Electrogenic proton translocation by the chloroplast cytochrome b_6/f complex reconstituted into phospholipid vesicles

Eduard C. Hurt, Günter Hauska* and Yosepha Shahak[+]

Institut für Botanik, Universität Regensburg, 8400 Regensburg, FRG and [+]Biochemistry Department, Weizmann Institute of Science, Rehovot 76100, Israel

When the cytochrome b_6/f complex from chloroplasts is incorporated into liposomes, reduction of external plastocyanin by plastoquinol is stimulated under uncoupling conditions. An extra H^+/e^- is ejected from the vesicles during the reaction, in addition to the scalar proton liberated from plastoquinol. This is stimulated by valinomycin/K^+ and abolished under uncoupling conditions. Furthermore, the formation of a membrane potential during the reaction, negative inside the vesicles, is observed with the help of carbocyanine dyes. We conclude that the cytochrome b_6/f complex, like the cytochrome bc_1 from mitochondria, functions as an electrogenic proton translocator.

Cytochrome b_6/f *complex*   *Chloroplast*   *Electron transport*   *Proton translocation*
Membrane potential   *Carbocyanine*   *Antimycin A*

1. INTRODUCTION

Oxidation of ubiquinol by cytochrome c_1 in the membranes of mitochondria and photosynthetic bacteria is catalyzed by the cytochrome bc_1 complex, which comprises two hemes b, one heme c, an iron–sulfur center and possibly one bound ubiquinone [1–4]. The complexes have been isolated in functional form from mitochondria of several organisms [1,5–7] and from *Rhodopseudomonas sphaeroides GA* [8]. When the mitochondrial complex was reconstituted into liposomes reduction of external cytochrome c by ubiquinol was stimulated by uncouplers [9,10], and vectorial proton translocation out of the vesicles was observed, in addition to the scalar protons liberated during quinol oxidation [9–11].

* To whom correspondence should be addressed

A similar complex with identical redox-center composition catalyzes the oxidation of plastoquinol by plastocyanin in the chloroplast membrane [12–15]. This cytochrome b_6/f complex has been isolated and characterized [16–20].

Here, we report on the reconstitution of isolated cytochrome b_6/f complex into liposomes. As with the mitochondrial complex, stimulation of oxidoreductase activity by uncouplers and extra proton translocation is found. In addition, the formation of a membrane potential during plastoquinol–plastocyanin oxidoreduction is detected with the help of carbocyanine dyes [21,22].

2. METHODS

The cytochrome b_6/f complex from spinach chloroplasts was prepared as in [16] with the omittance of Triton X-100 in the final sucrose density gradient [19]. The cytochrome b_6/f complex used in the reconstitution experiments was suspended in 30 mM octylglucoside, 0.5% cholate, 30 mM Tris–succinate (pH 6.5), 0.1% soybean lecithin and 18% (w/v) sucrose.

Proteoliposomes were prepared by sonication.

For the procedure, 0.45 ml cytochrome b_6/f complex, 25 μM in cytochrome f, was mixed with 50 mg soybean lecithin (Sigma) and sonicated for 5 min in a small violax tube with a Branson sonicator using the microtip with full output at position 1. The tube was cooled with ice water during sonication. After sonication proteoliposomes were further incubated for 1 h on ice before the experiments were started.

Reduction of plastocyanin (590–500 nm, $\epsilon = 4.2 \, mM^{-1}.cm^{-1}$) was measured as in [16], with an Aminco DW 2 spectrophotometer, in 2.3 ml, at 20°C with stirring. The reaction was started by addition of plastoquinol-1 or ubiquinol-1 in ethanol. The detailed reaction mixtures are given in the legends. If proton release was measured simultaneously to plastocyanin reduction, a pH-electrode (Ingold, FRG) was installed into the stirred cuvette. The response time of the electrode was 4-times faster than the fastest proton-release rates measured. The amount of protons released was calibrated by addition of known amounts of acid. The initial rates of proton release and plastocyanin reduction were corrected for the uncatalyzed rates (fig. 1) to obtain H^+/e^- stoichiometries or oxidation control ratios (table 1). The formation of a membrane potential was measured with the help of the carbocyanine dyes diO-C_5-(3) or diS-C_3-(5) [21,22] as specified in the legends to fig. 2 and fig. 3.

