Study on Conjugated Redox Membranes Containing an Intercellular Component of Wool as an Electron Carrier

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To realize artificially such a conjugated redox reaction as that which is drived across the biological cell membrane, two enzymic redox reactions were conjugated across a hydrogel membrane prepared with urethane prepolymer holding stabilized isocyanate groups. As redox couples, glucose 6-phosphate (G6P)/glucose 6-phosphate dehydrogenase (G6PD)/gluconolactone 6-phosphate (GL6P) and glutathione oxidized form (GSSG)/glutathione reductase (GRD)/glutathione reduced form (GSH) were chosen together with their common coenzyme, $NADP^+$/NADPH. The driving of a system consisting of two redox couples separated with the hydrogel membrane containing the coenzyme $NADP^+$ was intiated by the reduction of $NADP^+$ on the membrane surface in contact with the G6PD/G6P solution. The conjugated redox reaction proceeded by electron exchange between coenzymes in the membrane and the reproducing of $NADP^+$, which was accompanied with the GRD/GSSG solution. The rate of electron transfer across the urethane membrane was markedly accelerated by incorporating a nonkeratinous intercellular cement compound extracted from wool into the membrane. From cyclic voltammograms of a methyl viologen mediator, using a bare electrode and an intercellular compound modified carbon electrode, it was confirmed that the intercellular component could surely act as a good electron carrier.

In a previous paper[1] it was shown that methylene blue (MB) can be reduced to its leuco-form in an aqueous bath containing untreated wool fibers. However, no reduction occurred in the presence of wool fibers lacking an intercuticular cement material (δ_L component) by extraction with formic acid. In order to clarify this phenomenon a model experiment was contrived using thioglycolic acid (TGA) as a simple model compound having an -SH group like wool. According to expectations, the decolorization rate of MB was accelerated markedly by the addition of the δ_L component, indicating that the reduction of MB by -SH groups of wool could be mediated by the δ_L component.

Such a consideration was also made by Koga et al.;[2] they had observed a reverse oxidation reaction of leuconaphthazarin to naphthazarin on wool fibers in an aqueous bath and explained it by considering the reduction of disulfide (-SS-) groups of wool mediated by the δ_L component.

These two experimental facts described above suggest such a possibility that the δ_L component can work as an electron carrier for a redox system.

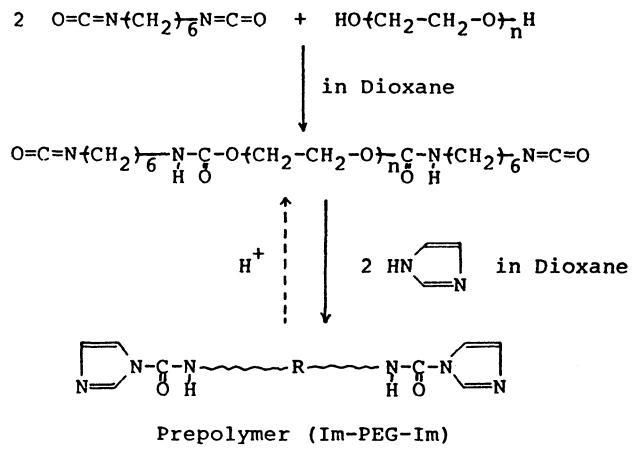
On the other hand, in connection with cell membranes there were a number of redox systems across membrane models such as micelles,[3] bilayer membranes,[4] and liquid membranes.[5] Such redox systems are, however, not suitable for practical uses because of such disadvantages as leakage of carrier or solvents from these membranes. To overcome such disadvantages, Endo et al., have devised some new systems for electron transfer across a synthetic solid membrane with a covalently bound electron mediator[6-8] and demonstrated redox reactions across the membrane. However, in the above-mentioned investigations enzymes were not used at all, which surely participate for driving the redox in real biological cell membranes.

