Proton-pump activity of *Nitrobacter agilis* and *Thermus thermophilus* cytochrome *c* oxidases

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The purified cytochrome *c* oxidases from *Nitrobacter agilis* and *Thermus thermophilus* were reconstituted into phospholipid vesicles and their H^+ pumping activity upon addition of ferrocytochrome *c* was examined. *Thermus* cytochrome *c* oxidase pumped H^+, while the *Nitrobacter* enzyme did not. The process of H^+ movement was successfully simulated.

Cytochrome c *oxidase* *Proton pumping* *Chemiosmosis* Nitrobacter agilis
Thermus thermophilus *Proteoliposome*

1. INTRODUCTION

Since 1979 cytochrome *c* oxidases (EC 1.9.3.1) containing heme *a* and copper as prosthetic groups have been highly purified from various bacteria [1,2], such as the thermophilic bacterium PS3 [3,4], *Thiobacillus novellus* [5,6], *Nitrobacter agilis* [5,7], *Thermus thermophilus* [8,9], *Paracoccus denitrificans* [10] and *Rhodopseudomonas sphaeroides* [11]. These bacterial cytochrome *c* oxidases are found to be composed of 2 [5] or 3 kinds of subunits [4], while the mitochondrial enzyme is known to consist of at least 7 kinds of subunits [12].

Observations on bovine cytochrome *c* oxidase reconstituted into liposomes, have shown that the enzyme pumps H^+ in addition to catalysis of electron transfer across membrane [13–15]. In this respect, cytochrome *c* oxidases from the thermophilic bacterium PS3 [3,16] and *P. denitrificans* [17] have been shown to pump H^+ with cytochrome *c* oxidation. The PS3 enzyme contains 3 subunits (M_r 56000, 38000, 22000) [4], while the *Paracoccus* enzyme contains 2 subunits (M_r 45000, 28000) [10]. An observation that the *Rhodopseudomonas* enzyme did not pump H^+, however, suggests that bacterial cytochrome *c* oxidases do not always act as a redox H^+ pump [11].

Nitrobacter cytochrome *c* oxidase is composed of 2 subunits (M_r 40000, 27000) [7,18] and the *Thermus* enzyme purified by an improved method consists of two kinds of subunits (submitted). Here, we report H^+ pumping activity by these 2 bacterial cytochrome *c* oxidases when reconstituted into liposome vesicles. A mathematical analysis of the H^+ movement upon addition of ferrocytochrome *c* is also described.

Abbreviations: Mops, 4-morpholinopropanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone

2. EXPERIMENTAL

Nitrobacter cytochrome *c* oxidase was purified from *N. agilis* as in [7] and *Thermus* cytochrome *c* oxidase from *T. thermophilus* HB8 by the modified method (submitted) of [9]. Tween 20 or Triton X-100 in these preparations was removed by precipitating the oxidase with ammonium sulfate in the presence of 1.5% sodium cholate as in the case of PS3 cytochrome *c* oxidase [3]. Cytochrome

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c oxidase vesicles were reconstituted from these oxidases and partially purified soybean phospholipids [19] by the freeze–thaw method [16] or by the dialysis method [14].

N. agilis cytochrome *c*-550 was prepared as in [20]. Yeast cytochrome *c* from *Candida krusei* was a product of Sankyo Co. (Tokyo). The reduced form of cytochrome *c* was prepared by addition with a small amount of $Na_2S_2O_4$ and subsequent centri-column of Biogel P-6 and its concentration was determined spectrophotometrically as in [16]. For *N. agilis* cytochrome *c*-550, ϵ_{mM} of 29.4 at 550 nm was used [20].

The pH change induced by a ferrocytochrome *c* pulse was measured in an open vessel (1.5 ml) with a Beckman combination pH electrode no. 39030. Oxidation of cytochrome *c* was measured in a Union Giken spectrophotometer (model SM 401) using a 1 cm light-path cell (1.5 ml). Oxygen uptake activity of the reconstituted vesicles was measured with an oxygen electrode in a 3 ml cell. These cells were all kept thermostatically and the contents were magnetically stirred.

3. RESULTS

3.1. *Reconstitution of cytochrome* c *oxidase vesicles*

Two methods have been known for successful reconstitution of cytochrome *c* oxidase into liposome vesicles capable of H^+ pumping; vesicles which show good respiratory control ratio and H^+ pump activity approaching 1 $\vec{H}^+/e^-$ have been prepared from bovine mitochondrial cytochrome *c* oxidase by the cholate-dialysis method [13–15], and the freeze–thaw method gave the best results to reconstitute vesicles capable of active H^+ pumping from PS3 cytochrome *c* oxidase [16].

