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RECONSTITUTION OF *b*-TYPE CYTOCHROME OXIDASE FROM *RHODOPSEUDOMONAS CAPSULATA* IN LIPOSOMES AND TURNOVER STUDIES OF PROTON TRANSLOCATION

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Purified *b*-type cytochrome oxidase from *Rhodopseudomonas capsulata* was incorporated into phospholipid vesicles to measure proton extrusion with pulses of ferrocytochrome *c* for one oxidase turnover. In accordance with the pH shift of its midpoint potential, the purified oxidase showed a proton extrusion of 0.24 H^+/e^- with uptake of 1 H^+/e^- from the liposomes for the reduction of oxygen to water. This proton translocation could only be observed in the presence of valinomycin + K^+ and was not inhibited by DCCD. Oxidase preparations from the first purification step, which contain other protein compounds especially a membrane-bound cytochrome *c* but not the ubiquinol-cytochrome c_2-oxidoreductase showed a pumping activity of 0.9 H^+/e^-, which was inhibited by DCCD for nearly 75%. Inhibition of the electron transfer was not observed, which could be explained by a 'molecular slipping' of proton extrusion and electron transfer. Proton extrusion from two oxidase-turnovers was only 80% of that from one turnover. The proton pumping of the *b*-type oxidase strongly depended on the enzyme/phospholipid ratio.

Introduction

Rhodopseudomonas capsulata, a facultative phototrophic bacterium, produces ATP under chemotrophic conditions by oxidative phosphorylation [1–3]. Only *b*-type cytochromes function in the two terminal oxidases [1] which differ in their midpoint potential, sensitivity to KCN and to CO [4,5].

We have purified the high potential cytochrome oxidase (ferrocytochrome *c*-oxidoreductase, E.C. 1.9.3.1) 160-fold [6]. The native structure of the enzyme consists of two polypeptides each with an apparent M_r of 65 000 and contains 1 mol protoheme per dimer [7].

A midpoint potential of 385 ± 15 mV was determined by redox titrations of the purified cytochrome oxidase at pH 7 and 25° [7]. This value indicated a functional cytochrome oxidase. The midpoint potential was strongly pH-dependent. When the pH was increased to a value of 9, the potential decreased approx. 180 mV per Δ pH [7], indicating three redox-linked acidic groups participating in the electron transfer. These redox-linked shifts in the p*K* of acid/base groups (redox Bohr effect) are often implicated in discussions of proton pump mechanisms [8], mainly for reasons of kinetic versatility and enzymatic efficiency. The observed modulation favoured the protonation of the reduced and deprotonation of the oxidized form of the transducing cytochrome oxidase.

ATP formation driven by NADH and succinate oxidation has been demonstrated in membranes

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DEAE, *N,N*-diethylaminoethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride $\Delta\tilde{\mu}_{H^+}$, electrochemical proton gradient.

from aerobically grown cells of *Rps. capsulata*. Baccarini-Melandri et al. [2] reported P/O ratios of 0.45 and 0.15 for NADH and succinate respiration, respectively, suggesting that the respiratory chain is branched having two pathways of oxygen reduction with different efficiency of phosphorylation. The high potential *b*-type cytochrome oxidase seems to function as the third coupling site between cytochrome c_2 and oxygen [2] although it is still of some controversary [9].

In the current study we report experiments with purified *b*-type cytochrome oxidase reconstituted into liposomes to measure proton translocation [10,11] with pulses of ferrocytochrome *c* for controlled turnovers of the enzyme as they were already described for the a/a_3-type cytochrome oxidase [12,13]. If one accepts the interpretation of the experimental data of Wikström and Kraab [11,14] and Casey and Azzi [15] our results provide strong evidence that the high potential *b*-type cytochrome oxidase of *Rps. capsulata* functions as a proton pump carrying out true proton translocation.

