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ARTIFICIAL LIPOSOME FUNCTIONALIZED WITH CYTOCHROME c_3 AS A MODEL FOR H_2-METABOLIZING BACTERIA

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The artificial liposome functionalized with cyt c_3 from Desulfovibrio vulgaris Miyazaki transports electrons across the membrane from the external H_2 to $K_3Fe(CN)_6$ in its interior in the presence of colloidal Pt. This electron transport simultaneously produces the considerable H^+ gradient across the membrane large enough for the ATP synthesis.

Although many organisms live by O_2 respiration, some microorganisms live on $SO_4^=$ or NO_3^- which have the hydrogenase[1] to catalyze the electron transport from molecular H_2 to these acceptors where the small amount of energy thus obtained is used for the ATP synthesis. These microorganisms catalyze the metabolism of dihydrogen by the use of hydrogenases and cytochrome-c_3 (abbreviated as cyt-c_3). This cyt-c_3 has an unique structure to keep 4 heme units in a single protein (Mw, ca 14,000 for Desulforibrio vulgaris, Miyazaki[2]), where each heme is interacting with others as shown by Mössbauer[3] or EPR[4] spectrum. This intramolecular heme-heme interaction seems to be very important for efficient electron transport by cyt-c_3 in H_2-metabolizing bacterias. We have succeeded to modify an artificial monolayer liposome with cyt-c_3 to find out this c_3-liposome was an extremely efficient electron transport system where self-aggregation of cyt-c_3 afforded an efficient electron channel. We now wish to report that combination of c_3-liposome with colloidal Pt,[5] as an efficient catalyst to weaken H-H bond, (Pt acts just like a hydrogenase) affords an excellent model for a H_2-metabolizing bacteria. This model system transported electron from external H_2 to internal $K_3Fe(CN)_6$ across the liposomal membrane to produce a large proton gradient across the membrane, just ready for the ATP synthesis.[6]

Recently, much attention has been paid for modification of an artificial liposome with an electron transporting protein.[7-11] The present cyt-c_3 membrane, however, showed unique characteristics, of second order dependence of the electron transport rate from outside $Na_2S_2O_4$ to inside $K_3Fe(CN)_6$ on the cyt-c_3 concentration as shown in eq(1).

$$-\frac{d[Fe^{3+}]}{dt} = k[\text{cyt-}c_3]^2[Fe^{3+}] \qquad \text{eq(1)}$$

As a consequence, at higher concentration of cyt-c_3 in membrane, electron transport rate became very fast.

Table I. Electron Transport Rate Across The Liposomal Bimolecular Membrane Or Rate Of Membrane-Solution Reaction.

artificial membrane	reaction	membrane-solution reaction (substrate)	solution-solution reaction across the membrane[a]
cyt c_3		immeasurably fast($Na_2S_2O_4$)	0.149 s^{-1}
cyt c		1.6 x 10^2 $M^{-1}s^{-1}$(ascorbate)	——
reduced coenzyme Q-cyt c reductase (complex III)		3.1 μmol/min/mg (cyt c)	——
NADH-coenzyme Q reductase (complex I)		0.35μmol/min/mg (NADH)	——

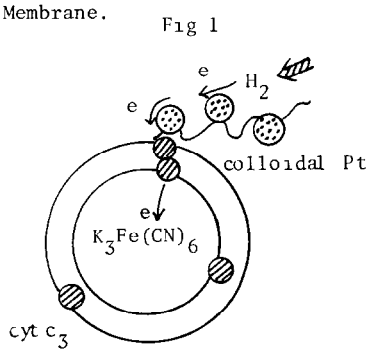
[a] ——; not measured.

Thus, 80 mg of egg-lecithin carefully purified[12] and 20 mg of bovine heart cardiolipin were dissolved in 10 ml of $CHCl_3$ and the solvent was removed under Ar in vacuo. The resulting lipid film was re-suspended in a 50 ml of 0.5 M solution of $K_3Fe(CN)_6$ (5 mM Tris-HCl pH 7.0) and the mixture was sonicated (5 min x 6) with ice cooling in a box filled with Ar to avoid any air oxidation of lecithin.[13] Unbound $K_3Fe(CN)_6$, smaller lecithin aggregate and multilayer liposome were removed by the centrifugation and the gelfiltration on the Sepharose 4B column and the artificial monolayer liposome containing $K_3Fe(CN)_6$ in its interior and having negative charges of cardiolipin on the membrane (abbreviated to $Fe^{III}(1)|Lip^-$ hereafter) was isolated. This artificial liposome showed reasonable stability and $K_3Fe(CN)_6$ was leaking out only in less than 2 % during 72 h at 4°C. To this freshly prepared artificial liposome solution (1.0 ml), 0.22 ml of 60 μM solution of cyt c_3 which was prepared from Desulfovibrio vulgalis, Miyazaki and carefully purified as described previously[2] and/or 0.05 ml of 400 μM solution of C_4V^{++} (abbreviation of N,N'-di-n-butylviologen) were added dropwise with gentle stirring for 1 min at r. t.

