UV-spectral characterization in Tris-washed chloroplasts of the redox component D_1 which functionally connects the reaction center with the water-oxidizing enzyme system Y in photosynthesis

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Amplitude and relaxation kinetics of UV-absorption changes induced in Tris-washed chloroplasts by the second flash of repetitive double flash groups were found to be dependent on the time between the flashes of each group. An analysis of these data leads to the conclusion that after oxidation by $P680^+$ the donor component, D_1^{ox}, becomes mainly reduced by the primary (Q_A^-) and secondary (Q_B^-) plastoquinone in non-B-type and B-type system II centers, respectively. D_1^{ox} reduction with Q_A^- occurs with $t_{1/} = 60–100$ ms, and D_1^{ox} reduction with Q_B^- is biphasic with half-times of 0.1–0.5 and 5–10 s. The difference spectrum of the oxidized *vs* reduced form of D_1 is presented in the range 250–370 nm. It is characterized by positive bands peaking at 265–270 and 300–305 nm with a smaller band around 350 nm; negative bands are not observed.

Photosystem II *Tris-washing* *Redox equivalent recombination* *Donor component D_1* *UV-difference spectrum D_1^{ox}/D_1*

1. INTRODUCTION

Photosynthetic water oxidation to molecular oxygen is realized by cooperation within the water-oxidizing enzyme system Y of 4 sufficiently oxidizing redox equivalents that are produced by photooxidation of a special chlorophyll *a*, P680, within the system II reaction center complex. P680 and system Y are functionally coupled via a redox component referred to as D_1 (or Z) (review [1]). D_1 has been characterized by its EPR spectrum [2], but its chemical nature still remains to be clarified. The reaction pattern of the system II donor side can be significantly modified by procedures (e.g., Tris-washing) that cause selective destruction of system Y without preventing D_1 oxidation by $P680^+$, but markedly retarding its electron transfer kinetics [3]. D_1 was found to act as a one-electron redox component [3] so that under repetitive flash excitation its donor capacity becomes readily exhausted if system Y does not function. Under these conditions $P680^+$ becomes reduced via a back reaction with the reduced primary plastoquinone acceptor, Q_A^-, characterized by a half-time of 100–200 μs [3,4]. In the dark, D_1 restoration is achieved by comparatively slow reactions with as yet not unambiguously identified electron sources.

This study is an attempt: (i) to clarify the donor components for D_1^{ox} reduction in Tris-washed chloroplasts, and (ii) to measure the difference spectrum of D_1^{ox} *vs* D_1 in the UV range.

Based upon recent findings about the existence of two types of reaction centers [5,6], our results lead to the conclusion that D_1^{ox} becomes reduced by Q_A^- in non-B-type centers and predominantly by Q_B^- in B-type centers. A difference spectrum infer-

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red to reflect the couple D_I^{ox} *vs* D_1 is presented in the range of 250–370 nm.

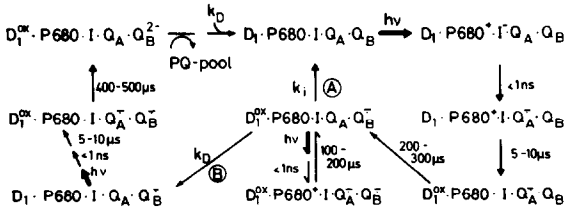
2. MATERIALS AND METHODS

Class II spinach chloroplasts were prepared from market spinach as in [7], except that 10 mM ascorbate was present in the grinding medium. Tris-washed chloroplasts were obtained from isolated chloroplasts by incubation with 0.8 M Tris–HCl (pH 8.0) by the procedure in [8]. The standard reaction mixture contained chloroplasts (10 μM chlorophyll), 10 mM KCl, 2 mM $MgCl_2$, 20 mM Tricine–NaOH (pH 7.5). Absorption changes were recorded by a repetitive flash photometer [9] equipped with a pulsed measuring light beam as described in [10]. Other experimental details were as in [10], except that the sample was not renewed during the course of one experiment.

