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RESTORATION OF HIGH-POTENTIAL CYTOCHROME *b*-559 IN LIPOSOMES

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Cytochrome *b*-559, purified from spinach leaves as a low-potential, ascorbate-reducible form, was incorporated into liposomes made from digalactosyldiacylglycerol and phosphatidylcholine. Approximately half of the cytochrome was restored to a high-potential, hydroquinone-reducible form in the liposome suspensions.

Introduction

The high-potential form of cytochrome *b*-559 ($E_{m,7} \approx 380$ mV) which is present in intact chloroplast membranes is converted to lower potential forms when those membranes are disrupted. The purpose of the present work was to determine conditions which would restore the midpoint potential of purified cytochrome *b*-559, present as a low-potential ascorbate-reducible form, to higher potentials. The rationale for this work rests on the assumption that a high-potential form of cytochrome *b*-559 is involved in oxygen evolution [1] and that attempts to reconstitute an oxygen-evolving system from chloroplast components may be enhanced by conditions which increase the midpoint potential of the isolated cytochrome *b*-559.

To date, only circumstantial evidence links the high-potential cytochrome *b*-559 to oxygen evolution. A wide variety of seemingly gentle treatments which inhibit oxygen evolution by blocking photosynthetic electron transport on the water side of Photosystem (PS) II also cause the high-potential form to be converted to lower potential forms [2]. Also, in experiments where the capacity for oxygen evolution was restored to heptane-extracted chloroplasts by reconstitution with β-carotene and plastoquinone [3–5], the low-potential cytochrome *b*-559 which resulted from the extraction procedures was restored to higher potential forms in the reconstituted fragments.

A hypothesis for the function of cytochrome *b*-559 in photosynthesis was developed a few years ago [1] in an attempt to reconcile some of the controversial aspects of this component. While it is generally accepted that cytochrome *b*-559 is closely associated with the photochemical apparatus of PS II, its precise role has remained elusive. Different kinds of evidence have suggested that it functions on the reducing side of PS II, on the oxidizing side of PS II or in a cycle around PS II [6]. The hypothesis [1] resolved some of the anomalies by proposing that cytochrome *b*-559 has a proton-binding function operating on the oxidizing side of PS II which is integrally linked to its electron-transfer function which operates on the reducing side of PS II and that the protons bound are among those which are released in the splitting of water. The high-potential form of cytochrome *b*-559 was assumed to be the protonated form. Simple thermodynamics specifies that if the reduced form of the cytochrome binds a proton more strongly than the oxidized form then the proto-

Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosydiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; PQ, plastoquinone.

nated form will have a higher midpoint potential than the unprotonated form by an amount related to the difference in the pK values of the reduced and oxidized forms [8]:

$$E_{m,prot.} = E_{m,unprot.} + 0.059(pK_{red} - pK_{ox})$$

Thus, if the pK values differed by 5 units, the midpoint potentials of the high-potential (protonated) and low-potential (unprotonated) forms would differ by approx. 300 mV. The observation that the midpoint potential of the low potential cytochrome *b*-559 has a pH dependence of approx. −60 mV/pH unit [8] shows that proton binding does occur. However, one should not confuse this pH dependence with the explanation of the potential difference between the high- and low-potential forms. The pH dependence would remain even if the pK values of the oxidized and reduced forms were the same so that there was no potential difference between the protonated and unprotonated forms.

Cytochrome *b*-559 is a large (M_r = 111 000) lipoprotein (55% lipid) [9] which could span much of the thylakoid membrane. It is assumed that the protein binding site is in the lipophilic part of the molecule and that this site is buried deep within the hydrophobic regions of the membrane in close proximity to the oxygen-evolving apparatus. The redox changes occur in the heme which is reasonably accessible to the outside of the thylakoid membrane [10]. According to the hypothesis the redox and proton-linked functions are integrally linked to one another because the redox state of the heme affects the strength of proton binding and the pK of the proton-binding site influences the midpoint potential of the heme. It was further assumed that a high pK value for the reduced form (which results in a high midpoint potential for the protonated form) requires a very hydrophobic environment around the proton-binding site [1]. Treatments which disrupt the membrane integrity of chloroplasts invariably cause the high-potential form of cytochrome *b*-559 to be converted to lower potential forms, presumbly because the pK of the reduced form is lower in a less hydrophobic environment.

