2 evolution (Burda and Schmid, 1996). The parameter $F_v = F_m - F_0$ is the maximum variable fluorescence in the state when all non-photochemical processes are at minimum (Holzwarth, 1993; Trissl *et al.*, 1993). The temperature dependence of F_v (Fig. 2c) is practically the same as that of the d parameter (Fig. 4b) in the range of 0 °C to 25 °C. This could mean that the efficiency of charge recombination on the acceptor side is the higher the higher the probability for the fast transition of S_3 to S_0 (d) is. At temperatures higher than 25 °C d is very small and almost constant; therefore, at these temperatures the increase of α_t could be well correlated with the decrease of F_v with increasing temperatures.

We have performed the same mathematical analysis of the oxygen pattern for *Oscillatoria chalybea*. The calculated initial S-state distribution at different temperatures are shown in Fig. 5. The S_4 state is always equal to 0 as it cannot be metastable. The populations of the S_i states exhibit the same discontinuities at the same temperatures as in the case of chlorella namely at 25 °C, 21 °C, 16 °C, 11 °C and 5 °C. The S-state distribution is in the whole range of the measured temperature very sensitive to temperature changes. Thus, in the cyanobacterium the dark stabilization of the oxygen evolving system seems to be more influenced by changes of the redox conditions on the acceptor

side than in the case of chlorella. In Fig. 6a and 6b the “total miss” parameter (α_t) and the d parameter are shown for *Oscillatoria chalybea*. The miss parameter decreases with decreasing temperature in the characteristic temperature ranges, whereas the d parameter increases (except in the range of 5 °C to 11 °C). Thus, the more efficient the transfer between the $S_i \rightarrow S_{i+1}$ states is (increase of successful transitions) the more the pattern of oxygen yield is improved in the case of *Oscillatoria chalybea*. In the case of *Chlorella kessleri* it is the increasing dark occupation of the S_1 state and the increase of the fraction of fast transitions from $S_3 \rightarrow S_0$ which are responsible for the improvement of oxygen oscillations under short saturating flashes (the oxygen pattern is similar to that of higher plant thylakoids). At temperatures below 17 °C the damping of the amplitudes of oxygen evolution in chlorella cells with increasing flash numbers seems to be due to the inferior connectivity with photosystem I. This circumstance has been taken into account in the mathematical analysis. This phenomenon is not observed with the cyanobacterium.

The temperature dependence of different parameters characterizing the oxygen evolving complex have been carried out with different plant species in the temperature range of 0 °C to 30 °C (Koike *et al.*, 1987; Messinger *et al.*, 1993; Kazanawa *et al.*, 1992; Zhong *et al.*, 1993). For example a breakpoint for $Y_2/Y_{\Sigma 3-6}$ (using a Joliot-type electrode) at around 21 °C was observed in spin-