3. RESULTS AND DISCUSSION

3.1. Reduction of donor D_I^{ox}

If, for the sake of simplicity, the functional groups of system II in Tris-washed chloroplasts are summarized by the complex $D_1 \cdot P680 \cdot I \cdot Q_A \cdot Q_B$, the following reaction pattern has to be considered for double flash group excitation (protonation/deprotonation reactions have been omitted):



D_I^{ox} could be reduced either directly by Q_B^- (pathway A) or by an alternative donor (pathway B). For the moment, the possibility of a reaction between D_I^{ox} and Q_A^- will be postponed (vide infra). The reductive formation of both plastoquinone anion radicals, Q_A^- and Q_B^-, is characterized by an increase of absorption, peaking around 320 nm [11]. Accordingly, two different patterns of 320 nm absorption changes are expected to arise for pathway A and B, respectively if Tris-washed chloroplasts are excited with repetitive double flash groups with the time between the flashes of each group being short, but the time between the repetitive flash groups being long compared to D_I^{ox} reduction (extensive fluorescence studies revealed that the reoxidation of Q_A^- by Q_B and Q_B^- occurs in the 150–300 μs time domain [12,13]). The expected patterns are schematically depicted in fig.1 (left). The case of Q_B^- reoxidation being faster than D_I^{ox} reduction, which gives rise to the same pattern as pathway A, has been tacitly omitted because Q_B^- was found to be rather stable in the absence of oxidants (see [6]). Fig.1 (right) shows typical traces of experimentally measured absorption changes. The data readily indicate that only pathway A is consistent with these findings. Furthermore, at constant dark time between the flash groups the relaxation kinetics of the absorption changes caused by the second flash of each group should be dependent on the time between the first and second flash of each group. If the degree of D_I^{ox} reduction after the first flash of each group increases, participation of the back reaction between $P680^+$ and Q_A^- should be diminished and, therefore, the amplitude of the slow relaxation kinetics in the second flash increases at the expense of the 100–200 μs kinetics (see reaction scheme). The data of fig.2 show that this really does occur. A more careful inspection

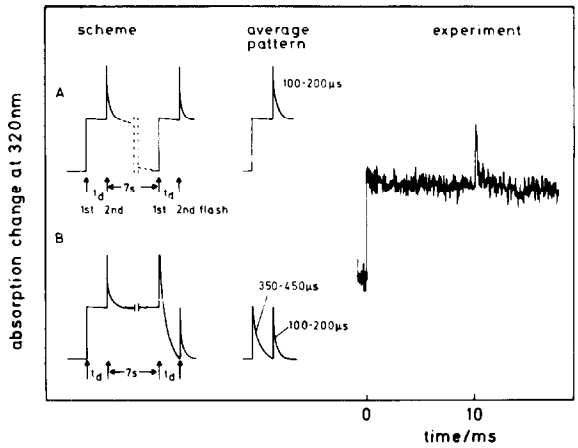


Fig.1. Absorption changes at 320 nm induced by repetitive double flash group excitation in Tris-washed chloroplasts. (Left) Schematic pattern of absorption changes according to pathways A and B of the reaction scheme (for details see text). (Right) Experimental data: 32 samples were averaged; time between flashes of a group, t_d = 10 ms; time between the flash groups, 7 s. Experimental conditions: see section 2.

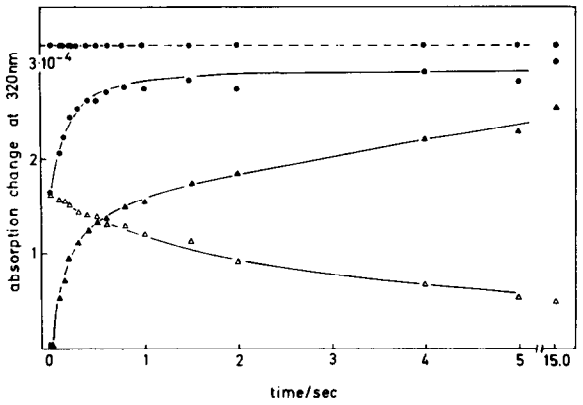


Fig.2. Initial amplitude of 320 nm absorption changes induced by the second flash of a group as a function of time between flashes. (●) Total extent, (△) component with 100–200 μs relaxation kinetics, (▲) component with 'slow' relaxation ($t_{1/2} \geqq 10$ ms); for comparison the extent of the first flash is depicted (●, – – –). Experimental conditions as described in section 2 except that the sample was renewed after each flash group.