In this study, we purposed to construct a new conjugated redox membrane system in which dehydrogenase and reductase separately adhere to both sides of a positively-charged polyurethane membrane containing $NADP^+$/NADPH as an electron mediator, and to demonstrate the driving of a redox reaction across the membrane. The intended purpose of this investigation involved improving a hitherto known conjugated redox system,[9] in which both enzymes are mixed together with their substrates and, hence, both products are also obtained in the mixture. In addition, the effect of incorporating the δ_L component into the membrane on the driving rate was investigated. It is worth noting that the δ_L component extracted from wool can truly work as an electron carrier. Except for cytochrome C, this is a unique example regarding the use of a natural electron carrier, although a large number of synthetic ones have been used.[10]

Experimental

Materials. Glucose 6-phosphate dehydrogenase (from yeast) G6PD and glutathione reductase (from yeast) GRD, nicotinamide-adenine dinucleotide phosphate $NADP^+$ and its reduced form NADPH were purchased from Oriental Yeast Co., Ltd. Glutathione, oxidized form GSSG, its reduced form GSH and glucose 6-phosphate **G6P** were all obtained commercially (Sigma) and were not further purified. The other reagents were analytical grade and used without further purifications.

Membrane. The requisites of a membrane for a conjugated redox system are as follows: the membrane should be hydrophilic because of use in aqueous system, it is charged positively to hold the negative charged coenzyme $NADP^+$/NADPH in it, and the pore size in the swollen membrane should be so adequate as to permit the thermal motion of the δ_L component in the membrane so as to diminish the leakage of enzymes, substrates, and products through the membrane.



Scheme 1. Synthesis of urethane prepolymer.

For this purpose the recently developed method to prepare an enzyme-fixed hydrophilic urethane gel membrane[11] was modified to make the membrane at lower temperature. The preparation process of the prepolymer is shown in Scheme 1.

Polyethylene glycol (PEG) (#600, Nakarai Chemicals Co., Ltd.,) (46.7 g) and hexamethylene diisocyanate (HMDI) (Tokyo Chem. Ind. Co., Ltd.,) (26.2 g) were dissolved in dioxane and heated with stirring up to 80—90 °C. The reaction was continued until the amount of an isocyanate (-NCO) group in the reaction mixture was reduced to one-half its initial value to prevent any further reaction, allophanation. The -NCO content was determined by the Sigga-Hanna method.[12] To protect the end -NCO group of produced poly(oxyethylene) bis(6-isocyanatehexylcarbamate), it was reacted with 2 molar imidazole in dioxane at 40 °C for 24 h. The prepared prepolymer (Im-PEG-Im) was stable for at least 6 months under dry conditions. Considerably strong and thin hydrogel membranes could be prepared by casting the mixture of the prepolymer Im-PEG-Im and 20% excess of aqueous solution of diethylenetriamine (DETA 0.5 mol dm^{-3}) as a cross-linking agent on a glass plate framed by a circled silicone rubber at room temperature for 2 days. The membrane was immersed in water for 1 to 2 weeks to remove dioxane and released imidazole.

Electron Carrier. The extraction of the nonkeratinous δ_L component was carried out by the usual method, described by Bradbury et al.[13] The Cu^{2+} content in the δ_L component was determined to be 1.65 mmol g^{-1} by using the Bathocuproin method.[14] From the amino acid analysis it became clear that the δ_L contained a relatively large amount of histidine, compared with the other fractions of wool.[1]

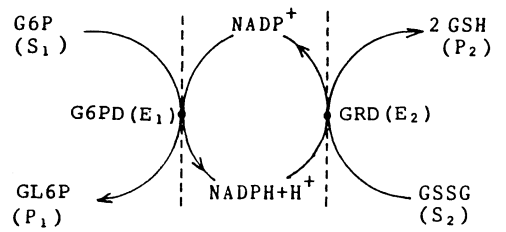
The incorporation of the δ_L component into the membrane was carried out as follows: the δ_L component (10 mg) dissolved in an aqueous solution of DETA (0.5 mol dm^{-3}, 1 ml) was mixed with Im-PEG-Im (1 g); the mixture was then cast on a glass plate to form a membrane.

