# TRANSMEMBRANE ELECTRON TRANSFER IN AN ENZYME-PHOSPHOLIPID COMPLEX

Katsuyuki Imai* and Arnold F. Brodie

Department of Biochemistry
University of Southern California School of Medicine
Los Angeles, California 90033

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SUMMARY: Purified malate-vitamin K reductase (MKR) from Mycobacterium phlei, which requires phospholipid and FAD for the enzymatic reduction of dye electron acceptors, was used as an electron transfer system in combination with synthetic phospholipid vesicles. Ferricyanide entrapped inside the sonically prepared vesicles was enzymatically reduced by MKR. The rate of reduction of ferricyanide by MKR was dependent upon a lipid soluble mediator. The rate of the enzymatic reduction of ferricyanide by malate-vitamin K reductase was increased by the addition of phenazine methosulfate (PMS) or benzoquinone.

INTRODUCTION. Electron transfer across artificial membranes has been reported with phospholipid vesicles (liposomes) which contained either ferricyanide (1, 2) or cytochrome c (3). These experiments demonstrated that externally added ascorbate could reduce the internal electron acceptor only when an appropriate electron carrier was present in the membrane phase. Malate-vitamin K reductase (MKR) has been recently purified (4) and shown to be a phospholipid-requiring enzyme which requires FAD as a cofactor for enzymatic activity. In the absence of the electron transport particles, the oxidation of malate by the MKR can be accomplished by several dye acceptors including ferricyanide (4, 5). It was of interest to determine if transmembrane electron transfer could be elicited in a synthetic phospholipid system reconstituted with MKR.

*Present address: Institute for Enzyme Research, School of Medicine, Tokushima University, Tokushima, Japan.



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MATERIALS AND METHODS. Purification and assay of MKR were described elsewhere (4). Ferricyanide entrapped liposomes were sonically prepared by a modification of the method of Hinkle (1). Purified asolectin (500 mg of soy bean phospholipid), in 10 ml of a solution containing 0.2 M ferricyanide, 20 mM Tris-HCl (pH 7.7), and 1 mM EDTA, was sonicated at 6° for 30 minutes under a nitrogen atmosphere. Reduction of the internal ferricyanide was assayed in a system containing 0.2 ml of liposomes and 1.8 ml of 50 mM Tris-HCl (pH 7.7) containing 0.3 M NaCl. Absorbance changes of ferricyanide were measured at 420 nm with a Cary spectrophotometer, Model 14, equipped with a 0.1 OD slide wire.

RESULTS AND DISCUSSION. As reported by Hinkle (1), the reduction by ascorbate of ferricyanide entrapped in the liposomes was observed after the addition of tetraphenylboron ($TPB^-$) and dicyclopentadienyl iron (ferrocene) (Fig. 1A). Ferrocene is considered to act as a slightly lipophilic electron carrier with the anion tetraphenylboron (1). The rate of reduction was further stimulated by the uncoupler pentachlorophenol (PCP). The addition of ascorbate to the liposomes without a mediator resulted in the reduction of a small quantity of ferricyanide. This level of reduction is an index of the concentration of external ferricyanide (Fig. 1A). Figure 1B and 1C show similar experiments with liposomes which were reconstituted with MKR and FAD. The addition of malate to the system resulted in a very slow, but significant rate of ferricyanide reduction (Fig. 1B), which was dependent upon enzyme concentration (Fig. 1B [1.75 μg MKR] and Fig. 1C [8.8 μg MKR]). The further addition of MKR resulted in an increase in the rate of reduction of the ferricyanide entrapped within the liposomes. Reduction of ferricyanide did not occur with the addition of malate if FAD was not present. In contrast to the effects observed with $TPB^-$ and ferrocene when ascorbate was used as the electron donor with the non-reconstituted liposomes, the further addition of $TPB^-$ (Fig. 1B) or ferrocene (Fig. 1C) did not stimulate the rate of the malate-supported reduction of ferricyanide with the MKR-liposome. The slight inhibitory effect observed

