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OBSERVATION OF TWO QUENCHERS OF CHLOROPHYLL FLUORESCENCE IN CHLOROPLASTS AT −196°C

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Summary

Fluorescence induction at −196°C has been monitored in chloroplasts rapidly frozen after poising at different redox potentials at room temperature. It was found that, as at room temperature, the initial level of fluorescence observed upon shutter opening (F_o), relative to the final level observed after 10 seconds of illumination (F_m) increased as the redox potential of the chloroplasts was lowered. Redox titration revealed the presence of two quenching components with $E_{m,7.8}$ at −70 mV and −275 mV accounting for approx. 75% and 25% of the variable fluorescence (F_v). Parallel observation of fluorescence yield at room temperature similarly gave two components, with $E_{m,7.8}$ at −95 mV and −290 mV, also accounting for approx. 75% and 25%. Simultaneous measurement of fluorescence emission at −196°C at 695 nm and 735 nm indicated that both emissions are quenched by the same redox components.

Introduction

The fluorescence yield of normally active chloroplasts at room temperature is determined by the redox state of the primary electron acceptor of Photosystem II, which was therefore termed Q, the quencher [1]. It is now well established that Q consists of more than one redox component [2—7]. Two quenchers with $E_{m,7}$ around −250 mV and 0 mV, termed Q_L and Q_H [4] or Q_1 and Q_2 [5] respectively, have been shown to be photoreducible and there-

Abbreviations: E_m, midpoint oxidaton reduction potential; Q, the fluorescence quencher of Photosystem II; PS I, Photosystem I; PS II, Photosystem II; F_o, initial fluorescence level; F_m, final fluorescence level; F_v, variable fluorescence, $F_m - F_o$.

fore to have some role in photosynthesis [4—7]. All experiments demonstrating more than one Q component have been performed using assays of room temperature photoactivity. At −196°C, determinations of the E_m of the primary acceptor of Photosystem II by measurement of photo-oxidation of cytochrome *b*-559 [8], photoreduction of *C*-550 (9) or light induced variable fluorescence [9] have shown only a single redox transition. This apparent difference in behaviour of Photosystem II at room temperature and −196°C prompted discussion as to whether only one of the two Q components functions at low temperature [4] and is of obvious relevance to the validity of various models for primary photochemistry in Photosystem II based on fluorescence measurements at this temperature [10]. It therefore seemed that a further investigation of the effect of redox potential on fluorescence emission at −196°C was warranted. In particular, because of the known difficulties in obtaining adequate redox equilibration of the inaccessible Q molecule (see a discussion in Ref. 4) it seemed important to establish that the samples taken for assay at −196°C were in equilibrium at room temperature at the time of sampling. In this paper data from parallel analyses of fluorescence induction at −196°C and of the room temperature F_o level recorded under continuous low intensity light [4] are compared.

Materials and Methods

Growth of peas and isolation of chloroplasts was as described previously [11]. Redox titration of the yield of chlorophyll fluorescence at 21°C was performed according to the procedure of Horton and Croze [4] except that the mediators present were 1,4-naphthoquinone, indigotetrasulfonate, 2-hydroxy-1,4-naphthoquinone and anthraquinone 2-sulfonate. In addition, the reaction medium contained 0.1 M sucrose, 3 mM $MgCl_2$, 10 mM NaCl, 10 mM Tricine (pH 7.8) and chloroplasts at 20 μg/ml chlorophyll. Emission at 685 nm was measured continuously using a low intensity measuring beam [4]. After both the redox potential and fluorescence intensity had stabilised (2—10 min), 0.2-ml aliquots were rapidly transferred by syringe from the redox cuvette to the pre-cooled sample chamber of the −196°C apparatus. The chamber had, in addition, been flushed with O_2-free argon for 2—3 minutes. Freezing took place within 5 s and equilibration to −196°C in about 1.5 min. Kinetics of fluorescence emission at 695 and 735 nm were measured simultaneously for samples frozen to −196°C using a trifurcated fibre optic apparatus [12]. Excitation radiation of 632.8 nm was produced by a 10 mW helium-neon laser and fluorescence emissions at 695 and 735 nm were monitored through a Balzers 695 nm interference filter and an Oriel monochromator with a 5 nm band width, respectively. Fluorescence induction was initiated by the opening of a Uniblitz electronic shutter (0.6 ms opening time) and recorded on a Datalab 905 Transient Recorder operating in a dual time base mode such that measurement of both F_o and F_m could be made from a single trace. When 695 nm and 735 nm emission were to be simultaneously measured the data instead was recorded on two channels of a Datalab 4000B Signal Averager.

