ACID-BASE INDUCED REDOX CHANGES OF THE CHLOROPLAST CYTOCHROME *b*-559

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1. Introduction

At least 80–90% of the cytochrome *b*-559 in dark adapted, well coupled spinach chloroplasts has been found to be in a high potential, hydroquinone-reducible state [1,2], whose midpoint is approximately +350 mV [3–6]. It has been shown that illumination with actinic light preferentially absorbed by photosystem I can induce oxidation of cytochrome *b*-559 in the presence of either 4-trifluoromethoxyphenylhydrazone [1,7,8] or low concentrations of *N*-methyl phenazonium methosulphate [9], and after pre-illumination with high intensity red light [2]. The inhibition of photooxidation by the plastoquinone antagonist 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) in each case implies that a low potential cytochrome *b*-559 species is being oxidised [1,2,8,9]. The very positive dark midpoint potential of cytochrome *b*-559 is very unusual for a *b* cytochrome. Because of the known lability of the high potential strate, it would appear to be the consequence of a positive ionic environment in the neighborhood of the heme, created by an organised membrane structure. The mechanism of a reversible light-induced decrease in cytochrome *b*-559 potential may involve a change in the charge environment in the membrane near the heme. (See [10] for a detailed discussion). During illumination a large proton flux occurs into or across the thylakoid membrane [11]. The possibility arises that local membrane pH changes occurring during illumination are responsible for the decrease in midpoint potential of cytochrome *b*-559. In this paper dark, acid-base induced redox changes of cytochrome *b*-559 are described, which can be interpreted in terms of a reversible decrease in midpoint potential at low pH.

2. Materials and methods

Chloroplast isolation [12] and room temperature cytochrome measurements using dual wavelength spectrophotometry [13] were performed exactly as previously described. Wavelength calibration was obtained with a mercury lamp (546.1 nm line). Difference spectra at 77°K were obtained using a single beam spectrophotometer interfaced to a mini-computer [13]. The optical path through the sample was horizontal using a 'cold finger' sample holder of the Shimadzu MPS-50L spectrophotometer. Four spectra were added to improve signal to noise ratios. Chloroplasts showing less acid/base-induced scattering changes were prepared by resuspension in a hypotonic medium (25 mM tricine–NaOH, pH 7.8, 5 mM K_2HPO_4, 5 mM NaCl and 2 mM $MgCl_2$), incubation for 10 min at 0°C, followed by homogenization and centrifugation before resuspension in a medium containing 0.4 M sorbitol, 2% bovine serum albumin, 25 mM tricine-NaOH, pH 7.8, 10 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA and 1 mM $MnCl_2$. The reaction medium contained 10 mM NaCl and 2 mM $MgCl_2$ and was only lightly buffered by tricine medium added with chloroplasts (approx. 1 mM). The pH of the reaction mixture was continuously monitored by a combination electrode immersed in the spectrophotometer cuvette and was pertubed by microlitre additions of 0.5 M succinic acid or NaOH.

3. Results and discussion

Upon addition of succinic acid to chloroplasts large scattering changes occur which make it impossible to follow cytochrome absorbance changes. Using less

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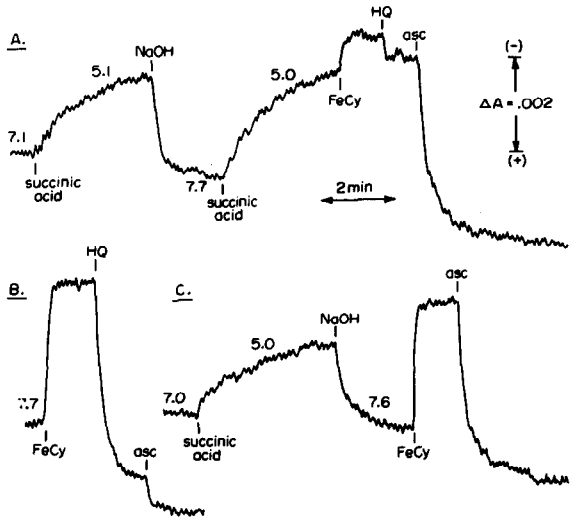

Fig.1. Absorbance changes at 560 nm during acid/base transitions. (A) Reversible acid-base transition and ferricyanide-induced oxidation at low pH. (B) Ferricyanide added before any acid transition. (C) ferricyanide-induced oxidation after reversal of absorbance change by addition of alkali. Reference, 570 nm. Chlorophyll, 80 μg/ml. Ferricyanide, 250 μM. Hydroquinone, 1 mM, Ascorbate, 2mM.

