Spectrophotometric Isolation of Kinetically Different Pools of P-700 and Their Correlation to the Reduction of NADP by Isolated Chloroplasts*
I. The Effect of Light Quality and Intensity

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Excitation of isolated chloroplasts in the presence of ferredoxin and NADP by repeated short flashes yields a polyphasic absorption change at 700 nm. Assuming first-order reactions, the signal may be resolved into three distinct components with average relaxation times of approximately 20 µs, 150 µs and 20 ms. Their relative magnitude is dependent on experimental conditions; their spectral characteristics indicate that all three components may be ascribed to P-700.

Concurrent measurements of Y-NADPH, the flash yield of NADP reduction with an enzymatic recycling method, allowed Y-NADPH to be compared to the magnitude of each of the three P-700 components and to total P-700. In general, the data show a good correlation of NADP reduction with the sum of the µs-phases but not with the ms-phase or total P-700.

Analysis of light intensity curves (blue or far red flashes) with a mathematical model which yields maximum values for all parameters at infinite light intensity shows that in both cases approximately two moles of the microsecond component of P-700 turn over for each mole of NADPH formed. In contrast, the molar ratio of the ms-component to the yield of NADP reduction is approx. 0.2 in blue and approx. 6.3 in far red light. The data suggest that only that portion of the P-700 pool which relaxes in the microsecond range may be involved in the reduction of NADP while the ms-component is funtionally isolated from linear electron transport.

Introduction

Since its discovery by Kok [1], P-700 has been considered as the reaction center of photosystem I in photosynthetic electron transport. In the present and commonly accepted Z-scheme [2], it plays an integral part in the light-induced reactions leading to the reduction of NADP and, alternatively, in the presence of non-physiological cofactors such as PMS, in cyclic electron transport with ATP as the sole reaction product.

Kok [3] first reported data which showed that the rates of P-700 turnover and NADP reduction were equivalent. In later work, Rurainski and co-workers [4, 5] suggested that this was a fortuitous result since they observed antagonistic relationships between the rates of electron transport through P-700 and of NADP reduction. After addition of mono- or divalent salts, for example, the former decreased whereas the latter increased. Conversely, DCMU inhibited pyridine nucleotide reduction in isolated chloroplasts and oxygen evolution in intact algae while stimulating the electron flux through P-700. The authors proposed that P-700 and the site of NADP reduction were located in parallel light reactions.

Work in several other laboratories also showed that the relationship between P-700 and NADP reduction was not as simple as implied by the Z-scheme. Thus, using DCPIP/ascorbate as artificial electron donor, Hiyama *et al.* [6] reported evidence for the existence of two types of P-700 in fragments of the blue-green alga *Nostoc muscorum*. Of these, only the high (light) intensity component was correlated with the reduction of NADP by the donor, while a low intensity component was apparently not so involved.

Haehnel [7] concluded from measurements of absorption changes in isolated chloroplasts that only 75% of P-700 is coupled to photosystem II via the rate-limiting step between the light reactions and

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, dichlorophenol indophenol; PMS, phenazine methosulfate; MES, morpholino ethane sulfonic acid; Y-NADPH, flash yield of NADP reduction.

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that the remaining 25% were functionally isolated. Several other authors (*e.g.* Grahl and Wild [8] and Egneus *et al.* [9]) investigated electron transport and P-700 content in plant material of different physiological states and found little correlation between them.

The work of Rurainski *et al.* [4, 5] cited above was carried out with a steady-state relaxation spectrophotometer having a time resolution limit of about 1 ms. Therefore, the authors could not exclude the possibility that the observed decrease or increase in electron flux through P-700 was due to a decrease or increase in the relaxation time and that under some of their experimental conditions, the absorption change had not been detected because of this limitation [4]. They consequently amended their interpretation later and proposed that the observed *20 ms component* of P-700 was not involved in *linear electron transport* [10].

Relaxation times of P-700 in the sub-millisecond range were reported by Haehnel and Witt [11] who isolated from absorption changes at 703 nm three components with average $t/2$-values of approx. 20 μs, 200 μs, and 20 ms. In their interpretation these authors suggested that the slow component was due to a rate limiting electron transport from plastoquinone to the primary donor of P-700 and the faster components due to the reduction of the pigment by the primary donors (esp. plastocyanin).

In the present communication, we report further on the involvement of P-700 in the reduction of NADP. Using a flash spectrophotometer with resolution in the microsecond range, we confirm previous reports [11] concerning the existence of three kinetically distinguishable components of P-700 if one assumes first-order reactions. Additionally, in the same sample we measured the amount of NADP reduced and correlated these data to the individual components and to total P-700. We suggest that only that portion of P-700 which is reduced in the microsecond range may be involved in the reduction of NADP. In line with previous reports by Rurainski and co-workers [4, 5, 10] we find little indication for a participation of the 20 ms-component in this reaction.

