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Nonphotosynthetic Reduction of the Intersystem Electron Transport Chain of Chloroplasts Following Heat Stress. The Pool Size of Stromal Reductants¶

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ABSTRACT

The properties of a negative transient signal (negative peak) observed during the first seconds of the induction of the photoacoustic (PA) signal in dark-adapted barley leaves treated with methyl viologen (MV) and diuron and then exposed to high temperatures have been examined. Under those conditions no electron donation from photosystem II (PSII) occurred, and electron flow through PSI could be supported only by soluble reductants located in the chloroplast stroma. The negative peak was observed only if the PA signal had been monitored at low, and not high, frequencies. The peak obviously originated from the oxygen consumption by PSI. The size of the peak increased as the temperature of preheating was raised from 39 to 45°C. The size of the peak decreased exponentially with a half-time of 3.7 s during illumination under low light. This decrease was found to be much faster under strong light. The recovery of the peak during dark acclimation required several minutes. It is concluded that the negative peak reflects the oxygen consumption supported by stromal reductants, their pool being rapidly exhausted under light in the presence of MV. The maximal size of the pool was calculated as 140 eq: P700 in dark-adapted leaves.

INTRODUCTION

Electron donation to photosystem I (PSI)† from reductants located in the chloroplast stroma constitutes a common phenomenon in green algae as well as in higher plants. As the input of electrons to PSI from a source alternative to PSII occurs continuously, some pool of electron equivalents must accumulate in the stroma. Hence, in the mesophyll chloroplasts of maize, a C_4 plant, the pool of stromal reductants that are capable of rapid electron donation to $P700^+$, the oxidized form of the primary electron donor of PSI, was found to be as high as 225 eq:P700 (1). In C3 plants the size of such a pool was much smaller but still exceeded 2–3 times the size of the usual pool of membrane-bound electron carriers between PSII and PSI (2).

The properties of the pool of stromal reductants capable of electron donation to PSI are not well known. The assessment of this pool becomes complicated also by the recycling of electrons around PSI (3). Hence, there are no data available in the literature on the kinetics of light–dark transformations of the pool and on the influence of environmental factors on its properties.

The rate of electron donation from stromal reductants to PSI is known to be enhanced by heat pretreatment of the intact leaves (4,5). The size of the pool of such reductants is expected to be enlarged compared with that of the nonstressed leaves as well. It must be pointed out, however, that the approaches commonly used to determine the pool of stromal reductants available for the reduction of $P700^+$ (1,2,6) cannot be applied in heat-stressed leaves as it requires the active PSII. The activity of PSII is known to be greatly weakened by heat stress (7). For this reason we made an attempt to use photoacoustic (PA) techniques (8,9) to characterize this in heat-stressed leaves. The rationale of the study was that, in the presence of effective electron acceptors of PSI, the pool has to be rapidly exhausted under sufficient light and then reaccumulated in the dark again.

In this report a strong negative transient signal (negative peak) observed during the first seconds of induction in the PA signal measured in heat-exposed leaves treated with methyl viologen (MV) will be documented to represent the yield of oxygen consumption by PSI supported by stromal reductants. The large area of this peak after dark adaptation and its light-induced decrease are related to the building up and the exhaustion of the pool of stromal reductants, respectively. Thus, it can be used to assess the pool size of those reductants. The maximal size of the pool was calculated as 140 eq:P700 in leaves exposed to 45°C.

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†*Abbreviations:* Chl, chlorophyll; MV, methyl viologen; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PA, photoacoustic; PSI, photosystem I.

MATERIALS AND METHODS

Primary leaves of barley (*Hordeum vulgare* L.) seedlings (7–9 days old) were used in the experiments. The plants were grown on vermiculite in a climatic chamber (Forma Scientific, Marietta, OH) at 20°C under continuous illumination with white fluorescence tubes. MV was introduced into leaves through the transpiration stream by placing the lower part of the leaf blades into a 1 m*M* MV solution, while transpiration was stimulated by a fan, and the leaves were irradiated with dim green light. Prior to the measurements, MV-treated leaves were additionally adapted to darkness for at least 20 min. Treatment of leaves with diuron was done by floating the segments of dark-adapted untreated or MV-treated leaves on 200 μ*M* diuron solution. The segments were kept with diuron in complete darkness for at least 1 h. Heat treatment was obtained by immersing the whole leaves or the leaf segments for 5 min into water kept at a given temperature.

