Analysis of Light-Induced Absorbance Changes in the Near-Infrared Spectral Region

I. Characterization of Various Components in Isolated Chloroplasts

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Light-induced absorbance changes in the near-infrared (NIR) spectral region (700–950 nm) are analyzed in isolated spinach chloroplasts using pulsed light-emitting diodes as modulated measuring light sources. With chloroplasts under coupled conditions the light-induced signal is dominated by a relatively slow scattering change displaying a flat difference spectrum. More specific changes can be distinguished by faster induction and relaxation kinetics and in the presence of valinomycin/nigericin which prevents scattering changes. Besides the dominant P 700 change, with a narrow bleaching band around 703 nm and a broad absorbance increase between 750 and 850 nm (peak at 815 nm) the following absorbance changes were identified in the NIR:

1) An absorbance increase caused by plastocyanin (PC) oxidation, with a relatively flat spectrum.
2) An absorbance decrease gaining amplitude towards longer wavelengths, which reflects reduction of a low potential acceptor of PS I different from ferredoxin.
3) A field indicating absorbance decrease peaking around 730 nm, the properties of which correspond to those of P 515.
4) An uncoupler insensitive absorbance decrease stimulated by dark adaptation and anaerobic conditions, the difference spectrum of which resembles that of ferredoxin reduction.

The relative contributions of P 700 and PC to the overall oxidized-reduced difference spectrum are determined by redox titration. At 706 nm, 815 nm and 950 nm the P 700/PC is −119/19, 67/37 and 31/69, respectively. From these ratios and the molar extinction coefficients a molar P 700/PC stoichiometry of 1/3 is determined.

Introduction

The components of the photosynthetic electron transport chain in thylakoid membranes of plant chloroplasts display numerous types of light-induced absorbance changes which have been extensively studied in the past, contributing decisively to our present knowledge of photosynthetic mechanisms (see *e.g.* ref. [1]). The largest and most specific absorbance changes occur in the UV- and visible spectral region, as *e.g.* caused by redox changes of plastoquinone, the cytochromes and P 700. So far, not much attention has been paid to absorbance changes in the near-infrared region (NIR), where published light-dark difference spectra are rather flat and non-specific (see *e.g.* ref. [2]). In principle, however, absorbance measurements in the NIR have the great advantage that there is very low absorption by the leaf tissue and chloroplast pigments; such absorption in the UV- and visible region makes sensitive measurements with highly scattering leaves and intact chloroplasts problematic. Also, strong solid-state light sources exist for the NIR which can be applied for measuring light without concern about possible actinic effects. To profit from these advantages for the study of *in vivo* photosynthesis in intact leaves, methods for separation and quantification of various broad-band absorbance changes in the NIR have to be developed. The dominant change is that of P 700, the reaction center chlorophyll of PS I, which upon oxidation shows bleaching around 705 nm [3] and a wideband absorbance increase between 750 and 900 nm, peaking at 810–830 nm [4, 5]. Measurements of P 700 at 705 nm have been

Abbreviations: P 700, reaction center chlorophyll of photosystem I; DCMU, 3-(3,4-dichlorophenyl-)1,1-dimethylurea; NIR, near-infrared wavelength region (700–950 nm); PC, plastocyanin.

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complicated by the fact that this wavelength excites chlorophyll fluorescence, the changes of which are overlapping the overall signal. This complication is avoided with measurements around 820 nm. Also components of PS II (*e.g.* P680, pheophytin) and, in general, excited states of chlorophylls and carotenoids (triplets) give rise to transient absorption changes in the NIR. However, in most cases these absorption changes can be neglected for kinetical reasons.

Recently, the usefulness of P700 measurements around 820 nm with intact leaves has been demonstrated by a number of researchers, with particular interest on regulatory processes in photosynthesis [6–12]. The ratio of $P700/P700^+$ determines the quantum yield of PS I and, hence, an accurate determination of this ratio is of considerable interest. However, besides P700 there are other components contributing to absorbance changes around 820 nm. Upon illumination of dark-adapted leaves there is a transient absorbance decrease, the origin of which is unknown [8, 11]. Also, oxidation of plastocyanin (PC) causes a broad band absorbance increase which reaches into the NIR [13]. This was estimated to contribute about 35% to the total change at 820 nm when P700 and PC become fully oxidized [11].

We have set out for a detailed analysis of the light-induced absorbance changes in the NIR under a variety of physiological conditions, with the aim to obtain basic information for practical applications of NIR measurements, in particular with intact leaves. Such information is not only essential for a quantitative assessment of the P700 redox state, but also may lead to new insights into other aspects of *in vivo* photosynthesis. The outcome of this investigation will be presented in a series of publications. In the present first contribution we will concentrate on light-induced absorbance changes observed in isolated chloroplasts, which allow the use of artificial electron acceptors, inhibitors and ionophores, and with which redox titration experiments can be performed. The obtained information will serve as a basis for an analysis of NIR absorbance changes in intact leaves, which will be reported on in later publications.

Materials and Methods

Intact chloroplasts were isolated from spinach by the method of Jensen and Bassham [14]. To increase the proportion of intact chloroplasts, a further purification by percoll gradient centrifugation was carried out as described before [15].

Chloroplasts were suspended isotonically in a reaction medium containing 330 mM sorbitol, 50 mM K-Tricine pH 7.6, 1 mM $MgCl_2$ and 0.25 mM Na_2HPO_4. Class D chloroplasts were prepared from intact chloroplasts by 1 min exposure to a hypotonic buffer containing 10 mM $MgCl_2$ and 5 mM K-Tricine pH 7.6, followed by isotonic resuspension by addition of an equal volume of the double concentrated reaction medium. For each measurement a fresh chloroplast suspension was prepared, which was illuminated only once. Temperature was kept at 18 °C. For difference spectra determination and redox titration measurements a 30 ml stock suspension of class D chloroplast was prepaired and stored on ice, from which 1,3 ml aliquots were taken to minimize variations of the chlorophyll concentration. Redox titrations were carried out by appropriate additions of 1 mM ferro-/ferricyanid mixtures at various ratios, adjusted to give defined redox potentials, which were determined in the cuvette with a redox electrode (Platin/Ag/AgCl, Ingold) calibrated with two redox buffers (U_H = 427 mV and 675 mV, Ingold). The chloroplast suspension was allowed to equilibrate for 15 min after addition of the ferro-/ferricyanid mixture. The temperature of the sample was kept at 18 °C.