Materials and Methods

Chloroplasts from peas were prepared as previously described [5]. They were resuspended in 15 ml 1 mM MES buffer, pH 7.5 and incubated at ice temperature for 15 min. This preparation was centrifuged at $1500 \times g$ for 5 min to remove debris. The supernatant was again centrifuged at $20\,000 \times g$ for 5 min and the sediment was resuspended in a medium containing 0.4 M sucrose, 10 mM NaCl and 20 mM Tris (pH 7.5). Prior to a measurement, chloroplasts were diluted to approx. 10 μg chlorophyll/ml sample using the same medium. Ferredoxin was isolated from spinach according to Buchanan and Arnon [12] and added in saturating amounts. The concentration of NADP was 0.25 mM.

The flash spectrophotometer used was constructed with commercially available parts. A measuring beam (700 nm, intensity approx. 80 ergs $(cm^2\ sec)^{-1}$ from a B + L monochromator (1200 grooves/mm) passed through the sample cuvette (light path 1 cm) and impinged upon the photomultiplier (EMI 9558, Extended S−20) which was shielded with a narrow-band interference filter (λ_{max} 700 nm, half-bandwidth 1.8 nm). Signals from the PMT were amplified and temporarily stored in a transient recorder (Biomation, Mod. 802) with 1024 addresses and a minimum sweep time of 0.5 μs/address. The time base of this instrument was split such that different portions of an input signal could be recorded at two independent sweep rates (see Fig. 1). The signals were transferred to an instrument computer (Nicolet, Mod. 1072) with 1024 addresses. Here, a preset number of signals were averaged for an improvement of the signal/noise ratio.

Actinic flashes impinged upon the sample perpendicular to the measuring beam. The light source was a Xenon tube (Verre et Quartz, VQX CAD 62) whose emission (approx. 8 Ws) was filtered through a broad-band interference filter transmitting between 550 and 650 nm (Balzers K 5), a blue glass filter (Schott, BG 23) or a steep cut-off filter (Schott, RG 715). The two first named filters were combined with 4 color subtraction filters (Balzers, Filtraflex DC-rot). The half-time of the flashes was either 2 or 10 μs; they were generally pulsed at a frequency of 2 Hz using a home-built pulse generator. To minimize disturbances due to scattered actinic light and fluorescence, the sample was about 60 cm removed from the face of the PMT. Also, after the desired number of signals including the remaining disturbance was recorded, the measuring beam was turned off and an equivalent number of signals (disturbance only) was subtracted. During subtrac-

tion, a light-emitting diode placed *after* the sample illuminated the PMT with approx. the same intensity as provided by the measuring beam. At the end of a measurement the content of the memory was recorded on a x/y-recorder.

The yield of NADP reduction was measured in the same sample by enzymatic recycling [13]. This method involved stopping of the reaction after the last flash with enough NaOH to bring the pH of the sample to 12.5. The mixture was incubated at 65 °C for 10 min to quantitatively destroy excess NADP. Then the pH value was brought back to 8.5 and the samples were centrifuged at 2500 × g for 10 min. An aliquot was added to 2 ml of a reaction mixture containing 4 mM glucose-6-phosphate, 0.15 mM DCPIP, 0.07 mM PMS in the medium described above. The reaction was started by adding glucose-6-phosphate dehydrogenase (EC 1.1.1.49) equivalent to 20 µg protein and the time-dependent reduction of DCPIP was recorded at 600 nm. Absolute values of NADP reduction were obtained from a calibration curve. If necessary, the value of a dark sample was subtracted from those of the illuminated samples. Also, in each set of measurements the recycling method was checked with an internal standard.

Results

An example of a representative signal recorded at 700 nm is shown in Fig. 1. Following a brief dark period, the exciting flash is triggered causing a rapid decrease in absorption. The subsequent recovery to the dark level occurs in two time ranges. To record both in one sweep, the time base of the recorder was split at the upward-pointing arrow. In the trace shown, for example, the sweep time was 2 µs/address on the left and 200 µs/address on the right side of the arrow. The signal is totally abolished by 10 µM DCMU.

The traces were manually smoothed and evaluated by plotting arbitrarily selected points on a semi-logarithmic scale (Fig. 2). The (right-hand) ms-component yields a straight line indicating a first-order reaction with a relaxation time of 17 ms, while the (left-hand) µs-component is biphasic. Following Haehnel and Witt [11] and assuming first-order kinetics also in this case, the trace may be resolved into two components. The respective relaxation times are 20 and 130 µs. Thus, we conclude in support of other work [11] that with this assumption

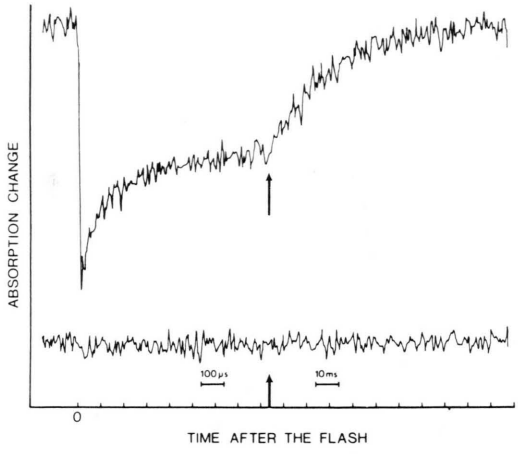

Fig. 1. Flash-induced absorption change at 700 nm as a function of time. The sample contained chloroplasts equiv. to 10 µg chlorophyll/ml, a saturating concentration of ferredoxin and 0.25 mM NADP. 1024 red flashes (half-time 10 µs) were fired. Dark time between flashes 300 ms. Upper curve: Control, lower curve: after addition of 10 µM DCMU. Note that the time scale of the sweep has been changed at the upward-pointing arrow.