Measurements. Cyclic Voltammetry: In order to confirm that the δ_L component can act as an electron carrier, cyclic voltammetry was employed. A Yanaco Voltammetric Analyzer P-1000/Function Generator FG-121B was used with an X-Y recorder for cyclic voltammetry. The electrode assembly consisted of a carbon electrode GC-P2, Yanaco P_{010} (not-modified or δ_L-modified) as a working electrode, a sodium chloride saturated calomel electrode (SSCE) as a reference electrode and a platinum spiral as a counter electrode. 1.0-M $NaClO_4$ solutions (1 M=1 mol dm^{-3}) adjusted to pH 7.0 with 0.02-M phosphate buffer, was used as the supporting electrolytic solution. The δ_L modified carbon electrode was prepared by casting a formic acid solution of the δ_L component (10 mg/g) on the electrode surface and drying it in air. The concentration of MV was 0.25-mM. As a model of δ_L, a 1-mM aqueous solution of Cu^{2+}-histidine (mixture of $CuSO_4$ and aqueous histidine solution in a molar ratio of 1 : 10) was also measured using a bare carbon electrode as the working electrode.

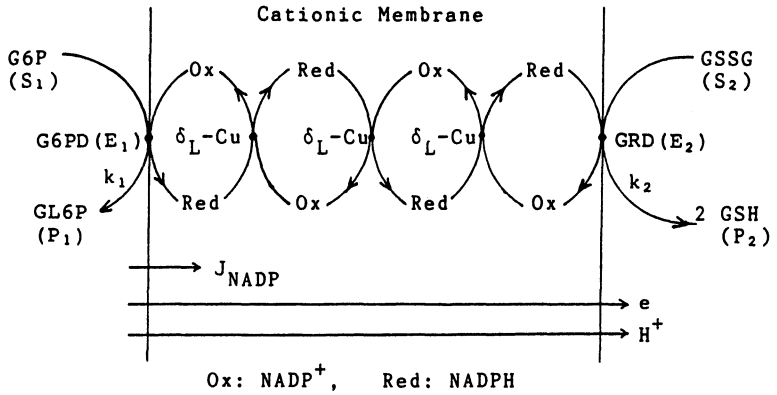
Differential Pulse Polarograhy: The reaction kinetics was followed by measuring the concentration of GSH and GSSG in the reaction system by means of a differential pulse polarographic method (DPP method), which is based on the fact that the reduction peak for GSSG in the DPP chart is shifted to a larger negative potential upon adding $NADP^+$; however, that for GSH it is not shifted.[15] The instrument used was a Yanaco Voltammetric Analyzer P-1000 with a Watanabe X-Y recorder WX4401. A stock solution of 4-M $NaNO_3$ containing a 0.08-M phosphate buffer was mixed with a sample solution of GSSG, GSH or both mixture and NADPH in appropriate ratio; the solution was then transferred to an electrochemical cell. The differential pulse polarograms of the sample solutions were measured at the scan rate 5 mV s^{-1}. From the corresponding peaks the amounts of GSSG, GSH, and NADPH were determined.

Membrane Potential: The membrane potential was determined in a cell that consists of two compartments as shown previously.[16] The potential was measured by means of a Digital Multimeter TR6843 (Takeda Riken Co., Ltd.) with pin-hole type of Ag/AgCl/sat. KCl electrodes (Horiba Co., Ltd. #2010). The mean value of two measurements obtained by exchanging the electrodes in both compartments was taken as a reliable membrane potential. The concentration of the KCl solution was kept constant in one compartment at 1,0 $\times$ 10^{-3} M and in the other compartment at 1.0$\times$10^{-4} M. The temperature was controlled at 25$\pm$0.2 °C.

Driving of Conjugated Redox Reaction: A conjugated redox reaction was performed by using a two-compartment cell, as shown in Scheme 2. Both compartments **I** and **II** were filled with a 35.0 ml phosphate buffer solution of pH 7.8, an optimum pH for activating both enzymes. To support the driving of the conjugated reaction a coenzyme, NADPH or $NADP^+$ was added to compartment **I** and adsorbed in the membrane. At the same time, the diffusion flux $J_{NADP(H)}$ of NADP(H) through the membrane was measured. The NADP(H) concentration was determined spectrophotometrically (molar extinction: 6.20$\times$10^3 l mol^{-1} cm^{-1} at 340 nm). After this measurement, the solutions were taken away and a 0.25-mM $NADP^+$ aqueous buffer solution was again freshly filled in both compartments and the 1 mmol



Scheme 2. A conjugated redox-enzyme system.



