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CHANGES IN LIGHT ABSORBANCE AND CHLOROPHYLL FLUORESCENCE IN SPINACH CHLOROPLASTS BETWEEN 5 AND 80 K

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Summary

A study was made of the properties of system 2 of photosynthesis below 77 K by measurements of light-induced changes of absorbance and chlorophyll *a* fluorescence in spinach chloroplasts. The chloroplasts were suspended in a non-crystallizing medium containing sucrose and glycerol.

The maximum yield (F_{max}) of chlorophyll *a* fluorescence during illumination increased by a factor of 3—4 upon cooling from 100 to 5 K. This increase was mainly due to an increase of the initial yield (F_0) upon illumination, suggesting a decrease of the efficiency of energy transfer between chlorophyll molecules upon cooling. Two different exponential components could be distinguished in the fluorescence rise during illumination.

The amplitudes of the absorbance changes due to C-550, the 'indicator pigment' for the reduction of the primary acceptor, and to cytochrome *b*-559 were not significantly altered by cooling from 80 to 5 K. However, the apparent half time of C-550 increased by a factor of 1.5—1.8, whereas that of cytochrome *b*-559 remained about the same. Both at 80 and 5 K, ferricyanide abolished the photooxidation of cytochrome *b*-559 and decreased the extent of the fluorescence increase. The rate, but not the amplitude of the absorbance change due to C-550 was markedly lowered.

The results indicate that the properties of the reaction center of system 2 are basically similar at 80 and at 5 K. In addition they indicate a heterogeneity of the reaction centers at low temperature with respect to the identity of secondary electron donors and the rates of donor reactions.

Introduction

Experiments have been done in various laboratories to study the photochemical properties of system 2 of photosynthesis at low temperatures [1—5].

In addition to a reversible photooxidation of the primary electron donor *P*-680, these studies have demonstrated an accumulation of the reduced primary acceptor Q^-, measured either directly or by means of the photoconversion of the 'indicator pigment' C-550, and an irreversible oxidation of cytochrome *b*-559. The latter reactions are accompanied by an increase in the yield of chlorophyll fluorescence [1,6].

Reactions of *P*-680 have been studied by means of electron spin resonance (ESR) down to 10 K [4,5], but optical measurements to study the other components have been restricted so far to temperatures above 77 K. Since the ESR measurements of $P\text{-}680^+$ suggest that the rates of secondary reactions may change significantly below 77 K [4], we have studied absorbance and fluorescence changes in spinach chloroplasts at temperatures between 5 and 77 K. The results of these experiments are presented in this paper.

Materials and Methods

Chloroplasts were obtained from market spinach as described elsewhere [7], suspended in a medium containing 50 mM tricine (pH 7.8), 0.4 M sucrose, 10 mM KCl and 2 mM $MgCl_2$ and stored in the dark on ice until use. Just before the measurements, aliquots of the chloroplast suspension were mixed with a solution of 1 M sucrose in glycerol in a final concentration of 55% (v/v), transferred in the dark to a helium-cooled continuous flow cryostat (Oxford Instruments F200) and cooled to the desired temperature. The cryostat was precooled to 80 K, and the rate of cooling of the sample was roughly 50 degrees per min in the range 300—100 K. The samples remained transparent upon cooling. The cryostat was equipped with four vertical windows. The sample holder consisted of a stainless steel tube equipped with a brass frame. A perspex vessel of 20 × 14 × 1 mm was placed in this frame. The temperatures were routinely measured by means of a thermocouple (gold —0.03% iron/chromel) positioned in the heat exchanger. This device was calibrated against a similar thermocouple positioned in the sample.

The cryostat was placed in a single-beam spectrophotometer, of similar design as used earlier [3]. The actinic light was provided by a tungsten-iodine lamp and filters. The angle between the measuring beam and the actinic beam was 90°. The cuvette was placed at an angle of 45° with respect to both beams; fluorescence, excited by the actinic beam, was measured with the same geometry of cuvette and photomultiplier. For the actinic light, the filter combination consisted of two Schott AL 630 nm interference filters, a 644 nm longwave cut-off interference filter and a Balzers Calflex C heat-reflecting filter. For the absorbance measurements the photomultiplier was protected by two Corning CS 4-96 glass filters. For the fluorescence measurements these were replaced by a CS 4-77 and an AL 692 nm filter. The intensity of the actinic light was measured with a Yellow Springs radiometer (model 65).