PA measurements were made with a laboratory-built PA spectrometer (10). The light beam from a 150 W xenon lamp (ILC Technology, Sunnyvale, CA) passing through a monochromator (Photon Technology International Inc., Brunswick, NJ; model PTI 101-001SF) to obtain light with wavelength of 680 ± 8 nm was modulated with a mechanical chopper at frequencies of 35 or 200 Hz. Leaf cuttings placed into the closed PA cell (MTEC Photoacoustic, Ames, IA) were also illuminated by pulses of strong white light or strong far-red light provided by the KL 1500 projector (Walz, Effeltrich, Germany) using the system of fiber branches. Far-red light was obtained by passing the white light from the projector through an RG9 filter (Schott, Mainz, Germany). These pulses were used to close the PSII centers due to reduction of all the primary acceptors, Q_A, or to close the PSI centers due to P700 oxidation. The PA signal was preamplified and then analyzed by a lock-in amplifier (EG&G Princeton Applied Research, Princeton, NJ; model 5210). The output of the latter was connected to a computer for further treatment and printing.

Chlorophyll (Chl) fluorescence was measured (11) simultaneously with the PA signal using a PAM Chl fluorometer (Walz) the output of which was connected to a computer. Absorbance changes at 820 nm were measured using the special emitter–detector unit (ED-P700DW) of the PAM Chl fluorometer (12).

RESULTS

Figure 1 shows the kinetics of the PA signal measured in leaves treated with MV and diuron and then exposed to 45°C. If the measuring light was modulated at a frequency of 35 Hz, the initial increase in the PA signal, developing simultaneously with the onset of measuring light, was followed by a transient negative peak. Figure 1 demonstrates that this negative peak has not been detected at high frequencies of modulated light at which the PA signal is determined solely by the thermal component (13). Hence, the PA signal induced at 35 Hz in heat-stressed MV-treated leaves represents a superposition of two components, a thermal one, which dominates after irradiation periods longer than 30 s, and a negative photobaric component. As the negative peak has not been observed in heated leaves not treated with MV, it is likely caused by an MV-induced oxygen consumption that proceeded intensively during the first seconds of leaf irradiation. The negative peak was observed after the heat exposure of MV-treated leaves, irrespective of whether they had been treated with diuron or not.

As the negative signal demonstrated a transient pattern, we propose that it reflects the PSI-driven oxygen consumption supported by electron donation from a pool of electron equivalents accumulated within the chloroplast during the prolonged dark adaptation of the leaves. This pool was rapidly exhausted under light by PSI-driven electron transport to oxygen mediated by MV. In the trace obtained at 35 Hz

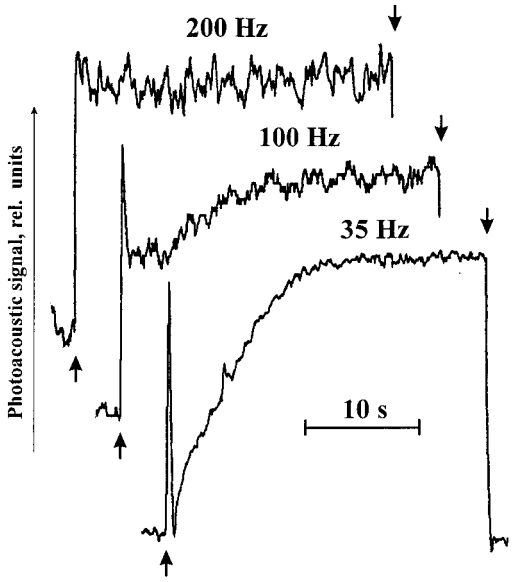

Figure 1. PA transients measured at the various indicated frequencies of modulated light in leaves treated with MV and diuron and exposed for 5 min to 45°C. Measurements were done with the same sample after a 5 min dark adaptation. Here and in the following figures the upward and downward arrows indicate modulated light (35 Hz, 680 nm, 61 μmol m^{-2} s^{-1}) turned on and off, respectively.

(Fig. 1) the recovery of the negative peak toward a steady-state PA signal level could be fitted (R^2 = 0.996) with a single exponential first-order kinetic equation:

$$\text{PA signal} = A[1 - \exp(-kt)] + c$$

where A = 1.038, k = 0.1895 and c = 0.02. The half-time of the exponential rise coinciding with the exhaustion of the pool of stromal reductants was calculated to 3.7 s.

