THE INFLUENCE OF MEMBRANE POTENTIAL ON MEASUREMENTS OF C-550 AT ROOM TEMPERATURE

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1. Introduction

Knaff and Arnon [1] first described a light-induced absorbance change in chloroplasts in the region of 550 nm which was closely associated with the primary photochemical activity of photosystem II. The light-induced bleaching, which occurred in the presence of ferricyanide added to oxidize cytochrome *f*, was sensitized more efficiently by red light than far-red light, was not blocked by DCMU and occurred at the temperature of liquid nitrogen as well as at room temperature. They suggested that the bleaching was due to a new photosynthetic electron carrier, C-550, which, on the basis of the action of electron donors to Tris-washed chloroplasts, was photoreduced by photosystem II.

Erixon and Butler [2] showed that the light-induced absorbance change of C-550 did represent a reduction and could be achieved chemically in the dark with strong reductants such as dithionite or borohydride but not with ascorbate. They also correlated the absorbance change with photosystem II activity by showing that the change was absent in photosystem I particles and in a high fluorescence mutant of Scenedesmus, no. 11, which lacked photosystem II activity. A redox titration of the light-induced absorbance changes of C-550 at $-196°$ and the light-induced fluorescence yield changes at $-196°$ showed a one-to-one correspondence [3]. On the basis of the isomorphic relationship between C-550 and fluorescence yield found in low temperature measurements Erixon and Butler proposed that C-550 was analogous to Q, the fluorescence quencher, and that C-550 was either the primary electron acceptor of photosystem II or an accurate indicator of the redox state of the primary acceptor.

The correlation between C-550 and Q, however, was challenged by Ben-Hayyim and Malkin [4] who examined the relationship between C-550 and fluorescence yield in room temperature measurements in the presence of DCMU. They found a reasonably good correlation when low intensities of red light were used but concluded, on the basis that the correlation broke down in far-red light and under other conditions, that the correlation in low intensity red light was fortuitous and the C-550 did not correspond to Q.

The purpose of the present work was to re-examine the room temperature measurements of C-550 in an effort to resolve the differences between the room temperature and low temperature measurements. At low temperature C-550 responds to the primary photochemistry of photosystem II and indicates the redox state of the primary electron acceptor. In the work reported here it is shown that at room temperature C-550 responds not only to the primary photochemistry but also to membrane potential. Since membrane potential does not have a direct influence on fluorescence yield the correlation between C-550 and fluorescence yield may not hold at room temperature whereas at low temperature, where only the effects of the primary photochemistry are manifest, the correlation is valid.

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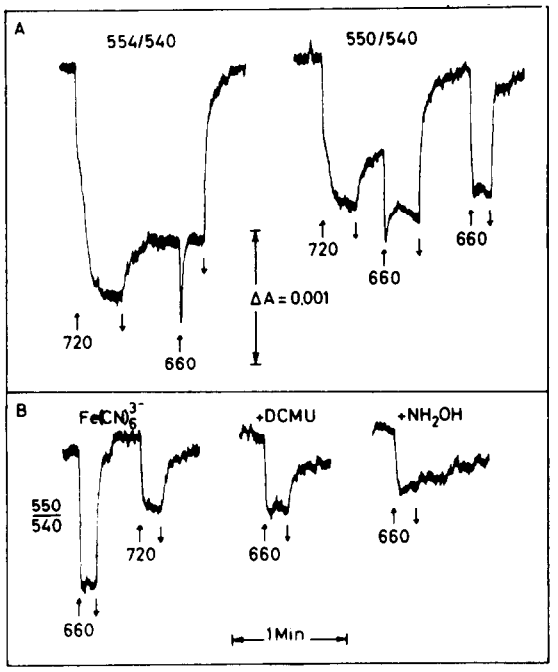

Fig. 1. Light-induced absorbance changes of spinach chloroplasts (100 μg/ml) at 554 and 550 nm relative to 540 nm due to irradiation with 720 nm light (1 μwatt/cm²) and 660 nm light (3 μwatts/cm²). A) With no additions. B) With sequential and cumulative additions of 3.3 mM ferricyanide, 5 μM DCMU and 100 μM hydroxylamine.