Cytochrome *b*-559 was purified from spinach as a large lipoprotein which was reducible by ascorbate. Incorporation of the cytochrome into liposomes caused approximately half of it to become hydroquinone reducible.

Materials and Methods

Cytochrome *b*-559 was purified from spinach leaves by the methods of Garewal and Wasserman [11] and Zielinski and Price [12]. 5 mM dithiothreitol was present throughout the purification procedure. The purified cytochrome *b*-559 was stored at −20°C in 10 mM Tris-HCl buffer, pH 7.2, with 1 mM dithiothreitol and 15% glycerol. The purified solution gave a single protein on polyacrylamide gel electrophoresis which corresponded to the high molecular weight lipoprotein. Calibration of the molecular weight of this material was carried out by gel filtration [13] on a 1.1 × 25 cm column of Sepharose CL-6B which had been equilibrated with 5 mM Tris-HCl buffer, pH 8.0, with 1 mM NaCl. A molecular weight of 110 000 was indicated, using the marker proteins chymotrypsinogen (25 000), bovine serum albumin (67 000), aldolase (158 000), catalase (232 000) and ferritin (440 000). Chromatography on an SDS gel gave a single band corresponding to the 6000 M_r polypeptide monomer [9]. Concentrations of the cytochrome were calculated using a 1 cm, millimolar extinction coefficient of 16 for the reduced form between 559 and 600 nm [11].

Liposomes were prepared from DGDG which had been purified from spinach [14] and PC purified from egg yolks [15]. 20 μl of 100 mM DGDG and 5 μl of 100 mM PC were flushed with nitrogen and kept in a vacuum desiccator at 4°C to remove organic solvents. 22 μl of plastoquinone A (1.72 mM) were also included when liposomes containing plastoquinone were desired. 300 μl of the purified cytochrome *b*-559 (6.3 μM) were added and the mixture was gently stirred with a magnetic stirring bar for 2 h at 4°C under nitrogen. 300 μl of 10 mM Tris-HCl buffer, pH 7.2, containing 15% glycerol were then added to give the final liposome preparation. The liposomes were generally used the same day that they were prepared but they could be stored at −20°C with no apparent ill effects.

Absorption spectra were measured with our single-beam spectrophotometer on line with a

computer [16]. A cylindrical cuvette was used which gave a vertical path of 1 cm for a 0.3 ml sample. Spectra were measured at room temperature after sequential additions of 0.2 mM potassium ferricyanide, 20 mM recrystallized hydroquinone, 20 mM sodium ascorbate and a few grains of sodium dithionite and difference spectra between the different states of oxidation were plotted out by the computer.

Results

In the terminology used here, the high-potential cytochrome *b*-559 is defined operationally as that cytochrome *b*-559 which is reduced by a relatively high concentration of hydroquinone (20 mM). Essentially the same results were obtained using a 5 mM concentration except that, in some cases, somewhat more cytochrome *b*-559 was reduced with 20 mM hydroquinone than with 5 mM. The term low potential will refer primarily to cytochrome *b*-559 which is reduced by ascorbate but may also be used to refer to even lower potential forms which require stronger reductants such as dithionite. Some care must be exercised to obtain and maintain the purified cytochrome *b*-559 in an ascorbate-reducible state. Prolonged treatments or use of the cytochrome tend to degrade it to forms which require stronger reductants. We do not intend that this terminology should imply well defined chemical species which have a definite midpoint potential. Rather, we assume from redox titration experiments on Tris-washed chloroplasts [17] that cytochrome *b*-559 may be present in a continuum of states extending over a broad range of midpoint potentials under our experimental conditions.