The rate of the transient PA signal changes was dependent on the actinic light intensity. Indeed, Fig. 2 shows that the uphill phase of the negative peak was greatly accelerated by strong actinic light applied at the dip (compare traces 1 and 2). When strong light was turned on during the development of the downhill phase of the negative peak, it first accelerated the decrease of the PA signal (Fig. 2, trace 3). Significantly, strong white actinic light and far-red light had a similar effect on the negative peak, thus indicating that the above peak reflects the processes related to electron transport within PSI.

The negative PA peak signal observed after a prolonged dark period represents a reversible phenomenon. As shown in Fig. 3, after the first illumination it recovered progressively during a subsequent period of dark adaptation. The kinetic curves of Chl fluorescence measured simultaneously with the PA signals as shown in Fig. 3 additionally argue that the recovery of the negative PA signal is not related to the residual donation from PSII to PSI in diuron-treated leaves. On the first illumination Chl fluorescence rapidly reached a maximum level, demonstrating an F_v/F_m ratio of 0.71. On further irradiation only small F_V/F_m ratios of 0.04–0.05 were observed, while the area of the negative peak varied greatly.

Since the steady-state magnitude of the PA signals presented in Fig. 3 did not vary with the period of dark adap-

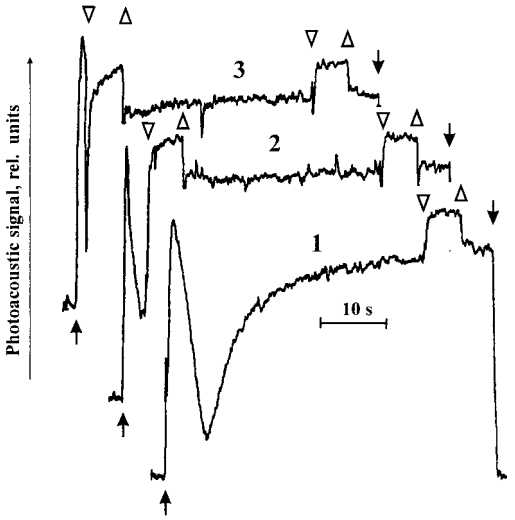

Figure 2. PA transients measured with 680 nm light (61 μmol m^{-2} s^{-1}, 35 Hz) in leaves treated with MV and diuron and exposed for 5 min to 45°C. Trace 1—a single 5 s saturating pulse was given 40 s after the onset of modulated light; traces 2 and 3—two 5 s saturating pulses were given, the first started at the dip of the negative PA signal (trace 2) or immediately after modulated light had been turned on (trace 3) and the second 40 s after the onset of modulated light. The downward and upward triangles indicate the 5 s white saturating pulses on and off, respectively. All the measurements were done with the same sample, and 5 min dark intervals were given between each irradiation.

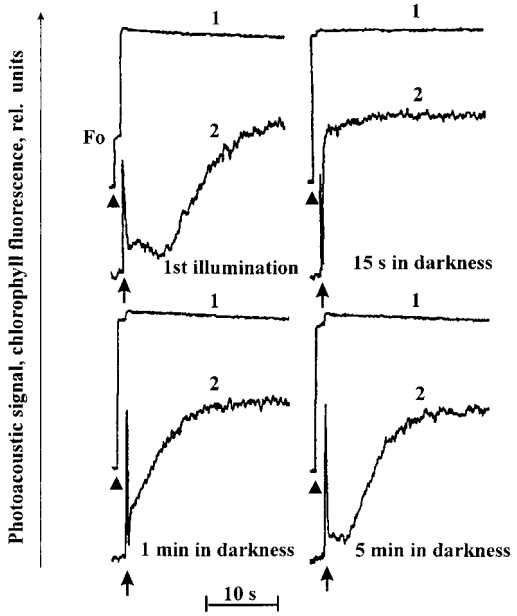

Figure 3. Chlorophyll fluorescence (curves 1) and PA (curves 2) transients measured in the same leaf treated with MV and diuron and exposed to 45°C after a dark adaptation period of 1 h (first illumination), 15 s, 1 min or 5 min following a previous 30 s irradiation with 680 nm light of 61 μmol m^{-2} s^{-1}. All the measurements were done with the same sample. The upward dark triangles indicate the onset of the very weak Chl fluorescence probe beam modulated at a frequency of 1.6 kHz. F_0 indicates the initial level of Chl fluorescence.

