Comparison of energy-transducing capabilities of the two- and three-subunit cytochromes aa_3 from *Paracoccus denitrificans* and the 13-subunit beef heart enzyme

Richard W. Hendler,* Komanduri Pardhasaradhi,* Baltazar Reynafarje,* and Bernd Ludwig‡
*Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA; and ‡Institute of Biochemistry, Medical University of Lubeck, Lubeck, Germany

ABSTRACT In the accompanying paper, we have shown that the two-subunit cytochrome aa_3 isolated from *Paracoccus denitrificans* displays the same kind of complex and interactive redox behavior as the 13-subunit cytochrome aa_3 from beef heart. Therefore, the redox characteristics are not dependent on the additional 11 subunits. In the current work, we have examined the energy-transducing capabilities of both the two- and three-subunit enzymes obtained from *Paracoccus denitrificans* in relation to that of the 13-unit mammalian enzyme. We have found that in all of the tested functions, which included the development of $\Delta\Psi$ and ΔpH, and the pumping of protons, that the two-subunit enzyme is at least as efficient as the structurally more complex mammalian enzyme. There is thus a correlation between the complex redox behavior and energy transducing capabilities of the two enzymes. There was also no difference in energy-transducing capabilities between the two- and three-subunit forms of the bacterial enzyme. It seems that only 2 subunits are required for an efficient energy-transducing cytochrome aa_3. The most likely role of the additional subunits in the mammalian enzyme, therefore, seems to be in regulation.

INTRODUCTION

In a series of papers from this laboratory, we have defined a complicated interactive redox behavior for beef heart cytochrome aa_3 (see Sidhu and Hendler, 1990 for references). Since all of the known redox centers reside on only two out of the 13 subunits that constitute the mammalian enzyme (Kadenbach et al., 1986), we wondered whether the cooperative interactions involved some of the other 11 peptides. Accordingly, we studied the redox behavior of a two-subunit cytochrome aa_3 isolated from *Paracoccus denitrificans* and found that the same kind of complicated interactive redox behavior was evident (Pardhasaradhi et al., 1991). The purpose of the present investigation was to see if there was a loss of functional capability in the two-subunit enzyme compared with the more complicated mammalian enzyme. In other words, is there a correlation between the kind of cooperative redox behavior evident in both enzymes and their ability to convert redox energy into a $\Delta\tilde{\mu}_{H^+}$. We report here no loss in functional ability of the two-subunit enzyme. The nonessentiality of the subunit III for energy transduction was independently confirmed by comparing the two- and three-subunit forms of the bacterial enzyme and finding no differences. Earlier, Gregory and Ferguson-Miller (1988*a*) concluded that subunit III is not involved in the control of mammalian cytochrome *c* oxidase activity by pH and the electrical potential gradient.

METHODS

Preparation and characteristics of cytochrome oxidase liposomes

Mammalian cytochrome *c* oxidase was purified as described by Yoshikawa et al. (1977). The concentrated enzyme (0.88 mM heme A, 88 mg/ml) was stored in 22 μl aliquots at −80°C. The two-subunit *Paracoccus denitrificans* cytochrome *c* oxidase was isolated as previously described (Ludwig and Schatz, 1980) and further purified by affinity chromatography on a cytochrome *c* column (Bill et al., 1980). The concentrated enzyme (0.36 mM heme A, 12 mg/ml) was stored in 50 μl aliquots at −80°C. The three-subunit enzyme from *Paracoccus denitrificans* was isolated in a manner similar to that of Berry and Trumpower (1985) and Haltia et al. (1988). Essential details were as follows. Bacterial membranes (10 mg/ml) were solubilized at 4°C with dodecyl maltoside (DM, 1.5 g/g protein) in 20 mM potassium phosphate (KPi, pH 8.0), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride. A clear supernate (after ultricentrifigation) was subjected sequentially to four stages of column chromatography. (*a*) DEAE cellulose eluted with a gradient 0.1–0.6 M NaCl in KPi and 0.5 g/l DM. (*b*) DEAE-sepharose eluted with 0.1–0.4 M NaCl in KPi and DM as above. (*c*) Ultragel AcA34 eluted with 0.1 M NaCl, KPi and DM as above. (*d*) Repeat step (*b*) on DEAE-sepharose. The material was then checked for spectral data, heme to protein ratio, activity, and purity on SDS gels. The concentrated enzyme (0.18 mM heme A, 8 mg/ml) was stored in 100 μl aliquots at −80°C.

Asolectin obtained from Fluka (Switzerland) was further purified by treatments with acetone and ether (Darley-Usmar et al., 1987) and stored as a dried powder in small aliquots at −80°C. Lipsomes were prepared by the cholate dialysis procedure as described by Wrigglesworth et al. (1987). In brief, hydrated, purified asolectin (100 mg) was sonicated to clarity in 2 ml 50 mM potassium phosphate (pH 7.4) containing 40 mg sodium cholate (Fluka) and 0.5 mM pyranine (8-hydroxy-1,3,6-pyrene-trisulfonic acid, trisodium salt; Eastman Kodak

Co, NY). One aliquot of enzyme (i.e., 19.4 nmol heme A, ~2 mg protein beef heart enzyme; 18.0 nmol heme A, ~0.7 mg bacterial enzyme) was added to 1.5 ml of the above solution. Dialysis (Spectrapore membrane 12,000–14,000 m.w. cutoff) was conducted at 4°C for a total period of ~24 h against four changes of 250 ml each of 50 mM potassium phosphate (pH 7.4) containing 0.5 mM pyranine. The liposome suspension (~2 ml) was passed over a 10 ml G-25 column preequilibrated with 50 mM potassium phosphate, and the void fraction collected in 2 ml. This suspension contained ~24 mM lipid-soluble phosphate and ~2.6 μM cytochrome aa_3. An orientation assay using cytochrome c± TMPD (Nicholls et al., 1980) showed ~75% correct orientation for the mammalian and bacterial enzymes.