Liposomes containing cytochrome *b*-559 can be separated from cytochrome *b*-559 in solution by gel filtration on a Sepharose CL-6B column (Fig. 1). In the case of the liposome-containing fractions, the 429 nm absorbance measurement was due primarily to turbidity so that the relative concentrations of cytochrome *b*-559 in the liposome and soluble fractions are indicated by the difference spectra shown in the inset of Fig. 1. During the course of the gel filtration most of the cytochrome *b*-559, which was present initially in hydroquinone- and ascorbate-reducible forms, was

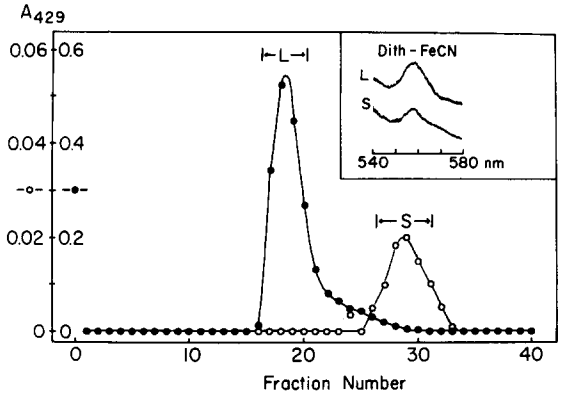

Fig. 1. Separation of liposomes by gel filtration on a 1.1×25 cm Sepharose CL-6B column. The equilibrating and eluting buffer was 10 mM Tris-HCl, pH 7.2, containing 15% glycerol and 5 mM dithiothreitol. The relative concentrations of cytochrome *b*-559 in the liposome and soluble fractions are indicated by the dithionite-minus-ferricyanide difference spectra (inset).

denatured to forms which required dithionite for reduction so that dithionite-minus-ferricyanide difference spectra were used to measure the amount of cytochrome *b*-559 in these fractions. In the subsequent experiments we did not attempt to isolate the liposomes because of the deleterious effect of the isolation procedures on cytochrome *b*-559.

Difference spectra of cytochrome *b*-559 in solution and in the liposome suspensions are shown in Fig. 2. The cytochrome *b*-559 was stored in the presence of dithiothreitol so that ferricyanide was added to the samples initially to oxidize the cytochrome. Sequential additions of 20 mM hydroquinone, 20 mM ascorbate and a few grains of dithionite were then made with spectra taken after each addition. The difference spectra of the purified cytochrome *b*-559 in solution (Fig. 2A) show that there was no hydroquinone-reducible cytochrome *b*-559 present but that most of it was reducible by ascorbate. In other preparations all of the cytochrome *b*-559 was ascorbate reducible. In a control experiment in which the cytochrome *b*-559 was stirred under N_2 for 2 h in the absence of lipid (but under the same conditions that were used to form liposomes in the presence of the lipid), virtually all of the cytochrome *b*-559 was converted to a dithionite-reducible state (Fig. 2B).