The low temperature measurement of C-550 is important because it provides an assay to determine whether particular treatments or mutations affect the photosystem II reaction centers directly. The assay focuses on a very limited region of the electron transport chain: measurements of C-550 at low temperature are not affected by DCMU or Tris washing which block electron transport on either side of photosystem II. The low temperature assay method does not require that experiments be done at low temperature. In general the experiments are carried out at room temperature and samples are taken and frozen to −196° to determine the state of, or the amount of, C-550.

2. Methods

Spinach chloroplasts with a good yield of "Class 1 chloroplasts" were prepared by the method of Kraayenhof [5]. The chloroplasts were suspended at 100 μg chlorophyll/ml in 0.4 M sucrose, 10 mM KCl, 2 mM $MgCl_2$, 50 mM tricine buffer, pH 7.5, in a 1-cm cuvette. For measurements of C-550 3.3 mM ferricyanide was added to oxidize cytochrome *f*.

Spectrophotometric measurements were made with an Aminco–Chance dual wavelength spectrophotometer with the measuring beams set at 554 and 540 nm for measurements of cytochrome *f*, at 550 and 540 nm for C-550, at 518 and 540 nm for the 518 nm change, and at a variable wavelength and 540 nm for difference spectra. Filters placed between the sample and the phototube transmitted the green measuring beams but not the red or far-red actinic light. The actinic light from a tungsten lamp through 660 or 720 nm interference filters was orthogonal to the measuring beams.

3. Results and discussion

Light-induced absorbance changes in spinach chloroplasts at 554 and 550 nm relative to 540 nm measured in the absence of an electron acceptor are shown in fig. 1. The general pattern of absorbance changes at 554 nm due to irradiation with 720 and 660 nm light has been reported previously [6, 7]. Cyt *f* which is normally in the reduced state in the dark is fully oxidized by far-red light and, in the absence of an electron acceptor, remains largely oxidized in the subsequent dark period. After an irradiation with red light, cyt *f* returns to its fully reduced state in the dark. It is apparent, however, by comparing the relative steady state levels of bleaching in red and far-red light measured at 554 and 550 nm (fig. 1A) that a photobleachable component other than cyt *f* is involved in the changes measured at 550 nm. Knaff and Arnon demonstrated such a component, which they labeled C-550 [1], in chloroplasts by showing that a photobleachable component with an absorption maximum near 550 nm remained in the presence of ferricyanide which oxidized cyt *f* prior to the actinic irradiation. Fig. 1B shows the light-induced bleaching at 550 nm (relative to 540 nm) in the presence of 3.3 mM ferricyanide. The bleaching at 550 nm induced by irradiation with 660 nm light was almost as great in the pres-

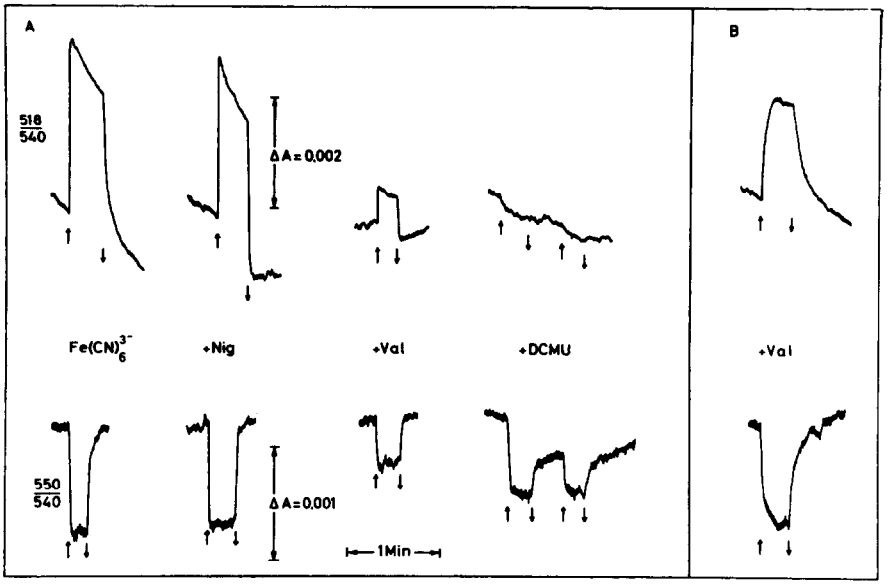

Fig. 2. Light-induced absorbance changes at 518 and 550 nm relative to 540 nm due to irradiation with 660 nm light (3 μwatts/cm^2); on at upward arrows, off at downward arrows. A) With sequential and cumulative additions of 3.3 mM ferricyanide, 0.5 μM nigericin, 0.1 μM valinomycin, and 5 μM DCMU. B) With 3.3 mM ferricyanide and 0.1 μM valinomycin.

