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# THE REDOX STATE OF CYTOCHROME $b_{559}$ IN SPINACH CHLOROPLASTS

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## SUMMARY

Spinach chloroplasts suspended in a mixture of redox buffers were titrated under anaerobic conditions by adding small amounts of oxidant (potassium ferricyanide) or reductant (reduced methyl viologen). At specific redox potentials, measured with a platinum electrode, samples of chloroplasts were removed, frozen to –196 °C, and single-beam absorption spectra were measured before and after actinic irradiation at –196 °C. The extent of the low temperature light-induced reduction of C-550 and oxidation of cytochrome $b_{559}$ was determined as a function of redox potential. Difference spectra between samples at different redox potentials were used to determine titration curves for the chemically induced changes. With intact chloroplasts at 0 °C the midpoint potential of cytochrome $b_{559}$ was found to be + 450 mV. Titration of Tris-washed chloroplasts at 20 °C showed that cytochrome $b_{559}$ is oxidized at lower potentials and over a wider range of potentials than in intact chloroplasts. A cytochrome $b_{559}$ with a still lower midpoint potential (dithionite reducible but not ascorbate reducible) was also demonstrated in intact chloroplasts which had not been subjected to treatments which modify the normal high potential cytochrome $b_{559}$.

## INTRODUCTION

The observations that cytochrome $b_{559}$ is photooxidized by Photosystem II at –189 °C[1] and that the low temperature photooxidation is stoichiometrically related to the photoreduction of the primary electron acceptor of Photosystem II[2] requires a reexamination of the role of cytochrome $b_{559}$ in photosynthetic electron transport. Earlier studies[3] showed cytochrome $b_{559}$ oxidation by far-red light and reduction by red light at room temperature which implied a function in the electron transport chain between two light reactions. The midpoint potential of cytochrome $b_{559}$, which should be an important clue to its function in electron transport, has been the subject of several investigations. Bendall[4] reported a value of + 370 mV from a titration of pea chloroplasts with the ferri–ferrocyanide couple under aerobic conditions. Fan and Cramer[5] titrated spinach cytochrome $b_{559}$ under anaerobic conditions and reported a midpoint potential of + 80 mV. Using particles from spinach chloroplasts enriched

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in cytochrome $b_{559}$ after fractionation by Triton X-100, Hind and Nakatani[6] reported a midpoint potential of + 55 mV under anaerobic conditions. Knaff and Arnon[7] reported a value of + 325 mV and Ikegani *et al.*[8] reported a value of + 320 mV for cytochrome $b_{559}$ in *Euglena*.

The wide range of values reported for the midpoint potential of cytochrome $b_{559}$ appears to be due to the susceptibility of the redox properties of this cytochrome to change. A number of treatments lower the midpoint potential of cytochrome $b_{559}$. Triton X-100[9], FCCP, antimycin A or high pH[10] converts some of the cytochrome $b_{559}$ that was reducible by hydroquinone to a form which is reduced by ascorbate but not by hydroquinone. Extraction of chloroplasts with hexane[11] or heptane[12] converts the hydroquinone reducible form to an ascorbate but non-hydroquinone reducible form. More extensive structural disruption of the chloroplasts may cause the midpoint potential of cytochrome $b_{559}$ to shift to even lower potentials so that reductants stronger than ascorbate are needed to reduce the cytochrome. Hind and Nakatani[6] noted that the midpoint potential of cytochrome $b_{559}$ is lowered to -65 mV after acetone extraction of the chlorophyll. Digestion of spinach chloroplasts with lipase converts cytochrome $b_{559}$ to a form which is reduced by dithionite but not by ascorbate and digestion of *Chlamydomonas* chloroplasts either destroys part of the high potential cytochrome $b_{559}$ or renders it nonreducible even with dithionite[13].