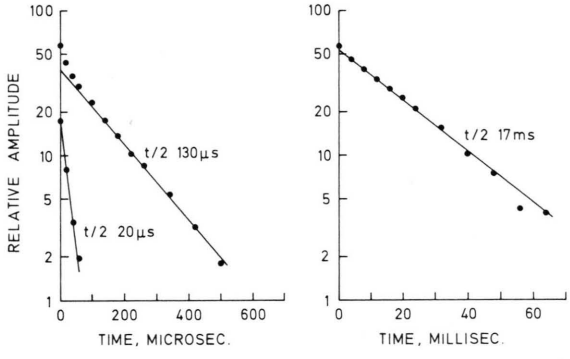

Fig. 2. Semi-logarithmic computer plot of the absorption change in Fig. 1. The biphasic µs-sweep was resolved into two components by linearly extrapolating the last portion of the plot to $t=0$ followed by substraction of this line from the actual data.

there exist three kinetically distinguishable components in the light-induced absorption change at 700 nm.

The relative proportions of the three components, expressed as per cent of the total absorption change, as well as their relaxation times varied from one chloroplast preparation to the next. Statistical evaluation of a number of individual measurements yielded the results in Table I. Please note that similar results were obtained with a 2 µs and 10 µs flash and with either red or blue light. The com-

Table I. Relative proportions of P-700 components and their relaxation times in pea chloroplasts.

Flash length	No. of measurements	% A_1	$t/2$ [μs]	% A_2	$t/2$ [μs]	% A_3	$t/2$ [ms]
2 μs	18	11.9 ± 7.6	13 ± 9	43.5 ± 4.9	155 ± 20	44.6 ± 4.5	16 ± 2
10 μs	22	18.3 ± 7.6	18 ± 7	39.2 ± 7.5	144 ± 25	41.7 ± 9.5	18 ± 4
All values	40	15.4 ± 7.3	16 ± 8	41.6 ± 6.4	149 ± 23	43.0 ± 7.9	17 ± 3

Data are means of the number of measurements indicated. All numbers are expressed as percent ± standard deviation of the total absorption change at 700 nm. The samples contained saturating concentrations of ferredoxin, 0.25 mM NADP and chloroplasts equiv. to between 10 and 15 μg chlorophyll/ml and were usually excited with 1024 flashes of saturating intensity. 10 μs flashes: broad-band interference filter (550–650 nm); 2 μs flashes blue light (BG 23).

ponents with average relaxation times of 150 ± 23 μs and 17 ± 3 ms which contribute approximately evenly to the total absorption change, were rather stable. Only the fastest component with a mean relaxation time of 18 ± 7 μs varied considerably. Note also, that its relative contribution amounts to only 10–20% of the total signal. Quite irregularly but in a significant number of measurements, this component was even absent, *i.e.* also the μs-sweep yielded a straight line in a semi-logarithmic plot. At present, we cannot account for this phenomenon.

It is to be emphasized that the data shown in Table I apply to "standard" conditions of measurement including saturating concentrations of ferredoxin and NADP and saturating flash intensities of red or blue light. As we will show here and in a following paper, the relative proportions of the components may be manipulated by a variety of exogeneous treatments.

An unequivocal assignment of the absorption change to P-700 can only be made on the basis of its spectral properties. As Fig. 3 shows, all three components exhibit a major peak at 703 nm and a satellite band centered around 685 nm. The apparent shift in this band of one of the components to lower wavelengths may well be fortuitous. At wavelengths near 680 nm measurements are somewhat difficult because of strong, overlapping signals due to fluorescence. Also, in contrast to the very narrow-banded interference filter shading the photomultiplier during routine measurements at 700 nm, the half-bandwidth at other wavelengths was approx. 15 nm. Despite the small uncertainty on the short wavelength side of the spectrum we feel that all three components may be ascribed to P-700.