3. RESULTS AND DISCUSSION

The incorporation of the cytochrome b_6/f complex into lipid vesicles by sonication was tested by centrifugation on sucrose density gradients. More than 90% of the material banded as a distinct, brownish zone between the densities characteristic for free complex and plain liposomes. Negatively stained proteoliposomes appeared in electron micrographs as vesicles with diameters of 40–80 nm.

Traces of plastocyanin reduction by plastoquinol and concomitant proton release, catalyzed by cytochrome b_6/f liposomes are shown in fig. 1. The reduction rate was stimulated by valinomycin, and even more by valinomycin/nigericin – a combination which totally dissipates electrochemical proton potentials across membranes. Proton release was also stimulated by valinomycin/-
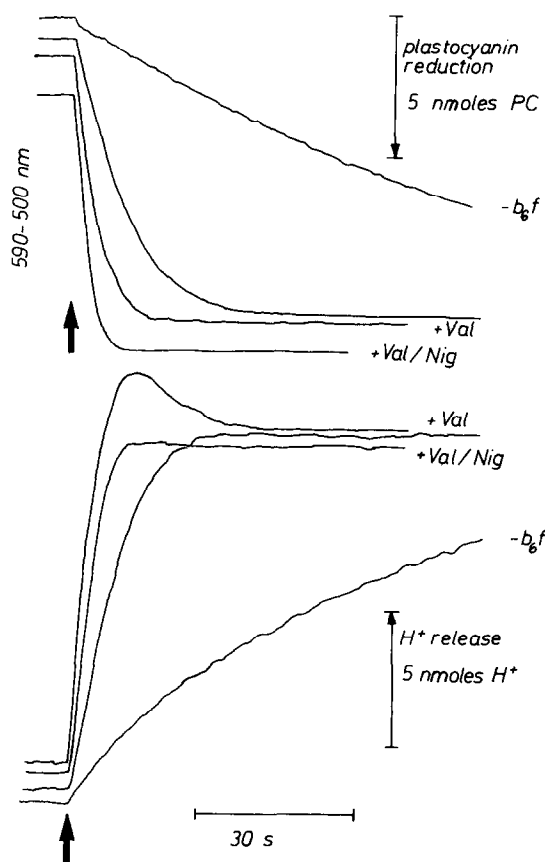

Fig. 1. Simultaneous measurement of plastocyanin reduction and proton release by the cytochrome b_6/f complex in liposomes. The measurements were done as in section 2. The reaction mixture contained in 2.3 ml, 50 mM NaCl, 10 mM KCl, 4.7 μM plastocyanin and proteoliposomes equivalent to 53 nM cytochrome f. The pH was adjusted to 6.9. The reaction was started by addition of plastoquinol-1 to 17 μM ($\rightarrow$). Where indicated valinomycin (val; Calbiochem) and nigericin (nig; Eli Lilly/Indianapolis) were present in 0.5 μg/ml. Controls were run in the absence of cytochrome b_6/f liposomes ($-b_6/f$).

nigericin, but even more so by valinomycin alone leading to an overshoot of acidification. Initial rates of plastocyanin reduction and proton release catalyzed by free and incorporated cytochrome b_6/f complex are compared in table 1. These were recorded at higher chart speeds than shown in fig. 1. With the free complex the rates were slightly inhibited by valinomycin which could reflect its reported inhibitory effect on chloroplast electron transport [23]. Also FCCP

Table 1

Comparison of plastocyanin reduction and proton release with free and incorporated cytochrome b_6/f complex

Addition	Rate of plastocyanin reduction	Oxidation control ratio	Rate of H^+ release	H^+/e^-
(A) Free cytochrome b_6/f complex				
no addition	36.0	–	34.0	0.94
+ val.	31.5	0.88	27.9	0.89
+ val./nig.	33.9	0.94	34.8	1.02
(B) Incorporated cytochrome b_6/f complex				
no addition	14.3	–	22.0	1.53
+ val.	39.8	2.78	75.3	1.89
+ nig.	16.5	1.15	17.2	1.04
+ val./nig.	49.9	3.48	55.4	1.10
+ FCCP	39.3	2.74	49.9	1.26
+ X-100	36.1	2.52	42.9	1.19