sence of ferricyanide as in the absence but 720 nm light was much less effective in the presence of ferricyanide. The sharp negative spike seen in fig. 1A on turning on the 660 nm light may be ascribed to a rapid bleaching of C-550 followed by a photoreduction of cyt *f*. Likewise the small recovery of absorbance observed on turning off the 720 nm light should be due in part to dark oxidation of the C-550 photoreduced by the 720 nm light. Knaff and Arnon reported that DCMU did not limit the extent of the light-induced bleaching of C-550 in the presence of ferricyanide, but did slow the dark recovery. In our experiments (fig. 1B) DCMU did limit the extent of the bleaching as well as slow the dark recovery. The dark recovery was slowed much more in the presence of DCMU plus hydroxylamine.

The slower dark recovery of C-550 in the presence of DCMU is consistent with DCMU blocking electron transport between C-550 and subsequent electron transport carriers between C-550 and photosystem I. The much prolonged dark recovery of C-550 in the presence of DCMU and hydroxylamine is probably due to a redox reaction between hydroxylamine and the oxidized primary electron donor, Z^+, which prevents the back reaction between reduced C-550 and Z^+. It was shown previously that DCMU plus hydroxylamine prevents the dark decay of the fluorescence yield [8] and that C-550, as measured by the low temperature assay, remains fully reduced in the dark after irradiation [3].

Light-induced absorbance changes in the presence of 3.3 mM ferricyanide were measured at 550 and 518 nm relative to 540 nm with and without ionophorous antibiotics and DCMU (fig. 2). Nigericin, a potent uncoupler of chloroplasts in the presence of potassium, had essentially no effect on either light-induced absorbance change (fig. 2A). However, nigericin plus valinomycin markedly decreased both absorbance changes. (The addition of reagents was cumulative so that the curves marked Val in fig. 2A represent the absorbance changes measured in the presence of ferricyanide, nigericin and valinomycin). Valinomycin without nigericin (fig. 2B) decreased the magnitude of the 518 nm change to a small extent, had little effect on the magnitude of the C-550 change and slowed the initial rates of both light-induced absorbance changes. Subsequent addition of nigericin to chloroplasts in the presence of valinomycin

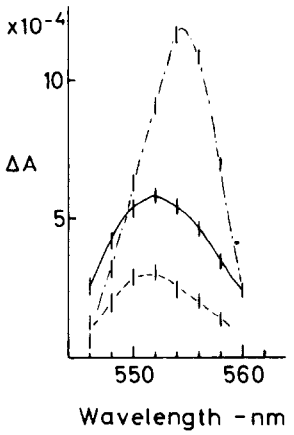

Fig. 3. Spectra of steady state light-induced absorbance changes relative to 540 nm. Absorbance changes induced by 660 nm light (3 μwatts/cm^2) in the presence of 3.3 mM ferricyanide (——); absorbance changes induced by 660 nm light in the presence of 3.3 mM ferricyanide and 5 μM DCMU (- - - -); absorbance changes induced by 720 nm light (1 μwatt/cm^2) in the presence of 5 μM DCMU and 1 mM ascorbate (– · – · –). The latter spectrum should be due predominantly to cyt *f*.

produced the same results as the addition of valinomycin to chloroplasts containing nigericin. Addition of DCMU to chloroplasts containing nigericin and valinomycin (fig. 2A) or to untreated chloroplasts (not shown) eliminated steady state changes of the 518 nm absorbance. The light-induced absorbance change at 550 nm, however, remained when DCMU was added in addition to valinomycin and nigericin and were of about equal amplitude to changes measured in the presence of DCMU without valinomycin and nigericin (fig. 1B). The effects of nigericin and valinomycin on the 518 nm change in spinach chloroplasts are similar to those reported by Jackson and Crofts [9] for bacterial chromatophores in continuous illumination.