The following experiments are concerned with correlating the P-700 absorption change and the flash yield of NADP reduction (Y-NADPH) measured in the same sample. In preliminary investigations, Y-NADPH was fitted to each of the three phases. In this case, however, neither component nor total P-700 yielded a completely satisfactory match. A qualitatively better correlation was obtained when the two μs-phases were taken together. This observation indicates that the μs-phase may in fact not consist of two separate components but that it may

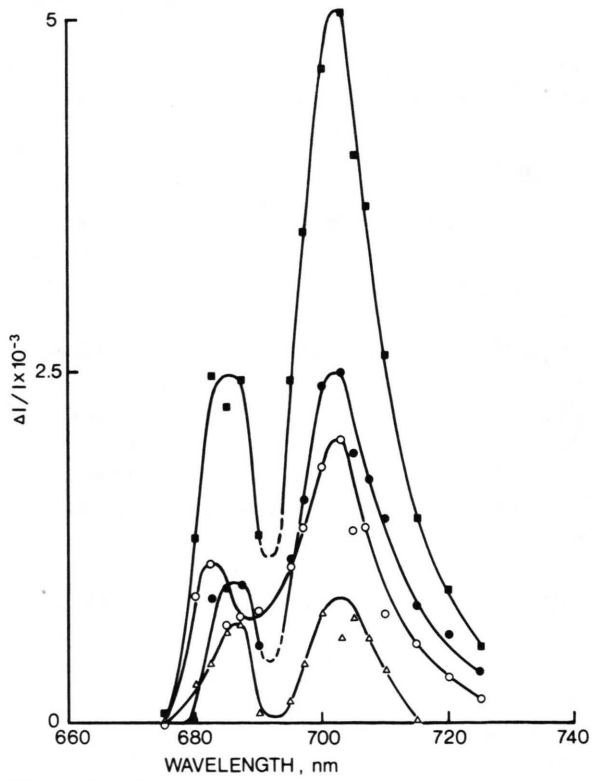

Fig. 3. The absorption change as a function of wavelength. Experimental conditions as in Fig. 1. 512 flashes (red light). Triangles: fast μs-component ($t/2 = 20$ μs); open circles: slow μs-component ($t/2 = 180$ μs); dots: ms-component squares: total absorption change.

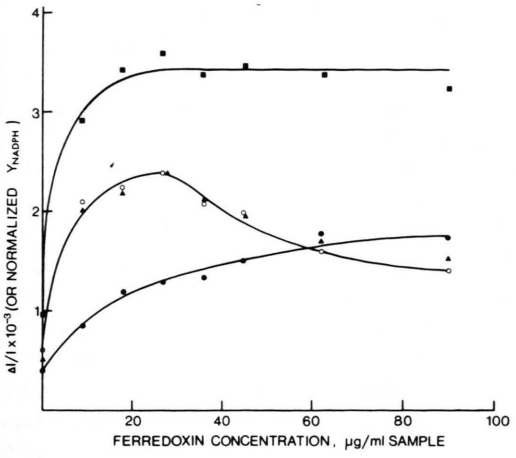

Fig. 4. Magnitudes of the μs- and ms-components, of total P-700 and of the flash yield of NADP reduction as a function of ferredoxin concentration. The samples contained 0.25 mM NADP and chloroplasts equiv. to 16 μg chlorophyll/ml. 1024 exciting flashes (10 μs) with broad band interference filter. Triangles: μs-component; dots: ms-component; squares: total P-700; open circles: Y-NADP normalized to the μs-component.

reflect a higher-order reaction [14, 15]. At any rate, in the remainder of this report we will designate the entire absorption change which relaxes in the μs-range as "μs-component".

During the preparation of chloroplasts, ferredoxin, a co-factor necessary for the reduction of NADP is lost and has to be added to the reaction medium. Fig. 4 shows that with increasing concentration, the flash yield of NADP reduction increases to a maximum value. Further additions lead to a gradual decline of the yield. The microsecond-component of P-700 closely follows the profile of this curve and thus may participate in this reaction as postulated in the currently accepted electron transport scheme [2]. In contrast, the magnitude of the millisec-component increases somewhat less markedly, without reaching a saturation value over the concentration range studied and, like total P-700, shows little correlation to Y-NADPH.

The continuing increase in the ms-portion at ferredoxin concentrations greater than approx. 20 μg/ml may be related to the decrease in the μs-component. Apparently, the fast relaxing portion can be converted to the slow relaxing one at high levels of the co-factor. This conclusion follows from the observation that the total signal (the sum of the μs- and ms-component) has reached a steady value.

These measurements were made with a flash frequency of 5 Hz, *i.e.* the dark time between flashes was 200 ms. Quite similar curve profiles were obtained at other frequencies between 1 and 10 Hz (data not shown). In all cases, the maximum value of all measured parameters were quite similar to those of Fig. 4 and only the μs-, but not the ms-component or total P-700 could be matched to Y-NADPH. Incidentally, we also observed that the concentration of ferredoxin needed to reach maximum values varied in a quasi-exponential form from 10 μg/ml at 1 Hz to 45 μg/ml at 10 Hz, *i.e.* as the dark time between flashes increased, the concentration of ferredoxin required for saturation of each of the parameters decreased.