Respiratory control ratios (R.C.I.) were determined in 50 mM potassium phosphate (pH 7.4) using ascorbate, TMPD (150 μM), and cytochrome c as the electron source and a combination of 2 μM valinomycin and 1 μM FCCP for uncoupling. These ratios were ~6.0, ~3.7, and ~5.6, respectively, for liposomes made with mammalian, two-subunit bacterial, and three-subunit bacterial enzymes and were not changed by varying ascorbate from 10 to 30 mM or cytochrome c from 10 to 20 μM. Actual respiratory rates were about double at the higher cytochrome c concentration compared with the lower. The R.C.I. data indicates that the loss of subunit III results in a lower control ratio. This same effect has been seen in other laboratories (Hinkle et al., 1972; Thelen et al., 1985; Prochaska and Fink, 1987). Gregory and Ferguson-Miller (1988*b*), however, reported that removal of subunit III from the beef heart enzyme resulted in a rise of R.C.I. of proteoliposomes from 5.9 to 11.8. Although our results on the effect of a loss of subunit III on R.C.I. differs from that of Gregory and Ferguson-Miller, we find in agreement with them, that the maximum uncoupled respiration rate at pH 7.4 is approximately doubled by the presence of the third subunit (Gregory and Ferguson-Miller, 1988*a*, *b*).

Realtime measurements of [TPP^+], [O_2], external pH, internal pH, $\Delta\Psi$, and ΔpH

Specific details describing the reaction cell, electrodes, electronic apparatus, computer interfaces, and software are presented separately (Hendler, 1991). A brief description is presented here.

A water-jacketed quartz cell was fitted with a pH electrode, a fast-responding Davies O_2 electrode (Reynafarje et al., 1982), a TPP^+-selective electrode, and a fused silica fiber optics bundle (model PCS 600; Quartz Products Corp., Plainfield, NJ) at right angles to a collimated, spatially uniform light source. The incident light was passed through an Oriel Corp. (Stratford, CT) #54320 interference filter with a sharp maximum transmission centered at 460 nm. The cell and all of the probes were placed in a grounded metal Faraday cage. The signals from all electrodes were passed to a custom electronic interface which was able to provide gains, speed the response of the pH electrode, reduce noise by common mode rejection between grounded shields on the pH and reference electrodes, and electronic filters (Hendler et al., 1983; Setty et al., 1986). The optics signal was passed through an Oriel Corp. #53870 interference filter with a sharp maximum transmission centered at 520 nm to a photomultiplier tube (Aminco Cat. No. 4-6250) that was originally part of an Aminco Chance dual beam spectrophotometer. The resultant electric signal was filtered and amplified using a Dynamics Electronic Products (Chatsworth, CA) model 7526A differential DC amplifier. All four of the data signals were fed to a Burr-Brown/Intelligent Instrumentation, Inc. (Tucson, AZ) model PCI-20002M, 12-bit, programmable gain, A/D converter and then to an IBM AT/PC computer. In realtime, the computer used the input signals to compute $\Delta\Psi$ and ΔpH. The computed signals were then fed through a Burr-Brown/Intelligent Instrumentation, Inc., model PCI-20021M, 12-bit, D/A converter to a 6-pen Linseis Inc. (Princeton Junction, NJ), model L7065-6, time base recorder.

[TPP^+] was measured using a modified type of PVC electrode in which a thick plug of selective interface replaced the thin membrane. Electrodes made with this design are extremely rugged and have been in continuous use in our laboratory for several years. We have found that binding of TPP^+ to liposomes conforms to a relationship involving a combination of saturable and unsaturable sites. This binding is readily fit using a model with a K_D = 324 and the concentration of saturable sites = 310 μM per ml liposomes in 6 ml incubation volume. The distribution ratio for the nonsaturable sites was 0.1 for 1 ml liposomal suspension per 6 ml incubation volume. Binding corrections were made assuming an equal distribution of sites on the two halves of the membrane. Internal liposomal volumes were determined using a modification of the calcein method (Oku et al., 1982). The modification was introduced because the original method assumes a completely linear relationship between calcein concentration and fluorescence. We find, however, that there is an initial lag in this relationship below a concentration of ~0.02 μM. Because fluorescence quenched in the presence of cobalt falls in this range, we have used experimentally determined fluorescence/concentration curves rather than the assumed linear relationship. Internal volume, determined in this way was 16 μl per ml liposomal suspension or ~0.9 μl/mg phospholipid. $\Delta\Psi$ was computed in real time by converting electrode readings to external [TPP^+], computing apparent internal [TPP^+] from changes in external [TPP^+] and the known internal volume, correcting for both external and internal [TPP^+] binding to the exposed membrane surfaces, and then using the Nernst equation. ΔpH was computed as follows. First, an aliquot of pyranine-loaded lipsomes was used for calibration, during which the fluorescence vs. pH was determined in the presence and absence of valinomycin plus nigericin, which remove the permeability barrier of the liposomes to H^+/OH^-. It was thus possible to determine the total amount of fluorescence, the fraction of externally bound pyranine, and the true baseline for fluorescence measurements. With this information determined for each preparation of liposomes and the experimentally determined pK = 7.4 for internal pyranine, fluorescence values could be converted to internal pH values. ΔpH was computed from these values in relation to the external pH.