The conditions of measurements are given in section 2 and in fig. 1. Initial rates of plastocyanin reduction and proton release are given in μmol . nmol cytochrome $f^{-1} . h^{-1}$. 'Oxidation control ratio' stands for the ratio of initial plastocyanin reduction in the presence and absence of the additions indicated in the first column. H^+/e^- ratios were obtained by dividing the initial rates of proton release by the initial rates of plastocyanin reduction. Additions where indicated were: valinomycin, 0.5 μg/ml; nigericin, 0.5 μg/ml; FCCP (Boehringer) 5 μM; Triton X-100, 0.05%; ethanol was always present to 0.1%

and Triton X-100 [16] inhibited slightly (not shown). The stoichiometry of proton release to plastocyanin reduction calculated from the initial rates was always close to 1 with the free complex, according to 1 H^+/e^- liberated from plastoquinol. The reduction rate catalyzed by the vesicular complex was comparatively low, but was stimulated by valinomycin/nigericin even beyond the rate observed with the free complex. Also the uncoupler FCCP or dissolution of the vesicles stimulated by Triton X-100. Valinomycin alone increased the rate substantially, while nigericin alone was only slightly stimulatory, which suggests that the reaction is predominantly under control by a membrane potential, and less by a pH-difference across the membrane. The highest oxidation control ratio comparing the initial rates in the presence and absence of valinomycin/nigericin was 3.5. This is less than reported for the mitochondrial cytochrome bc_1 complex [9], which could be explained by the 10-fold higher maximal rates

observed in this system. In the presence of valinomycin ~2 H^+/e^- are released during plastoquinol/plastocyanin oxidoreduction catalyzed by cytochrome b_6/f liposomes, when initial rates are compared. In 7 experiments a ratio of 1.98 ± 0.20 H^+/e^- was determined. This shows that, in addition to the liberation of a scalar proton from plastoquinol during oxidation, an extra proton is released from the vesicles. Also in the absence of valinomycin >1 H^+/e^- are released, although no overshoot of acidification is observed (fig. 1). The release of the extra proton is inhibited by nigericin, uncoupler or detergent. These findings are strikingly similar to the results obtained with the mitochondrial cytochrome bc_1 complex in liposomes [9–11].

Reconstitution of oxidation control was also obtained with cytochrome b_6/f proteoliposomes prepared by the detergent-dilution procedure [24], but was not observed when the cholate dialysis method was tried [9].
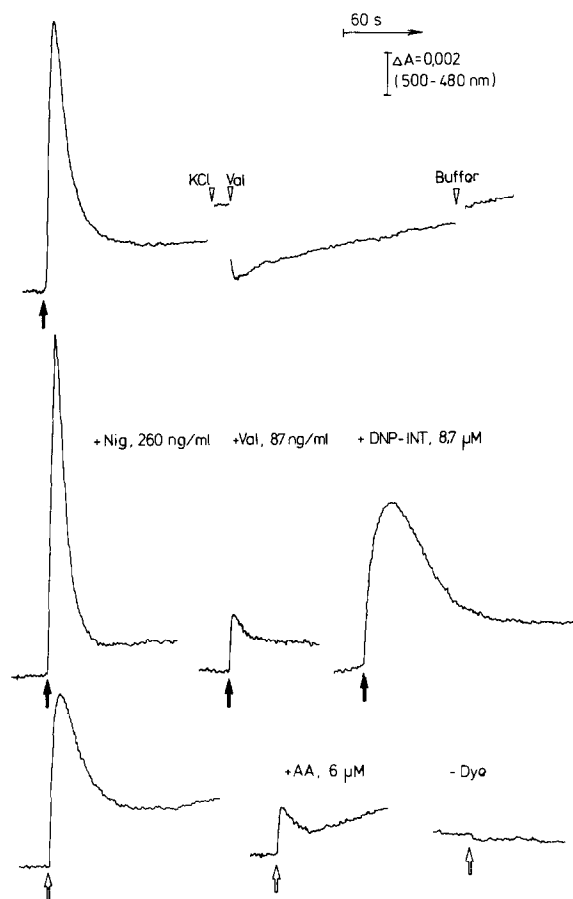
Fig. 2. Membrane potential formation during oxidation of plastoquinol-1 or ubiquinol-1 by plastocyanin in proteoliposomes containing the cytochrome b_6/f complex. The reaction mixture contained in 2.3 ml, 50 mM NaCl, 10 mM KCl, 3.6 μM plastocyanin, 2.2 μM of the carbocyanine diO-C5-(3), and 10 μl proteoliposomes containing 1 mg lipid and 36 μg protein corresponding to 108 nM cytochrome f. The pH was adjusted to 6.9. The reaction was started by the addition of either plastoquinol-1 ($\rightarrow$) or ubiquinol-1 ($\Rightarrow$) to 18 μM. Absorption changes were measured at 500–480 nm. In the upper trace KCl (to 60 mM) and valinomycin (val.; to 0.17 μg/ml) were added, followed by the addition of dilute buffer in the same volume as KCl to indicate the dilution effect. The other additions indicated were made before injection of quinol. Nig, Val and AA stand for nigericin, valinomycin and antimycin A, respectively. The last trace was run in the absence of indicator dye. The initial rates of plastocyanin reduction by plastoquinol, or ubiquinol were 8.5 and 8.0 μmol . nmol cytochrome f^{-1} . h^{-1}, respectively. Addition of uncoupling concentrations of valinomycin plus nigericin (table 1) stimulated the rates to 33 and 15, respectively.