Flash intensity curves with blue light are shown in Fig. 5. The μs-component of P-700 increases gradually without reaching light saturation over the intensity range studied. In contrast, the ms-component rises more steeply and after going through a maximum, decreases again. As in the observation made with ferredoxin (Fig. 4), this decrease in the millisec.-component appears to be related to the continual increase in the μs-phase since the total signal remains at a nearly constant level. The normalized flash yield of NADP reduction yields an excellent match with the fast component but not with either the slow phase or the total signal suggesting again

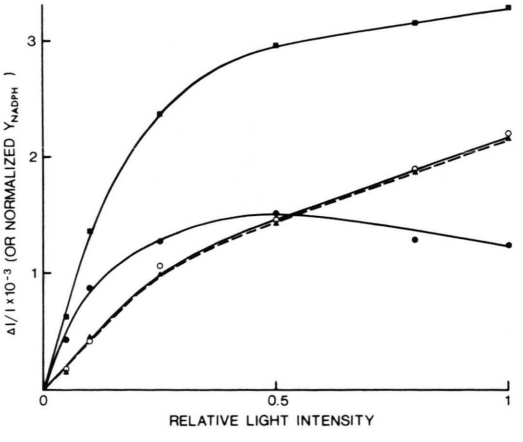

Fig. 5. Magnitudes of the μs- and ms-components, of total P-700 and of the flash yield of NADP reduction as a function of flash intensity (blue light). The samples contained 0.25 mM NADP, saturating concentrations of ferredoxin and chloroplasts equiv. to 13 μg chlorophyll/ml. 1024 blue exciting flashes of 2 μs duration. Symbols as in Fig. 4. Dashed line: Y-NADPH normalized to the μs-component. The absolute yield at the highest intensity used was 7.7 pmol NADPH/ml · flash.

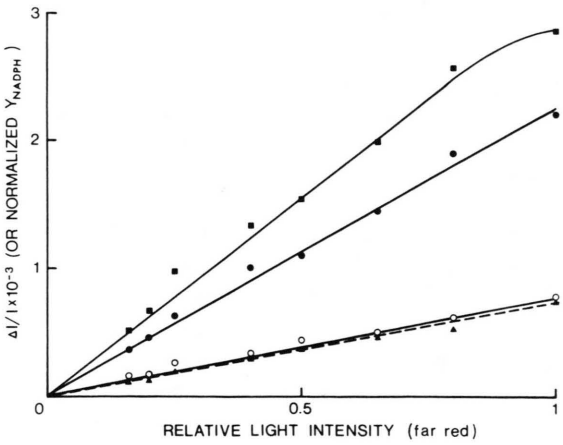

Fig. 6. Magnitudes of the μs- and ms-components, of total P-700 and of the flash yield of NADP reduction as a function of flash intensity (far red light). Experimental conditions and symbols as in Fig. 5, except that the duration of the flash was 10 μs and that two flashes were triggered simultaneously from two sides of the cuvet.

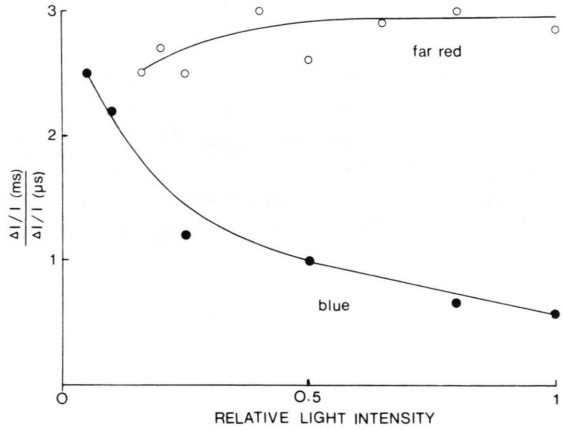

Fig. 7. Ratios of the ms- to μs-components as a function of light intensity (blue and far red light). Data were taken from Figs. 5 and 6. Note: the light intensity plotted refers to those in Figs. 6 and 7. They are not necessarily equivalent.

that only the fast component may be involved in the reduction of NADP.

Measurements in far-red light, which supposedly excited primarily photosystem I, are shown in Fig. 6. Although in this case the sample was illuminated from two sides by two simultaneously triggered flashes, the excitation intensity was too low to obtain complete saturation; all measured parameters increase in an quasi-linear fashion. The flash yield of NADP reduction in this figure is normalized to the μs-component, yielding a good, qualitative correlation. A similarly good fit, however, could be obtained also with the ms-component and total P-700. Thus, in this case the simple correlation provides little knowledge about the relationship between either of the P-700 components, of total P-700, and of NADP reduction. That there exist quantitative differences will be shown below.

The data of both intensity curves indicate further that the relative proportions of the P-700 components may deviate from those shown in Table I for "standard" conditions. As shown in Fig. 7, with low intensities of blue light and with all intensities of far red light, the magnitude of the ms-component greatly exceeds that of the μs-phase. Whereas their ratio remains reasonably constant over all intensities of far red light, it decreases as the intensity of blue light is increased. In other words, when the yield of NADP reduction is low (as in low intensitites of blue and in far red light), the magnitude of P-700 turnover in the ms range is relatively high. This antagonism has previously been observed under a variety of other experimental conditions and reported on in detail [4, 10].