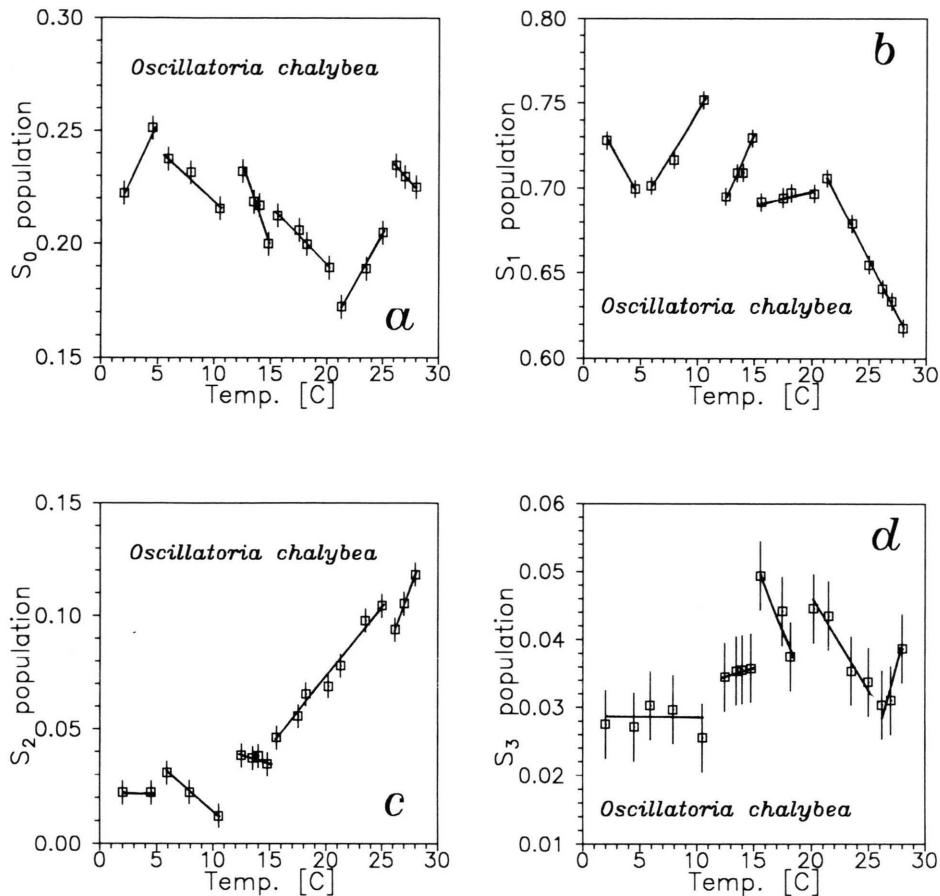


Fig. 5. Temperature dependence of the initial S-states distribution: (a) the S_0 state, (b) the S_1 state, (c) the S_2 state and (d) the S_3 state in *Oscillatoria chalybea*. The S_i states are calculated from a heterogenous 5 S-state model. There is no population of the S_4 state. Error bars are given in the figures.

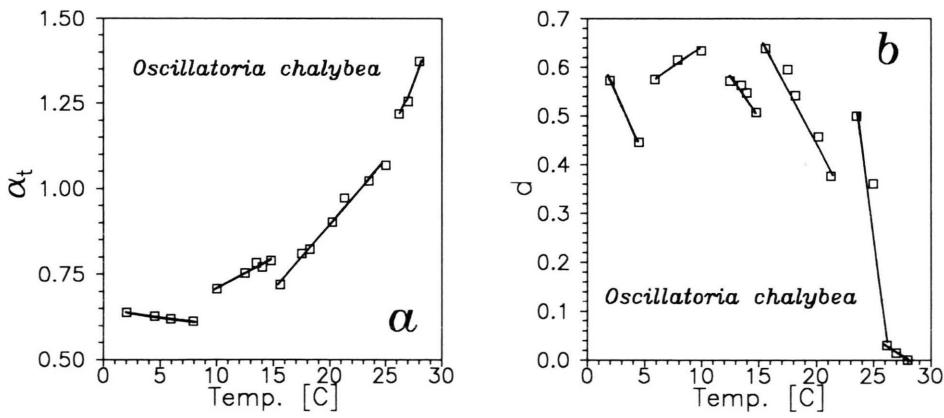


Fig. 6. The total miss parameter, α_t , (a) and the probability for the fast transition $S_3 \rightarrow S_0$, d , (b) as functions of temperature in *Oscillatoria chalybea*. The parameters are calculated from a heterogenous 5 S-state model. The maximal error bars for α_t and d are within the sign size.

ach thylakoids and PS II membrane fragments (Messinger and Renger, 1990). By measuring time resolved absorption changes at 355 nm in dark adapted PS II membrane fragments from spinach, a breakpoint at 5.9 °C for the reaction $Y_Z^{ox}S_3 \rightarrow Y_ZS_0 + O_2$ was detected by Renger and Hanssum (1992). The temperature-induced changes in Hill activity for chloroplasts isolated from barley at 9 °C, 20 °C, 29 °C and 36 °C have been reported (Nolan and Smille, 1976). The transitions at 9 °C and 29 °C coincide with temperature-induced changes in the fluidity of chloroplast thylakoid membranes. In the range of 10 °C to 30 °C, only 5–10% of polar lipids from spinach thylakoids undergo a phase transition as has been shown by Law *et al.* (1984) using differential scanning calorimetry.