structurally intact chloroplasts prepared as described in methods above, this problem is largely eliminated. On lowering the pH of the medium to 5 an oxidative absorbance change at 560 nm is seen, which can be reversed by subsequent addition of NaOH and restoration of the initial pH (fig. 1A). Ferricyanide addition at pH 5 gives a much smaller oxidative change than at pH 7 (compare fig. 1, A and B). The amplitude of the total reductive change caused by addition of ascorbate or hydroquinone plus ascorbate is unchanged, indicating that a pH-

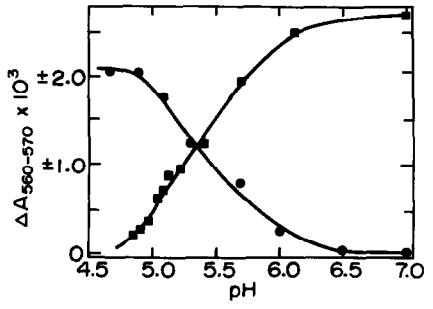

Fig.2. Amplitudes of acid-induced cytochrome *b*-559 oxidation (•) and its reduction by ferrocyanide (▪) as a function of pH. Conditions as in fig.4 A–C. pH poised by addition of different quantities of succinic acid.

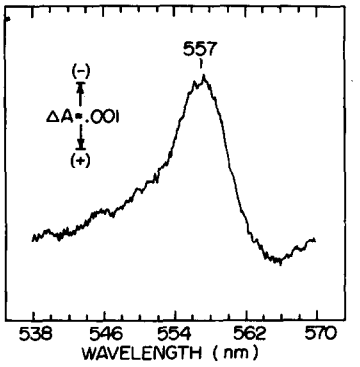

Fig.3. pH 5 minus pH 7 difference spectrum at 77°K. Two samples, at pH 5 after succinic addition and at pH 7 after subsequent addition of NaOH were rapidly frozen in liquid nitrogen before scanning.

induced change in the cytochrome oxidation state has occurred, rather than the alternative possibility of a pH-induced alteration in extinction coefficient. The reversibility of the acid-induced oxidation is shown in fig.1C. The amplitude of ferricyanide oxidation is clearly increased if the pH is raised again after succinic acid-induced oxidation. The amount of acid-induced oxidation varies between 40 and 70% of the total ferricyanide oxidisable cytochrome, with the pH of the medium at 4.5–5.0 after addition of succinic acid. The increase in the extent of the acid-induced cytochrome *b*-559 oxidation occurs between pH 5 and 6, with an approximate pK of 5.5 (fig.2). That most of the absorbance decrease seen at pH 5 arises from cytochrome *b*-559 oxidation is seen in a pH 5 minus pH 7 difference spectrum recorded after rapid cooling of samples to 77°K (fig.3), at which temperature the characteristic α-band maximum is 556–557 nm. There is a small amount of cytochrome *f* oxidation and this proportion can be somewhat larger, particularly in aged chloroplasts.

Oxidation of cytochrome *b*-559 in the dark could conceivably occur either because of a decrease in its mid-point potential or because of an increase in potential of plastoquinone or other redox pools resulting in a new redox equilibrium. However, the data in figs.2,4 and 5 indicates that a decrease in mid-point potential occurs on lowering the pH to 5. At pH 5, after succinic acid addition, ferrocyanide causes little reduction of ferricyanide-oxidised cytochrome *b*-559 (fig.4B) compared to pH 6-7 (fig.4A and C). The reversibility of the acid-induced change in ferrocyanide reducibility is clearly

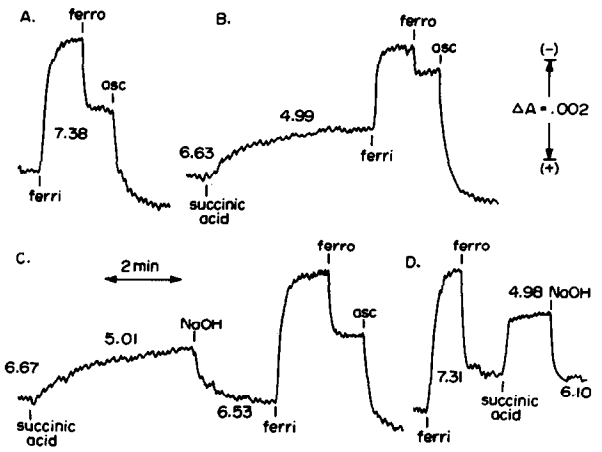

Fig.4. Effect of acid/base transition on ferrocyanide-induced absorbance changes at 560 nm. (A) Ferrocyanide-induced reduction without acid transition. (B) after acid transition and (C) after acid-base transition. (D) Succinic acid induced oxidation after redox poising at pH 7 with ferri-ferrocyanide. Ferricyanide, 250 μM; ascorbate, 2 mM; ferrocyanide, 1 mM in A, B and C, 2 mM in D. Chlorophyll, 80 μg/ml. Reference, 570 nm.