The remainder of this report is concerned with attempts to show a quantitative correlation between the two components and the terminal reaction. In connection with their work on the relationship between P-700 and NADP reduction in DCMU-poisoned fragments of *N. muscorum* in the presence of an artificial electron donor, Hiyama *et al.* [16] developed a mathematical model for the analysis of light intensity curves of the two parameters. A useful equation derived from this model is similar to the Lineweaver-Burk equation for enzymatic reactions. Plots of the reciprocal values of the P-700 concentration turning over or the yield of NADP reduction against the reciprocal values of the light intensity result in a straight line. Its intercept with the *y*-axis yields the maximum activity of each parameter at infinite light intensity. The intercept with the negative *x*-axis was termed $I_{1/2}$ and corresponds to the light intensity at which the parameters are saturated half-maximally.

When plotted accordingly, the data of Figs. 5 and 6 result in the graphs of Figs. 8 and 9. Statistically best-fitting lines as well as the intercepts with the ordinate and abscissa were calculated by linear regression analysis. As regards $I_{1/2}$, the analysis reveals that in blue light both NADP reduction and the μs-component of P-700 extrapolate to the same

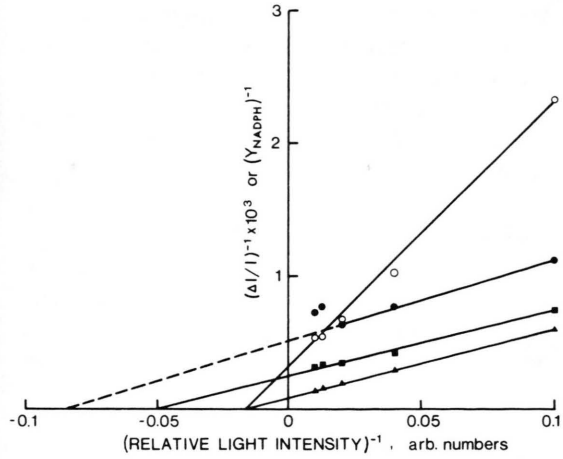

Fig. 8. Reciprocal plots of the light intensity curves in Fig. 5 according to Hiyama *et al.* [17]. The lines drawn are statistical best fits as determined by linear regression analysis.

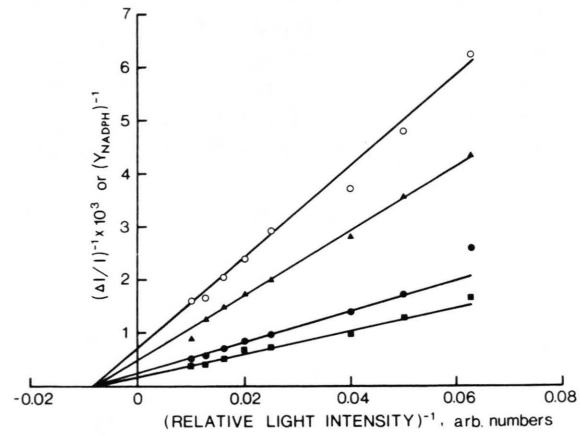

Fig. 9. Reciprocal plots of the light intensity curves in Fig. 6 according to Hiyama *et al.* [17]. The lines drawn are statistical best fits as determined by linear regression analysis.

value. In contrast, the ms-phase and total P-700 attain half-saturation at a considerably lower intensity. This result is in line with the previous postulate the only the μs-component and Y-NADPH may be connected. A similar conclusion cannot be drawn for the data in far red light since all parameters extrapolate to the same $I_{1/2}$.

The *y*-intercepts were evaluated quantitatively by calculating molar concentrations of NADP reduced and of P-700 turning over. The calculations are based on an extinction coefficient for P-700 of 64 (mM cm)$^{-1}$ [17] and an average molecular weight for chlorophyll of 900.

Table II shows the results of this evaluation. The flash yield of NADP reduction is higher in blue than in far red light, a result which agrees with numerous investigations in continuous light. The μs-component follows this trend. Conversely, the ms-component shows a rather low yield in blue and a very high yield in far red light. Apparently, the ms-phase is largely reduced by the activity of the pigment system of photosystem I which is primarily excited by far red light. The magnitude of total P-700 turnover is similar in both lights and amounts to 450 and 490 chlorophyll molecules per mole P-700. This size of the chlorophyll/P-700 unit has been

Table II. Analysis of reciprocal light intensity curves.