reveals that the recovery of the slow kinetics does not exactly fit the time-dependent decline of the extent of the 100–200 μs component. The significantly faster recovery ($t_{1/2} = 60$–100 ms) of the slow kinetics, which amount to ~30–40% of the total extent, is not counterbalanced by a corresponding disappearance of the 100–200 μs kinetics. On the basis of a recently proposed hypothesis [5], this effect can be explained by assuming that 30–40% of the centers lack the component Q_B (non-B-type centers) so that Q_A^- serves as donor for D_1^{ox} reduction. This explanation seems reasonable in view of the findings in [6]. It is further supported by recent measurements indicating that in DCMU-blocked Tris-washed chloroplasts Q_A^- becomes reoxidized by D_1^{ox} via a 50–100 ms kinetics [14]. Analogous results have been obtained from hydroxylamine-treated chloroplasts in the presence of DCMU [15].

The biphasic decline of the extent of the 100–200 μs kinetics which, according to the reaction scheme (pathway A), reflects the reduction of D_1^{ox} by Q_B^- is characterized by half-times of 0.5–1 and 5–10 s. This result fits EPR measurements [6] quite well, which indicate a similar biphasic reduction of D_1^{ox}. Interestingly enough, in normal chloroplasts Q_B^- reduces the redox state S_2 in the water-oxidizing enzyme system Y [16]. If one assumes that in Tris-washed chloroplasts Q_B^- also reacts with D_1^{ox} via equilibration with Q_A [17,18], the biphasic D_1^{ox} reduction would suggest that some heterogeneity might exist for the equilibrium between $Q_AQ_B^-$ and $Q_A^-Q_B$ giving rise to constants of the order of 10 and 100, respectively. This estimation differs significantly from previous ones based on fluorescence measurements [11]. For the time being we cannot decide whether this discrepancy provides an argument against the involvement of the above-mentioned equilibration in D_1^{ox} reduction by Q_B^-.

3.2. *Difference spectrum of D_1^{ox}/D_1*

On the basis of the simplified scheme, we also tried to determine the difference spectrum of D_1^{ox} *vs* D_1. The amplitudes of excitation changes 3.5 ms after induction by the first flash were measured as a function of wavelength. The spectrum depicted in fig.3 (open symbols) is due to the superposition of the absorption differences of couples D_1^{ox}/D_1 and Q_A^-/Q_A (non-B-type centers) and Q_B^-/Q_B (B-type centers) plus possible contribution due to Photosystem (PS) I turnover. The PS I effect can be eliminated by subtracting absorption changes

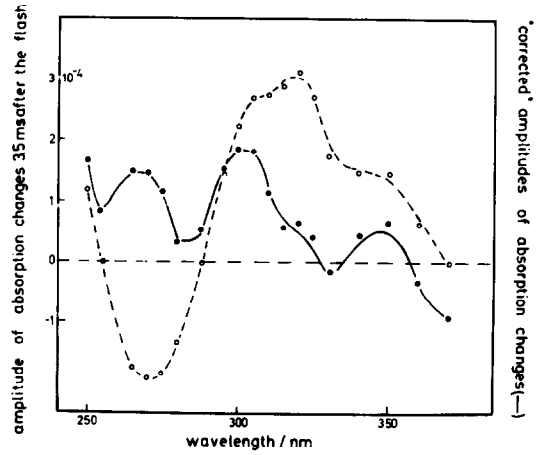


Fig.3. Amplitude of absorption changes measured 3.5 ms after excitation with the first flash of each group as a function of wavelength in Tris-washed chloroplasts. (○) Experimental data, (●) data after subtraction of the Q_B^-/Q_B difference spectrum (see [12]) and of the PS I contribution measured in the presence of 3 μM DCMU and 1 mM NH_2OH. Dark time between the repetitive flash groups: 8 s. Other experimental conditions as described in section 2.