Protocol for typical experiment

Potassium phosphate buffer (5.6 ml, 50 mM, pH 7.4) was placed into the reaction vessel and the [O_2] content reduced by bubbling with N_2 gas. The vessel was closed and argon was streamed across the top of the magnetically stirred solution at a flow rate of 100 ml/min. TPP^+ (12.5 μM) was added followed by an additional aliquot of 12.5 μM amount to verify the response of the TPP^+-electrode. Liposomal suspension (0.35 ml) was added and after all of the measured input signals were stabilized, the computer was activated to start computing $\Delta\Psi$ and ΔpH with the assumption that the starting values were zero in the absence of O_2, substrates, and cytochrome c. Sequential additions of microliter aliquots of ascorbate (10 mM), cytochrome c (3 μM), and TMPD (50 μM) were made using Hamilton syringes. The addition of cytochrome c resulted in the formation of a $\Delta\Psi$. During the course of the experiment, O_2 was introduced into the gas stream using a Matheson Gas Prods. (Secaucus, NJ) series 7300 Gas Proportionater mixer. The flow rate of argon was reduced to 10 ml/min and the O_2 was at 36 ml/min. The O_2 pulse was terminated by closing the valve controlling its flow and increasing the argon flow back to 100 ml/min.

When present, nigericin was at 0.08 μM and valinomycin in the indicated amounts up to 1.9 μM concentration.

Measurement of proton-pumping activity

The liposomes were prepared in 125 mM KCl and 3 mM Hepes, pH 7.0. Reactions were initiated by adding aerated buffer to a 1.56-ml solution containing reduced enzyme, cytochrome *c* (60 μM), ascorbate (6 mM), valinomycin (1 μM), in the KCl/Hepes buffer. Temperature was maintained at 25°C and the solution was stirred with a magnetic bar at 2,000 *g*. pH was measured with a Beckman Instrs. (Fullerton, CA) model 39522 electrode having a 90% response time of ~500 ms. [O_2] was measured with a rapid-response, membraneless electrode (Reynafarje et al., 1982).

RESULTS

Five pens were used to follow the [O_2], [TPP^+], fl (i.e., internal pyranine fluorescence), $\Delta\Psi$, and ΔpH. In the actual recorder traces, a different color pen is used for each output. There is, however, a displacement in the alignment of the pens so that simultaneous events are not seen at the same coordinate position. To facilitate the viewing of the data for reproduction in black and white, each trace has been separated and redrawn in register. Fig. 1 shows the record of an experiment performed with reconstituted mammalian enzyme. At the start of the experiment (far right), liposomes were added ("L"). The O_2-pen responded to the O_2 that was present in the aliquot; the TPP^+-pen to the dilution and initial binding of TPP^+; and the fl-pen to the pyranine in the liposomes. During the next 16 min, the [O_2] decreased, the TPP^+ signal stabilized, and the fl-pen recorded the slow equilibration of the pH of the internal phase with that of the external medium. The next event was the addition of cytochrome *c*("C") which was preceded by ascorbate. Although the O_2 level was quite low (~5 μM), it was sufficient to allow the immediate formation of a significant membrane potential, which was marked by a decrease in external [TPP^+] and the recording of a $\Delta\Psi$ of 130 mV. At the same time, there was a transient rise in ΔpH (inside alkaline) of ~0.08 units (4.8 mV) followed by a fall of 0.04 units. The O_2 level dropped to its baseline on the chart, but a $\Delta\Psi$ of 130 mV was maintained. In all cases the extremely low level of O_2 which could leak back through a small injection port in an otherwise closed vessel that was continually flushed with argon, was sufficient to maintain a substantial level of membrane potential in the liposomes. The O_2 valve was then opened and the O_2 pen recorded the slow rise of [O_2]. The TPP^+ pen showed the decrease in external [TPP^+]. It is always seen that the decrease in external [TPP^+] precedes the rise in [O_2] as recorded by the O_2 pen. This is not due to a slow relaxation of the O_2 electrode because a rapid response Davies type electrode is used. Most likely, it reflects the strong binding affinity of the liposomal cytochrome oxidase for O_2 at a concentration level where the electrode is not fully responsive[1] (i.e., <2 μM). The $\Delta\Psi$-pen showed that the magnitude of the $\Delta\Psi$ increased to 164 mV, while the ΔpH increased by 0.05 units followed by a slow decline. The addition of 83 nM nigericin (denoted by "N") collapsed the ΔpH by 0.1 units while building the $\Delta\Psi$ to 177 mV. When the O_2 valve was closed, and the O_2 trace slowly fell to its baseline level, the $\Delta\Psi$ dropped 37 to 140 mV while the ΔpH rose by 0.11 units. The level of $\Delta\Psi$ remained high until the O_2 trace actually returned to baseline. This is consistent with the findings discussed above that only trace levels of O_2 appear sufficient for sustaining near maximal levels of $\Delta\Psi$. This condition of near anaerobiosis is denoted in the figure by "A". A subsequent opening of the O_2 valve caused the return of the $\Delta\Psi$ to a value of 173 mV and the loss of 0.08 units of ΔpH. After another cycle of anaerobiosis followed by exposure to O_2 and the reestablishment of a $\Delta\Psi$ of 171 mV, a final addition of 1.9 μM valinomycin, in three stages, (denoted by "V") was made. This caused a total collapse of the $\Delta\Psi$ and a rise in the ΔpH of ~8 mV. A summary of the kinds of changes shown in this figure as obtained in six experiments with reconstituted mammalian cytochrome oxidase is presented in Table 1.