Quantity	Blue light				Far red light			
	Y_{NADPH}	μs-component	ms-component	Tot. P_{700}	Y_{NADPH}	μs-component	ms-component	Tot. P_{700}
$I_{1/2}$ (rel. values)	0.63	0.63	0.12	0.20	0.13	0.13	0.13	0.13
nmol/mg chlorophyll · flash	0.89	2.04	0.21*	2.24	0.29	0.63	1.81	2.44
mol chlorophyll/mol activity	1235	540	5240	490	3790	1750	600	450
mol P_{700}/mol NADPH	–	2.21	0.24	2.45	–	2.17	6.25	8.42

The reciprocal light intensity curves (Figs 8 and 9) were evaluated for the following parameters: $I_{1/2}$, obtained from the intercept of the lines with the negative abscissa, is the light intensity at which the measured parameters were excited to one-half of their maximum. The maximum values of P-700 components and of Y-NADPH were obtained from the intercepts of the lines with the ordinate ($I = \infty$). Intercepts were determined by linear regression analysis. Molar concentrations of P-700 were calculated with an extinction coefficient of 64 (mM cm)$^{-1}$ [17].

* Since the reciprocal values of the ms-component increase at high light intensities, the y-intercept cannot be determined unambiguously. Therefore, the yield in this case was taken as the difference between max. total signal and max. μs-component.

reported repeatedly for spinach or pea chloroplasts and indicates that all P-700 is accounted for.

The quantitative relationship between the extrapolated P-700 components and NADP reduction is shown in the last line of Table II. Present concepts of photosynthetic electron transport [2] predict that two moles of P-700 turn over for each mole of NADPH formed. Implicit in this formulation is furthermore that the total pool participates in the reaction. The data show, however, that the ratio of total P-700 to Y-NADPH is somewhat higher in blue light and considerably higher in far red light, suggesting that a portion of the total pool is functionally isolated from the electron transport pathway leading to the reduction of NADP. On the other hand, since ratios of approximately 2 are found with the μs-component, this pool may in fact be coupled to linear electron transport. It follows that the ms-pool, the difference between the total and μs pools, is functioning in a different reaction. Such a proposal has previously been made [5, 10].

Discussion

In agreement with, and in support of, previous reports [7, 11], we show in this communication that, following a short flash of light, P-700 is reduced to the dark state with a polyphasic time course. Approximately one-half of the total pool relaxes in a first-order reaction with a relaxation time of about 20 ms. The other half, relaxing in the μs-range, is biphasic and may be resolved into two components with relaxation times of 20 and 150 μs if one assumes first-order behavior.

The assumption of two first-order reactions prevailing in the μs-range is favored by Haehnel *et al.* [18] who concluded that the kinetically isolated 20 μs-component is reduced by an electron transfer from complexed plastocyanin and the slower, 200 μs-component, by an electron transfer from a more mobile pool of plastocyanin to P-700. In contrast, several other authors (*e.g.* Delosme *et al.* [14] and Olsen *et al.* [15]) interpreted their kinetic data in terms of a second-order reaction between P-700 and its electron donor. Our own results are at present insufficient to distinguish decisively between these and other possible mechanisms. However, we clearly obtained better correlations between the flash yield of NADP reduction and the *entire* μs-component of P-700 than the individual kinetically resolved μs-portions. This observation is consistent with a single reaction of higher order. Alternatively, if there exist two monomolecular electron donation reactions between different pools of plastocyanin and P-700 they should both lead to the reduction of NADP.

A second aspect of this work is concerned with the prevailing interpretation concerning the electron donors of the μs- and ms-components of P-700. Haehnel and Witt [11] suggested a linear sequence of electron transfers between the two light reactions of photosynthesis which, in an abbreviated notation, may be written:

$$\text{PS II} \rightarrow \text{PQ} \xrightarrow{20\ \text{ms}} \text{PC} \xrightarrow{200\ \mu\text{s}} \text{P-700} \rightarrow \text{NADP}\,.$$

In the view of these authors, the microsecond relaxation is due to an electron transfer from reduced plastocyanin (PC) to P-700. If PC exists in the oxidized form, electrons first have to traverse a rate-limiting step from plastoquinone (PQ) to PC resulting in a relaxation time for P-700 of approx, 20 ms. Such a sequential arrangement of reaction steps implies that the total pool of P-700 participates in the reduction of NADP. Our data show, however, that this requirement is not fulfilled: the best correlation of NADP reduction with P-700 is obtained if only the μs-component is used. It is still possible that electron transfers from plastochinon or plastocyanin give rise to the different relaxation times observed, but these reactions cannot be in series.

A different interpretation concerning the origin of the μs- and ms-component was proposed by Bouges-Bocquet [19]. In her investigations of the reduction of methyl viologen by photosystem I, this author observed a biphasic relaxation with half-times of 400 μs and 10 – 20 ms. The former was ascribed to the relaxation of the reaction center (*viz.* P-700), whereas the latter was explained by double quantum hits in the duration of the exciting flash. The probability of double hits is, however, dependent on the length and/or the intensity of the flash. As we show, shortening of the flash length from 10 to 2 μs had no effect on the ratio of the ms- and μs-components (Table I). Moreover, with decreasing light intensity, the proportion of the ms-component increased rather than decreased. We conclude, therefore, that the double-hit theory cannot be applied in this instance.