obtained in the presence of DCMU and NH_2OH, a procedure which totally blocks PS II turnover [19]. After subtraction of the PS I contributions which were found to be rather small under our conditions (not shown) and the corresponding Q_A^-/Q_A (30%) and Q_B^-/Q_B (70%) difference spectra [11] on the basis of 500 chlorophylls per reaction center II, the data of fig.3 (full symbols) are obtained. (As the difference spectra for Q_A^-/Q_A and Q_B^-/Q_B are very similar [11], the percentage of B-type and non-B-type centers has only a marginal influence on the shape of the D_1^{ox}/D_1 difference spectrum.) This difference spectrum ascribed to D_1^{ox} *vs* D_1 exhibits positive peaks at 265–270 and 300–305 nm with a smaller band at 350 nm; bleaching bands are not observed.

The positions at 265–270 and 300–305 nm are in line with latest data obtained with special PS II preparations, but the absolute oscillator strength differs [20,21]. This might be mainly due to uncertainties in the contribution of the PS II acceptor side, a problem that also arises for the data reported in [20]. Regardless of these details, the spectral data confirm very well our previous conclusion that the absorption changes at 320 nm, oscillating synchronously with the oxygen yield and characterized by a 1 ms decay kinetics, predominantly reflect the oxidative turnover of the precursor state of photosynthetically evolved oxygen within system Y rather than the concomitant D_1^{ox} reduction [10]. Based upon EPR spectral analysis, D_1^{ox} was inferred to be a special plastoquinone, but the protonation state was not unambiguously clarified [22,23]. Recently, in Tris-washed inside-out thylakoids D_1 oxidation was found to be coupled with an H^+ release [24]. The results were confirmed by a different approach in Tris-washed thylakoids [25]. These findings might favor D_1^{ox} being deprotonated. This idea has been questioned on the basis of EPR data which were claimed to reflect the back reaction between Q_A^- and D_1^{ox} [26]. However, for two reasons it seems worthwhile to confirm this conclusion by independent lines of evidence:

(i) It has to be shown that the equilibrium between $P680^+D_1$ and $P680(D_1^{ox})^+$ is really pH-independent, because the functional groups of P680 and D_1 are embedded in protein matrices, which could be modified by pH, thereby giving rise to a relative redox potential shift.

(ii) The variations at the same pH of the half-lifetimes by a factor of up to 5 reported for the reaction between D_1^{ox} and Q_A^- under different conditions (see [14,15,20,26]) suggest that the system is more complex than assumed for the analysis of the data in [26].

Regardless of these problems, the substantiation of D_1 with a special plastoquinol poses serious problems. In the case of D_1^{ox} being a specifically complexed neutral semiquinone, the oxidizing power of D_1^{ox} has to be enhanced significantly by a special mechanism (e.g., by the microenvironment of D_1 or by approprimate complexation, see [27,28]) to support water oxidation. On the other hand, a semiquinone cation radical could satisfy the redox conditions but the pK value is expected to be negative and, therefore, deprotonation has to be prevented by a special mechanism. Therefore, the substantiation of D_1 as a special plastoquinol implies, in any case, the existence of a very special protein matrix that determines the specific reactivities of the oxidized species. A further problem should also be taken into account. If the shape of the EPR signal is really unique for a hydroquinone cation radical [23] then signal II_s giving rise to the same EPR spectrum as signal II_f has to be necessarily assigned as being the same species. This would imply the existence of a long-lived, strongly oxidizing species within the membrane, which is slowly formed simply by a transition from pH 8.5 to pH 6.0 [29]. In the light of these data it seems premature to us to consider the identification of D_1 as a completely resolved problem.

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