Results

Chlorophyll fluorescence

The temperature dependence of the intensity of chlorophyll fluorescence of

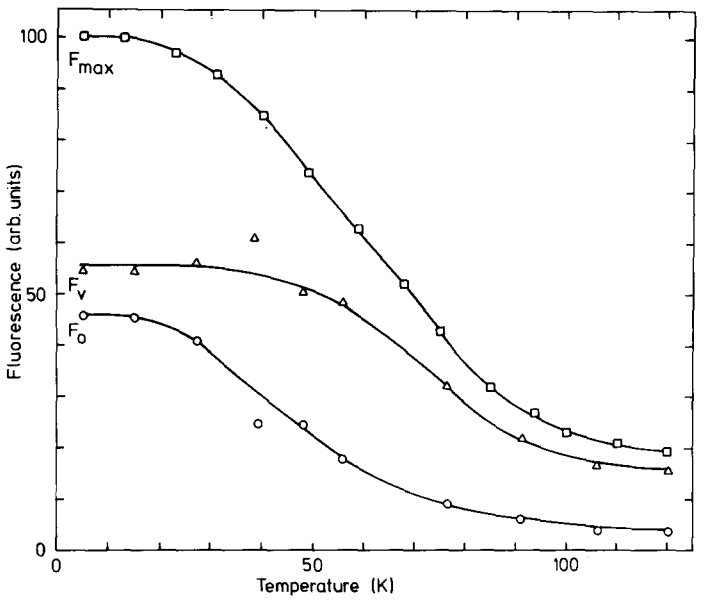

Fig. 1. Temperature dependence of the fluorescence yield of chloroplasts (1 mg chlorophyll/ml), measured at 692 nm upon continuous illumination (630 nm, 0.24 mW/cm^2). ○———○, initial fluorescence yield (F_0); △———△, variable fluorescence yield (F_v); □———□, maximal fluorescence yield (F_{max}).

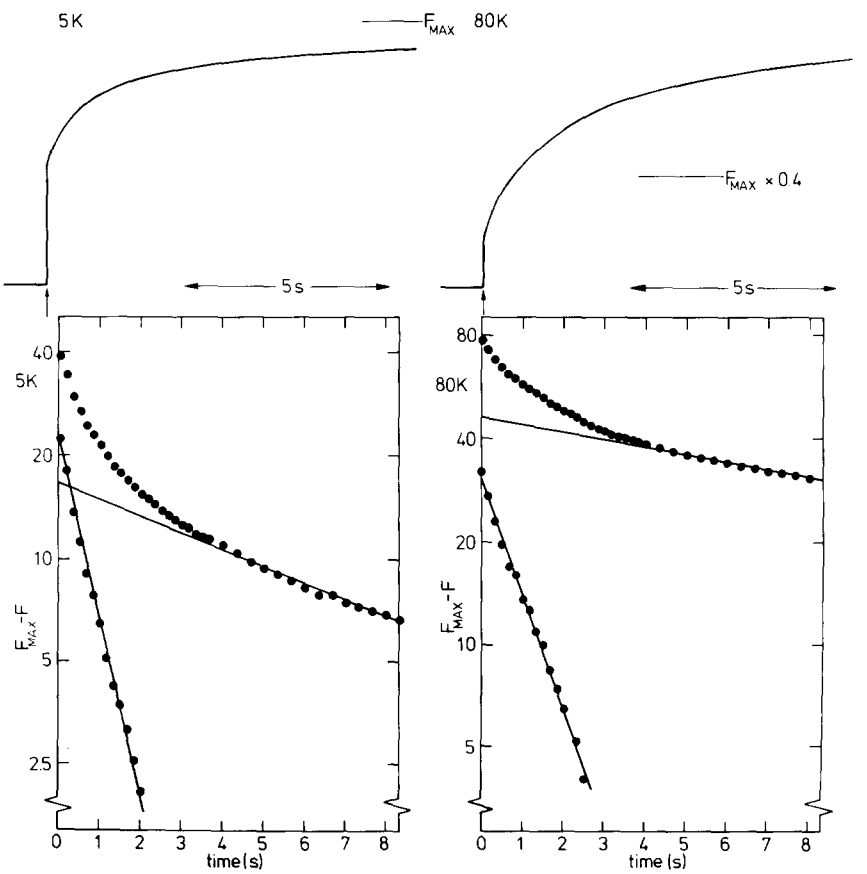

Fig. 2. Upper part: time course of the fluorescence yield at 5 and 80 K. Actinic light on at upward pointing arrows. In the lower part of the figure the difference between F_{max} and the fluorescnece yield is plotted on a logarithmic scale. Conditions as for Fig. 1, except chlorophyll concentration: 0.1 mg/ml.