Table II. Pseudo First Order Rate Constants For Internal Fe^{III} Reduction With External H_2 Across The Modified Bimolecular Membrane.

membrane modification	k(sec^{-1})
without modification	∿ 0
6.5 μM of cyt c_3	20 x 10^{-3}
6.5 μM of cyt c_3 + 10 μM of C_4V^{++}	65 x 10^{-3}
10 μM of C_4V^{++}	1.5 x 10^{-3}
6.5 μM of cyt c	∿ 0 (< 0.03x 10^{-3})

Fig 1



and the volume of the solution was adjusted to 2.0 ml with buffer (5 mM Tris-HCl pH 7.0) (see Table II). The artificial liposome, thus obtained, $Fe^{III}(1)|Lip^{-}$·cyt c_3, or $Fe^{III}(i)|Lip^{-}(C_4V^{++})$·cyt c_3, was separated and purified through a Sephadex G-50 column. The purified artificial liposome was treated with colloidal Pt, which was prepared from 25 mg of K_2PtCl_4 and 50 ml of 1 % aqueous solution of PVA(n=1500) according to the reported procedure.[14] Hydrogenolysis was carried out by the rapid mixing of the solution of colloidal Pt (final concentration was 14 μ eqv/mol in terms of K_2PtCl_4) with the solution of $Fe^{III}(1)|Lip^{-}(C_4V^{++})$·cyt c_3 described above to which vigorous H_2 bubbling was applied just before mixing (Fig 1).

All measurements were made at 27°C, pH 7.0 and the change of the absorbance at 435 nm characteristic to $K_3Fe(CN)_6$ was followed by use of a specially designed stopped-flow apparatus.[15] In the case of $Fe^{III}(1)|Lip^{-}$·cyt c_3 (cyt c_3=6.5 μM), very rapid reduction of cyt c_3 was observed within 0.6 sec, reaching to the stationary state where $c_3^{II}/c_3^{III} \geq 0.80$. This was followed by the pseudo first order decrease of $K_3Fe(CN)_6$ and the rate gradually slowed down with time. Typical pseudo first order rate constants obtained are listed in Table II, clearly demonstrating that cyt c_3 with or without C_4V^{++} as a cocatalyst is a very efficient electron transport catalyst. While the liposome modified with cyt c, $Fe^{III}(1)|Lip^{-}$·cyt c, instead of cyt c_3 exhibited only very slow reduction of cyt c ($\tau_{1/2}$=70 sec) and the reduction of $K_3Fe(CN)_6$ across the membrane was too slow to measure. These trends were also observed for the $Na_2S_2O_4$ reduction of artificial liposomes,[16] where the formation of the effective "electron channel" of cyt c_3 aggregate was concluded to be responsible to this facile electron transport.

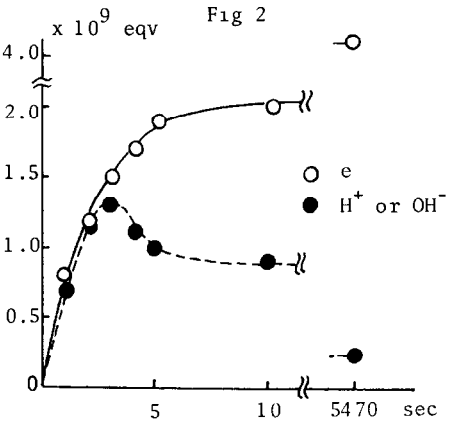
Observed rate decrease with time is concluded to be due to potential difference generated across the membrane by the co-transport of H^+ or counter transport of OH^- associated with the electron transport from outside to inside of the liposome based on the following experiments. Thus, the functionalized artificial liposome containing 2-naphthol-3,6-disulfonic acid disodium salt (abbreviated as NDSA hereafter), $Fe^{III}(1)$·NDSA(1)$|Lip^{-}$ C_4V^{++}, was prepared by the same method as described in the text, except that 522 mg of NDSA was added to the solution of $K_3Fe(CN)_6$ as a pH indicator. In the reduction of $Fe^{III}(1)$.NDSA(1)$|Lip^{-}$ C_4V^{++} with H_2/colloidal Pt at pH 9.0, spectral change was monitored at 315 nm characteristic to dissociated form of NDSA. The estimated amount of fluxed H^+ or OH^- coupled with the electron transport is shown in Fig 2. Obviously, the amount of transported H^+ or OH^- was approximately equal to that of transported electron in early stages of the reaction (Table III). Then OH^- (or H^+) gradient gradually decreased through a broad maximum.

According to our independent preliminary experiments, OH^- transport should be much more important than H^+ transport at pH 9.[17] Therefore, the present results clearly indicates that, in the present electron transport system, the "charge neutrality" is preserved mainly by the (active) counter transport of OH^-. The pH gradient thus produced causes the passive transport of OH^- and/or H^+. At the same time the produced pH gradient decelerates the electron transport rate (more details will be discussed in a full length article). A superposition of these effects certainly leads to the observed complex pH gradient change in the present artificial cell.

It was demonstrated by Jagendorf[18] that an artificial proton gradient (Δ pH $\gtrsim$ 3) set up across a membrane is sufficient for the ATP synthesis and the ATP synthesis was observed from ADP and Pi by the irradiation of the artificial liposome functionalized with ATPase and

Table III. H^+ Gradient Produced, Coupled With Electron Transport.

Δ[e]influx (eqv)	$\Delta[H^+]$influx (eqv)	pH(i) t = 0	pH(i) t = t
1.5×10^{-9}	1.3×10^{-9}	7.00	4.06 (expected)



bacteriorhodopsin[19] or other related systems.[20,21] Assuming that approximately the equal amount of electron is transported at pH 7.0, the coupling H^+ or OH^- transport may change the interior pH value from 7.0 to 4.0 (Table III). Therefore, a conclusion is presently drawn that an artificial liposome appropriately modified by cyt c_3 can "digest" hydrogen to produce the pH gradient sufficient enough for the ATP synthesis.

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