Isolated spinach plastocyanin was obtained from Prof. G. Hauska (Regensburg), and spinach ferredoxin was a kind gift of Prof. D. Bendall (Cambridge). Their oxidized minus reduced difference spectra were measured in the split beam mode with an Aminco DW-2a spectrophotometer, the wavelength range of which was extended to 930 nm by mounting a RG 9 filter at the entrance slit of the sample compartment. Half-band width was 4 nm. The concentration of the stock solution of ferredoxin was determined by assuming an extinction coefficient of 4.65 mM^{-1} cm^{-1} at 420 nm for the oxidized minus dithionite-reduced form [16].

Measurements of absorbance changes were carried out using a laboratory-built pulse modulation system similar to a commercial system described before [8] with the following differences: The present system was constructed to process simultaneously two transmission signals compensated by

a constant offset or in difference to a third transmission signal. This is achieved by alternating two or three pulse-light sources with a frequency of 64 kHz in cyclic operation and by synchronous detection of the amplified pulse signals. The pulsed light is obtained from light emitting diodes (LED) in combination with interference filters (Schott, A 7-0.5) of 3–5 nm half-band width. The following LED types for the corresponding wavelength regions were chosen:

650–685 nm: Toshiba TLRA 105-C
690–720 nm: Stanley SPR 5501
730–750 nm: Hitachi HLP 30 RGA
760–780 nm: Hitachi HLP 40 RA
805–855 nm: Stanley DN 305
915–950 nm: Siemens SFH 415-2

In order to obtain a sufficiently high measuring light intensity in the 690–720 nm region, the emission of nine SPR 5501 LEDs was concentrated by a light-gathering plexiglass cone. The light passing through the interference filters was led by multi-branched fiber optics to a standard 1 × 1 cm thermostated cuvette, connected to the cuvette by a light-mixing glass rod. The transmitted light was picked up by another fiber bundle connecting to the detector. Continuous actinic light was provided by a laboratory-built halogen lamp (Type Xenophot XLX 64634, Osram) equipped with an electromagnetic shutter (Compur electronic-m). If not stated otherwise, the blue actinic light was selected by BG 18 (Schott), Calflex X (<700 nm, Balzers) and DT Cyan (<640 nm, Balzers) filters with an intensity of 30 W/m^2. The silicon PIN photodiode (type S 1732, Hamamatsu) of the detector was protected against actinic light by RG 630, RG 645, RG 665 or RG 780 cut-off filters (Schott) depending on the chosen measuring wavelength.

In the present study, generally two absorption signals at two different NIR wavelengths were measured simultaneously, with one wavelength being fixed at 815 nm, so that always comparison to the changes at 815 nm (peak of P700 changes) was possible. In some experiments simultaneous measurements of absorbance changes in the green spectral region were carried out, using a recently developed LED array spectrophotometer, the properties of which have been described previously [17]. The combined fiber optics system also connected to a modulation fluorometer for measuring chlorophyll fluorescence (PAM system, H. Walz, Effeltrich, F.R.G.).

The signals were recorded with a digital storage oscilloscope (Type Nicolet Explorer III), stored on floppy disc and plotted by a X-Y plotter. The extracted data were further analyzed by a microcomputer (80386 processor, with numerical co-processor) with the help of a calculation program (Excel 2.01, Microsoft).

Results and Discussion

Phenomenology of light-induced NIR absorbance changes in isolated chloroplasts

A first overview on the complex phenomenology of light-induced absorbance changes in the near-infrared spectral region is given in Figs. 1 and 2 for intact and broken chloroplasts, respectively, under a variety of conditions. A moderate intensity of continuous blue light is applied to drive both photosystems. The kinetic changes are shown for 706, 815, and 947 nm, *i.e.* for wavelengths varying by almost 250 nm. Still, it is apparent that the largest changes observed with isolated chloroplasts in many cases are very similar at these three wavelengths, *i.e.* they are dominated by a component with a rather flat difference spectrum in the NIR. The similarity is particularly evident in the example of Fig. 1 A, where intact chloroplasts without additions are illuminated. It may be noted that the apparent absorbance increase sets in with some delay upon illumination and continues for some time after light-off. The recovery of this change is rather slow (half-time of several minutes, not shown). This "non-specific" signal is likely to reflect a light-induced conformational change of the thylakoid membrane, associated with "membrane energization" and stimulation of light scattering, as previously studied by measurements around 540 nm [18, 19]. Actually, the slow component of the light-induced induction kinetics at 540 nm is almost identical to the changes at 947 nm (not shown). This "scattering change" is enhanced in presence of the Hill reagent methylviologen (Fig. 1 B), diminished to 1/3 by the protonophore nigericin (Fig. 1 C) and totally suppressed by full uncoupling, using a combination of nigericin and of the field dissipating ionophore valinomycin (Fig. 1 D). Valinomycin alone does not have a significant effect (Fig. 1 E).