The lipid composition of biological membranes is complex and therefore the transition of membrane lipids from the liquid crystalline to the mixed solid-liquid crystalline phase occurs steadily at higher temperatures (> 0 °C in the room temperature range). The break temperatures observed by us indicate very sharp transitions in the oxygen evolving system. Thus, it is clear that the detected transitions are the intrinsic feature of the PS II reaction center and represent conformational changes and interactions within the system. Of course the influence of the temperature changes in the membrane lipids should not be excluded.

We want to emphasize that our measurements have been carried out with intact species without any external acceptors or other reagents. The recorded transitions are not caused by pH changes. The pH changed monotonously from 7.28 to 7.5 for the cyanobacterium and from 8.15 to 8.22 for the chlorella medium with decreasing temperature. The discontinuities of F_0 and F_m appear at the same temperatures as in the case of the oxygenmetric measurements. The sharpness of the transitions depends at least in Chlorella somehow on the age of the algae but is always detectable at the same temperatures. The contribution of scattering effects due to changes of the condition of the sample, could only lead to monotonous changes of the fluorescence parameters.

Conclusions

We have detected 5 characteristic discontinuities of the pattern of oxygen evolution for *Chlorella*

kessleri and *Oscillatoria chalybea* in the range of 0 °C to 30 °C. The temperatures at which these discontinuities occur are: 5 °C, 11 °C, 15 °C, 21 °C and 25 °C. The calculated initial S-states distribution, the miss parameters and the fraction of the fast transition $S_3 \rightarrow S_0 + O_2$ show for both species discontinuities at the same transition temperatures. This is confirmed by fluorescence measurements. F_0 and F_m have the same temperature dependence as the oxygen measurements. However, it looks as if the temperature-induced changes have different characteristics suggesting different equilibria between the donor and acceptor sides of PS II in the cyanobacterium and in chlorella.

Based on these studies we can conclude that the non-vanishing signal under the first flash observed in *Oscillatoria chalybea* regardless whether the time of dark adaptation is more or less long cannot have its origin in interactions between oxygen-evolving complexes. The decrease of temperature should slow down the expected charge exchanges – improve the oscillations, lower the first two amplitudes and influence the transition temperatures for the conformational (redox) changes of the water oxidase. The improvement of oscillations has been observed for both species and in the case of the cyanobacterium the oxygen release under the first flash has not vanished even at temperatures closed to 0 °C (see also Messinger *et al.*, 1993). There is no doubt that such interactions do not exist in chlorella (Mauzerall and Dubinsky, 1993), and the oxygen pattern for *Chlorella kessleri* and *Oscillatoria chalybea* show the same transition temperatures. It is more probable that the phenomenon observed in cyanobacteria is the result of different redox equilibria between the donor and acceptor side of the reaction center. It might be that one should analyze the kinetics of the first two signals (Bader *et al.*, 1983). On the other hand, excitation energy transfer from closed reaction centers to open ones cannot be excluded. If there is a certain number of PS II units sharing a definite antenna or when all reaction centers are connected to an infinite sea of chlorophyll (Mauzerall and Greenbaum, 1989) the original Kok idea is still valid and models based on it can well describe oxygen evolution under short saturating flashes. The transfer of excitation energy from closed to opened reaction centers increases the probability of oxygen yield (as long as there is no

cyclic electron flow around PS II (Falkowski *et al.*, 1988; Gruszecki *et al.*, 1996) but the process of water oxidation remains a four electron act requiring the trapping of four photons by the reaction center.

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