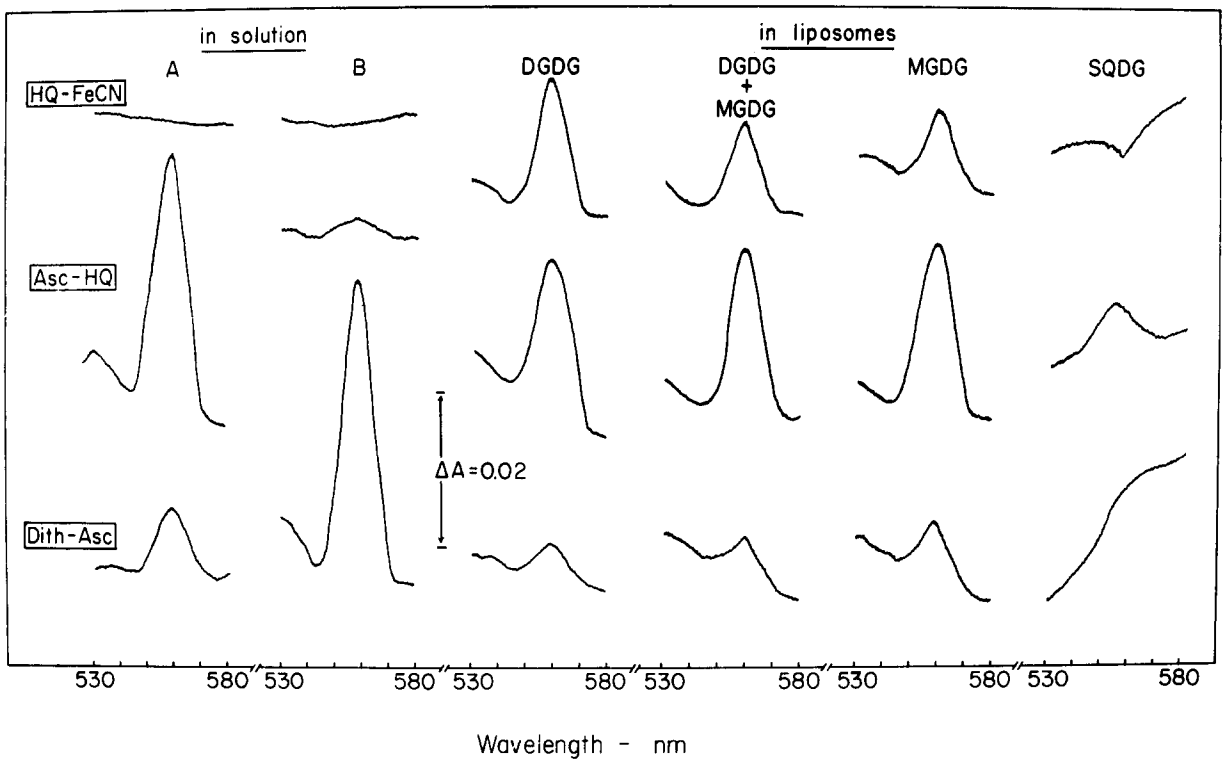

Fig. 2. Redox difference spectra of cytochrome *b*-559 in solution and in liposomes at 25°C. Sequential additions were made of 0.2 mM ferricyanide (FeCN), 20 mM hydroquinone (HQ), 20 mM ascorbte (Asc) and a few grains of dithionite (Dith) and single-beam absorption spectra were measured. Difference spectra between the different redox conditions are shown. (A) Cytochrome *b*-559 in solution; (B) cytochrome *b*-559 after stirring for 2 h under N_2 in the presence of 1 mM dithiothreitol (the same conditions that were used to make liposomes). (DGDG) Cytochrome *b*-559 after liposomes were allowed to form using DGDG as the lipid and PG as the detergent. (MGDG + DGDG) Same, except that a 50:50 mixture of the two lipids was used. (MGDG) Same, except that MGDG was the lipid. (SQDG) Same, except that SQDG was the lipid.

However, if DGDG and PG were present so that liposomes could form, approximately half of the low-potential, ascorbate-reducible cytochrome *b*-559 was converted to the high-potential, hydroquinone-reducible state with little or no degradation to dithionite-reducible forms. We assume from the distribution of cytochrome *b*-559 between liposomes and solution in Fig. 1, that only part of the cytochrome *b*-559 was incorporated into the liposomes but that most of that which was incorporated was converted to a high-potential form.

We experimented with different recipes for making the liposomes. In some experiments in which plastoquinone was incorporated into the liposomes in a molecular ratio DGDG:PQ:cytochrome *b*-559 of 1000:20:1, 75% or more of the cytochrome *b*-559 was high potential. However, if digitonin, Triton, deoxycholate or cholate was used as the detergent instead of PC, no high-potential cytochrome *b*-559 was formed (data not shown). We also tried mixtures of different galactolipids, all purified from spinach leaves. MGDG, alone or in a 50:50 mixture with DGDG, did not give as much high-potential cytochrome *b*-559 as DGDG and the acidic SQDG appeared to denature all of the cytochrome *b*-559 to a point where it was not even reducible by dithionite (Fig. 2).

Discussion

While the restoration of a high-potential form of cytochrome *b*-559 in liposomes by no means proves the hypothesis [1] on the role of cytochrome *b*-559 in oxygen evolution, it at least con-

firms the expectation from that hypothesis that the high-potential form requires a hydrophobic environment. It is certainly possible that the link between oxygen evolution and high-potential cytochrome *b*-559 is only circumstantial in that the hydrophobic conditions which lead to a high midpoint potential are also required for oxygen evolution. However, if we ask why do hydrophobic conditions lead to the high midpoint potential and recognize that the midpoint potential can be related to the strength of proton binding, a possible connection to oxygen evolution follows from the assumption that cytochrome *b*-559 may bind protons released in the splitting of water. In either case, the high-potential cytochrome *b*-559 may be taken to indicate conditions which are favorable for oxygen evolution. On this basis we would propose that liposomes made from DGDG and PC are preferable for chloroplast reconstitution studies over the other recipes we tried.

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