Fig.1A shows that vesicles prepared by the cholate-dialysis method from the *Nitrobacter* oxidase exhibits a respiratory control ratio as high as 6; the respiration rate in the presence of an uncoupler, FCCP was 6-times faster than the respiration rate without FCCP. The vesicles prepared by the freeze–thaw method showed a respiratory control ratio of 2.5 (not shown). In the case of the *Thermus* oxidase better results were obtained with vesicles reconstituted by the freeze–thaw method. Fig.1B shows that such vesicles give a respiratory control ratio of 2.7 when yeast cytochrome *c*

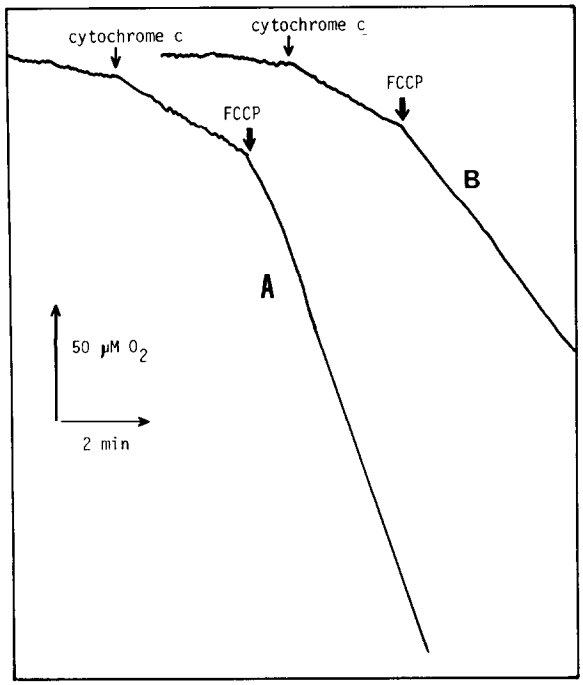

Fig.1. Respiratory control shown by vesicles containing bacterial cytochrome *c* oxidase. (A) Vesicles (0.5 ml) were reconstituted from *Nitrobacter* cytochrome *c* oxidase (1.5 nmol heme *a*) and soybean phospholipids (15 mg) by the cholate-dialysis method. (B) Vesicles (0.5 ml) were reconstituted from *Thermus* cytochrome *c* oxidase (1.4 nmol heme *a*) and soybean phospholipids (20 mg) by the freeze–thaw method. An aliquot (0.1 ml) of these vesicles was suspended in 3 ml 20 mM sodium phosphate buffer (pH 6.6) containing 5 mM sodium ascorbate, and oxygen uptake was followed at 25°C (A) or at 32°C (B). Additions were: (A) yeast cytochrome *c* (45 nmol) and FCCP (1.6 μg); (B) yeast cytochrome *c* (30 nmol) and FCCP (2.0 μg).

(10 μM) was added in addition to ascorbate.

3.2. *Proton pump activity*

If cytochrome *c* oxidase does not pump H^+ and H^+ conductivity of the liposomal membrane is very low, no pH change will be observed in the medium (outside) upon addition of ferrocytochrome *c*. Fig.2A is a typical trace of pH change when a small amount of yeast ferrocytochrome *c* was supplied as a reductant pulse to vesicles reconstituted from the *Nitrobacter* oxidase by the cholate-dialysis method. No H^+ ejection occurred except successive slow alkalinization. When the same experiment was carried out in the presence of

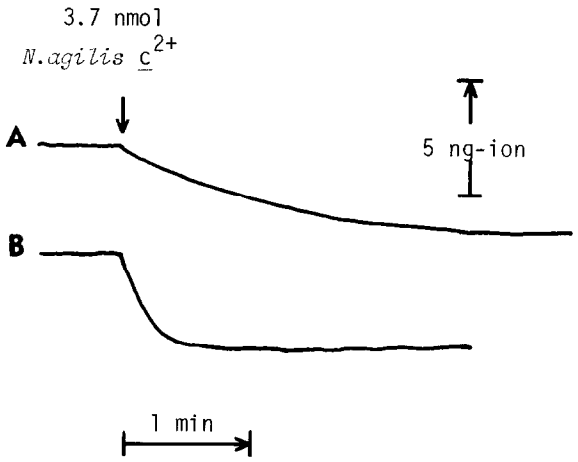

Fig.2. No H^+ pumping by *Nitrobacter* cytochrome *c* oxidase vesicles upon *N. agilis* ferrocytochrome *c* pulse. Vesicles (75 μl) prepared from *Nitrobacter* cytochrome *c* oxidase by the cholate-dialysis method as in fig.1A, were suspended in 1.5 ml the reaction medium containing 25 mM K_2SO_4, 2.5 mM $MgSO_4$, 0.1 mM Mops buffer (pH 6.6). Amount of H^+ ejected or taken in was measured with a pH meter at 25°C. Valinomycin (0.1 μg) was added; (A) control; (B) FCCP (0.6 μg) added.