Results

Induction curves for 695 nm fluorescence recorded at −196°C, with the redox potential of the thylakoids poised at +120 mV, −205 mV and −395 mV at room temperature, are shown in Fig. 1. Since the maximal level of fluorescence, F_m, of similarly treated samples was not constant due to unavoidable variations in the pattern of freezing producing differences in the optical properties of the samples, it was impossible to use the absolute variable fluorescence, F_v, as an indicator of the total amount of oxidised Q in the sample before irradiation. This problem could be overcome by using the ratio of two fluorescence parameters of a given sample [12]; F_o/F_m proved a particularly useful ratio in this respect. At +120 mV, Q was initially completely oxidised and F_o/F_m was at its minimal value; values of F_o/F_m ranged from 0.35 to 0.50 depending on the age of the chloroplasts at the time of cooling to −196°C. Stabilisation of PS II primary photochemical capacity could be achieved by addition of either 10 mM $MgCl_2$ or 100 mM NaCl to the resuspension medium [12]. When chloroplasts were reduced to −395 mV, almost all the F_v was eliminated and an F_o/F_m of between 0.98 and 1.00 was obtained. At an intermediate potential of −205 mV only about 70% of F_v was eliminated. A redox titration curve was constructed by taking the ratio of F_o/F_m at each redox potential and normalising to the value obtained for fully oxidised Q; thus a normalised F_o/F_m value of 100% indicates a total initial reduction of Q, whilst 0% represents a fully oxidised Q pool. Fig. 2 shows data points obtained from traces similar to those in Fig. 1; points from titrations performed both oxidatively and reductively are shown. Despite the spread of data, it is clear that a single component Nernst equation would not provide an adequate fit. Instead, a plateau region between −140 mV and −220 mV is discernable, separating two components with $E_{m,7.8}$ around −250 to −300 mV and −50 to −100 mV. In Fig. 2, Nernst plots with $E_{m,7.8}$ at −275 mV and −70 mV are shown, accounting for 26% and 74% of the fluorescence, respectively. There is approximately an error of ±20 mV on these values and ±5% on the ratio of the two quenchers. It is seen that both oxidative and reductive titrations fit the same curves.

Titration of the room temperature fluorescence emission was performed

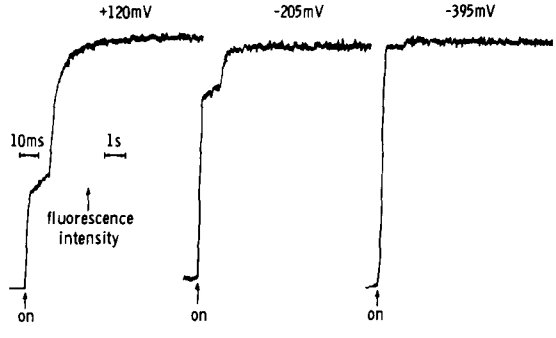

Fig. 1. Fluorescence induction at −196°C. Samples poised at the potentials indicated at 21°C were frozen and their induction curves were recorded as described in the text. Emission wavelength 695 nm. Two times bases were used as shown.