A parallel experiment with reconstituted 2-subunit *P. denitrificans* enzyme is shown in Fig. 2. The behavior of the bacterial enzyme was qualitatively the same as that of the mammalian enzyme, but the maximum $\Delta\Psi$ was 60 mV higher in the case of the bacterial enzyme. This was true even though the fully uncoupled rate of O_2 uptake in the bacterial liposome preparations was only ~60% of that of the mammalian preparations. Fig. 2 shows the initial dilution and binding of TPP^+, the rise and fall of [O_2], and fluorescence of internal pyranine upon addition of the liposomes to the medium (denoted by "L" in the figure). The addition of cytochrome *c* after ascorbate (denoted by "C") resulted in the formation of 185 mV of $\Delta\Psi$, even though, the O_2 trace was at baseline, confirming the fact that extremely low levels of O_2, are sufficient for forming and maintaining high levels of $\Delta\Psi$. The small spike in the TPP^+-trace just after the addition of cytochrome *c* is due to the addition of 50 μM TMPD.

[1]It should be noted that one of us (Dr. Reynafarje) has recently described four respiratory phases exhibited when the fully reduced cytochrome aa_3 is pulsed with O_2 (Reynafarje and Davies, 1990). The first phase which lasts ~0.3 s is the most rapid and it exhibits a K_m of ~30 μM.

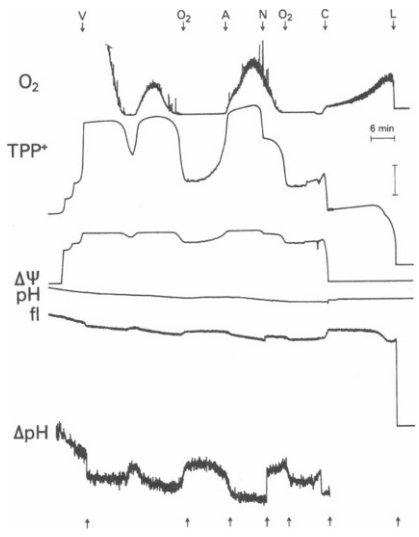

FIGURE 1 Energy transduction by beef heart cytochrome aa_3-containing liposomes. The realtime tracings of five pens are shown in a time course that proceeds from right to left. The vertical calibration bar represents 48 ngA of O_2/ml, 5 mV TPP^+ electrode signal, 100 mV of $\Delta\Psi$, units of external pH, 500 mV of fluorescence signal, and 0.1 units of ΔpH. At the start of the experiment, 0.35 ml of liposomal suspension containing ~24 mM lipid-soluble phosphate and ~2.6 μM cytochrome aa_3 were added to 5.6 ml of 50 mM potassium phosphate (pH 7.4) and 25 μM TPP^+, that had been thoroughly flushed with a stream of N_2 gas. Anaerobiosis was then maintained by a continuous flow of argon over the surface of the stirred suspension. The addition is indicated with an "L" at the top of the figure. The O_2 pen responded to the liposome addition with a sharp rise followed by a slow decline. A decrease in [TPP^+] is shown by an increase in signal. The rise in TPP^+ signal upon addition of liposomes is due to dilution and binding to the liposomes. The $\Delta\Psi$ and ΔpH pens are not activated until the system stabilizes after addition of the liposomes and just before energization by addition of ascorbate and cytochrome *c*. The pH pen shows not much change in external pH. The fluorescence species is the salt form, and therefore an increase in signal is associated with an increase in alkalinity. Upon addition of the pyranine-loaded liposomes, the signal shows a sharp rise followed by slower change as the interior of the liposomes equilibrates with the external medium. At ~16 min, the $\Delta\Psi$ and ΔpH pens were activated, 10 mM ascorbate was added followed shortly after by 3 μM cytochrome *c* (indicated by "C"). The O_2 trace dropped to baseline. The TPP^+ trace rose as TPP^+ was concentrated internally. TMPD (50 μM) was added just after cytochrome *c*. The TPP^+ electrode signal was processed by the computer which converted it to external concentration, corrected for binding to the external liposomal surface by both saturable and nonsaturable sites, computed the change in internal concentration, corrected for binding to the internal liposomal surface, and computed membrane potential from the external and internal free [TPP^+], using the Nernst equation. The computed $\Delta\Psi$ was sent to a D/A converter for output to a recorder pen. The $\Delta\Psi$ pen shows that after a slight overshoot, a $\Delta\Psi$ of ~130 mV was maintained. The computer processed the fluorescence signal to compute internal pH and ΔpH. The ΔpH signal was sent to a D/A converter for output to a recorder pen. It is seen that a brief development of ~0.08 units of ΔpH was followed by a decrease to ~0.04 units. During the course of the experiment, O_2 was periodically admitted to the argon gas stream (indicated by "O_2") or withheld (indicated by "A"). The added O_2 caused a further uptake of TPP^+ and an additional alkalinization of the vesicle interior (~0.03 units). After the first addition of O_2, 0.08 μM nigericin was added (indicated by "N"). This caused a sudden drop in ΔpH and a concomitant uptake of TPP^+ from the external medium. The rise of $\Delta\Psi$ is shown by the $\Delta\Psi$ pen. Subsequent cycles of O_2 withdrawal and replenishment caused changes of ~45 mV in $\Delta\Psi$ and ~5 mV in ΔpH. Finally, the addition of 1.9 μM of valinomycin at the end completely collapsed the $\Delta\Psi$ and relaxed the small ΔpH. A summary record of several experiments of this kind is shown in Table 1.