chloroplasts is shown in Fig. 1. Fluorescence was measured at 692 nm. The curve marked F_0 shows the temperature dependence of the initial fluorescence yield, measured upon onset of illumination, F_v denotes the increase in yield observed during continuous illumination of sufficiently long duration (the so-called variable fluorescence) and the curve marked F_{max} shows the maximum yield obtained in the light. Upon lowering the temperature from 100 to 20 K, F_{max} increased by a factor of 3—4; below 20 K it became approximately constant. A similar increase of F_{max} was observed earlier by Cho and Govindjee [8] and by Drissler et al. [9] with intact cells of *Chlorella pyrenoidosa*. The fluorescence increase induced by cooling was reversible: if the temperature was increased again to 100 K, F_{max} decreased again to its former value. Within experimental error the same temperature dependence of F_{max} was observed when the sample was either illuminated for the first time at 80 K and then cooled to 5 K or first illuminated at 5 K and then warmed to 80 K. The increase of F_{max} is mainly due to an increase in F_0; the ratio F_v/F_0 was 3.5 at 90 K and 1.2 at 5 K.

Fig. 2 compares the kinetics of the fluorescence at 80 and 5 K. In order to ensure uniform illumination, independent of the depth of penetration of the light, a dilute sample was used for these measurements. In addition to a larger F_0, the recording at 5 K also shows a shorter apparent half time for the fluorescence increase F_v than that at 80 K. Measurement of the absorption spectrum showed that the latter effect could not be explained by an increase in the amount of absorbed light. Semilogarithmic plots of the fluorescence as a function of time of illumination indicated the existence of two different exponential phases in the fluorescence increase. At 5 K, the half times differed by a factor of about 8. The slowest component appeared to be slower at 80 than at 5 K.

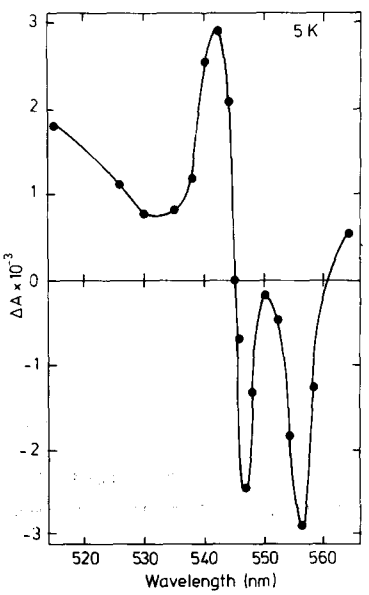

Fig. 3. Spectrum of the light-induced absorbance changes at 5 K. Conditions as for Fig. 1, except actinic illumination: 0.70 mW/cm^2.

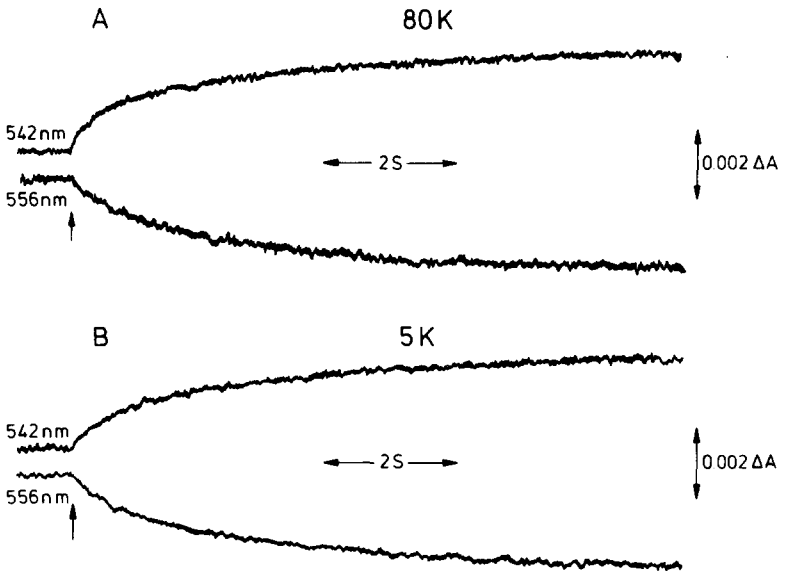

Fig. 4. Time course of the abosrbance changes at 542 and 556 nm at 80 K (A) and 5 K (B). Conditions as for Fig. 1, except actinic illumination: 0.70 mW/cm^2.