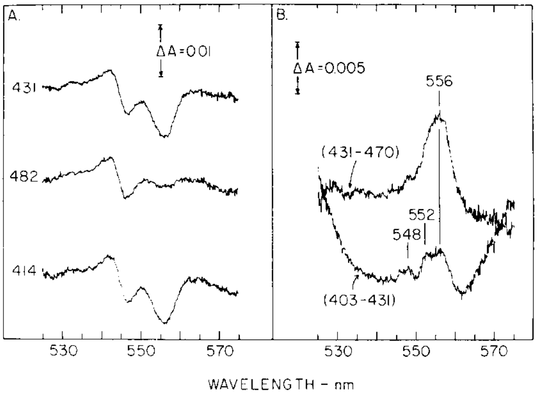
The purpose of the work reported here was to examine the redox state of cytochrome $b_{559}$ and the light-induced reduction of C-550 and oxidation of cytochrome $b_{559}$ at -196 °C in normal chloroplasts and in Tris-washed chloroplasts as a function of the redox potential of the chloroplast medium.

## METHODS

Normal and Tris-washed chloroplasts were prepared by methods described by Yamashita and Butler[14]. Procedures for anaerobic titration and liquid nitrogen temperature spectroscopy of chloroplasts were described previously[2]. The redox buffers in the titration experiments are indicated in the figure legends. Spectra of samples frozen to -196 °C at various redox potentials were taken before and after actinic illumination with red light ($1\ \mathrm{mW \cdot cm^{-2}}$ for 30 s) and stored in digital form in a computer. Difference spectra of samples at different redox potential could be calculated subsequently by the computer and plotted on an X–Y recorder. Chemical and light-induced difference spectra of samples frozen under aerobic conditions in the presence of ferricyanide, ascorbate, or dithionite were also measured. For complete reduction with dithionite the sample was allowed to incubate for 2 min before freezing; for partial reduction the sample was frozen 30 s after addition of a few grains of dithionite.

## RESULTS

The data presented in Figs 1 and 2 were obtained from an anaerobic titration in which the chloroplast suspension was maintained near 0 °C. The redox potential, initially near 100 mV, was increased in steps by the addition of small aliquots of ferricyanide, and then decreased with aliquots of reduced methyl viologen. After each addition, when the redox potential had come to a constant value, a sample was withdrawn and frozen to -196 °C. The absorption spectrum of each sample was measured before and after actinic illumination at -196 °C.