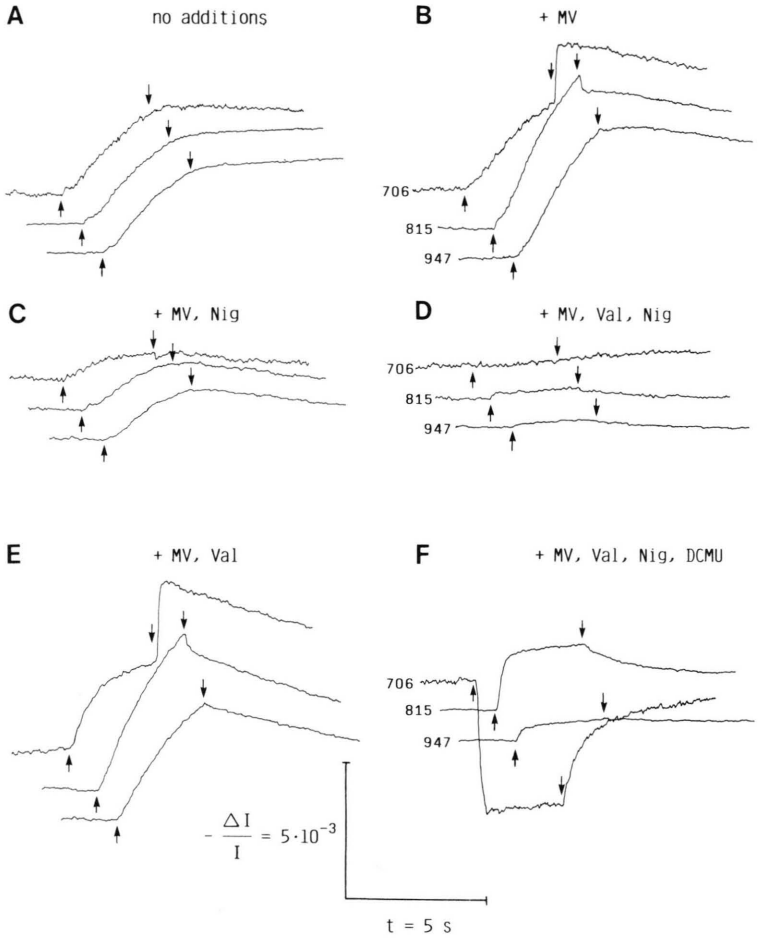

Fig. 1. Kinetics of light-induced absorbance changes of intact spinach chloroplasts in the NIR spectral region under a variety of experimental conditions. The changes at 706 nm (top traces), 815 nm (middle traces) and 947 nm (bottom traces) are displayed. The arrows mark the time points at which 30 $W \cdot m^{-2}$ blue light was switched on (↑) and off (↓). Chlorophyll concentration was 40 $\mu g \cdot ml^{-1}$. The chemical additions present under the various conditions are indicated in the figure for the different sets of traces A–F. The following concentrations were applied: methylviologen (MV), 5×10^{-4} M; nigericin (Nig), 5×10^{-7} M; valinomycin (Val), 5×10^{-7} M; DCMU, 5×10^{-5} M.

The PS II inhibitor DCMU stimulates the specific absorbance changes caused by oxidation of P700, namely a large bleaching around 706 nm, and an absorbance increase at longer wavelengths, which is approx. 3 times larger at 815 nm than at 947 nm (Fig. 1 F).

Light-induced absorbance changes due to P700 oxidation are also contributing to the kinetics in absence of DCMU; but then they are smaller and mostly hidden by the large non-specific changes (see Fig. 1 B, E). As can be judged from the rapid transients upon light-off, the relative contribution of P700 to the overall changes is largest at 706 nm, smaller at 815 and under the given conditions almost undetectable at 947 nm. Hence, in order to assess scattering changes with a minimal contribution of P700, a 947 nm measuring beam is advantageous.

By presence of the artificial acceptor methylviologen the overall electron transport rate and the transthylakoidal ΔpH are increased, which leads to a shift of the rate limiting step from the PS I acceptor side to the Cyt *b/f* complex and, consequently, to a stronger oxidation of P700 (compare Fig. 1 B with Fig. 1 A). Not unexpectedly, nigericin prevents P700 oxidation, even in presence of methylviologen, by removing the control at the *b/f* coupling site (Fig. 1 C). On the other hand, valinomycin even somewhat stimulates P700 oxidation (Fig. 1 E).

Fig. 2 A shows that a pronounced light-induced scattering change is also observed in broken chloroplasts (Class D), which have lost their stroma components and with it most of the ferredoxin. In addition, broken chloroplasts show a rapid light-induced bleaching, which becomes dominant when

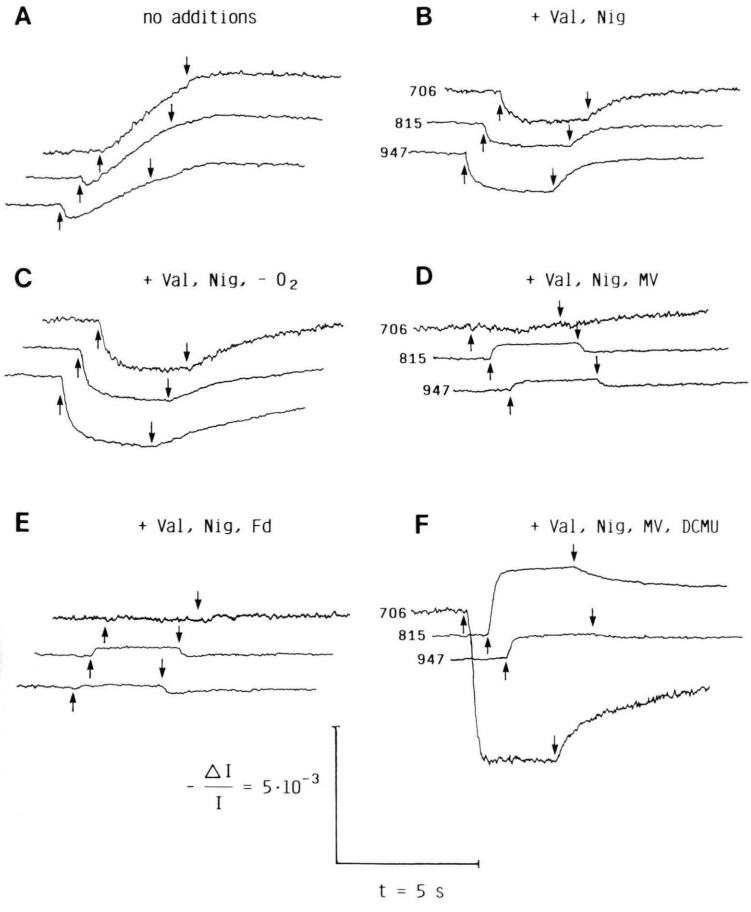

Fig. 2. Kinetics of light-induced absorbance changes of broken (Class D) chloroplasts in the NIR spectral region under a variety of experimental conditions. The conditions are indicated for the different sets of traces A–F in the figure. Concentrations were as detailed in Fig. 1. In (C) removal of oxygen ($-O_2$) was achieved by the glucose/glucose oxidase system, with 20 mM glucose, 30 units · ml^{-1} glucose oxidase and 3000 units · ml^{-1} catalase. In (E) the concentrations of ferredoxin (Fd) was 4×10^{-5} M.