tation in a given sample, we have postulated that the area above the kinetic curve constitutes a quantitative measure of the negative peak, and relative values can be obtained by normalizing the corresponding areas to the one measured on the first irradiation that follows a prolonged dark adaptation (1 h). In order to analyze the kinetics of the dark restoration of the negative peak, we have determined those areas after various periods of darkness following a 30 s irradiation with light of 61 μmol m^{-2} s^{-1} using experiments similar to Fig. 3. The data obtained from four individual samples are presented in Fig. 4A. It is clear that 75–80% of the initial area of the negative peak was restored in 5 min after the light had been turned off. Restoration of the remainder required a longer dark-adaptation period. In Fig. 4B the kinetic curves are represented in semilog plots. Assuming, for each individual leaf, the value obtained for 10 min in the dark as the quasi-asymptotic one, we have deconvoluted the rapidly relaxing phase of the kinetics as a single exponential term. Individual samples differed slightly in the rate of dark restoration of the peak; the averaged value for the half-time of this process was found to be 66 s in the experiment presented in Fig. 4. Similar values fluctuating around 1 min were also obtained in other experiments.

As stated earlier, the negative peak appeared only after heat exposure of MV-treated leaves. In order to determine which temperatures are effective in this process, we have examined the relative areas of the negative peak after 5 min exposures of leaves treated with MV and diuron to various temperatures. As the steady-state magnitudes of the PA signal varied among individual samples, direct comparison of the areas above the kinetic curves did not provide adequate characteristics of the negative peak. Thus, we normalized those areas (area A in the inset of Fig. 5) to the total areas above and below the kinetic curves measured during a fixed period of 30 s (area A + area B in the inset of Fig. 5). Such calculations enabled the direct comparison of the signals obtained with different samples. Exposures of leaves to temperatures below 39°C did not produce a negative peak. Fig. 5 shows that the relative area of the negative peak increased with the following rise in temperature up to 45°C. At higher temperatures it decreased slightly.

As shown in Fig. 6 the additional treatment of MV- plus diuron-treated leaves with 50 μ*M* rotenone (A) changed the kinetics of the PA signals after exposure to 45°C compared with the leaves not treated with rotenone (B). Rotenone reduced the area of the initial negative PA peak which is most obvious after short dark-adaptation periods. It should be noted that the effect of rotenone was observed only after prolonged treatments, which may be related to the slow penetration of that substance into the chloroplasts of intact leaves (see also Field *et al.* [14]).

DISCUSSION

The above study of the negative PA peak observed in leaves treated with MV plus diuron and then exposed to heat demonstrates that the formation of this peak results from a pool of stromal reductants accumulated in the dark and capable of supporting the electron flow through PSI. In fact, the peak has not been observed in heat-exposed leaves treated with diuron in the absence of MV. Oxygen is known to be a weak electron acceptor (15). Thus, unlike in MV-treated leaves, a large light-induced consumption of the pool of native reduc-