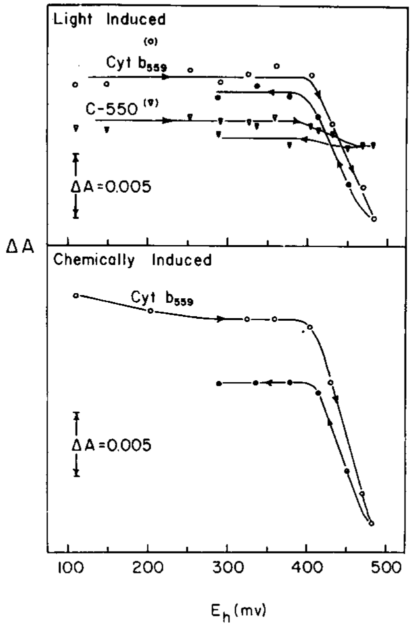


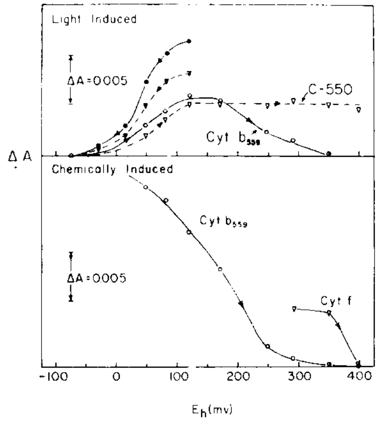
Fig. 1. Difference spectra of samples at −196 °C frozen in liquid nitrogen after equilibration at the potentials indicated (in mV, measured at 0–5 °C). Each sample contained 40 μg chlorophyll in 0.3 ml medium including the following redox buffers: 10 μM anthroquinone-2-sulfonate, $E_{m_7} = -225$ mV; 10 μM 2-hydroxy-1,4-naphthoquinone, $E_{m_7} = -145$ mV; 10 μM indigotetrasulfonate, $E_{m_7} = -46$ mV; 10 μM 1,4-naphthoquinone, $E_{m_7} = +60$ mV; 5 mM EDTA + 50 μM $FeCl_3$, $E_{m_7} = +120$ mV; 100 μM 2,5-dimethyl benzoquinone, $E_{m_7} = +180$ mV; 80 μM 1,2-naphthoquinone-4-sulfonate, $E_{m_7} = +215$ mV; 40 μM hydroquinone, $E_{m_7} = +280$ mV. A, light-*minus*-dark difference spectra of samples measured first in the dark, then after actinic irradiation with red light. B, difference spectra between unirradiated samples frozen at different redox potentials.

Fig. 2. Redox titration of low temperature light-induced (top) and redox potential induced (bottom) absorption changes derived from the data of Fig. 1 *plus* additional samples from the same titration experiment for which the spectra are not shown. The extent of the low temperature light-induced cytochrome $b_{559}$ change at 482 mV, the highest potential used, was assumed to represent the amount of cytochrome $b_{559}$ present in the reduced state at that potential.

Low temperature light-induced difference spectra at three redox potentials are shown in Fig. 1A. The samples were taken at 431 and 482 mV of the oxidative titration and at 414 mV of the reductive titration. Data from such curves taken over a range of redox potentials were used to plot the redox titration curves for the light-induced changes of C-550 and cytochrome $b_{559}$ presented in the upper part of Fig. 2. The absorbance changes of C-550 were calculated from the absorbance difference between the maximum at 543 nm and the minimum at 547 nm and the changes of cytochrome $b_{559}$ were determined as the bleaching at 556 nm relative to an estimated baseline. The titration curve for the photooxidation of cytochrome $b_{559}$ indicates a midpoint potential of about 450 mV. The light-*minus*-dark difference spectrum at 482 mV shows a small amount of cytochrome $b_{559}$ was still available for photooxidation so that the redox titration curve which indicates the amount of cytochrome $b_{559}$ in the reduced state does not go to zero at that potential.

Difference spectra between the unirradiated spectra taken at different redox potentials indicate the chemically induced redox changes. Two such difference spectra are shown in Fig. 1B. The difference spectrum between the samples at 403 and 431 mV shows that a major portion of cytochrome *f*, as seen by the split α band at 548 and 552 nm, was oxidized in going from 403 to 431 mV and that a small portion of cytochrome $b_{559}$ was oxidized as indicated by the peak at 556 nm. There was little or no cytochrome change in the prior redox step from 358 to 403 mV. Continuing from 431 to 470 mV, most of the cytochrome $b_{559}$ was oxidized. In the reductive titration from 482 mV downward, cytochrome $b_{559}$ was reduced before cytochrome *f* in the same potential regions. The redox titration curves for the chemically induced changes of cytochrome $b_{559}$ are presented in the lower part of Fig. 2. The cumulative absorbance changes obtained over the course of the titration are plotted relative to the redox state of the most oxidized sample measured. The cytochrome *f* spectral changes are masked too much by the larger cytochrome $b_{559}$ changes to permit a titration curve for cytochrome *f* to be determined. However, it is apparent from the two difference spectra in Fig. 1B that the midpoint potential of cytochrome $b_{559}$ in that chloroplast preparation was more positive than that of cytochrome *f*.