Opening of the O_2 valve resulted in an increase of the $\Delta\Psi$ to 230 mV and a transient rise in the ΔpH of 0.05 units, followed by a drop of 0.08 units indicating the "suck-back" of protons under the influence of the high electric field. As was seen in Fig. 1, the rise in $\Delta\Psi$ preceded the rise in the O_2-electrode signal. Closing the O_2 valve resulted in a drop of $\Delta\Psi$ by 40 mV and reestablishing the O_2 flow caused a rise in $\Delta\Psi$ by 40 mV. During the exposure to the higher [O_2], the ΔpH slowly rose by 0.07 units. The addition of 83 nM nigericin (denoted by "N") in the presence of high [O_2], resulted in the drop of ΔpH by 0.13 units (~8 mV) and a rise in $\Delta\Psi$ of about the same magnitude. Subsequent cycles of aerobiosis and near-anaerobiosis caused fluctuations in $\Delta\Psi$ of ~40 mV and in ΔpH of ~0.16 units. It is interesting to note in both Figs. 1 and 2 after the additions of nigericin, that the changes in ΔpH after each change from high to rate-limiting concentrations of [O_2] are opposite from what would be expected for a respiration-linked proton pump. The stimulation of respiratory activity by O_2 resulted in the acidification of the liposome interior and the slowdown of respiration resulted in its alkalinization. It would appear that the ability of the $\Delta\Psi$ to draw protons in from the outside was quantitatively more significant than the ability of the pump to move protons in the other direction. When the $\Delta\Psi$ was first neutralized by the presence of valinomycin (discussed below), the expected changes in ΔpH after stages of increased and decreased respiration were observed. A summary of results for five experiments conducted with reconstituted two-subunit bacterial enzyme using the protocol shown in Fig. 2 is presented in Table 1. Similar experiments were performed with the reconstituted three-subunit bacterial enzyme. A summary of five such experiments is presented in Table 1.

Additional experiments were performed with the reconstituted mammalian and bacterial enzymes to quantify the extent of ΔpH that could be formed and maintained in the presence of valinomycin which removes the effects of the membrane electric field (Table 2). In all cases, the addition of 1.2 μM valinomycin to the liposomes in the presence of electron donors and O_2 collapsed the $\Delta\Psi$ to zero, while increasing the ΔpH by ~0.1 units (i.e., ~6 mV). Subsequent cycles of high and rate-limiting concentrations of O_2 caused fluctuations of ΔpH by ~0.1 units. Although the magnitude of the changes in ΔpH were not much different from those seen in Fig. 1, the directions of the changes were now consistent with expectation for a respiration-driven proton pump. Enhanced respiration led to alkalinization of

TABLE 1 **Comparison of $\Delta\tilde{\mu}_{H^+}$ formation by liposomes reconstituted with mammalian or bacterial cytochrome c oxidases**

		P. denitrificans	
	Beef heart total	Two-subunit total	Three-subunit total
	mV	*mV*	*mV*
+Cytochrome *c*			
$\Delta\Psi$	131 ± 3	161 ± 14	142 ± 7
ΔpH	0	0	0
$\Delta\tilde{\mu}_{H^+}$	131	161	142
$+O_2$			
$\Delta\Psi$	175 ± 4	205 ± 8	184 ± 6
ΔpH	2	4	4
$\Delta\tilde{\mu}_{H^+}$	177	209	188
+Nigericin			
$\Delta\Psi$	184 ± 4	222 ± 8	208 ± 8
ΔpH	−2	−3	−5
$\Delta\tilde{\mu}_{H^+}$	182	219	203
"Anaerobic"			
$\Delta\Psi$	138 ± 3	159 ± 15	147 ± 10
ΔpH	3	3	2
$\Delta\tilde{\mu}_{H^+}$	141	162	149
O_2			
$\Delta\Psi$	180 ± 3	217 ± 8	191 ± 11
ΔpH	−2	−3	−1
$\Delta\tilde{\mu}_{H^+}$	178	214	190
"Valinomycin"			
$\Delta\Psi$	0	0	0
ΔpH	0	0	0
$\Delta\tilde{\mu}_{H^+}$	0	0	0

the liposomal interior, whereas a slowdown in respiration led to acidification. Table 2 shows the summarized results for five experiments with reconstituted mammalian and two-subunit bacterial enzymes, and four experiments with reconstituted three-subunit bacterial enzyme.