Absorbance changes of C-550 and cytochrome b-559

Fig. 3 shows the light-induced absorbance difference spectrum obtained at 5 K. The spectrum is similar to those earlier obtained at higher temperatures [2,3,10]. At 556 nm cytochrome *b*-559 oxidation was observed and the maxima and minima at about 542 and 547 nm, respectively, are due to the band shift caused by the conversion of C-550 [1]. The maximum amplitude of the absorbance changes was approximately the same at 5 K and 90 K. However, the kinetics of the absorbance changes were clearly different (Fig. 4). Whereas at 90 K the rate of reaction of C-550 measured by the apparent half time upon illumination, was significantly faster (up to a factor of two) than that of cytochrome *b*-559 [1,2], the rates were much less different, and often even about equal at 5 K. This effect was mainly due to an increase in the apparent half time of C-550 upon cooling. The same was observed at 515 nm. Table I summarizes measurements done with different samples.

In order to obtain a sufficient sensitivity of measurement, it was necessary to use a ten times higher concentration for the absorbance than for the fluorescence measurements. Use of a dilute suspension and averaging was not possible because the absorbance changes were almost irreversible. Thus an analysis in exponential components was not possible, because the intensity was different for different layers of the sample.

The decrease of the overall rate of the C-550 conversion upon cooling to 5 K was also clearly demonstrated in an experiment where the absorbance change caused by a flash was compared with the total change due to continuous actinic illumination. At 90 K the increase due to the flash was 25—30% of the total increase for C-550, whereas it was 10—15% for cytochrome *b*-559, in agreement with earlier reports [3,12]. However, at 5 K, the flash-induced increases were about 15% of the total change for both components.

TABLE I

Rates of absorbance and fluorescence changes in chloroplasts (1 mg chlorophyll/ml) at 5 K upon continuous illumination (630 nm; 0.70 mW/cm^2).

Sample No.	Apparent half time in s at			
	542 nm (*C*-550)	556 nm (cytochrome *b*-559)	515 nm	692 nm (fluoresence)
1	1.5	1.5		0.7
2	0.8	1.2	1.0	0.6
3	1.1	1.0	1.0	
4	1.1	1.3		
5	1.0	1.0		0.7
6	1.0	1.4		0.9

Effects of ferricyanide

Potassium ferricyanide, when added to a final concentration of 1 mM before cooling, decreased the extent of the variable fluorescence (F_v) at 5 K by a factor of about two (Fig. 5). The same effect has been reported earlier for temperatures around 80 K [6]. The different components in the kinetics of the fluorescence increase at 5 K were reduced in approximately the same proportions. Fig. 5 also shows that ferricyanide (applied at a concentration of 10 mM to a more dense suspension) also abolished cytochrome *b*-559 photooxidation at 5 K. The amplitude of the absorbance change of C-550 was not affected, but the overall rate of C-550 conversion decreased strongly, corresponding to a marked increase of the apparent half time, both at 5 K (Fig. 5) and at 80 K (not shown).

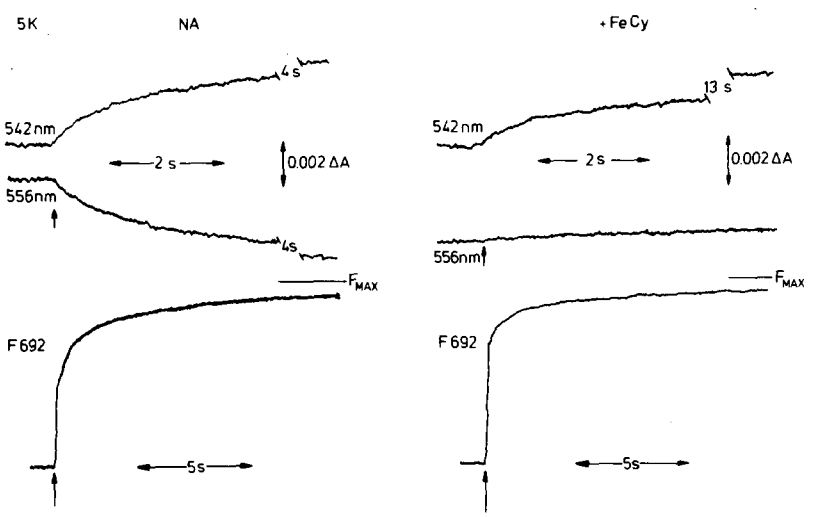

Fig. 5. Time course of the absorbance changes at 542 and 556 nm and of the fluorescence yield at 5 K without (NA) and with ferricyanide. Note the different time scales for the fluorescence and absorbance measurements. For the absorbance changes a sample containing 1 mg chlorophyll/ml and a ferricyanide concentration of 10 mM was used. Both concentrations were 10 times lower for the fluorescence measurements. The fluorescence recordings with ferricyanide were obtained at a 1.35 times higher amplification. Actinic illumination: 1.0 mW/cm^2 for the absorbance measurements and 0.24 mW/cm^2 for the fluorescence measurements.