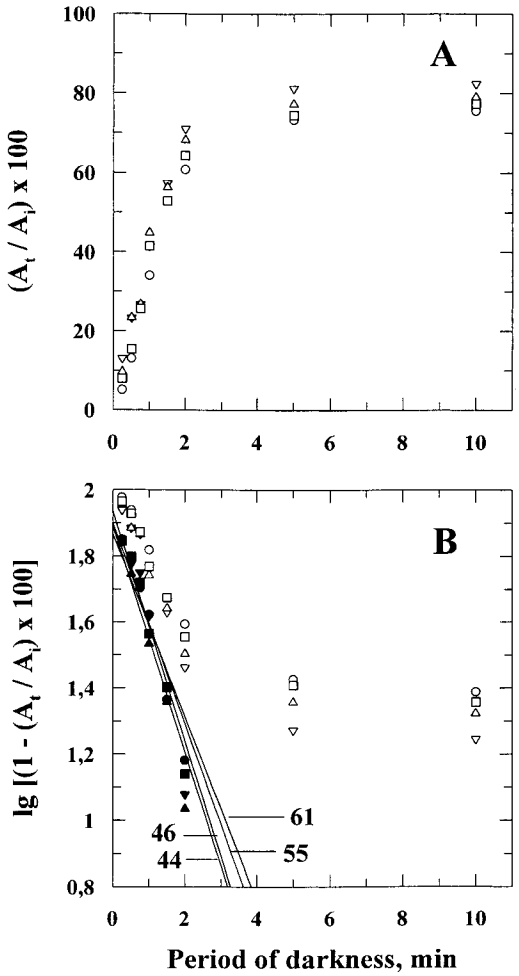

Figure 4. Kinetics curves of the dark restoration of the area above the negative PA peak following a 30 s irradiation of leaves treated with MV and diuron and exposed to 45° (A) and their semilog transformations (B). The areas (A_t) of the negative peak measured after various periods of darkness were normalized to the area (A_i) measured on the first irradiation of leaves adapted for at least 1 h to the dark. In (A) the different symbols represent the data for four individual samples (each included two segments from different leaves). In (B) the open symbols represent raw data, and the closed symbols represent the fast component of the dark restoration deconvoluted assuming, for each individual sample, the value obtained after 10 min of darkness as the offset. The numbers adjacent to the traces represent the half-times of the fast component of dark restoration (in seconds). The PA signals were measured with light of 61 μmol m^{-2} s^{-1} modulated at 35 Hz. It should be stressed that the order of the dark intervals used was randomized to avoid any systematical trend related to a possible decay of the negative signal with time after heat treatment.

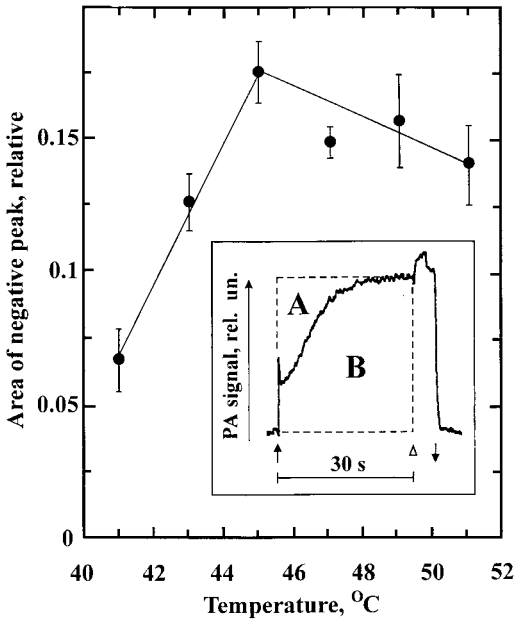

Figure 5. Relationship between the temperature of the 5 min heat treatment and the relative area of the negative peak in the PA signal measured at 35 Hz in leaves treated with MV and diuron. Inset: illustration of the normalization procedure; the relative area of the negative peak was obtained by normalizing the area above the kinetic curve measured during 30 s to the total area above and below the kinetic curve.

tants in the presence of oxygen as the sole electron acceptor is not expected. However, in the presence of effective electron acceptors the pool of stromal reductants disappeared within a few seconds of illumination (see Fig. 3, 35 Hz), while its dark recovery required several minutes (Fig. 4A). Increasing the temperature of preheating enlarged the size of the negative PA peak (Fig. 5), which coincides with the stimulating action of high temperatures on the rate of P700 rereduction (3) and on the rate of steady-state electron flow through PSI from stromal reductants.

We have found (16) that a steady-state light-induced electron flow through PSI enhanced by heat treatment can be supported by stromal reductants for at least several tens of minutes under conditions which prevent electron recycling owing to the presence of MV (15). Thus, the total number of electrons which can be delivered through PSI from the stromal components to the exogenous acceptors seems relatively large. As the steady-state rate of electron transport to PSI was estimated to be as high as 3.6 μmol m^{-2} s^{-1} in leaves exposed to 45°C (10), the total number of electrons delivered through PSI during the measurement of the PA signal exceeded 350 μeq m^{-2} or 900 electrons per a single PSI reaction center. Moreover, no symptoms of weakening were observed for that donation to the end of the experiment, which indicated an even larger capacity of the donor system. The above estimation agrees well with the data of Field *et al.* (14) who reported that the potential chlororespiratory electron donor pool size is 75-fold larger than the size of the plastoquinone pool. The pools of reductants potentially capable of donating electrons to PSI, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) (17,18), glycolate (19) and even ascorbate for which the intrachloroplast concentration was found to be several micromolars (20,21), all have much smaller sizes. Therefore, we must assume that the stromal reductants are regenerated continuously during light exposure, while their optimal accumulation in the stroma may be observed after long dark periods. The inhibitory action of rotenone (Fig. 6) indicates that the pool of reductants is regenerated *via* NAD(P)H dehydrogenase (14).