Proton pumping

Fig. 3 (*top*) shows the acidification of external medium coupled to the uptake of O_2 for both the mammalian enzyme and for the two-subunit bacterial enzyme. The magnitude of the measured H^+/O stoichiometry ratio is influenced by the number of enzyme takeovers and therefore by the amount of O_2 injected (Fig. 3, *bottom*). These and similar experiments show that the two enzymes show comparable abilities for respiration-dependent proton translocation. In a previous comparison of the proton pumping abilities of the reconstituted *Paracoccus denitrificans* and beef heart cytochrome *c* oxidases, Solioz et al. (1982) reported a H^+/e^- stoichiometry of 0.6 for the bacterial enzyme and slightly above 1 for the mammalian enzyme. These were extrapolated zero time values obtained from reductant (ferrocytochrome *c*) pulses added to the oxidized enzyme in the presence of atmospheric O_2. Our stoichiometries were obtained using small pulses of O_2 added to the reduced enzyme. It is important to emphasize that there are a number of experimental and theoretical considerations which account for differences in numerical stoichiometries reported in different laboratories (Hendler and Shrager, 1987). The possibilities for experimental variation are somewhat greater in liposomal systems in which the particular character of the lipids used could influence the net pumping ratio. For this reason, no particular significance is attached to the actual numerical values shown in Fig. 3. The purpose of the figure is to compare relative pumping efficiencies for the two enzymes when tested at the same time and under the same conditions. A second series of liposomal experiments were conducted to compare the proton pumping ratios of the two- and three-subunit forms of the *P. denitrificans* enzyme. These experiments were initiated 6 mo after the completion of the experiments comparing the 13-subunit mammalian and two-subunit bacterial enzymes. Fig. 4 shows that the pumping ratios of the two forms of the bacterial enzyme were also similar. If there is a real

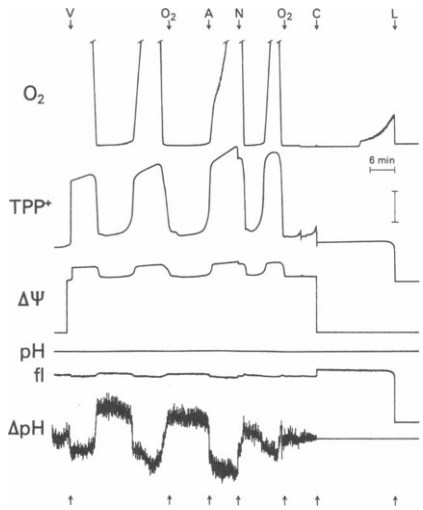

FIGURE 2 Energy transduction by *P. denitrificans*-containing liposomes. The description for Fig. 1 applies to Fig. 2 except that the 0.35 ml of liposomal suspension contained ~2.4 μM *Paracoccus denitrificans* cytochrome aa_3. Results of several experiments of this type are summarized in Table 1.

TABLE 2 **Comparison of $\Delta\tilde{\mu}_{H^+}$ formation, in the presence of valinomycin, by liposomes reconstituted with mammalian or bacterial cytochrome c oxidases**

		P. denitrificans	
	Beef heart total	Two-subunit total	Three-subunit total
	mV	*mV*	*mV*
+Cytochrome *c*			
$\Delta\Psi$	128 ± 4	163 ± 9	143 ± 9
ΔpH	0	0	0
$\Delta\tilde{\mu}_{H^+}$	128	163	143
$+O_2$			
$\Delta\Psi$	174 ± 2	209 ± 8	175 ± 10
ΔpH	Transient	Transient	5
$\Delta\tilde{\mu}_{H^+}$	174	209	180
+Valinomycin			
$\Delta\Psi$	0	0	0
ΔpH	8	6	9
$\Delta\tilde{\mu}_{H^+}$	8	6	9
"Anaerobic"			
$\Delta\Psi$	0	0	0
ΔpH	1	0	0
$\Delta\tilde{\mu}_{H^+}$	1	0	0
O_2			
$\Delta\Psi$	0	0	0
ΔpH	8	4	6
$\Delta\tilde{\mu}_{H^+}$	8	4	6
"Anaerobic"			
$\Delta\Psi$	0	0	0
ΔpH	2	0	0
$\Delta\tilde{\mu}_{H^+}$	2	0	0