The formation of a membrane potential during reduction of plastocyanin by plastoquinol in the cytochrome b_6/f liposomes is indicated by the effect of valinomycin (fig. 1, Table 1). More direct evidence for it was obtained using the carbocyanine dyes diO-C5-(3) and diS-C3-(5), which are suitable indicators of negative membrane potentials inside lipid vesicles [21,22]. Recently, diS-C5-(5) has been successfully employed to indicate membrane potential formation by the cytochrome o complex from E. coli reconstituted into liposomes [25]. Both carbocyanines were suitable in our system. Oxonol VI, which is the indicator of choice for positive membrane potentials inside the vesicles [26,27], gave no response.

In fig. 2, the transient membrane potential is reflected by absorption changes of diO-C5-(3) during reduction of plastocyanin by plasto- or ubiquinol-1. The absorption change corresponds to an electric field negative inside the vesicles. This is indicated by an absorption change of the dye into the opposite direction caused by a diffusion potential, positive inside the liposomes, induced by addition of external K$^+$/valinomycin (fig. 2, upper). The transient extent of the membrane potential during oxidoreduction is stimulated by nigericin and inhibited by valinomycin. It is inhibited but still observable by the electron transport inhibitor DNP-INT, at concentrations which inhibited plastocyanin reduction below the uncatalyzed reaction with plastoquinol-1 (fig. 1). Probably the direct reaction is suppressed in the presence of the cytochrome b_6/f complex. Like the extra proton release (table 1), membrane potential formation is fully inhibited by 2 μM FCCP, 0.1% Triton X-100, 2 μM gramicidin, 40 μM 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO) and 20 μM 5-(n-undecyl)-6-hydroxyl-4,7-dioxobenzothiazole (UHDBT). It is insensitive to myxothiazol. No potential-indicating absorption changes occurred with the free cytochrome b_6/f complex, or with plain liposomes, not even at pH 8.0 where the direct reduction of plastocyanin by plastoquinol-1 was faster than the catalyzed reaction at pH 6.9. Ubiquinol-1 is also accepted as electron donor by the cytochrome b_6/f complex [16], although the oxidoreductase activity with cytochrome b_6/f liposomes was < 0.5 the one with plastoquinol-1 under uncoupled conditions. Correspondingly, the transient membrane potential formation was smaller (fig. 2, lower traces). In-