the scattering change is abolished by uncoupling with nigericin/valinomycin (Fig. 2B). The spectrum of this bleaching seems to be rather flat in the given wavelength region, increasing towards longer wavelengths. Comparison of the data presented in Fig. 1A and 2A, B leads to the suggestion that the bleaching signal is stimulated by a loss of PS I acceptors, as occurring with the rupture of the chloroplast envelope. This suggestion is supported by the observation that the amplitude of the bleaching is doubled by removal of O_2 (Fig. 2C). Conversely, the signal disappears in presence of suitable electron acceptors, as methylviologen (Fig. 2D) and ferredoxin (Fig. 2E). Under these conditions a rapid absorbance increase becomes apparent particularly at 815 nm. As no corresponding change is found at 706 nm, on first sight this absorbance increase appears not to be due to a P700 change. However, as will be discussed below, a combination of P700 and PC oxidation can well account for the observed kinetic and spectral behaviour (see text accompanying Table I).

In Fig. 3 the amplitudes of the light-induced absorbance changes at 815 and 947 nm of broken, uncoupled chloroplasts are plotted *versus* ferre-

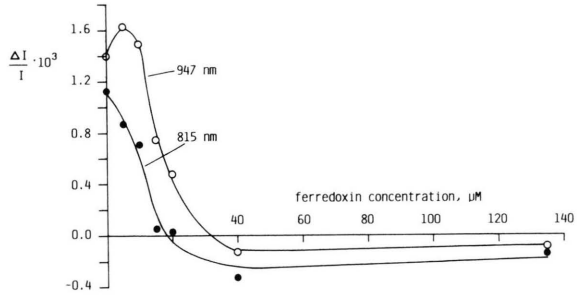

Fig. 3. Dependence of the light-induced absorbance changes at 815 and 947 nm in Class D chloroplasts on the concentration of added ferredoxin. Conditions as for Fig. 2B, E. The amplitudes of the changes observed 1 s after light-on are plotted.

doxin concentration. Half-maximal suppression of the absorbance increase is observed at 10–15 μM ferredoxin. This concentration agrees well with that found to catalyze linear and cyclic electron flow [20]. It may be noted that the 947 nm curve is showing still another bleaching reaction which is stimulated at 5 μM ferredoxin. The origin of this bleaching is not clear and will not be further discussed in the present communication. With the present stand of information, it appears that the rapid broad band bleaching, which is observed between 700 and 950 nm is reflecting the reduction of a PS I acceptor before ferredoxin. Because of its extremely negative redox potential this component will not accumulate in its reduced form under normal conditions, *i.e.* in presence of ferredoxin and other secondary acceptors present in the stroma.

As with intact chloroplasts (see Fig. 1F) also with broken chloroplasts the most pronounced absorbance changes due to P700 oxidation are found in presence of DCMU (Fig. 2F). This PS II inhibitor prevents P700 reduction by electrons from water splitting. Furthermore, in the example of Fig. 2F, full P700 oxidation is ensured by presence of methylviologen, and energy-dependent conformational changes are suppressed by uncoupling conditions. Under these conditions, not only P700 but also all PS I donors, and PC in particular, will become fully oxidized upon illumination. PC also causes an absorbance increase in the NIR upon oxidation [13], the contribution of which to the overall change can be determined by redox titration, as will be shown below (see section on *Redox titration of P700 and PC changes*).

Identification of a field-indicating absorbance change around 730 nm

It is well-known that a field across the thylakoid membrane is indicated by an absorbance increase around 515 nm, the so-called P515 change [1, 21, 22]. Following transmembrane charge separation by a light flash, formation and relaxation of the membrane potential can be monitored *via* P515. As shown in Fig. 4, there is a rapid flash-induced bleaching around 730 nm which relaxes with kinetics very similar to those of P515. For comparison the P515 response was also measured at 535 nm, the lowest wavelength presently available with a recently developed LED array spectrophotometer

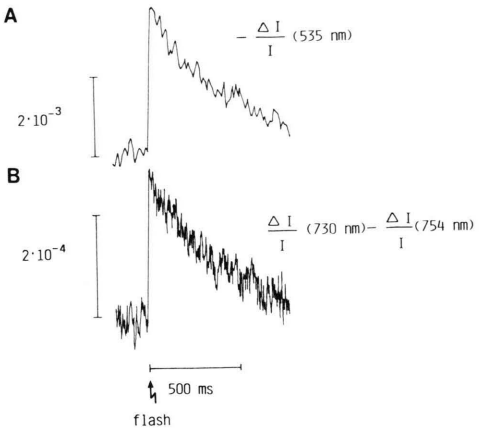

Fig. 4. Comparison of field-indicating, flash-induced absorbance changes in the green and NIR spectral region. A. Measurement with LED array spectrophotometer (see Materials and Methods) at 535 nm. B. Dual wavelength measurement (730–754 nm) to eliminate broadband changes caused by P700 and PC. Intact chloroplasts in the presence of 5×10^{-7} M nigericin; repetitive illumination (3 s dark intervals) with single turnover saturating flashes; the averages of 8 individual relaxation kinetics are displayed. Chlorophyll concentration 40 μg/ml.

[17]. The flash-induced bleaching around 730 nm as well as the P515 change, are fully suppressed by 10^{-6} M valinomycin (data not shown).