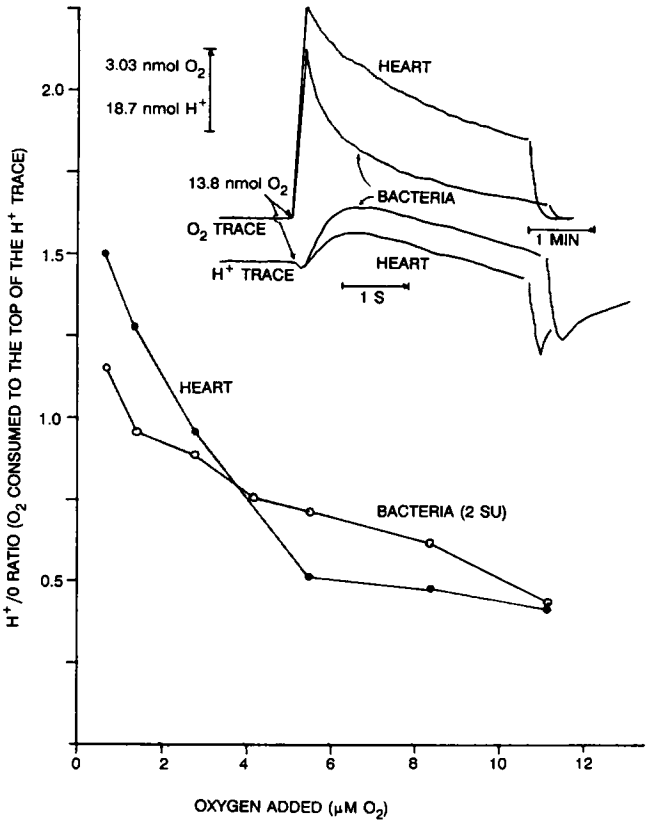

FIGURE 3 Proton pumping by liposomes containing beef heart- and two-subunit *P. denitrificans*-cytochrome oxidase. (*Top*) In each case, 1.56 ml of liposomal suspension containing 4.8 mM lipid-soluble phosphate and ~1 μM cytochrome aa_3 in 125 mM KCl, 3mM Hepes (pH 7.0), 60 μM cytochrome *c*, 6 mM ascorbate, and 1 μM valinomycin was made anaerobic and stirred at 2,000 *g* with a magnetic flea at 25°C. The reaction was initiated by the injection of 60 μl of aerated KCl/hepes buffer. [O_2] and pH were recorded as described in Methods. H^+/O pumping ratios were measured for different sized O_2 pulses using the peak levels of H^+ ejection and amounts of O_2 consumed at the time of the H^+ ejection peak. These ratios are shown in the bottom of the figure, with the solid circles representing the mammalian enzyme and the open circles representing the bacterial enzyme.

quantitative difference, it appears to be in favor of the two-subunit species. The safest conclusion is that the presence of the third subunit does not enhance the net proton pumping ability of the enzyme.

Control of respiration

The concentration of cytochrome aa_3 in both mammalian and bacterial liposome suspensions was ~2.6 μM. When fully uncoupled by 2 μM valinomycin and 1 μM FCCP, the respiratory rates, in the presence of 20 μM cytochrome *c*, were ~160 ng A O/s and ~98 ng A O/s per nmol cytochrome aa_3, respectively, for the mammalian and two-subunit enzyme bacterial liposomes. According to this criterion, the bacterial liposomes had ~61% of the enzyme activity of the mammalian liposomes. Table 3 shows that the respiratory control index (R.C.I.) was ~6 for the mammalian system and ~3.5 for the bacterial system. The "coupled" respiratory rate (i.e., before addition of uncouplers) in the bacterial system was usually ~10% higher than that of the mammalian system as indicated. For an R.C.I., the coupled respiratory rate is taken as 1 unit. Therefore, an R.C.I. of 6 means a release of 5 units of respiration. When valinomycin was added alone to the mammalian system, the relative control index (i.e., ratio of rates after and before the addition) was 1.5 (Table 3). This represents a release of 0.5 units or only 10% of the amount released by the combination of both uncouplers. FCCP added alone, released 60% of the total. These results are not expected

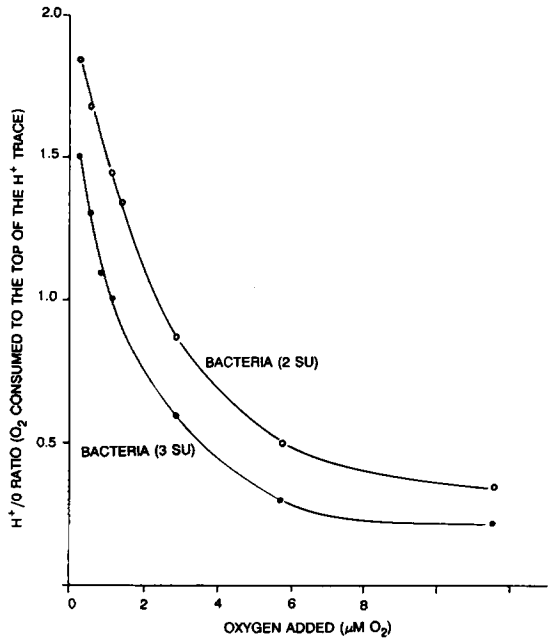

FIGURE 4 Proton-pumping by liposomes containing two-subunit and three-subunit *P. denitrificans* cytochrome oxidase. The conditions described in the legend to Fig. 3 were used.

since the $\Delta\Psi$ component is ~175 mV and the ΔpH component only ~6 mV (Tables 1 and 2). Dissipation of ~3% of the $\Delta\tilde{\mu}_{H+}$ which is in the form of ΔpH released 60% of respiration, while dissipation of $\Delta\Psi$ which represents ~97% of the $\Delta\tilde{\mu}_{H+}$ released only 10% of the respiration. Table 3 shows another interesting observation. The relative control index of each uncoupler is the same either when added along or added after the addition of the other uncoupler. This suggests that $\Delta\Psi$ and ΔpH each has a unique mechanism for controlling the activity of the enzyme and is consistent with any earlier studies leading to the same conclusion (Hinkle and Mitchell, 1970; Gregory and Ferguson-Miller, 1988*a*, 1989; Papa et al., 1988; Nicholls, 1990). The control situation for the bacterial enzyme is quite different. First of all, valinomycin alone released 53% of the respiration, and FCCP alone released 78%. The sum of these individual effects is 131% compared with only 70% for the mammalian enzyme. Because of the ability of each uncoupler to release some of the control exerted by the other in the bacterial system, the relative control index was less when one of the uncouplers was added second than when it was added first (Table 3).