Scheme 3. Effect of δ_L-Cu as electron carrier on the conjugated redox system.

G6P and 0.6 mmol GSSG in compartments **I** and **II**, respectively. The reaction was started by adding 0.3 mg of G6PD to compartment **I** and 0.35 mg GRD to the compartment **II** at same time, respectively. The concentration change of GSSG and GSH in both compartments with time was followed by the method described above. Before the measurements it was confirmed that both the enzymes G6PD and GRD could not permeate through the membrane, perhaps because of their large molecular sizes and their high affinity against positively charged membranes.

The cyclic voltammetry, the differential pulse polarography and the conjugated redox reaction were performed in the O_2-free solutions.

Results and Discussion

Some characteristics of the membranes prepared are summarized in Table 1. Membrane Code 0 was prepared without the incorporation of the δ_L component, while Membrane Code δ was obtained by mixing the δ_L component. The thickness of these membranes was almost the same (ca. 120 μm). However, the pore sizes of the membranes were considerably different from each other: 0.22 and 0.12 μm for Membrane Code 01 and δ2, respectively. It is clear from the large negative membrane potential that both membranes were charged positively. This means that negative charges in the δ_L component, appearing at the adjusted pH of the test solution, could not overcome positive charge in the urethane membrane.

Figure 1 shows cyclic voltammograms of MV using (a) a bare carbon electrode and (b) a δ_L-modified carbon electrode. The electrode reaction proceeds as

Table 1. Properties of Prepared Urethane Membranes

Membrane code	Thickness	Pore size	Membrane potential
	μm	μm	mV
01	120	0.22	−54.8
02	148	0.18	−51.0
δ1	106	0.14	−52.6
δ2	123	0.12	−49.6

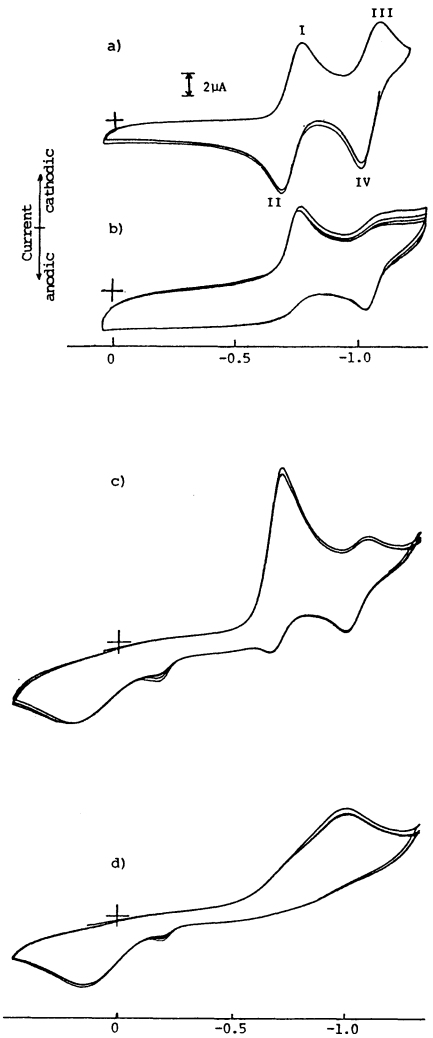


Fig. 1. Cyclic voltammograms of methyl viologen, supporting electrolyte: 1 M $NaClO_4$, pH 7.0 (0.02 M phosphate buffer), sweep rate: 150 mV s^{-1}.
a) Bare carbon electrode, 0.25 mM MV. b) carbon electrode modified with δ_L, 0.25 mM MV. c) Bare carbon electrode, 0.2 mM MV+0.8 mM Cu^{2+}-histidine*. d) Bare carbon electrode, 1 mM Cu^{2+}-histidine*. * molar ratio, Cu^{2+}: histidine=1:10.