Measurements of the absorbance at 518 nm are taken as indicating light-induced changes of membrane potential [10]. The comparison of absorbance changes at 550 and 518 nm indicates that a part of the absorbance change at 550 nm is sensitive to the membrane potential but not to the pH gradient, since nigericin in the presence of KCl has no effect on either the 518 or 550 nm absorbance changes. Under conditions where the membrane potential is largely or completely dissipated, as indicated by the 518 nm change, the 550 nm change is decreased but not eliminated. A part of the 550 nm change is independent of the membrane potential. It is not surprising that DCMU should eliminate that part of the C-550 change which responds to membrane potential but it should not eliminate the part due to the photochemical reduction of the primary electron acceptor. DCMU may change the extent of the C-550 change which is related to the primary photochemistry. The block of electron transport could cause the C-550 change to increase because the primary electron acceptor would become more fully reduced during irradiation but the intensity of the measuring beams may be sufficient in the presence of DCMU to keep the primary electron acceptor partially reduced so that less is available for photoreduction by the actinic light. The latter effect appears to be relatively small, however, with the monochromator slits set at 0.75 mm since changing the intensity of the measuring beam 16-fold by increasing the slits from 0.25 to 1.0 mm did not decrease the light-induced C-550 change in the presence of DCMU by more than about 10%. The C-550 changes measured in the presence of valinomycin and nigericin should be less subject to these uncertainties. A more precise separation of the C-550 change into the parts due to the primary photochemistry and to the membrane potential presumably could be made in a detailed study of the effects of DCMU and ionophorous antibiotics on the fluorescence yield changes as well as the absorbance changes but the measurements of fluorescence yield and absorbance would have to be made under identical conditions with the same (or identical) samples, measuring beams and actinic light. The purpose of the present paper is not to determine quantitatively the amounts of the C-550 change due to the primary and secondary photochemical processes but only to show that both processes contribute to the C-550 measurements at room temperature.

Ben-Hayyim and Malkin [4] suggested there might be two types of C-550 molecules on the basis of biphasic kinetic data. The fast phase which accounted for about half of the C-550 change in high light intensities (the two phases were not so clearly resolved with low light intensities) followed the reciprocity law over a wide range of light intensities while the slower phase saturated at moderate intensities. They found no significant spectral differences between these two components.

Two types of C-550 changes are also reported here: one type which is independent of membrane potential and may ascribed tentatively to primary photochemical activity of photosystem II on the assumption that it has the same basis as the C-550 change measured at -196°, and an additional change which responds to membrane potential. Difference spectra for the C-550 changes measured in the presence of ferricyanide with and without DCMU are presented in fig. 3 (and compared against the spectrum of cyt *f* obtained from measurements of the far-red light-induced absorbance changes in the presence of DCMU and ascorbate). The two difference spectra for C-550 are essentially the same, except for magnitude, and are very similar to the difference spectra reported by Ben-Hayyim and Malkin. The difference spectrum measured in the presence of ferricyanide, nigericin and valinomycin (not shown) was the same as that measured in the presence of ferricyanide and DCMU.

At liquid nitrogen temperature the correlations between C-550 and fluorescence yield are excellent and have been established in a wide variety of experiments [3, 11–13]. The low temperature measurements indicate that C-550 is either the primary electron acceptor of photosystem II or a completely isomorphic indicator of the redox state of the primary acceptor. The measurements of C-550 at room temperature reported here indicate that it responds to the membrane potential as well as to the primary photochemical electron transfer. On this basis alone it should be suspected that correlations between C-550 and fluorescence yield might not be as good at room temperature as at low temperature. However, these correlations may be even further complicated by the findings of Murata and Sugahara [14] and Wraight and Crofts [15] that the fluorescence yield of chloroplasts at room temperature in the presence of a cofactor for cyclic photophosphorylation is dependent on the energy state of the membrane, specifically the pH gradient, as well as the redox state of Q. Thus at room temperature photosystem I activity may influence both the C-550 absorbance change and the fluorescence yield. An increase of the pH gradient by photosystem I activity would be expected to quench chlorophyll fluorescence (at a given redox level of Q) while the build up of a membrane potential would increase the C-550 absorbance change resulting in a negative correlation between fluorescence yield and C-550 bleaching. Other secondary photochemical effects of photosystem I on fluorescence yield in whole cells in the absence or presence of DCMU are also known [16, 17]. The lack of correlation between C-550 and fluorescence yield in room temperature measurements does not invalidate the correlation established at low temperature where the secondary influences due to membrane potential and pH gradient should not occur.

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