Fig. 3 shows the titration curves of the light-induced and chemically-induced redox changes of Tris-washed chloroplasts. The chloroplasts were taken first through a reductive titration (solid symbols) and then through an oxidative titration (open symbols). Some of the C-550 appears to be lost or inactivated during the reductive titration (see upper part of Fig. 3). Any loss of C-550 should result in a proportionate decrease in the amount of cytochrome $b_{559}$ photooxidized since the photooxidation of cytochrome $b_{559}$ at low temperature is stoichiometrically related to the amount of C-550 photoreduced[2]. The entire titration experiment required approx. 11 h at 20 °C



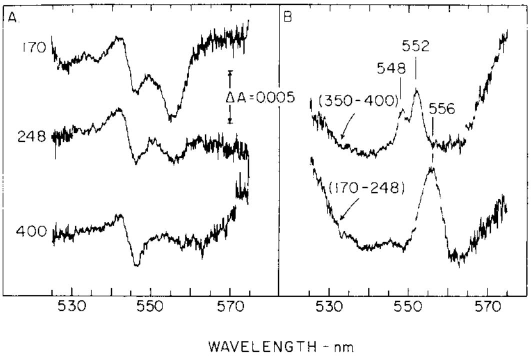


Fig. 3. Redox titration of Tris-washed chloroplasts. Redox buffers for this experiment were: 10 μM 2-hydroxy-1,4-naphthoquinone, $E_{m7} = -145$ mV; 10 μM indigotetrasulfonate, $E_{m7} = -46$ mV; 10 μM 5-hydroxy-1,4-naphthoquinone, $E_{m7} = +33$ mV; 10 μM 1,4-naphthoquinone, $E_{m7} = +60$ mV; 20 μM 1,2-naphthoquinone-4-sulfonate, $E_{m7} = +215$ mV. Potentials were measured at 20 °C.

Fig. 4. Difference spectra between some of the samples of the titration shown in Fig. 3.

so that the chloroplasts were subjected to aging as well as to the Tris washing. The primary influence on cytochrome $b_{559}$ in the experiment indicated in Fig. 3 can be attributed to the Tris washing since the changes in the redox properties of cytochrome $b_{559}$ are apparent immediately after the Tris washing treatment. In other experiments (data not shown) normal chloroplasts at 20 °C were titrated in a manner similar to the Tris-washed chloroplast titration of Fig. 3. The potential of cytochrome $b_{559}$ in these experiments was intermediate between that of Tris-washed chloroplasts and normal chloroplasts at 0 °C. The shape of the titration curve was broad, resembling that for Tris-washed chloroplasts.

Fig. 4A shows the light-induced absorbance changes at —196 °C of three samples taken at different redox potentials during the oxidative titration of the Tris-washed chloroplasts. The light-*minus*-dark difference spectrum of the sample taken at +170 mV shows the normal ratio of cytochrome $b_{559}$ oxidized to C-550 reduced. At 248 mV most of the cytochrome $b_{559}$ was oxidized chemically so little was available (*i.e.* in the reduced state) for the photochemical oxidation and at 400 mV all of the cytochrome $b_{559}$ was oxidized. As previous experiments have shown[15,16], the photoreduction of C-550 at low temperature does not require the concomitant photooxidation of cytochrome $b_{559}$.

Difference spectra between unirradiated Tris-washed chloroplast samples at different redox potentials are presented in Fig. 4B. Titrating from 170 to 248 mV, only cytochrome $b_{559}$ was oxidized although some cytochrome $b_{559}$ as well as cytochrome $b_{563}$ was oxidized at lower potentials. Only cytochrome *f* was oxidized in going from 350 to 400 mV.

The presence of a low potential, dithionite but not ascorbate reducible, cytochrome $b_{559}$ can be demonstrated in fresh chloroplasts. Fig. 5 shows difference spectra of intact chloroplasts at —196 °C. The chloroplasts were frozen in the dark to —196 °C after no addition, after addition of 3 mM ascorbate, after partial reduction with dithionite, after complete reduction with dithionite, or after addition of 3 mM ferri-