Materials and Methods

Cytochrome *c* oxidase from membranes of chemotrophically grown cells of *Rhodopseudomonas capsulata*, strain 37b4 (DSM 938), was prepared as in Ref. 6. Buffer exchange and desalting of the preparations were done as in Ref. 16. Reconstituted cytochrome oxidase-containing vesicles were prepared by a modification of the cholate-dialysis method of Hinkle et al. [10] as described in Ref. 17 using soybean phospholipids (Sigma, type II-S). Cytochrome oxidase from the first purification step after DEAE-chromatography and highly purified preparations were added to the suspension (0.75–0.15 mg of protein equals 5.8–1.16 nmol oxidase/ml liposomes). The concentrations were calculated from the native M_r of 130000, from the $\Delta A_{560-575nm}$ using an extinction coefficient of 22 $mM^{-1} \cdot cm^{-1}$ [18] and from the pyridine-hemochrome preparations [19]. To reduce the amount of Triton X-100 (Merck) the oxidase preparations were extensively dialysed against 100 mM Hepes (Sigma), pH 7.2. The purity of the reconstituted oxidase was controlled by SDS-polyacrylamide gel electrophoresis [20]. Ferrocytochrome *c* from horse heart (Sigma, type II-A or type VI) was prepared as in Ref. 16.

Changes in pH were measured by using a pH electrode (Ingold, 405M5) with a Philips ion activity meter (PW 9413) and a LKB-recorder (type 2210) or the pH-indicator dye Phenol Red (Merck). The absorbance change of Phenol Red was monitored at 556.5–504.5 nm [17] with a Perkin-Elmer split beam spectrophotometer model 330 (Überlingen). Extreme care was taken to exclude cytochrome *c*- and *b*-induced absorbance changes not linked to changes in pH. The assay contained in a final volume of 2.01 ml 70 mM choline-chloride (Merck)/30 mM KCl/50 μM Phenol Red/1 μM valinomycin (Sigma)/1.16 nmol reconstituted cytochrome oxidase. With the pH electrode – using 45 mM choline-chloride/55 mM KCl/1 μM valinomycin/1.16 nmol reconstituted cytochrome oxidase – the stoichiometry of the reaction was followed by the oxidation of ferrocytochrome *c* at 550–540 nm ($\epsilon = 19.6$ $mM^{-1} \cdot cm^{-1}$ [21]) and by the reduction of oxygen using a YSI 53 Biological Oxygen Monitor (Yellow Springs Instruments Co., Yellow Springs, OH). The assays were incubated at 18°C in a cuvette stirred below the light path. 0.25 mM Antimycin A (Sigma) was added when the activity of the oxidase preparation after DEAE-chromatography was tested. CCCP (Sigma) was added to a final concentration of 5 μM. The system was calibrated with 1 mM HCl (Titrisol, Merck).

The cytochrome oxidase was incubated for 10 min with 10 mM DCCD to inhibit proton translocation and for testing the inhibition of electron transfer.

Ubiquinol-cytochrome c_2-oxidoreductase was measured with sodium-dithionite reduced duroquinone (Serva, Heidelberg) as described by Ruzicka and Crane [22]. The reduction of ferricytochrome *c* was followed at 30°C with an Eppendorf-spectrophotometer (type 1101M) at 546 nm using an extinction coefficient of 21 $mM^{-1} \cdot cm^{-1}$ [23]. As a control of specificity the enzyme reaction was inhibited by 0.25 mM antimycin A.

Results

The proton pump inhibitor DCCD did not inhibit the electron transfer of the *b*-type cy-

tochrome oxidase. Even at a molar ratio of DCCD to cytochrome oxidase of $1 \cdot 10^7$ the ferrocytochrome *c* oxidizing activity remained unaffected. This is in contrast to the results with the a/a_3-type oxidase from mitochondria, where DCCD inhibited not only proton translocation but also oxidation of ferrocytochrome *c* [24]. Therefore, we incorporated different preparations of the high potential *b*-type cytochrome oxidase of *Rps. capsulata* into liposomes to measure directly proton translocation and DCCD-inhibition.