follows:[17,18]

$$MV^{2+} + e \underset{II}{\overset{I}{\rightleftharpoons}} MV^{+\cdot} \quad (1)$$

$$MV^{+\cdot} + e \underset{IV}{\overset{III}{\rightleftharpoons}} MV^{\circ} \quad (2)$$

The numbers in **I—IV** correspond to those on the peaks of the curves in Fig. 1. The second reduction peak and the second oxidation peak were found to be smaller for the electrode (a), probably because the $MV^{+\cdot}$ produced in the first reduction step could be consumed by Cu^{2+} ions contained in the δ_L component. This could be confirmed from the fact that the same decrease in the peaks of CV was observed for a measurement of an aqueous MV solution containing a Cu^{2+}-histidine complex (Fig. 1c).

A typical feature of the conjugated redox reaction is shown in Fig. 2 for the case of a membrane containing

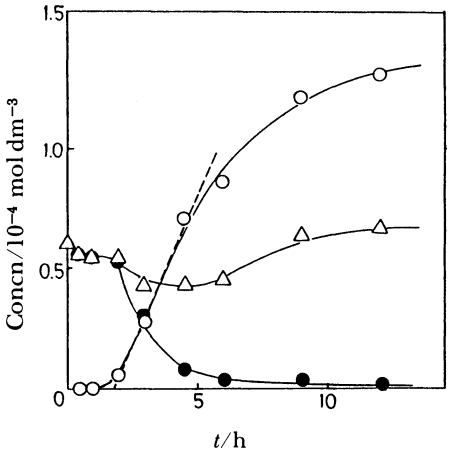


Fig. 2. Reduction of GSSG(●) and production of GSH(○) in compartment II in a conjugated redox reaction across the membrane containing δ_L component.
Membrane thickness; 90 mm(sustained Cu-treatment). △ shows the sum of GSSG and GSH (reduced to GSSG form).

δ_L. In this figure the concentrations of GSSG and GSH in components II are plotted against time. From the fact that the concentration of GSSG decreased and that of GSH increased after elapsed time, and the latter approached nearly double the initial concentration of GSSG, it is suggested that the recycle reaction between $NADP^+$ and NADPH occurs in the membrane, even if only small amounts of $NADP^+$ or NADPH (much fewer than one equibalent of the substrate) were added to the system. It should be noted that two GSH molecules are produced from one GSSG molecule. In this figure, the total concentration of glutathione in GSSG-form in both compartments was also plotted. This concentration decreases after a certain time, increases again and approaches a constant value equal to the initial concentration. This could be considered to be caused by the interaction of GSSG with the coenzyme adhering in the membrane (the interaction of GSSG with $NADP^+$ observed in DPP measurement).

For cases of membranes without δ_L, similar results were obtained, except that the concentration change of GSSG and GSH were much slower. The maximum production rate of GSH and the permeation time-lag were obtained from the slope and the intercept on the time-axis of the asymptote of the GSH-production curve (see Fig. 2), respectively. The effect of the electron carrier was estimated using the electron-transfer efficiency (ETE value),

$$ETE = J_{NADP(H)\leftarrow GSH} / J_{NADP(H)}, \quad (3)$$

where $J_{NADP(H)\leftarrow GSH}$ is an imaginary diffusion flux of NADP(H) reduced under the assumption that the production of GSH proceeds stoichiometrically according to the amount of NADPH diffused out through the membrane ($GSSG+NADPH+H^+ \rightarrow 2\ GSH+NADP^+$).

Kinetic data on the driving of the conjugated redox reaction through the membrane under various conditions are summarized in Table 2. Experiments No. 1 to 3 were made using membranes without the electron carrier δ_L component. No. 1 was driven without a prepermeation of the coenzyme, while Nos. 2 and 3 were driven after prepermeations of $NADP^+$ and NADPH, respectively. All of the values, d[GSH]/dt,

Table 2. Kinetic Data on the Driving of Conjugated Redox Reaction through the Urethane Membrane