In Fig. 5 the ± valinomycin difference spectrum of the absorbance change observed 50 ms after a

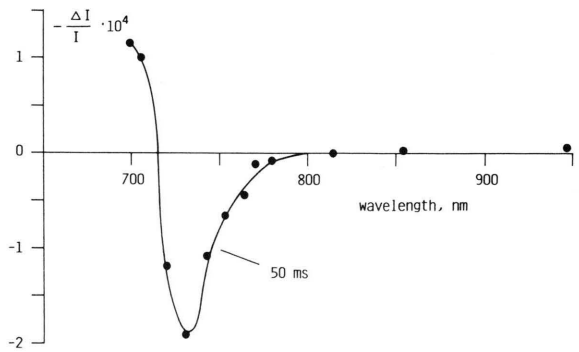

Fig. 5. Difference spectrum of field-indicating absorbance change around 730 nm, derived from flash-induced changes in the absence and presence of valinomycin. Conditions as for Fig. 4B, except that chlorophyll concentration was 20 μg/ml. For each data point two fresh samples were used, one without valinomycin and one with 5×10^{-7} M valinomycin. The plotted points correspond to the differences between the absorbance changes, 50 ms following a flash in the absence and presence of valinomycin.

single turnover saturating flash is shown. The field-indicating absorbance band peaks around 730 nm and displays a half-band width of approx. 25 nm. The differential extinction coefficient at 730 nm is about one order of magnitude smaller than at 515 nm. Still, the significance of the 730 nm change should not be underestimated. First, very strong measuring light may be applied without actinic effect. Second, the spectrum of background signals in the 730 nm region is flat, so that deconvolution of the field-indicating change can be easily achieved. And, third, due to an enhanced pathlength of 730 nm light in highly scattering intact leaves, there the apparent differential extinction coefficient of the 730 nm change is significantly larger than in isolated chloroplasts (Klughammer and Schreiber, in preparation).

The field-indicating 730 nm change is also readily detected upon continuous illumination, if conditions are chosen to minimize the contributions of other rapid changes, *i.e.* of P700, PC and of the component at the PS I acceptor side described above (see Fig. 2A–C). In the experiment of Fig. 6 intact chloroplasts in presence of nigericin were illuminated by continuous blue light. As may be expected from the difference spectrum shown in Fig. 5, in the absence of valinomycin there is a rapid light-induced absorbance decrease at 730 nm, reflecting the formation of a membrane potential. In addition there is the slow scattering change discussed above (see Fig. 1), which is not completely suppressed by nigericin. In presence of valinomycin/nigericin the rapid bleaching and the slow scattering change have disappeared, but now a relatively slow bleaching can be distinguished, which remains to be explained. The light-dark difference spectra displayed in Fig. 7 (obtained in presence and absence of valinomycin) reveal that the valinomycin-insensitive absorbance change decreases towards longer wavelengths. The difference spectrum of this valinomycin-insensitive bleaching resembles the reduced minus oxidized spectrum of ferredoxin, which is also displayed in Fig. 7 (dotted line). For a definite identification of ferredoxin it would be necessary to extend the absorbance measurements below 700 nm, which is a difficult task because of the artifacts caused by fluorescence excitation. As will be shown below, in principle with isolated chloroplasts the fluorescence contribution can be minimized by the use of an efficient fluorescence quencher. But, the commonly used quinone-type quenchers at the same time act as electron acceptors and, hence, do not allow accumulation of reduced ferredoxin. Also dinitrobenzene, another efficient fluorescence quencher, turned out to prevent ferredoxin-reduction (data not shown), which may not be surprising in view of the fact that it induces photochemical fluorescence quenching [23].

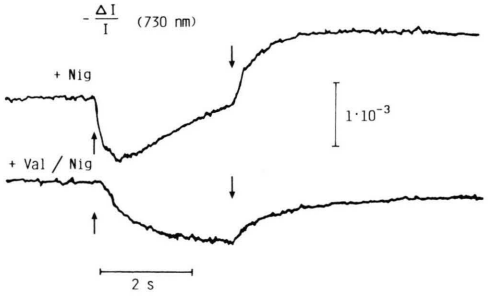

Fig. 6. Effect of valinomycin on light-induced absorbance changes at 730 nm in intact chloroplasts. Continuous illumination with 30 $W \cdot m^{-2}$ blue light. Chlorophyll concentration, 40 $\mu g \cdot ml^{-1}$. Nigericin (Nig), 5×10^{-7} M; valinomycin (Val), 5×10^{-7} M.

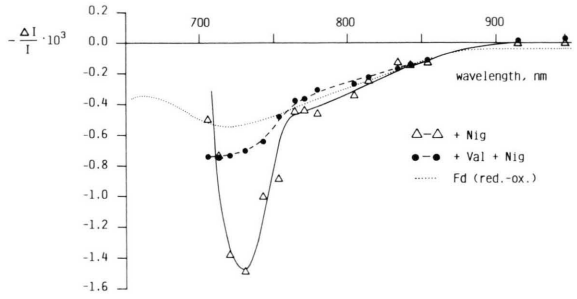

Fig. 7. Light-dark difference spectra of absorbance changes induced by continuous illumination of intact chloroplasts and reduced minus oxidized difference spectrum of ferredoxin. The data points were obtained from kinetic traces as shown in Fig. 6, the amplitudes corresponding to the changes observed 0.5 s following light-on. The dotted line represents a difference spectrum obtained upon reduction of 0.9 μM ferredoxin by dithionite.

Light-induced changes by P700 and PC

After having characterized a number of possible absorbance changes in the NIR by the experiments summarized in Fig. 1–7, it is possible to define

conditions to minimize all contributions except for those of P700 and PC, which then can be investigated separately in more detail. Such conditions include presence of valinomycin/nigericin, to suppress the scattering and field-indicating changes, presence of methylviologen, to prevent accumulation of reduced PS I acceptors; and in order to obtain maximal P700 and PC oxidation it is necessary to inhibit PS II by DCMU (see Fig. 2F for typical kinetic responses). Under these conditions, a detailed light-dark difference spectrum was measured, which is shown in Fig. 8. In order to extend the spectrum into the wavelength region of chlorophyll fluorescence excitation, 10^{-4} M juglone was added, which is an effective quencher of fluorescence [24]. Under the given conditions, this quinone quenched 50% of initial fluorescence and 95% of variable fluorescence (data not shown). The depicted difference spectrum corresponds to the absorbance changes induced by 16 $W \cdot m^{-2}$ blue light within 3 s. The spectrum has been corrected for particle flattening, using the differential flattening factors published by Pulles [25] at wavelengths below 710 nm (see broken line in Fig. 8). For $\lambda > 710$ nm the factor was assumed to be unity.