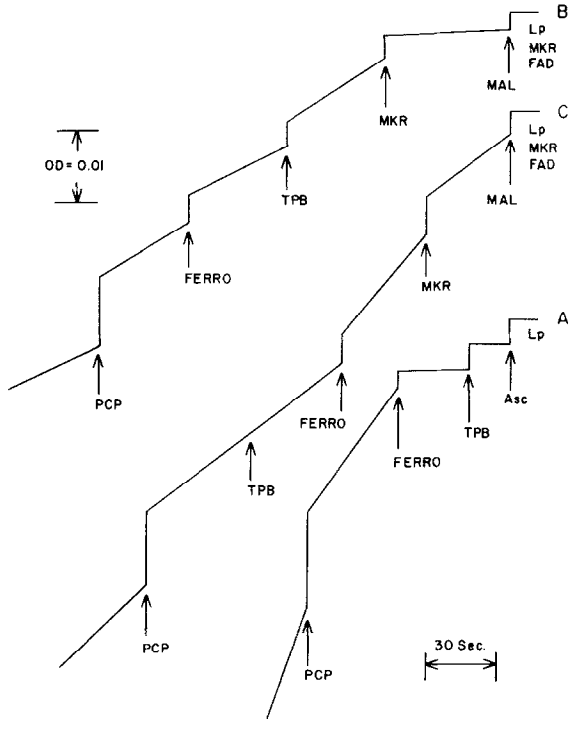


Fig. 1. Reduction of ferricyanide entrapped in liposomes.

The reaction mixture was described in the text. Lp: liposomes. The following additions were made as indicated: (A) 10 μmoles of sodium ascorbate (Asc); 0.04 μmole of TPB; 0.04 μmole of ferrocene (Ferro) in ethanol; 0.05 μmole of PCP. The initial reaction mixture in (B) contained 5 nmoles of FAD and 1.75 μg of MKR. The following additions were made as indicated: 10 μmoles of malate (Mal); 8.8 μg of MKR; and the same amounts of TPB, ferrocene, and PCP as described above in (A). The reaction conditions in (C) were the same as those in (B) except that 8.8 μg of MKR was present initially.

with PCP may be due to the inhibitory effect of PCP on MKR activity (5). The inability of ferrocene to act as a mediator may be explained by the relatively high electron potential of this compound (0.40V) (1).

Since MKR can efficiently reduce dichloroindophenol (DCIP) via phenazine methosulfate (PMS), and since PMS is a moderately lipid soluble cation (2), the effect of PMS on transmembrane electron transfer was examined. As shown in Fig. 2A, the addition of malate to the MKR-reconstituted liposome resulted in a rapid reduction of external ferricyanide, which was followed by a slower, but constant rate of reduction of internal ferricyanide. The removal of external ferricyanide was incomplete in these experiments even after dialysis. How-

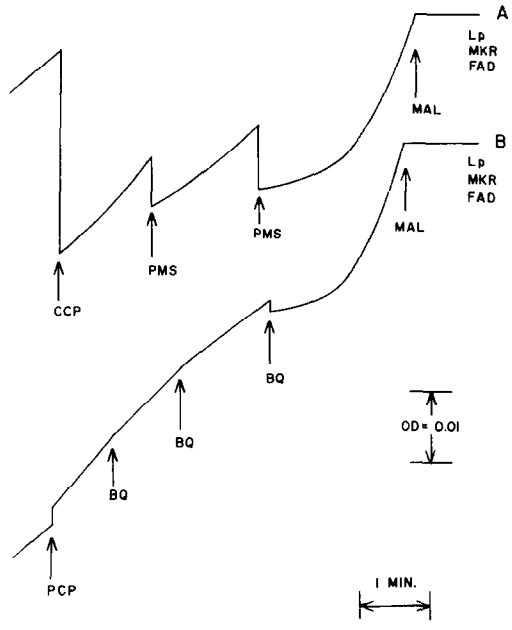


Fig. 2. Effects of phenazine methosulfate and benzoquinone on the reduction of ferricyanide entrapped in liposomes.