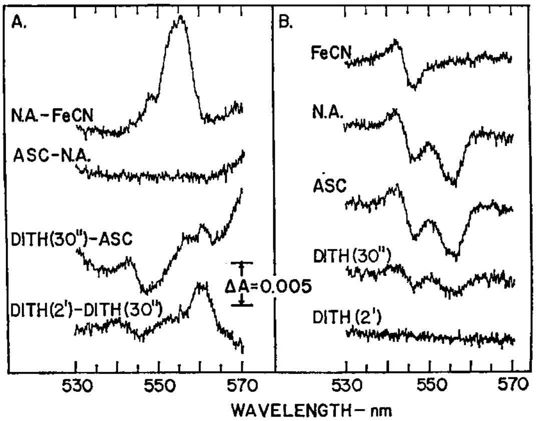


Fig. 5. A, chemical and B, low temperature light-induced difference spectra of fresh chloroplasts (50 μg chlorophyll) frozen under aerobic conditions in 0.3 ml of 0.1 M tricine-KOH (pH 8) in the presence of no addition (N.A.), 3 mM ascorbate (ASC), 3 mM ferricyanide (FeCN) or a few grains of dithionite incubated for 30 s (DITH (30")) or 2 min (DITH (2')) before freezing as indicated.

cyanide. Difference spectra between the various samples are shown in Fig. 5A. Cytochrome *f* and the normal high potential cytochrome $b_{559}$ are in the reduced state in the chloroplasts and adding ascorbate has no effect on the cytochromes (Asc-N.A.). Partial reduction by dithionite (dith (30″)-Asc) reduced some C-550, a cytochrome with a low temperature absorption band at 556–557 nm, and some cytochrome $b_{564}$ with a low temperature absorption band at 560 nm. Further reduction with dithionite (dith (2′) *minus* dith (30″)) completes the reduction of C-550 and cytochrome $b_{564}$. A small amount of a component which we refer to as the low potential cytochrome $b_{559}$ is seen in the dith (30″) *minus* ascorbate difference spectrum. Bendall[4] also reported the presence of a low potential cytochrome $b_{559}$ as well as the high potential form in normal chloroplasts. The amount of cytochrome *f* and the high potential cytochrome $b_{559}$ present in the chloroplasts is indicated by spectral changes induced by ferricyanide (N.A.-FeCN).

Light-*minus*-dark difference spectra due to irradiation of the various samples at —196 °C are shown in Fig. 5B. Normal light-induced difference spectra showing photoreduction of C-550 and photooxidation of cytochrome $b_{559}$ were obtained with the chloroplasts with no addition or with ascorbate. Partial reduction by dithionite left a little C-550 to be reduced photochemically and a stoichiometric amount of cytochrome $b_{559}$ photooxidized. When all of the C-550 is reduced chemically no electron acceptor is available and the photoreaction is blocked.

## DISCUSSION

The midpoint potentials for intact chloroplasts determined at 0 °C (Figs 1 and 2) may be somewhat higher than values measured at 25 °C either because of some systematic error in the calibration of the system or in the measurement of potentials at 0 °C or because of a real temperature dependence of the redox potential. The difference spectra in Fig. 1B indicate that the midpoint potential of cytochrome *f* in normal chloroplasts at 0 °C was between 403 and 431 mV while the midpoint potential in the Tris-washed chloroplasts at 20 °C was about 370 mV. The value of 450 mV for the midpoint potential of cytochrome $b_{559}$ is probably also higher than would have been obtained at 20 or 25 °C. The lower temperature, however, was needed to inhibit aging during the course of the titration. Boardman *et al.*[16] reported a value of +350 mV for cytochrome $b_{559}$ obtained by measuring extent of the photooxidation of cytochrome $b_{559}$ at —196 °C in the presence of different ratios of ferri- and ferrocyanide.