shown in fig.4C. The mid-point potential of ferri-ferrocyanide (Em_7 = +0.43 V at high ionic strength) is the same at pH 5 and 7 [14]. A ferro-ferricyanide ratio of four is expected to cause incomplete reduction of *b*-559 at the higher pH in fig.4A and C. The spectrum for ferrocyanide reduction at pH 7 has a maximum at 560 nm

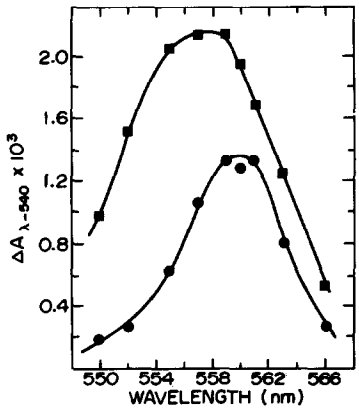

Fig.5. Spectrum for ferrocyanide (•) and ascorbate (▪) reduction at pH 6.5 after ferricyanide oxidation. Ferricyanide, 250 μM. Ferrocyanide, 1 mM. Ascorbate, 2 mM. Chloroplasts buffered at pH 6.5 with 50 mM. 2-(*N*-morpholino) ethane sulphonic acid. Chlorophyll 80 μg/ml.

(fig.5). Ferrocyanide seems to hardly reduce cytochrome *f* at all in these experiments. This is probably a consequence of the inaccessibility of cytochrome *f* within the membrane [13]. Titration of the amplitude of ferrocyanide reduction at 560 nm shows that, again, most of the change occurs between pH 5 and 6 (fig.2). The reciprocal relationship between the pH dependence for the acid-induced oxidation and the decrease in ferrocyanide reducibility suggests that the cytochrome *b*-559 oxidation occurring after succinic acid addition is a consequence of a decrease in mid-point potential. Although we may expect some decrease in membrane permeability at low pH, in these experiments there is no indication that the loss of ferrocyanide reducibility is due to a low pH-induced decrease in accessibility, since ferricyanide oxidation of cytochrome *b*-559 proceeds fast at pH 5 and pH 7.

The oxidation of cytochrome *b*-559 observed after succinic acid addition is slow in figs.1 and 3. This appears to be due to a slow rate of oxidation rather than a slow alteration in mid-point potential. If the redox state of cytochrome *b*-559 is poised with 250 μM ferricyanide and 2 mM ferrocyanide at pH 7, addition of succinic acid causes rapid oxidation of cytochrome *b*-559 (fig.4D). Other acids such as citric acid and HCl, as well as potassium phthalate, can substitute for succinic acid (data not shown). These acids also induce the slow cytochrome *b*-559 oxidation seen in figs.1 and 3, with rates and amplitudes equal to those seen with succinic acid. In this respect the conditions necessary for observing the change in redox state of cytochrome *b*-559 upon acid-base transition are different from those which stimulate acid-base induced ATP synthesis in chloroplasts [15]. In addition, the cytochrome *b*-559 changes are unaffected by the presence of the uncoupler gramicidin at 10 μg/ml (data not shown). Therefore, the question of whether changes in the redox state of cytochrome *b*-559 are involved in acid-base phosphorylation remains a subject for further experimentation.

A decrease in midpoint potential with decreasing pH has not been reported in studies on *b* cytochromes in different membrane systems [2]. In general, $\Delta E_m/\Delta pH \leqslant 0$. The pH region over which such redox measurements extend, however, rarely goes below pH 6. In the case of cytochrome *b*-559, the midpoint has been found to be the same at pH 6.5 and 7.5 [3]. A decrease in effective midpoint potential at very low pH values might then be more readily considered in terms of

changes in membrane structure occurring at low pH, rather than in terms of protonation-deprotonation of the cytochrome itself. Changes in chloroplast structure at low pH are well documented [16,17] and these are similar to membrane changes occurring upon illumination. Recent observation of pH dependent changes in the transition temperature of phosphatidic acid model membranes [18] provides a further molecular basis on which to consider structural changes induced by low pH. Changes in membrane structure at alkaline pH may also provide an explanation for the increased level of reduced mitochondrial *b*-566 [19].

Light induced acidification of the chloroplast membrane could provide a mechanism for a decrease in midpoint potential previously proposed to account for the DBMIB - sensitive photo-oxidation of cytochrome *b*-559 by photosystem I. The magnitude of a light-induced decrease in cytochrome *b*-559 potential required for it to act as a donor to plastoquinone will depend as well on the plastoquinone midpoint which will become more positive with decreasing pH.

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