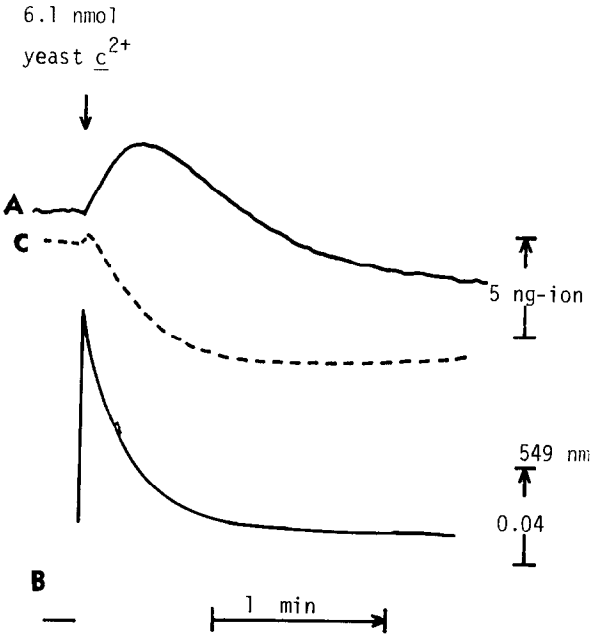

Fig.3. H^+ pumping by *Thermus* cytochrome *c* oxidase vesicles upon yeast ferrocytochrome *c* pulse. Vesicles (75 μl) prepared from *Thermus* cytochrome *c* oxidase by the freeze–thaw method as in fig.1B, were suspended as in fig.2 but at 32°C. (A) pH meter trace without FCCP; (B) spectrophotometer trace at 549 nm without FCCP; (C) (---) as in (A) with FCCP.

FCCP, rapid alkalinization of the expected final stoichiometry of -1 H^+/e^- took place (fig.2B). On the contrary, when vesicles reconstituted from the *Thermus* oxidase by the freeze-thaw method were used, H^+ ejection occurred upon addition of yeast ferrocytochrome *c* (fig.3A). Fig.3B shows the time course of the oxidation of cytochrome *c*. This trace just followed the first order kinetics. The $\vec{H}^+/e^-$ ratio, usually defined as the maximal amount of H^+ translocated/ferrocytochrome *c* added was 0.68. However, the $\vec{H}^+/e^-$ ratio obtained from the initial velocities (0–10 s) was 1.1 in these experiments. The dotted line (fig.3C) shows the pH meter trace when FCCP was present, which was very similar to the curve of cytochrome *c* oxidation.

3.3. *Simulation of H^+ movement upon ferrocytochrome* c *pulse*

This process can be analyzed mathematically. Instead of the equation of Krab and Wikström in which ascorbate and small amount of cytochrome *c* are present to keep a constant oxidation rate [21], we postulate that the oxidation proceeds according to the first-order kinetics in terms of ferrocytochrome *c* (fig.3B). Since back diffusion of H^+ may be linearly proportional to pH difference in the presence of valinomycin and K^+, the change in numbers of H^+ in the two water phases (N_1 and N_2) can be described as follows:

$$dN_1/dt = n_1 r[c^{2+}] + k(N_2/V_2 - N_1/V_1)$$

$$dN_2/dt = n_2 r[c^{2+}] - k(N_2/V_2 - N_1/V_1)$$

where:

n_1 and n_2 = the stoichiometry ($\vec{H}^+/e^-$) by which H^+ are released on outside (*c*-site) and inside;

k = a diffusion constant;

r = first-order rate constant of oxidation;

V_1 and V_2 = buffering capacities of the two aqueous phases with the dimension of volume, respectively.