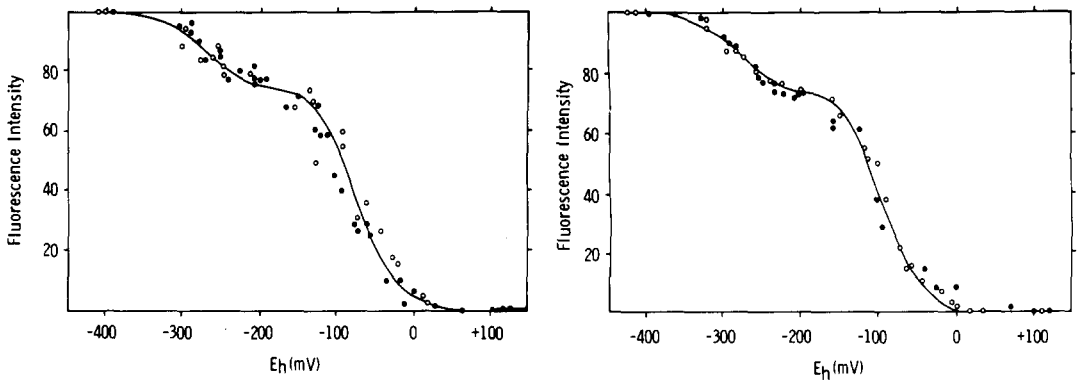

Fig. 2. Redox titration of the initial fluorescence level at −196°C. Induction curves at different redox potentials were recorded as in Fig. 1. Data from seven different oxidative (○) and reductive (●) titrations are shown. Each point was calculated first as a ratio F_o/F_m which was then normalised to the minimum value for that particular titration, so that a range of 'fluorescence intensity' of 0—100% was derived. The solid line shows a Nernst plot with $E_{m,7.8}$ = −70 mV and −275 mV accounting for 74% and 26%, respectively.

Fig. 3. Redox titration of the fluorescence intensity at 21°C. Data is derived from 3 separate oxidative (○) and reductive (●) titrations, recorded using the same samples used to provide the −196°C data. Each point represents the amplitude of fluorescence change above the fully oxidised F_o level normalised such that $F_m - F_o$ is 100%. The solid line shows a Nernst plot with $E_{m,7.8}$ at −95 mV and −290 mV accounting for 74 and 26%, respectively.

simultaneously to the low temperature experiment, by continuously monitoring the yield under low intensity illumination. This ensured an equilibrium condition at the time of sampling for −196°C analysis. Data from the room temperature titrations are shown in Fig. 3. Two quenchers with $E_{m,7.8}$ at −290 mV and −95 mV representing 26% and 74% of the fluorescence were present. This result closely resembles previously published data [4] and clearly shows the presence of Q_L (−290 mV) and Q_H (−95 mV).

It is well established that F_v at 735 nm and −196°C is directly proportional to F_v at 695 nm [10]; however it is not known whether both Q_H and Q_L are involved in determining F_v at 735 nm.

Measurement of fluorescence emission at 735 nm and 692 nm simultaneously at −196°C indicated that photochemical quenching of 735 nm emission was also controlled by the redox state of Q_L and Q_H. In order to compare the effect of closing PS II traps on F_v at 735 the compound ratio $(F_v/F_m)735/(F_v/F_m)695$ is plotted as a function of redox potential in Fig. 4. Between +100 mV and −300 mV, this ratio shows no significant deviation from an average value of 0.35. If the 735 nm variable emission was not a result of closing of PS II traps, significant deviation from this value would be expected; very high values would be expected if, for instance, 735 nm emission was controlled by closing of PS I traps at more negative redox potential than Q_L or Q_H. Similarly high values would be obtained if only Q_L were controlling 735 nm emission and conversely, values approaching zero would be obtained if Q_H and not Q_L were involved.