A low R.C.I. is usually taken to indicate poor coupling or leakiness of the membranes. Therefore, from the lower R.C.I. of the two-subunit bacterial system, we would expect to find lower $\Delta\Psi$'s. But, as shown in Table 1, $\Delta\Psi$'s were higher in the two-subunit bacterial liposomes than in the mammalian liposomes. Although the statistical uncertainties for the means in $\Delta\Psi$ and respiratory activities of the two systems do not allow a definite estimate of the magnitudes of these differences, both the "coupled" rates of respiration and levels of $\Delta\Psi$ were at least 10% greater in the bacterial system. This means that low R.C.I.'s cannot always be thought of in terms of dissipative leak pathways and uncoupled respiration.

A comparison of the relative respiratory control by valinomycin and FCCP on the cytochrome oxidase activity of the two- and three-subunit bacterial enzymes is presented in Table 4. The liposome preparations used

TABLE 3 Comparison of relative uncoupling abilities of valinomycin and FCCP toward liposomes containing mammalian or two-subunit *P. denitrificans* cytochrome aa_3

Added first	Rel control index	% Release of respiration	Added second	Rel control index	R.C.I.
		Mammalian			
Valinomycin	1.50 ± 0.13 (14)	10	FCCP	4.42 ± 0.56 (14)	6.0 ± 0.6
FCCP	4.06 ± 0.26 (6)	60	Valinomycin	1.49 ± 0.13 (6)	6.1 ± 0.7 (6)
		70			
		Two-subunit bacterial			
Valinomycin	2.27 ± 0.22 (11)	53	FCCP	1.54 ± 0.08 (11)	3.4 ± 0.2 (11)
FCCP	2.94 ± 0.30 (5)	78	Valinomycin	1.17 ± 0.06 (5)	3.5 ± 0.5 (5)
		131			

Relative control index is the ratio of respiratory rates before and after addition of an uncoupler. R.C.I. is the ratio of respiratory rates in the presence of both uncouplers compared with that in the absence of either. 100% of release of respiration is taken as the R.C.I. minus 1.0. The % release of respiration after the addition of a single uncoupler is figured as 100 × (relative control index − 1)/(R.C.I. − 1).

TABLE 4 **Comparison of relative uncoupling abilities of valinomycin and FCCP toward liposomes containing two- or three-subunit *P. denitrificans* cytochrome aa_3**

Added first	Rel control index	% Release of respiration	Added second	Rel control index	R.C.I.
		Two-subunit enzyme			
Valinomycin	2.25 ± 0.08 (3)	38	FCCP	1.91 ± 0.09 (3)	4.3 ± 0.4 (3)
FCCP	3.27 ± 0.12 (3)	91	Valinomycin	1.07 ± 0.02 (3)	3.5 ± 0.2 (3)
		129			
		Three-subunit enzyme			
Valinomycin	2.70 ± 0.4 (7)	35	FCCP	2.50 ± 0.3 (7)	5.9 ± 0.3 (7)
FCCP	4.53 ± 0.07 (3)	83	Valinomycin	1.16 ± 0.09 (3)	5.3 ± 0.3 (3)
		118			

Refer to the legend of Table 3.

in these studies were prepared 6 mo after the ones used for Table 3. Although the effect of valinomycin on the bacterial liposomes was less, relative to that of FCCP when compared with the data in Table 3, the distinction in control patterns between the mammalian and bacterial liposomes is still seen.

DISCUSSION

The two-subunit cytochrome aa_3 from *Paracoccus denitrificans* is every bit as capable as the 13-subunit enzyme from beef heart in using respiratory energy to pump protons and form a $\Delta\tilde{\mu}_{H+}$. In fact, the magnitude of the $\Delta\Psi$ attained was higher with the two-subunit bacterial enzyme (Table 1). These observations show that the other 11 subunits of the enzyme are not essential for proton pumping and energy transduction. We have observed, as did others (Shaughnessy and Nicholls, 1985; Singh and Nicholls, 1986; Kamp, 1990), that the ΔpH obtained in reconstituted liposomes is very much smaller than the $\Delta\Psi$ (i.e., ~8 mV compared with ~200 mV). The $\Delta\Psi$ that we have obtained with our procedures using a TPP^+-selective electrode, however, are significantly higher than reported earlier using a $BTPP^+$-selective electrode (Singh and Nicholls, 1986). We also confirm that the small ΔpH has a much greater effect in controlling respiration than $\Delta\Psi$ (Moroney et al., 1984; Shaughnessy and Nicholls, 1985). There is no obvious reason how this is accomplished nor why is is so.

The minimal two-subunit structure contained in the bacterial enzyme is sufficient for both the intricate redox behavior (Pardhasaradhi et al., 1991) and functional capabilities of this energy transducing enzyme. Nonetheless, there have been a number of publications pointing to an important role of subunit III in the proton-pumping capability of cytochrome *c* oxidase (see Prochaska and Fink, 1987, for a review). The comparison between the intact mammalian enzyme and the two-subunit bacterial enzyme shows that the subunit is not essential. That no apparent advantage results when subunit III is present is clearly seen in the direct comparisons between the two- and three-subunit forms of cytochrome *c* oxidase from *Paracoccus denitrificans.*

It is safe to assume, however, that the evolutionary selection of an enzyme with additional (nuclear coded) peptides must confer some advantage in mammalian energy conservation. It seems likely that this advantage must be regulational rather than functional. Our data on the relative control of respiration by the $\Delta\Psi$ and ΔpH components of $\Delta\tilde{\mu}_{H+}$ support this idea. Additional support is seen in recent work of Hüther and Kadenbach (1988). They have found that intraliposomal ATP increases and ADP decreases the kinetics of ferrocytochrome *c* oxidation of the bovine but not the Paracoccus enzyme.

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