terestingly, antimycin A drastically inhibited membrane potential formation at concentrations which hardly affected oxidoreduction. This is just the opposite behavior to DNP–INT. In control experiments antimycin A was found not to cause an additional leak in the vesicles (not shown). The dye diS-C_3-(5) gave identical responses at the wavelength combination 683–660 nm, which is especially suitable when cytochrome c-552 from *Euglena gracilis* was used as an alternative electron acceptor ([16]; not shown). To estimate the size of the transient membrane potential in our system negative diffusion potentials inside the vesicles
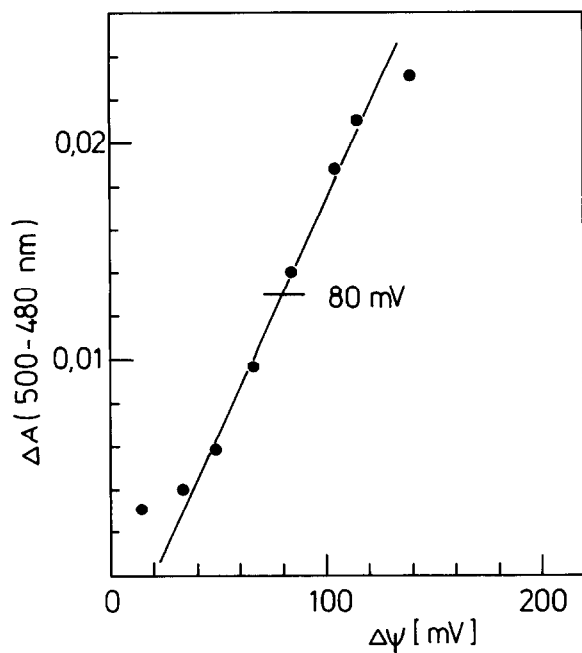

Fig. 3. Calibration of the carbocyanin absorption changes in proteoliposomes containing the cytochrome b_6/f complex by K^+-diffusion potentials. Proteoliposomes were prepared by the sonication procedure in section 2 except for the inclusion of 150 mM KCl in the medium. The assay mixture contained in 2.3 ml: 60 mM NaCl, 18% (w/v) sucrose, 10 μl proteoliposomes, 2.2 μM diO-C_5-(3) and KCl at concentrations which should lead to the diffusion potentials given along the abscissa. The absorption changes (500–480 nm) induced by addition of valinomycin (0.17 μg/ml) are plotted against the calculated membrane potentials. The bar at 80 mV corresponds to the peak of the absorption change observed in fig. 2 (upper trace).

were generated by addition of valinomycin to cytochrome b_6/f liposomes, preloaded with KCl, at various external KCl concentrations. The observed absorption changes of diO-C_5-(3) are plotted against the calculated diffusion potentials in fig. 3. From this graph 80 mV can be estimated for the size of the transient membrane potential during reduction of plastocyanin by plastoquinol-1 (fig. 2, upper trace).

These experiments allow us to conclude that, as found for mitochondrial cytochrome bc_1 complexes, electrogenic extra proton translocation is a basic feature of the isolated cytochrome b_6/f complex from spinach chloroplasts, oxidant-induced reduction of cytochrome b_6 as in [18,20]. Both features are not readily observed in the parent chloroplast membrane [14]. However, conditions for measuring extra proton translocation [28,29], a slow rise of the electric field [30,31], and redox changes of cytochrome b_6 [32] probably reflecting oxidant-induced reduction of cytochrome b_6, all attributable to the function of plastoquinol–plastocyanin oxidoreductase, have been established. Since we now know that these are properties of the basic functional mode of isolated plastoquinol–plastocyanin oxidoreductase, any loss of these properties in the chloroplast, if physiological, would require the regulatory action of additional components.

Oxidant-induced reduction of cytochrome *b* and extra proton translocation by isoprenoid quinol–cytochrome *c* (or plastocyanin) oxidoreductases have led to the formulation of the Q-cycle to explain their reaction mechanism [33]. An alternative is the *b*-cycle which requires a proteinaceous proton pump [34]. We hope that the cytochrome b_6/f complex, because of its relative simplicity, might lend itself to decide between the different views.

REFERENCES

[1] Rieske, J.S. (1976) Biochim. Biophys. Acta 456, 195–247.