Addition of 4.6 nmol ferrocytochrome *c* to 1.16 nM liposome-incorporated *b*-type oxidase from the first purification step after DEAE-chromatography resulted in an acidification of the medium of 0.03 pH-units (Fig. 1a). Although ubiquinol-cytochrome c_2-oxidoreductase-activity eluted from ion exchange column at higher ionic strength (0.5 M KCl) than the oxidase (0.3 M KCl), antimycin A was added to inhibit traces of ubiquinol-cytochrome c_2-oxidoreductase. The acidification corresponded to 4.5 nequiv. H^+ and could only be seen in the presence of valinomycin. Parallel experiments showed an oxidation of ferrocytochrome *c* and a stoichiometrical reduction of O_2. This pulse of respiration lasted 4–5 s.

The ratio of protons translocated in the medium to electrons transferred from ferrocytochrome *c* to O_2 via the oxidase was 0.9 ± 0.04 (mean value ± standard deviation). The presence of the lipophilic uncoupler CCCP caused an equilibration of protons between the interior of liposomes and the medium with a concomitant alkalinization of 4.5 nequiv. H^+. This indicated that 1 H^+/e^- was consumed for the reduction of O_2 to water (Fig. 1a). The maximal stimulation of the electron-transfer activity of the cytochrome oxidase after addition of CCCP to the liposomes was 2-fold.

The acidification of the medium was inhibited by addition of 1 mM NaN_3. Ferrocytochrome *c* was oxidized under these conditions by ferricyanide, thus by-passing the oxidase. Further addition of another 4.6 nmol ferrocytochrome *c* to the sample in the presence of CCCP only caused an alkalinization for the proton uptake during reduction of oxygen (Fig. 1a).

The proton translocation of the oxidase was inhibited for 72% (0.25 ± 0.03 H^+/e^-; Fig. 1a) when the oxidase preparation after DEAE-chromatography was incubated with the ATP-ase-inhibitor DCCD for 10 min. DCCD did not affect the electron transfer of the oxidase as already mentioned. On addition of CCCP a stoichiometrical proton uptake per O_2-reduction was observed.

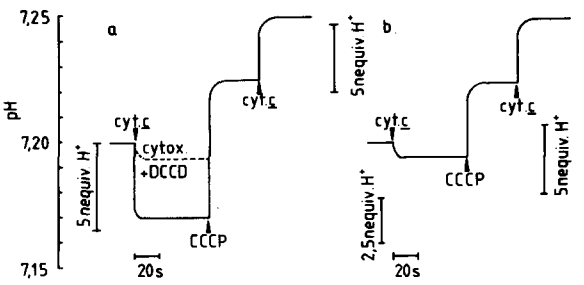

Fig. 1. Proton extrusion of 1.16 nmol reconstituted cytochrome oxidase incorporated in liposomes after addition of 4.6 nmol ferrocytochrome *c* (arrows). The system was adjusted to the indicated pH with 1 mM HCl. The broken line indicates the acidification after incubation of the oxidase with 10 mM DCCD for 10 min. The bars to the left and right indicate the pH change caused by 5 µl of 1 mM HCl in the absence and presence of 10 µl of 1 mM CCCP, respectively. The zero line is corrected for a slight shift in pH. The enzyme : lipid ratio was 0.03 nmol/mg. (a) Reconstituted oxidase from DEAE-preparation; (b) purified oxidase after affinity chromatography reconstituted into liposomes.

Similar experiments were performed with the highly purified cytochrome oxidase after affinity chromatography on cytochrome *c*-thiol activated Sepharose 4B [6]. In constrast to observations with the DEAE-preparation the ratio of protons translocated to electrons transferred from 4.6 nmol ferrocytochrome *c* to O_2 via 1.16 nmol cytochrome oxidase was only 0.24 ± 0.04 (Fig. 1b). Nevertheless, the alkalinization in the presence of CCCP was 1 H^+/e^- for the reduction of O_2 to H_2O. The proton translocation of the purified oxidase could not be inhibited by DCCD. It is worth noting that the observed acidification nearly corresponded to the translocating activity of the DEAE-oxidase preparation after inhibition by DCCD (Fig. 1).