Exp. No.	Membrane code	$J_{NADP(H)}\times10^{11}$ mol cm^{-2}s^{-1}	d(GSH)/d$t\times10^5$ mol l^{-1}s^{-1}	Time-lag h	$J_{NADP\leftarrow GSH}\times10^{11}$ mol cm^{-2}s^{-1}	Substrate conversion ratio (%)	ETE-value (ETE)[a]
1	01		3.10	2.5	2.76	90	1.6 (2.0)
2	01_{NADP^+}	1.75	6.20	2.0	5.68	94	3.2 (4.2)
3	01_{NADPH}	(1.36)	6.85	2.0	6.26	95	3.6 (4.6)
4	$\delta2_{NADP^+}$	0.613	2.55	4.5	2.33	37	3.8
5	$\delta2_{CudNADP^+}$	0.592	0.00	0.0	—	0	0.0
6	$\delta2_{Cu\ NADP^+}$	0.166	64.1	4.5	58.7	78	353

a) Calculated by using J_{NADPH}.

time-lag, $J_{NADP(H)\leftarrow GSH}$ and ETE-values were found to be almost same for both experiments. Of interest is the fact that the ETE-value is larger than 1. This means that the production of GSH in component II does not proceed based only on a simple diffusion of NADPH. Considering the fact that the time-lag of No. 3, in which NADPH was prepermeated, was not shorter than that of No. 2, an ETE-value larger than 1 can be explained considering either: that the electron exchange in the membrane took place between $NADP^+$ and NADPH, if they are ordered side by side in the membrane; or that not only the substrate, but the enzyme also could be condensed in the membrane because of the positive charge of the membrane. The latter should be meaningless, considering the following additional experiment: i.e. GSH was produced only in the compartment in contact with the membrane surface, on which GRD was previously adsorbed, when GSSG and NADPH were added to both compartments.

Of special interest are the results of experiments Nos. 4, 5, and 6 in the driving through the membrane entrapping the δ_L component. In these cases, the diffusion flux of $NADP^+$ is rather smaller and the time-lag are larger, compared with those in the cases of membranes without the δ_L component, perhaps because of a slightly smaller pore size, owing to the incorporation of the δ_L component in the membrane.

Experiment No. 5 was carried out by using the δ_L incorporated membrane after it was dipped in an aqueous $CuSO_4$ solution (1.6×10^{-6} mol dm^{-3}) for 24 h and slightly rinsed. In this case, GSH was not produced. An additional experiment showed that free copper ions inhibit the enzymic reaction in this system. Thus, the membrane was rinsed in water for 1 week and then used for driving experiment No. 5. A very interesting result was obtained wherein the rate of GSH-production was much larger, compared with those through the other membrane, although the diffusion flux J_{NADP} was considerably lower (1.6×10^{-12} mol cm^{-2} s^{-1}). Consequently, the ETE-value went up to over 300. In the case of the δ_L-incorporated membrane, the association of the substrates and coenzyme in the membrane could also be one of the causes of the extremely high ETE-value, though it does not seem likely considering the relatively small intake of coenzyme, and the considerably large time-lag. Therefore, it can be concluded that the large ETE-value for experiment No. 6 is achieved by the aid of the membrane-incorporated δ_L component catalyzing the electron-transfer reaction between the coenzymes. This consideration is shown schematically in Scheme 3. It is presumed that the electron can be transferred advantageously through the coenzymes probably adsorbed on the δ_L-component, a complex of Cu^{2+} with basic amino residues of polypeptides. However, further details of this electron-transfer mechanism have not yet been clarified.

Conclusion

Modeled on a biological cell membrane, an artificial conjugated redox membrane was constructed which incorporated dehydrogenase and reductase separately on both membrane surfaces. The conjugated redox reaction across the membrane was successfully demonstrated. The driving of redox reactions owes much to electron exchanges between $NADP^+$ and NADPH in the membrane. The electron-transfer efficiency (ETE value) could be improved by two orders of magnitude by incorporating the δ_L component extracted from wool fibers, compared with the fact that the ETE value was changed with the Cu^{2+} content in the δ_L component. The Cu^{2+} complex ions were regarded as being a cooperator for the electron transfer: they accelerate the electron-exchange reaction between coenzymes in the membrane.

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