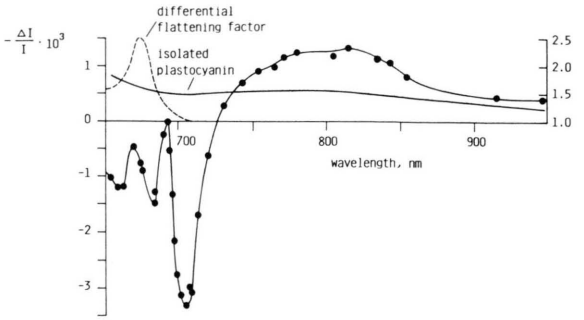

Fig. 8. Light-dark difference spectrum of absorbance changes of Class D chloroplasts in the red and near-infrared spectral region. The data points correspond to the changes observed 3 s after onset of actinic, blue illumination (16 $W \cdot m^{-2}$). Conditions essentially as for Fig. 2F, with presence of 5×10^{-4} M methylviologen, 5×10^{-7} M valinomycin, 5×10^{-7} M nigericin and 2×10^{-5} M DCMU. In addition, 10^{-4} M juglone was present to quench chlorophyll fluorescence and 1000 units $\cdot ml^{-1}$ catalase was added to decompose H_2O_2 formed in presence of methylviologen. Chlorophyll concentration, 20 $\mu g \cdot ml^{-1}$. For comparison, also a oxidized minus reduced spectrum of isolated plastocyanin is shown, normalized at 815 nm to 37% of the light-induced change (see text). The right-hand scale applies to the wavelength-dependent differential flattening factors of chloroplasts (broken line) (see text).

Comparison of this difference spectrum with that of Hiyama and Ke [26] for PS I particles confirms that it is dominated by the oxidized minus reduced P700 spectrum with its characteristic bleaching bands around 680 and 700 nm, and its broad absorption band between 750 and 850 nm. However, there are some differences between our spectrum and that of Hiyama and Ke, which shall be briefly discussed:

The ratio of maximum bleaching at 706 nm to the maximum absorbance increase at 815 nm is only 2.5, while it was 8 with the Hiyama–Ke preparation. One cause for this difference should be a significant contribution of a PC change in the case of our spectrum, by which an overall upward shift is produced. The difference spectrum of oxidized minus reduced isolated spinach PC is also shown in Fig. 8, normalized to a 37% contribution to the total change at 815 nm (for justification of this assumption, see section on *Redox titration of P700 and PC changes* below). When this PC change is subtracted, the 706/815 ratio is increased from 2.5 to 4.9. Compared to the ratio of 8, there still remains a factor of about 1.6 to be explained. We suggest that this results from a longer effective pathlength of the 815 nm measuring light as compared to that of the 706 nm light, because the probability for multiple scattering is enhanced at extremely low absorbance of the sample. This explanation is supported by the fact that an increase of the refraction coefficient by addition of 7% Ficoll to the reaction medium reduces the absorbance increase at 815 nm by 26% without affecting the bleaching at 706 nm (not shown). Furthermore, as will be shown in a consequent publication (Klughammer and Schreiber, in preparation) the 706/815 ratio is still lower than 2.5 in highly scattering intact leaves.

Redox titration of P700 and PC changes

P700 and PC are kinetically so closely linked that separation of their individual light-induced changes is only possible by flash relaxation experiments [27]. A determination of the maximal contribution of P700 and plastocyanin to the overall light-induced absorbance changes in the NIR can be achieved, however, on the basis of their different redox potentials. For this purpose, broken

chloroplasts are incubated in reaction media adjusted to different redox potentials by ferro-/ferricyanide mixtures. According to their difference in redox potentials, PC and P700 will be chemically pre-oxidized to different extents. The extent of pre-oxidation can be determined from the amplitudes of the complementary changes induced upon full oxidation by far-red illumination in presence of DCMU. As shown in Fig. 9, without addition of a redox mediator, an incubation time of 15 min proved necessary to reach full equilibration after addition of 1 mM ferricyanide to a broken chloroplast suspension.

Using this approach, we have carried out redox titration measurements in the 200–600 mV range of the light-induced absorbance changes at 706, 815 and 947 nm. The results are presented in Fig. 10A, B. The data correspond to two independent titration experiments, with the changes at 815 nm being measured in paralleled with those at 706 nm and 947 nm, respectively. The data points were fitted to titration curves on the basis of the Nernst equation assuming the involvement of two components with single electron steps. Fitting involved the assumption of different combinations of redox potentials and relative contributions of P700 and PC to the absorbance changes at the three wavelengths. The outcome is presented in Table I. The relative contributions of P700/PC are −119/19 at 706 nm, 63/37 at 815 nm and 31/69 at 947 nm, when mid-point potentials of 470 mV for P700 and 370 mV for PC are assumed. These potentials correspond closely to those reported in the literature [28, 29].

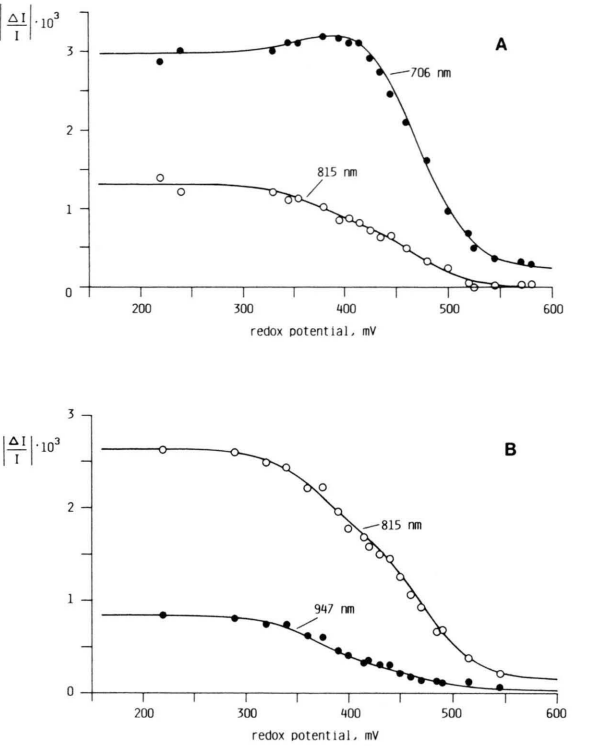

Fig. 10. Light-induced absorbance increases at 706, 815 and 947 nm of Class D chloroplasts in dependence of redox potential after 15 min incubation of appropriate ferro-/ferricyanide mixtures. A. Chlorophyll concentration, 20 μg·ml^{-1}. The 706 nm measuring beam was admitted only briefly before actinic illumination to avoid an actinic effect. B. Chlorophyll concentration, 40 μg·ml^{-1}. Other conditions as for Fig. 8 and 9, except that juglone was not present. See also Materials and Methods for redox potential measurements.