The response of the pH electrode used in these experiments was relatively slow. Under conditions of an appreciable decay of the ferrocytochrome *c*-induced acidification for more than one oxidase turnover the absorbance change of the pH indicator Phenol Red was used to measure the pH changes at the initial decay of the proton gradient.

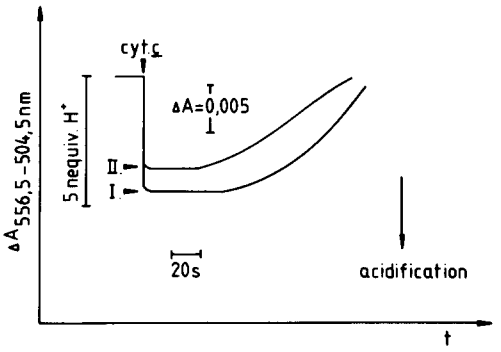

Fig. 2. Comparison of the proton translocation of 1.16 nmol oxidase from DEAE preparation incorporated in lipid vesicles after pulses of ferrocytochrome *c* for one (4.6 nmol) and two (9.2 nmol) turnovers. The pH of the suspension was adjusted to pH 7.2 and the absorbance was recorded as described in Material and Methods. The absorbance trace of two turnovers has been drawn on half-scale to facilitate its comparison with the trace for one turnover.

After addition of ferrocytochrome *c* sufficient for two turnovers (9.2 nmol) to 1.16 nmol *b*-type oxidase, only 80% of protons compared with proton extrusion of one turnover was translocated (Fig. 2). The stability of the proton gradient decreased at the same time. The gradient from one turnover started to decay 60 s after addition of ferrocytochrome *c*, but the gradient already began to equilibrate after 40 s when two turnovers were initiated.

The stability and decay kinetics of the ferrocytochrome *c*-induced acidification strongly depended on the ratio of cytochrome

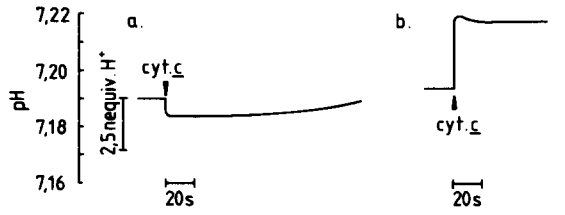

Fig. 3. Ferrocytochrome-*c*-induced pH changes of oxidase-containing vesicles with different enzyme : lipid ratios. The experimental procedure was the same as described for Fig. 1. The final oxidase concentration was 0.58 μM. (a) Cytochrome oxidase : lipid ratio, 0.03 nmol/mg; (b) cytochrome oxidase : lipid ratio, 0.15 nmol/mg.

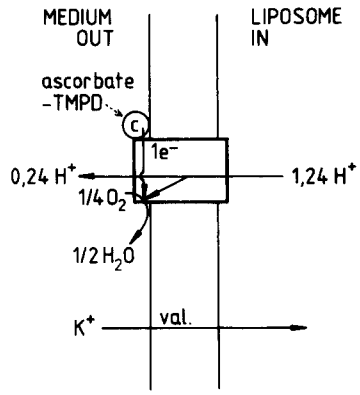

Fig. 4. Proposed scheme for the orientation of the reconstituted *b*-type cytochrome oxidase-containing vesicles. The figure summarizes the results for proton translocation of the purified oxidase.

oxidase/phospholipid employed in the reconstitution process (Fig. 3). At a ratio of 0.15 nmol purified oxidase/mg soy-bean phospholipids, only an alkalinization of the medium was observed (Fig. 3b) similar to that observed during proton uptake and O_2-reduction after addition of CCCP (Fig. 1b). No initial acidification could be observed. A stable acidification with a slow decay within 1 min was observed when a ratio of 0.03 nmol cytochrome oxidase/mg phospholipid was used (Fig. 3a). The kinetics of the gradient showed only a slow reentry of protons (see also Fig. 2). This was in contrast to the suboptimal ratio of 0.01 nmol oxidase/mg phospholipid causing an acidification followed immediately by an equilibration of protons with a hyperbolic kinetic (data not shown).