The exponential light-induced decay of the area of the negative peak (see Fig. 3, 35 Hz) allowed us to calculate the absolute size of the maximum pool of stromal reductants

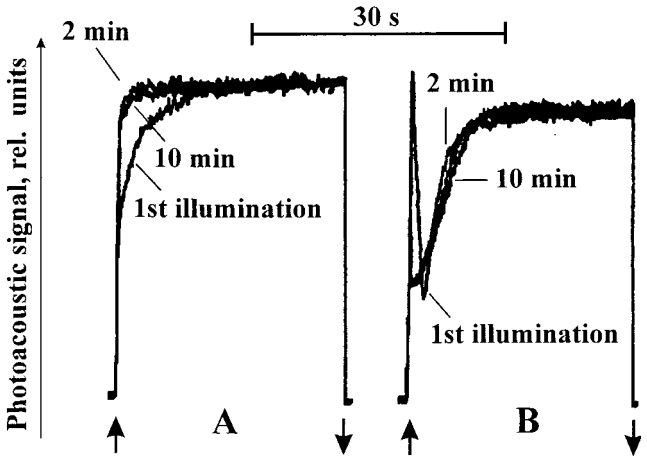

Figure 6. Kinetics of the PA signal modulated at a frequency of 35 Hz measured in MV- plus diuron-treated leaves which were additionally treated for 4 h with 50 μM rotenone (A) or stored in water for 4 h (B). All the leaves were exposed for 5 min to 45°C. Measurements were taken after a dark adaptation period of 1 h (first illumination), 2 min or 10 min following a previous 30 s irradiation with 680 nm light of 61 μmol m^{-2} s^{-1}.

accumulated in the dark as follows. The data reported above clearly show that the negative PA peak reflects the exhaustion of the pool of electron equivalents accumulated prior to the onset of light. The size of that pool, which is obviously proportional to the area of the negative PA peak, was found to be 50% reduced after about a 3.7 s irradiation of the leaves with a photon flux density (PFD) of red light of 61 μmol m^{-2} s^{-1}. Leaf absorbance is known to be about 0.85 at 680 nm. Since the relative Chl content in the PSI antenna was estimated for wild-type barley as 43% of the total Chl (22), PSI absorbed about 100 μmol quanta m^{-2} during the illumination period of 3.7 s. Certainly, not all those absorbed quanta induced charge separation in PSI reaction centers because a fraction of the P700 centers were kept in the oxidized state under light. In a separate experiment (results not shown) we have determined the relative fraction of reduced P700 integrated over 3.7 s. For this, dark-adapted barley leaves treated with MV and diuron and then exposed for 5 min to 45°C were first irradiated for 3.7 s with light of 61 μmol m^{-2} s^{-1} and then with intense far-red light provided by an RG715 filter (Schott) in order to oxidize P700 fully. The absorbance changes at 820 nm due to P700 redox transformations were monitored simultaneously. The absorbance at 820 nm increased rapidly during the first second of illumination with light of 61 μmol m^{-2} s^{-1} and then remained stable (data not shown). Far-red light increased the magnitude of the absorbance changes more than twice compared with the light of 61 μmol m^{-2} s^{-1}, demonstrating greater P700 oxidation. Three measurements provided a value of 0.55 ± 0.02 for the ratio of P700 kept in the reduced state (after the 3.7 s period under light of 61 μmol m^{-2} s^{-1}) to the total P700 content. Thus, P700 was able to deliver 55 μeq electrons m^{-2} during this period. As the P700 concentration was calculated to be 0.4 μmol m^{-2}, the number of electrons available for donation to PSI in the first moments of leaf irradiation has been quantified as 140 eq:P700 in leaves exposed to 45°C. Dark regeneration of the reductants did not affect the estimated value of the pool size since the rate constant of dark regeneration was found to be more than 10 times less than the rate of its light-induced exhaustion (compare Figs. 4 and 6B). Clearly, as the capacity of the membrane-bound redox carriers does not exceed 15–20 eq:P700 (2), the above number of electron equivalents could be obtained only from the soluble stromal reductants.

Thus, the pool size of reductants that are able to donate electrons rapidly to $P700^+$ was estimated as 140 eq:P700 in leaves exposed to 45°C. This value coincides remarkably with the size of the corresponding pool estimated by monitoring P700 redox changes in the cyanobacteria *Synechocystis* PCC 6803 (140 eq:P700, [23]). It is 1.6 times less than in maize leaves (225 eq:P700, [1]) but 3–5 times higher than in the nonstressed leaves of various C3 plants (20–30 eq:P700, [2]). Thus, heat not only stimulates the steady-state electron flow to PSI (10) but also enlarges the reservoir of soluble reductants capable of donating to the intersystem chain of thylakoids.

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