Discussion

The results reported here suggest that the properties of the reaction center of system 2 of photosynthesis are basically similar at 5 and at 80 K. At both temperatures, illumination causes a band shift of C-550, a photooxidation of cytochrome *b*-559, and an increase in the yield of chlorophyll fluorescence. The amplitudes of the absorbance changes were approximately the same at both temperatures. These results indicate that at 5 K the electron acceptor is the same (i.e. plastoquinone, refs. 13 and 14) and that cytochrome *b*-559 is oxidized in the same number of reaction centers as at 80 K.

Nevertheless, there are also clear differences. In agreement with earlier measurements on *Chlorella* [8,9] we observed that the fluorescence yield increased considerably upon lowering the temperature. This increase, which at 5 K amounted to a factor of about 3, compared to that at 80 K, is mainly due to a strong increase of the initial fluorescence level (F_0). This may be due to a lower efficiency of photochemical conversion at the reaction center, as suggested by the extent of C-550 conversion in a flash. In addition there may be a decreased efficiency of energy transfer between chlorophyll molecules. According to the simplest models, the increase of F_{max} upon lowering the temperature is not explained by these mechanisms. This indicates that other effects should be considered in addition, like energy transfer to system 1, fluorescence by a pool of chlorophyll not connected to the photochemical apparatus and a change in the rates of radiationless decay. That these effects are probably important is indicated by the observation that the F_0 level at 5 K even exceeds that of F_{max} at 120 K.

Analysis of the kinetics indicates that there are two first-order components in the rise curves of the fluorescence yield upon illumination. These were also recently observed by Bonnet et al. [15] at 77 K with *Chlorella*, and suggest that energy transfer between reaction centers [16] is negligible at 77 K and below (see also ref. 3). At the intensity used for Fig. 2 components with half times of 0.8 and 6.5 s could be discerned at 5 K. As discussed in the previous section, a similar analysis could not be applied to the absorbance measurements. Nevertheless, it is possible that the same components are also present in the absorbance changes. If so, the half times for fluorescence, C-550 and cytochrome *b*-559 indicate that the relative amplitudes are different.

Results obtained in the temperature region of 77—120 K have been explained by a model for charge stabilization involving a primary charge separation and a relatively slow donor reaction:

$$\mathrm{D}\,P\text{-}680\,\mathrm{Q} \underset{k_{-1}}{\overset{h\nu}{\rightleftharpoons}} \mathrm{D}\,P\text{-}680^{+}\,\mathrm{Q}^{-} \xrightarrow{k_2} \mathrm{D}^{+}\,P\text{-}680\,\mathrm{Q}^{-},$$

k_2 being several times smaller than k_{-1} [1]. A more refined model was proposed more recently [4]. Measurements of the ESR singal centered at $g = 2.002$ indicate that k_{-1}, the rate constant for the back reaction, is the same for all reaction centers and independent of temperature [3,5]. This indicates that the heterogeneity reflected by the kinetics of fluorescence and absorbance changes in continuous light is due to different rates of secondary reactions for differ-

ent reaction centers. In addition, our results indicate that at 5 K, like at higher temperatures [3], cytochrome *b*-559 is the ultimate electron donor in only half of the reaction centers, and that an unknown donor becomes oxidized in the other reaction centers. Thus at least four types of reaction centers may exist at 5 K with different electron donors and different rates of secondary electron transport. The faster rise kinetics of fluorescence than of the absorbance changes indicate that these reaction centers have different quenching properties. It is not known if the heterogeneity of reaction centers is an artifact induced by the preparation and cooling method of the chloroplasts or if it reflects a basic property of the system 2 reaction center that also exists under physiological conditions.

The results obtained with ferricyanide indicate that, as was earlier concluded from measurements at higher temperatures [6,17], cytochrome *b*-559 is oxidized by this substance in a dark reaction and that another donor is photooxidized instead of cytochrome *b*-559. The decrease in the amplitude of F_v may be explained in a similar way as was done by Okayama and Butler [6] by a quenching of chlorophyll fluorescence by an oxidized donor. The strong increase in the apparent half time of C-550 has not been reported so far; Visser [3] observed an increase of the apparent half time by only about 20% at 90 K. The reason for this discrepancy is not clear. The effect may be explained by a lower rate of oxidation of alternative donor(s) operating in the presence of ferricyanide. Quenching of chlorophyll *a* fluorescence by these donors in the oxidized state then may explain the absence of a marked effect of ferricyanide upon the relative proportion of the slow component in the fluorescence rise.

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