It was reported that mitochondrial cytochrome oxidase faced outwards with all the catalytic sites of the enzyme when incorporated into lipid vesicles and was accessible to ferrocytochrome *c*. [17,25]. In order to investigate the orientation, of the reconstituted *b*-type cytochrome oxidase we set up a test system which based on the experience that cytochrome *c* cannot penetrate the lipid layer of the vesicles [26,27] and that the *b*-type oxidase of *Rps. capsulata* cannot be directly reduced by ascorbate and the mediator TMPD without cytochrome *c* (Fig. 4). The half-diffusion time of ascorbate into liposomes was found to be 6.5 h under similar experimental conditions as in this work [29]. The absorption at 560 nm of the re-

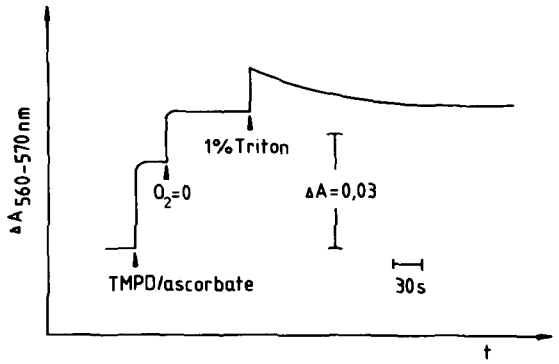

Fig. 5. Studies on the orientation of the incorporated oxidase by recording reduction of cytochrome *b*, with ascorbate and TMPD before and after addition of Triton X-100. The reduction of the *b*-type oxidase was measured at 560–570 nm. The assay contained 0.58 μM cytochrome oxidase/2.2 μM cytochrome *c*/25 μl 20 mM sodium ascorbate/25 μl 25 mM TMPD, in a final volume of 2.01 ml as described in Material and Methods. Another 20 μl of ascorbate was added when the absorption showed a constant level after Triton-addition to be sure of complete oxidase-reduction. The Triton solution was gassed with nitrogen to remove any O_2. The initial absorbance change after Triton addition was causedb y N_2-bubbles in the stirred suspension.

duced *b*-type cytochrome oxidase increased when ascorbate and TMPD were added to the oxidase-vesicles in the presence of cytochrome *c* (Fig. 5). The dissolved oxygen became reduced and the absorption increased after about 30 s, once more reflecting the reduction of all the 'right-side-out'-oriented oxidases. When a steady absorbance value had been reached the lipsomes were lysed by addition of 1% Triton X-100 and the internal faced oxidases were reduced by another 20 μl of 20 mM absorbate. The $\Delta A_{560-575nm}$ increased about 4%, indicating that 96% of the incorporated *b*-type cytochrome oxidase molecules were oriented with their active sites facing the external medium. The high portion of 'right-side-out'-oriented molecules made sure that the oxidases were catalysing no more than one turnover at the calculated quantities of ferrocytochrome *c*. The non-statistical incorporation of the *b*-type cytochrome oxidase may be due to the interactions of hydrophobic and polar regions of the polypeptide with the phospholipids [30] and may also be influenced by the negative surface charge [31] and the fluidity of the lipid layer [32].

Discussion

The presented data provide strong evidence for a proton translocation catalyzed by the high potential *b*-type cytochrome oxidase of *Rps. capsulata*. The stoichiometry of H^+/e^- uptake and release was shown to be dependent from the degree of purification and the protein : lipid ratio. At optimum enzyme : phospholipid ratio a stable acidification of the medium was observed. When the optimum ratio was exceeded, only alkalinization was observed, as though uncouplers were present. Additionally the proton : lipid ratio strongly influenced the decay kinetics of the proton gradient.