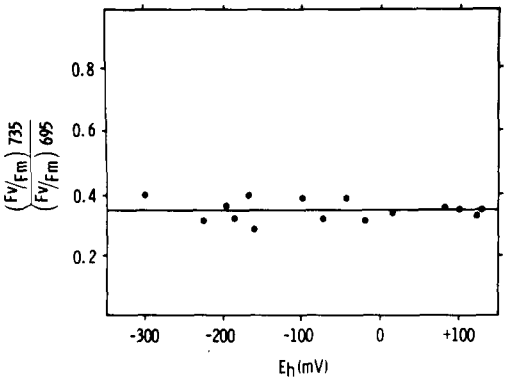

Fig. 4. A comparison of variable fluorescence emission at 735 nm and 695 nm at different redox potentials. Induction curves were recorded simultaneously at 695 and 735 nm as in Fig. 1 and the compound ratio $(F_v/F_m)735/(F_v/F_m)695$ calculated. Data from a mixture of 5 oxidative and reductive titrations are shown. The solid line represents the average value of 0.35 for the ratio.

Discussion

It has been shown in various laboratories that more than one electron acceptor associated with PS II has the property of quenching fluorescence at room temperature when in its oxidised state [2,13,14]. This heterogeneity of Q is reflected in the observation of two components in the redox titration of the fluorescence yield at room temperature [2—7]. At room temperature it has also been shown that charge separation associated with PS II titrates according to the presence of two primary acceptors [15]. Other assays of the E_m of the primary acceptor of PS II have been performed at —196°C and, in these cases, however, no evidence for more than one redox transition was shown [8,9]. In this paper, it has been demonstrated that, if care is taken to ensure proper redox equilibration by taking samples from a room temprature experiment known to be in a stable situation, two quenchers of fluorescence at —196°C can be observed. These quenchers have similar E_m values and are present in the same proportions as at room temperature. Failure to achieve adequate redox equilibration could lead to a disappearance of the plateau between Q_L and Q_H and a merger of the two components. This would be reflected in hysteresis when comparing oxidative and reductive titrations, a phenomenon reported by Erixon and Butler [9] in their titrations of *C*-550 and Q at —196°C. Another experimental problem is reflected in the greater degree of varibility seen in the data at —196°C, compared to 21°C (compare Figs. 2 and 3). This may be due, to some extent, to slight alterations in redox potential between the time of sampling and freezing. A more important source of error may be the apparent instability of photochemical activity when measured by —196°C light-induced assays. For example, chloroplasts 4 h after isolation still gave the same F_v/F_m at room temperature (measured as in Fig. 3) but a 25% decrease in F_v/F_m at —196°C (measured as in Fig. 1). We have, in fact, occasionally observed (2 experiments out of 30) the absence of appreciable quantities of Q_L at —196°C with normal amounts seen at 21°C. Changes in F_v/F_m for 695 nm emission at —196°C during ageing of chloroplasts have been attributed to decreases in the

rate constant for PS II primary photochemistry which result from alterations in the spatial relationships between the primary electron donors, *P*-680 and the two Qs [12].

However, the experiments of Knaff on photo-oxidation of cytochrome *b*-559 [8], in which a reversible, one component titration with good fit of data to the Nernst plot was measured, cannot be readily explained by these kinds of problems. It is possible, as discussed previously [4] that cytochrome *b*-559 may preferentially donate to Q_H. An analysis of the multiphasic induction kinetics at −196°C in the redox potential range of both donor and acceptor would provide an answer to this question.

In conclusion, it has been shown that Q_L and Q_H function as 'primary' electron acceptors at −196°C as at 21°C. This does not go any further towards answering the crucial question as to the nature or the function of the Q heterogeneity. However, the absence of any preferential control by Q_L of variable emission at 735 nm which is thought to be due to energy transfer from PS II to PS I [10] tends to argue against a confinement of Q_H to stromal membranes and Q_L to granal membranes, as recently speculated [4,16].

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