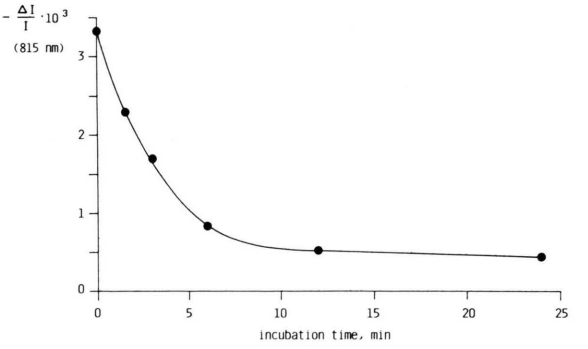

Fig. 9. Light-induced absorbance increase at 815 nm of Class D chloroplasts in dependence of the incubation time of 10^{-3} M ferricyanide. Data points correspond to the changes at 1 s after onset of 30 W·m^{-2} blue illumination. After ferricyanide addition, a redox potential of approx. 500 mV was established. Other conditions, as detailed for Fig. 8.

Table I. Information on specific absorbance changes by P700 and PC derived from the redox titration data of Fig. 10 and 11. For conditions, see legends of Fig. 10 and 11. The 706 nm data were corrected for the particle flattening effect (see Fig. 8 and accompanying text).

		P700	Plastocyanin
Relative	at 706 nm	−119	19
absorption	at 815 nm	63	37
change	at 947 nm	31	69
Midpoint potential E_0', mV		470	370
Slope (947 *vs.* 815)		0.16	0.60
Slope (706 *vs.* 815)		3.80	0.95

Additional information can be obtained when the titration data of Fig. 10 are plotted in a different way. In Fig. 11A, B the far-red induced changes at 706 nm and 947 nm are plotted against the simultaneously measured changes at 815 nm. Both graphs display two straight line segments which correspond to the sequential oxidation of PC and P700 with increasing redox potential. The slopes of these lines reflect the ratios of the differential molar extinction coefficients of PC or P700 at the two wavelengths. These ratios, which are listed in Table I, will be used below in the calculation of the molar ratio of P700/PC. In the plots of Fig. 11A, B, starting from the upper right corner, there is first selective oxidation of PC. At the crossing point of the two lines full PC oxidation is reached and P700 oxidation begins. Towards the lower left corner the data points merge into a line segment which corresponds to selective P700 oxidation.

At 706 nm P700 and PC cause opposite absorbance changes upon oxidation, while at 815 and 947 nm both changes go in the same direction. From these features it is clear that under certain conditions there may be distinct absorbance changes at 815 and 947 nm, reflecting joint contributions of P700 and PC oxidation, whereas no overall change is indicated at 706 nm, when the opposing contributions of P700 and PC are cancelling each other at this wavelength. This consideration may provide an explanation for the results of Fig. 2E. Obviously, the lack of bleaching at 706 nm cannot always be interpreted as a lack of P700 oxidation.

The presented data show that the contribution of PC oxidation to the overall changes is much bigger at 947 nm than at 815 nm (69% at 947 nm as compared to 33% at 815 nm). Hence, 947 nm is advantageous for monitoring preferentially PC, while 815 nm is more suited for monitoring P 700. Provided the molar ratio of P700/PC is constant, it should be possible to derive the selective P700 and PC changes from measurements at these two wavelengths. This approach should not be affected by varying degrees of sample scattering, as this should have equivalent effects on the effective pathlengths of 815 and 947 nm measuring light, for both of which absorbance is extremely low.

With the knowledge of the P700 and PC contributions to the overall absorbance changes, the molar ratios of these two components can be derived, when the differential molar extinction coefficients are known. Actually, it is sufficient to know the ratios of the coefficients which should not be influenced by the scattering properties of the sample. If one ratio is known (*e.g.* at 815 nm) the ratios at 706 nm or 947 nm can be calculated from the slopes of the plots in Fig. 11A, B, which correspond to the ratios of the molar extinction coefficients, as listed in Tab. I (see above). In order to

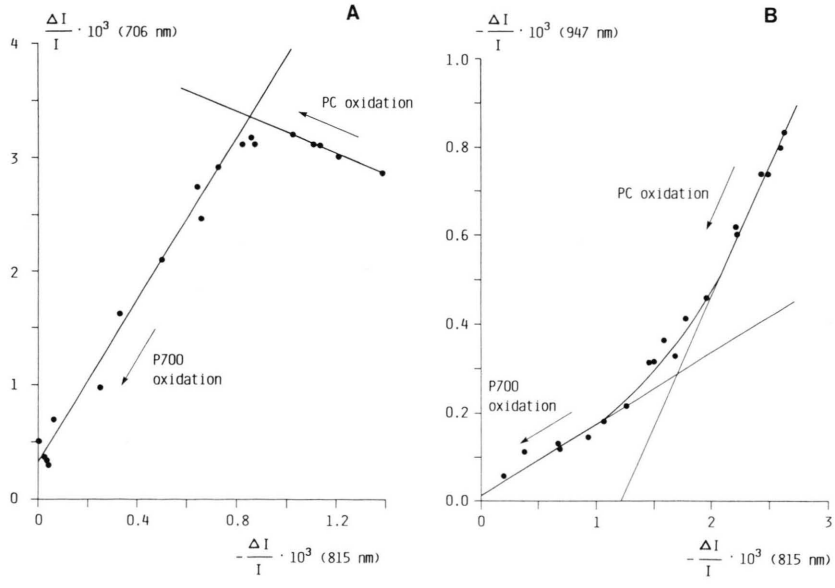

Fig. 11. Sequential oxidation of plastocyanin (PC) and P700 with increasing redox potential, as reflected by plots of remaining light-induced absorbance changes at 706 *vs.* 815 nm (A) and 947 *vs.* 815 nm (B). The original data points of Fig. 10 are replotted. The slopes of the straight line segments representing specific PC and P700 oxidation are listed in Table I. The crossing points mark full PC oxidation and start of P700 oxidation.