[2] Trumpower, B.L. and Katki, A.G. (1979) in: Membrane Proteins in Energy Transduction (Capaldi, R.A. ed) pp. 89–200, Dekker, Basel, New York.

[3] Bowyer, J.R. and Trumpower, B.L. (1981) in: The Proton Cycle (Skulachev, V. and Hinkle, P. eds) pp. 105–122, Addison Wesley, New York.

[4] Dutton, P.L. and Prince, R.C. (1978) in: The Photosynthetic Bacteria (Clayton, R.K. and Syström, W.R. eds) pp. 525–570, Plenum, London, New York.

[5] Engel, W.D., Schägger, H. and Von Jagow, G. (1980) Biochim. Biophys. Acta 592, 211–222.

[6] Weiss, H. and Kolb, H.J. (1979) Eur. J. Biochem. 99, 139–149.

[7] Palmer, G. (1978) Methods Enzymol. 53, 113–121.

[8] Gabellini, N., Bowyer, J.R., Hurt, E., Melandri, B.A. and Hauska, G. (1981) Eur. J. Biochem. 126, 105–111.

[9] Leung, K.H. and Hinkle, P.C. (1975) J. Biol. Chem. 250, 8467–8471.

[10] Guerrieri, F. and Nelson, B.D. (1975) FEBS Lett. 54, 339–342.

[11] Von Jagow, G., Engel, W.D., Schägger, H., Machleidt, W. and Machleidt, I. (1981) in: Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F. et al. eds) pp. 149–161, Elsevier Biomedical, Amsterdam, New York.

[12] Nelson, N. and Neumann, J. (1972) J. Biol. Chem. 247, 1917–1924.

[13] Wood, P.M. and Bendall, D.S. (1976) Eur. J. Biochem. 61, 337–344.

[14] Bendall, D.S. (1982) Biochim. Biophys. Acta in press.

[15] Hauska, G., Hurt, E., Gabellini, N. and Lockau, W. (1982) submitted.

[16] Hurt, E. and Hauska, G. (1981) Eur. J. Biochem. 117, 591–599.

[17] Hurt, E., Hauska, G. and Malkin, R. (1981) FEBS Lett. 143, 1–5.

[18] Hurt, E. and Hauska, G. (1982) Photobiochem. Photobiophys. in press.

[19] Hurt, E. and Hauska, G. (1982) J. Bioenerg. Biomembr. in press.

[20] Hurt, E. and Hauska, G. (1982) Biochim. Biophys. Acta in press.

[21] Sims, P.J., Waggoner, A.S., Wang, C.H. and Hoffman, J.F. (1974) Biochemistry 13, 3315–3330.

[22] Waggoner, A.S. (1978) Methods Enzymol. 55, 689–695.

[23] Voegeli, K.K., O'Keefe, D., Whitmarsh, D. and Dilley, R.A. (1977) Arch. Biochem. Biophys. 183, 333–339.

[24] Racker, E., Violand, B., O'Neal, S., Alfonzo, S. and Telford, J. (1979) Arch. Biochem. Biophys. 198, 470–477.

[25] Kita, K., Kasahara, M. and Anraku, Y. (1982) J. Biol. Chem. 257, 7933–7935.

[26] Schuurmans, J.J., Casey, R.P. and Kraayenhof, R. (1978) FEBS Lett. 94, 405–409.

[27] Bashford, C.L. and Smith, J.C. (1978) Methods Enzymol. 55, 569–586.

[28] Fowler, C.F. and Kok, B. (1976) Biochim. Biophys. Acta 432, 510–523.

[29] Rathenow, M. and Rumberg, B. (1980) Ber. Bunsenges. Phys. Chem. 84, 1059–1062.

[30] Bouges-Bocquet, B. (1981) Biochim. Biophys. Acta 635, 327–340.

[31] Crowther, D. and Hind, G. (1980) Arch. Biochem. Biophys. 204, 568–577.

[32] Velthuys, B.R. (1979) Proc. Natl. Acad. Sci. USA 76, 2765–2769.

[33] Mitchell, P. (1976) J. Theor. Biol. 62, 327–367.

[34] Wikström, M., Krab, K. and Saraste, M. (1981) Annu. Rev. Biochem. 50, 623–655.