The reaction conditions were same as in Fig. 1B except that 50 μg of a different batch of MKR were used. The following additions were made as indicated: 5.3 nmoles of PMS, 20 nmoles of m-chlorocarbanylcyanide phenylhydrazone (CCP) and 6 nmoles of benzoquinone (BQ).

ever, the level of the external ferricyanide was easily verified and measured by reduction with added ascorbate. The addition of PMS resulted in a stimulation of the rate of reduction of entrapped ferricyanide. The effect was dependent upon FAD and the amount of PMS added. Benzoquinone may also be used as an electron mediator for MKR (5). The addition of benzoquinone to the MKR reconstituted liposome resulted in the reduction of ferricyanide entrapped in the liposome similar to that observed with PMS (Fig. 2B). It should be noted that the different rates of ferricyanide reduction by MKR in Figures 1 and 2 were due to differences in the specific activity of the MKR used in the two experiments.

With either the PMS or benzoquinone supplemented systems, uncouplers did not stimulate the rate of reduction of ferricyanide. This should be considered in light of the fact that the ionic mediators, $TPB^-$ and ferrocene,

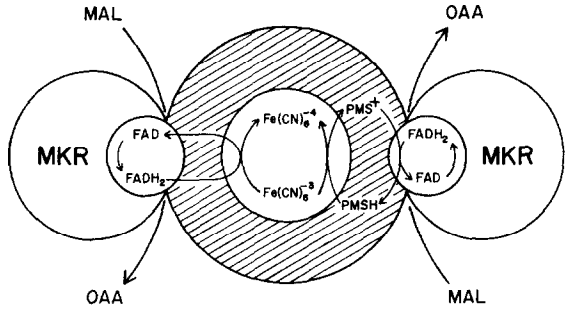


Fig. 3. Suggested reaction mechanism for the reduction of internal ferricyanide in the MKR system.

OAA represents oxaloacetate. Other abbreviations used are defined in the text.

did not affect the rate of ferricyanide reduction by the MKR-liposome system.

The proposed mechanism for electron transfer across the liposomal membrane is via a lipid soluble ion which acts as an electron carrier between the external and internal space (1, 2). In the systems described in the present study (Fig. 2), PMS and benzoquinone were acting as an electron carrier as suggested by Hinkle (1) and Deamer et al. (2). In the absence of such mediators, a measurable amount of ferricyanide was reduced (Fig. 1B and 1C). This would suggest that the enzyme cofactor, FAD, may function at the membrane phase (Fig. 3). This does not imply that FAD is a soluble mediator, although FAD is probably not insoluble in lipoidal membranes. FAD is very soluble in cresol or phenol and this property has been used for the separation of FAD from aqueous extracts (6). The rate of reduction of ferricyanide entrapped in liposomes (Fig. 1B) was calculated as 2.7 μmoles ferricyanide reduced/min/mg protein, whereas the rate assayed with the regular sonicated liposome with external ferricyanide was 32.0. The rate of reduction measured in the system in which ferricyanide was added externally to the ferricyanide entrapped liposomes was 5.8. These facts suggest that in this system, the solubility of FAD or $FADH_2$ is the rate limiting factor for the reduction of the internal ferricyanide. The fact that the addition of external ferricyanide to the

liposomes containing ferricyanide resulted in a lower rate of reduction than that observed with liposomes and externally added ferricyanide suggests a difference in the binding of MKR-FAD to liposomes prepared in the presence of ferricyanide. Actually, the difference in the method of preparing phospholipid vesicles influenced the enzymatic activity. Liposomes prepared in the presence of cholate, followed by a gradual removal of detergent by dialysis (7), had an activity 30-50% higher than the regular sonicated liposomes. However, phospholipid vesicles prepared by mechanical shaking for 2-4 min (without sonication) had less than 10% activity than the sonicated liposomes. Thus, the presence of ferricyanide during sonication may change the structure or the nature of the surface of the liposomes, causing a decrease in the binding sites or a decreased affinity for the enzyme.

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