determine the molar extinction coefficient of PC at 815 nm we have measured an oxidized minus reduced difference spectrum of isolated PC extending into the NIR. The spectrum is shown in Fig. 12. It is normalized at the 597 nm peak to a differential molar extinction coefficient of 4.9 mM^{-1} cm^{-1} [13]. A coefficient of 1.6 mM^{-1} cm^{-1} can be derived for 815 nm. For P700 we adopt a value of 8 mM^{-1} cm^{-1} which was determined previously with PS I particles [26]. On the basis of these molar extinction coefficients and the observed ratio of 63/37 for P700/PC in the 815 nm signal (Fig. 10, 11 and Table I) the molar ratio can be calculated according to the equation: P700/PC = (63/37) × (1.6/8) = 1/2.94. Equivalent calculations from the ratios at 706 nm and 947 nm give values of 1/3.15 and 1/2.93, respectively. In average, this corresponds to a molar stoichiometry of 3 PC per P700, in close agreement with a recent determination for spinach thylakoids by Haehnel *et al.* [30] using a combination of rocket immunoelectrophoresis and flash spectroscopy.

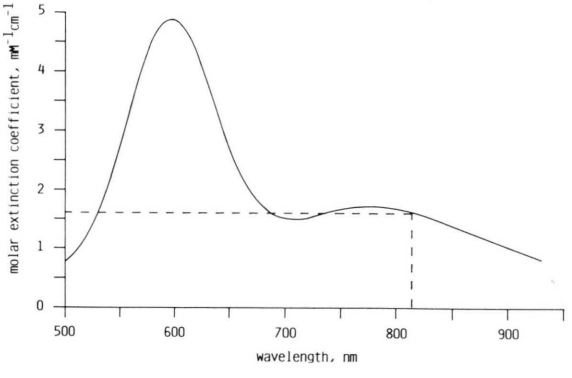

Fig. 12. Wavelength dependence of differential molar extinction coefficient of isolated plastocyanin derived from an oxidized minus reduced difference spectrum normalized at 597 nm to 4.9 $mM^{-1} \cdot cm^{-1}$. A 12 μM solution of PC in medium C (pH 7.6) was oxidized by 10^{-4} M ferricyanide and the difference spectrum measured in the split beam mode (see Materials and Methods).

Conclusions

The presented data reveal a remarkable complexity of different types of absorbance changes in the NIR. Part of these changes, like the bleaching reaction shown in Fig. 2A–C and 3, are unlikely to occur under *in vivo* conditions. Others, like the rapid bleaching characterized in Fig. 6, 7, will play a significant role under special conditions, as after dark adaptation or O_2 removal. Knowledge of the existence of such changes and of the conditions under which they are likely to occur will help to avoid misinterpretations of "P700 changes". Furthermore, these newly characterized absorbance changes may provide some important information on photosynthetic electron transport, in particular on aspects of the PS I acceptor side and possibly cyclic flow around PS I.

As was already realized by Harbinson and Hedley [11], PC contributes considerably to the overall absorbance change at 815 nm when P700 and its donors become oxidized. We have determined a 37% contribution by redox titration. While it is true that such contribution does not disturb kinetic measurements, as P700 and PC equilibrate rapidly, there is a problem with $P700^+$ determination. The redox potentials of P700 and PC differ by 100 mV and under moderately oxidizing conditions there will be a tendency of PC being more oxidized than P700. This aspect should be considered when estimating PS I quantum efficiency from the absorbance change at 815 nm. Also measurements of P700 at its bleaching peak around 703 nm have to account for a contribution of PC, which at this wavelength has an opposite direction (Fig. 8). As we showed in the analysis of Fig. 2D, opposite changes of P700 and PC may cancel each other, and care must be taken not to misinterpret such behaviour in terms of a lag phase in P700 oxidation.

The isolated chloroplasts used in the present study showed large scattering changes, which however displayed a very flat difference spectrum (Fig. 1, 2). Hence, they are easily removed in dual or multi-wavelength measurements. Also, such changes can be avoided by addition of valinomycin/nigericin. On the other hand, they may be a very useful tool for the study of membrane energization, similar to changes in the green region, characterized and applied by Heber and co-workers [18, 19, 31]. A possible advantage of NIR measurements would be the lack of other large absorbance changes in the 950 nm region and the possibility of using very strong measuring light without actinic effect.

Although the field-indicating absorbance change identified around 730 nm is relatively small

(Fig. 4, 5) it still may be expected to prove quite useful for *in vivo* analysis of photosynthesis. As was already concluded from a comparison of the apparent differential extinction coefficients of P700 at 706 and 815 nm in PS I particles and chloroplasts (see text accompanying Fig. 8), the absorbance changes are amplified considerably when the samples cause multiple scattering of the measuring light. This effect is particularly strong in intact leaves [32–34]. Furthermore, because of the extremely low absorption at 730 nm, a very strong measuring light can be applied without actinic effect, and the transmitted signal is high. It remains to be clarified from what type of pigment molecules the 730 nm change originates. The bleaching at 730 nm could be due to an electrochromic blue shift of an absorbance band of a rather long-wavelength absorbing species. An electrochromic blue shift resulting in bleaching around 505 nm has been considered before [35].

In the next publication of this series we will present an analysis of absorbance changes in the NIR region with intact leaves. Eventually, it is our aim to assess the various components in the NIR by multi-wavelength analysis using a similar approach as recently described for the green spectral region [17].

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