The redox potential of cytochrome $b_{559}$ is sensitive to the state of the chloroplasts. Conditions or treatments which inhibit electron transport between water and Photosystem II (these include washing with high concentrations of Tris or chaotropic agents, heat treatment, ultraviolet radiation, high pH and aging) cause the redox potential of cytochrome $b_{559}$ to shift to lower values and the cytochrome becomes autooxidizable. The modified cytochrome $b_{559}$ which results from these treatments does not have a well defined midpoint potential of a single species. Rather a continuum of midpoint potentials, perhaps dependent on the degree of modification, is indicated by the broad titration curves. The high potential form of cytochrome $b_{559}$ may require the structural integrity of the chloroplast membranes and treatments which inhibit between water and Photosystem II appear to have a disruptive influence on the membranes. We noted previously[17] that a number of treatments that inhibit between water and Photosystem II appear to disorganize the chloroplast membranes to some extent because the $Mn^{2+}$ ESR signal, which is not observed in normal chloroplasts because the $Mn^{2+}$ is chelated, appears after such treatments.

The modified cytochrome $b_{559}$ in the Tris-washed chloroplasts is also oxidized by Photosystem II at −196 °C. Knaff and Arnon[1] showed earlier that cytochrome $b_{559}$ was photooxidized by Photosystem II in Tris-washed chloroplasts at room temperature. It is apparent in Fig. 3 that at redox potentials where the amount of reduced cytochrome $b_{559}$ is less than the amount of oxidized C-550, such as at 248 and 290 mV, all of the reduced cytochrome $b_{559}$ is photooxidized at −196 °C by the Photosystem II reaction.

It appears that the high potential cytochrome $b_{559}$ is the principal form *in vivo*. It is converted to lower potential forms by various disruptive treatments. However, some dithionite but non-ascorbate reducible cytochrome $b_{559}$ is also present in fresh chloroplasts. The function of the low potential form of cytochrome $b_{559}$ in normal chloroplasts is unknown but it might participate in electron transport reactions between Photosystems II and I.

The functional role of the high potential cytochrome $b_{559}$ is also difficult to assess. It is clear, however, that this cytochrome $b_{559}$ is closely associated with the primary reaction center of Photosystem II and can serve as an electron donor to Photosystem II. It is possible that the high potential cytochrome $b_{559}$ functions on the water-splitting side of Photosystem II. Such a position is suggested by the observation that treatments which block electron transport between water and Photosystem II alter the redox potential of this cytochrome. The same functional role was indicated by mutants of *Chlamydomonas reinhardi* which were deficient in high potential cytochrome $b_{559}$ and were blocked between water and Photosystem II (B. Epel, W. L. Butler and R. P. Levine, unpublished). Bendall and Sofrova[18] presented a hypothetical scheme whereby the high potential cytochrome $b_{559}$ might function in the S states proposed by Kok *et al.*[19] to participate in the oxygen-evolving mechanism of photosynthesis.

While this manuscript was in the review process a paper by Wada and Arnon[20] appeared reporting three forms of cytochrome $b_{559}$ in spinach chloroplasts; a high potential (H) form which was reducible by hydroquinone, a middle potential (M) form which was reducible by ascorbate but not by hydroquinone and a low potential (L) form which was reducible by dithionite but not by ascorbate. The H form could be converted to the M or L forms by aging, sonication or heating. Our results with Tris-washed chloroplasts are largely in agreement with those of Wada and Arnon. We would suggest, however, that modification of the high potential cytochrome $b_{559}$ by the various disruptive treatments leads to a mixture of forms of cytochrome $b_{559}$ with a range of midpoint potentials, depending on the degree of modification, rather to specific M or L forms. We would also suggest that the low potential form of cytochrome $b_{559}$, first mentioned by Bendall[4], which we as well as Wada and Arnon find in fresh chloroplasts may represent a specific functional cytochrome rather than a modified form of the high potential cytochrome $b_{559}$.

For reasons we do not understand we think that our anaerobic redox titration of the high potential cytochrome $b_{559}$ in the mixture of redox buffers indicated a midpoint potential, 450 mV, which is too high. In more recent work an aerobic titration near 0 °C with mixtures of ferricyanide and ferrocyanide resulted in a midpoint potential of 365 mV which is more in agreement with the results of others.

## ACKNOWLEDGMENTS

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