Since amounts of ferrocytochrome *c* ($[c^{2+}]$) follow the equation:

$$[c^{2+}] = [c^{2+}]_{t=0} \cdot e^{-rt}$$

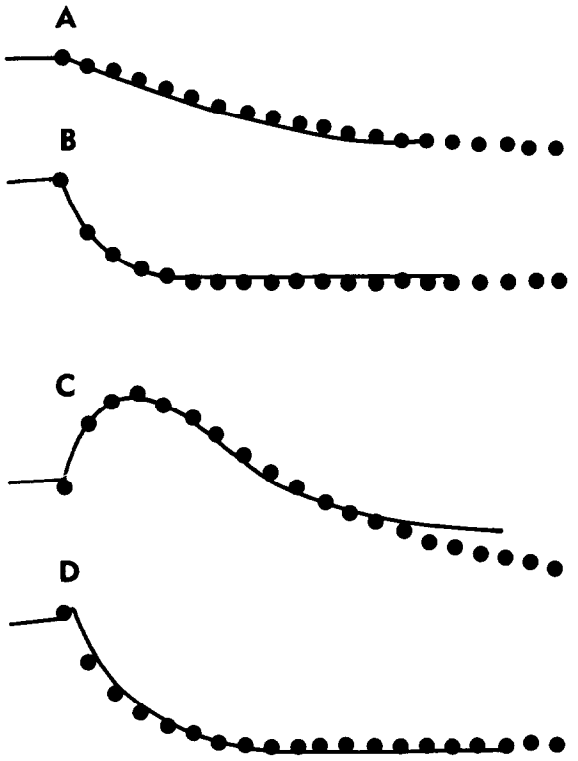

Fig.4. Simulation of H^+ ejection upon ferrocytochrome *c* pulse. Simulated values are plotted (○) on the data in fig.2 and 3: (A) for fig.2A, $n_1 = 0$, $n_2 = -1$, $r = 7.0\ s^{-1}$, $k = 0.15\ s^{-1}$; (B) for fig.2B, $n_1 = 0$, $n_2 = -1$, $r = 7.0\ s^{-1}$, $k = 5.0\ s^{-1}$; (C) for fig.3A, $n_1 = 1.2$, $n_2 = -2.2$, $r = 6.0\ s^{-1}$, $k = 0.1\ s^{-1}$; (D) for fig.3C; $n_1 = 1.2$, $n_2 = -2.2$, $r = 6.0\ s^{-1}$ and $k = 2.0\ s^{-1}$; $V_1 = 5$ and $V_2 = 0.1$ were assumed in all cases.

N_1 can be computed with Lunge–Kutta method by assuming proper constants.

Fig.4 shows that H^+ movement upon ferrocytochrome *c* pulse can be simulated well by the above equations, when $n_1 = 0$ and $n_2 = -1$ in the case of the *Nitrobacter* oxidase and $n_1 = 1.2$ and $n_2 = -2.2$ in the case of the *Thermus* enzyme, are postulated. The n_2 should be $-n_1 - 1$, since 1 H^+ disappears for water production per 1 e^- transfer.

4. DISCUSSION

This investigation has shown that *Thermus* cytochrome *c* oxidase has a good H^+ pump activity, while the *Nitrobacter* enzyme does not. We do not yet know why the *Nitrobacter* enzyme preparation does not pump H^+. The possibility that the reconstitution method is not suitable can be excluded, since the reconstituted vesicles showed a good respiratory control (fig.1A). In PS3, where good H^+ pumping has been observed, cytochrome *c* oxidase is composed of 3 kinds of subunit [4,16]. The data with mitochondrial cytochrome *c* oxidase also suggest the importance of the third subunit (subunit III) for H^+ permeation (as a channel) across the membrane [22,23]. Thus it might be possible that a component corresponding to subunit III has been lost from the *Nitrobacter* enzyme during the preparation. However, the 2-subunit cytochrome *c* oxidases from *T. thermus* and *P. denitrificans* have been shown to pump H^+. Thus, it seems likely that a kind of denaturation, which injure H^+ pump activity but not oxidase activity, takes place during preparation of the *Nitrobacter* enzyme. Purified PS3 enzyme loses H^+ pump activity at 60°C although the oxidase activity is intact (unpublished). Another possibility that the *Nitrobacter* enzyme does not pump H^+ intrinsically also remains. It is noteworthy that H^+ ejection is not observed with nitrite oxidizing *N. agilis* cells [24]. However, phosphorylating electron transport particles from *Nitrobacter winogradskyi* take up H^+ with nitrite oxidation [25]. If this is also the case for *N. agilis*, H^+ translocation may occur at cytochrome oxidase [26] or nitrite dehydrogenase [27].

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