Our data bear directly on previous observations by Rurainski and co-workers [4, 5, 10] who simultaneously measured P-700 turnover and NADP reduction in a steady-state relaxation spectrophotometer. Since the instrument used at that time had a resolution limit of about 1 ms, only the slow, 20 ms-component could be detected. In several instances, the authors observed that the rate of electron transport through P-700 (20 ms) was low when the rate of NADP reduction was high and *vice versa.* It was concluded that "the weight of the evidence shows that P-700 with a relaxation time of 20 ms is not directly involved in the reduction of NADP" [10].

This conclusion is supported by the extended work reported here. With the exception of the uncertain result in the experiment with far-red light, neither total P-700 nor the ms-component could be correlated to the flash yield of NADP reduction. Also, calculations based on the model of Hiyama *et al.* [16] resulted in P-700 (ms)/NADP molar ratios which are either too low or too high to be consistent with an involvement of the ms-component in the reduction of NADP as set forth in the currently popular Z-scheme [2].

On the other hand, in all experiments (and others reported elsewhere [20]) the μs-component not only was qualitatively well correlated with NADP reduction but also yielded P-700 (μs)/NADP ratios of approximately 2. These results strongly point to a participation of the μs-component in linear electron transport. However, these data alone are insufficient to tell whether this involvement is adequately described by the Z-scheme.

On the basis of these results we suggest that the total functional pool of P-700 is divided into at least two smaller pools which manifest themselves by different relaxation times. The data show that we are dealing with dynamic pools, *i.e.* their fraction of the total pool size may vary depending on external parameters, such as light intensity and light quality. Expressed differently, each P-700 may, depending on conditions, participate in one or the other pool. We consider these pools as "kinetic" entities and the relaxation time as a marker by which the pools can be differentiated. Whether they exist morphologically needs to be established. There have been previous reports on the existence of two types of photosystem I in the grana stacks and stroma lamellae of the thylakoid membrane [21].

The idea of the existence of at least two pools leads us to suggest further, that they react with different immediate or distant electron donors and that they may catalyze different terminal reactions. In the case of the μs-pool we provide evidence for its involvement in the reduction of NADP. The immediate electron donor is probably plastocyanin [18] and ultimately photosystem II. Further work, now in progress, must establish if the ms-pool plays a role in any terminal reaction. For the present, we cannot but speculate that it may participate in a "cyclic" electron transport which may catalyze the biosynthesis of ATP.

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[1] B. Kok, Biochim. Biophys. Acta **22,** 399 (1956).
[2] A. Trebst, Ann. Rev. Plant Physiol. **25,** 423 (1974).
[3] B. Kok, Photosynthetic Mechanisms of Green Plants (B. Kok and A. T. Jagendorf, eds.), p. 35, NAS-NRC Publication 1145, Washington, DC 1963.
[4] H. J. Rurainski, J. Randles, and G. E. Hoch, FEBS Lett. **13,** 98 (1971).
[5] H. J. Rurainski and G. E. Hoch, Proc. 2nd Int. Congr. Photosynth. Res., Stresa, Italy (G. Forti, M. Avron, and A. Melandri, eds) p. 133, Junk Publ., The Hague 1971.
[6] T. Hiyama, B. D. McSwain, and D. I. Arnon, Biochim. Biophys. Acta **460,** 76 (1977).
[7] W. Haehnel, Biochim. Biophys. Acta **459,** 418 (1977).
[8] H. Grahl and A. Wild, Z. Pflanzenphysiol. **67,** 443 (1972).
[9] H. Egneus, G. Sellden, and L. Andersson, Planta **133,** 47 (1976).
[10] H. J. Rurainski, Z. Naturforsch. **30 c,** 761 (1975).
[11] W. Haehnel and H. T. Witt, 2nd Int. Congr. Photosynthesis (G. Forti, M. Avron, and A. Melandri, eds). p. 469, Dr. Junk Publ., The Hague 1971.
[12] B. B. Buchanan and D. I. Arnon, Methods in Enzymology, **Vol. 23,** p. 413, Academic Press, New York 1971.
[13] H. Matsumura and S. Miyachi, Methods in Enzymology. **Vol. 69,** p. 465, Academic Press, New York 1980.
[14] R. Delosme, A. Zickler, and P. Joliot, Biochim. Biophys. Acta **504,** 165 (1978).
[15] L. F. Olsen, R. P. Cox, and J. Barber, FEBS Lett. **122,** 13 (1980).
[16] T. Hiyama, B. D. McSwain, and D. I. Arnon, Biochim. Biophys. Acta **460,** 65 (1977).
[17] T. Hiyama and B. Ke, Biochim. Biophys. Acta **267,** 160 (1972).
[18] W. Haehnel, A. Propper, and H. Krause, Biochim. Biophys. Acta **593,** 384 (1980).
[19] B. Bouges-Bocquet, Biochim. Biophys. Acta **396,** 382 (1975).
[20] H. J. Rurainski, Proc. 5th Int. Congress on Photosynthesis (G. A. Akoyunoglou, ed.), in press.
[21] C. J. Arntzen and J.-M. Briantais, Bioenergetics of Photosynthesis (Govindjee, ed.) p. 51, Academic Press, New York 1975.