The proton pumping activity of the *b*-type oxidase decreased from 0.9 H^+/e^- with DEAE-chromatography preparations to 0.24 H^+/e^- when purified enzyme preparation after affinity chromatography was used. A general destabilization of the oxidase activity would have influenced not only the proton translocation but also the electron transfer. In this case the inhibition by DCCD should be the same in all preparations. In addition, it could be excluded that the lower acidification of the medium was caused by a proton uptake from outside the liposomes for reduction of oxygen because 96% of the reconstituted oxidase was oriented righ-side-out. The rest of proton pumping activity of the purified oxidase, which was not inhibited by DCCD, could be explained by the separation of a proton pumping subunit with DCCD-reactive carboxy-groups – not necessary for electron transport activity – from the oxidase during affinity chromatography. The lack of proton pumping activity of the a/a_3-type oxidase from *Nitrobacter agilis* [33] and *Rps. sphaeroides* was explained in the same way [15]. The DEAE-preparation of the *b*-type cytochrome oxidase contained several contaminating proteins which were removed during affinity chromatography [6]. The identification of a possible proton pumping subunit of the oxidase was not possible but is under investigation. Although the DEAE-preparation was free of other respiratory activities and the properties of the observed reactions – ferrocytochrome *c* serves as electron donor, inhibition by KCN and NaN_3 – pointed to the high-potential oxidase of *Rps. capsulata* with a functional *b*-type cyto-

chrome, we cannot completely exclude that traces of other H^+-translocating redox proteins (antimycin-insensitive) were present in the DEAE-preparation.

We were unable to observe an irreversible acidification of the external medium caused by deprotonation of a phosphatidylserine-ferrocytochrome *c*-complex during oxidation as proposed by Mitchell and Moyle [34] or by a rupture of protonated salt-bridges in the oxidase-lipid complex as proposed by Papa et al. [35]. Such a scalar process should have resulted in a smaller alkalinization than the one observed after addition of the uncoupling agent CCCP because of the missing proton translocation into the liposomes [15]. A non-vectorial process of deprotonation of a phospholipid-cytochrome *c*-complex should also have led to an acidification of the medium even when the oxidase was inhibited by N_3^-, which could not be observed.

The proton extrusion of the oxidase has been seen only in the presence of the $\Delta\psi$-collapsing agent valinomycin $+K^+$ because of a more extensive and detectable proton translocation after the collapse of the charge differential. Calibrations of the system with HCl in the presence of CCCP showed a greater buffering capacity because of the higher ionic strength inside the vesicles.

A 'molecular slipping' [36], that means an intramolecular uncoupling of proton translocation and electron transfer, seemed to avoid the feedback inhibition of electron-transport activity by DCCD. This 'molecular slipping' may also refer to a regulation of proton translocation by the cytochrome oxidase in view of a second alternative oxidase with a reduced activity as a coupling side in the branched respiratory chain of *Rps. capsulata* [2,9].

In a recent study, Matsushita et al. [37] reported the incorporation of cytochrome *o* oxidase and *lac* carrier from *Escherichia coli* into liposomes and the generation of $\Delta\tilde{\mu}_{H^+}$ (interior negative and alkaline) that drove lactose transport against a concentration gradient into the vesicles. Our present study provides strong evidence for a real proton translocation catalyzed by a *b*-type cytochrome oxidase. Because of its lack of CO-binding this oxidase was defined to be an atypical *o*-type cytochrome [38]. In comparison to other bacterial cytochrome oxidases the high potential *b*-type cytochrome oxidase seems to function as energy coupling side for the generation of $\Delta\tilde{\mu}_{H^+}$ like the a/a_3-type oxidase. The translocation of protons is still doubtfull for other oxidases of the *bd*- and cd_1-type [39].

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