

NADH-Linked Ferricyanide and Cytochrome *c* Reduction Activities in Corn Root Plasma Membrane

Shu-I Tu, Deidre Patterson and David Brauer

*U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center,
600 East Mermaid Lane, Philadelphia, PA 19118, U.S.A.*

Corn root plasma membrane catalyzed NADH reduction of ferricyanide and cytochrome *c* over a wide pH range. At pH 7.5, apparent K_m s of NADH-cytochrome *c* pair were significantly lower than those of NADH-ferricyanide pair. FMN and polylysine respectively enhanced the reduction of ferricyanide and cytochrome *c*. Yet, polyaspartate decreased the ferricyanide reduction. NADH oxidation observed in the presence of both ferricyanide and cytochrome *c* was significantly slower than the sum of rates obtained with individual acceptors. The results suggest that the membrane may contain different but not totally independent reduction sites for cytochrome *c* and ferricyanide.

Key words: Corn roots — Electron transfer — NADH-linked reduction — Plasma membrane.

The plasmalemma of higher plant cells is now thought to contain a NADH-linked redox system. Evidence for its existence includes, but is not limited to, NADH-supported medium acidification of plant leaves (Ivankin and Novak 1980), and NADH-linked cytochrome *c* reduction (Larsson 1985) of purified plasma membrane vesicles. This redox system may contain many membrane components exhibiting NADH-cytochrome *c* reductase (Larsson 1985), flavin-NADH dehydrogenase (Ramirez et al. 1984), and b-type of cytochrome (Leong et al. 1981) activities. Particles containing multiple NADH-linked redox activities have been obtained from detergent treated corn root plasma membrane (Luster and Buckhout 1988). A 27 kilodalton, FMN stimulated, redox protein has also been identified and purified from the plasma membrane (Luster and Buckhout 1989). Based on the difference in surface properties, purified plant plasma membrane may be further separated into right-side-out and inside-out vesicles (Larsson et al. 1988). The availability of these vesicles has led to assignment of NADH oxidation, ferricyanide reduction, and cytochrome *c* reduction occurring mainly to the cytoplasmic face of plasma

membrane (Askerlund et al. 1988). Transmembranous electron transfer from cytoplasmic NADH to apoplasmic acceptors was recently described (Askerlund and Larsson 1991). Evidence for NADH oxidation and its associated reduction of ferricyanide occurring at the external surface (in-vivo) of plasmalemma is also available (Crane et al. 1985).

The exact physiological roles of NADH-linked electron transport in plasmalemma are yet to be firmly established. Proposed involvements of this process include the reduction of Fe^{3+} to Fe^{2+} for uptake (Møller and Crane, 1990), hormonal regulation of cell growth, and keeping —SH groups of membrane protein in a reduced state (Crane et al. 1985, Møller and Crane 1990). As mentioned, both cytochrome *c* and ferricyanide have been extensively utilized to follow the oxidation of NADH catalyzed by the membrane. Yet information on the possible relationship between the reductions of these two acceptors is lacking. To this end, we studied the properties of the electron transfer from NADH to these two acceptors under different conditions. The results suggest that the reduction sites of ferricyanide and cytochrome *c* are different but may not be totally independent in the plasma membrane.

Abbreviations: BSA, bovine serum albumin; TCA, trichloroacetic acid; DOC, deoxycholate; BTP, bis-tris-propane; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethyleneglycol-bis(amino-ethyl ether)*N,N*-tetraacetic acid; DTT, dithiothreitol.

Reference to brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Materials and Methods

Isolation of corn root plasma membrane—The plant materials were prepared according to the procedures described in our previous study (Tu et al. 1986). Briefly, the roots of 3-day old corn (Cross Hybrid WF7551) seedlings

germinated in the dark were homogenized in a buffer containing 25 mM BTP-MES pH 7.8, 10% (w/v) glycerol, 0.25 M sucrose, 0.5% (w/v) BSA, 2 mM MgSO₄, 2 mM ATP, 1 mM PMSF, 2 mM EGTA, 5 mM DTT. After filtering the homogenate through cheesecloth, the filtrate was centrifuged at 6,000 × *g* for 10 min. The supernatant was centrifuged again at 96,000 × *g* for 40 min. The membranes were suspended in 4 ml of homogenization buffer and applied to a discontinuous sucrose density gradient of 34 (10 ml) and 42% (10 ml) (w/w). After being centrifuged at 100,000 × *g* for 150 min, the plasma membrane was extracted from the interface of 34 and 45% (w/w) sucrose solutions. All the isolation procedures were carried out at 4°C. The protein content of the membrane was determined by a modified Lowry method after precipitation by TCA in the presence of DOC (Brauer and Tu 1991). In a previous report (Hsu et al. 1990), we concluded that root plasma membrane isolated from the corn hybrid by the use of glycerol and discontinuous sucrose density centrifugation contained only minimal contaminations of mitochondria, Golgi, and tonoplast.

Measurements of electron transfer—The reduction of ferricyanide and cytochrome *c* was monitored at 22°C by absorbance decrease at 420 nm and absorbance increase at 550 nm, respectively. The oxidation of NADH was determined from the decrease in absorbance at 340 nm. For quantitative analysis of the data, differences in extinction coefficients between oxidized and reduced forms of the substrates were used. The values used (reduced minus oxidized) for cytochrome *c*, ferricyanide, and NADH were 18.7, -0.93, and -5.6 mM⁻¹ cm⁻¹, respectively. Unless specified otherwise, the assay media contained 1.5 ml of a 0.25 M sucrose solution containing 20 mM KCl and 20 mM buffers (pH 4 to 7, MES, and pH 7 to 9.3, HEPES), 70 μg plasma membrane, and various concentrations of other additions including FMN and polyelectrolytes. After a 10 min incubation at 22°C, the redox reactions were initiated by the additions of either NADH or appropriate electron acceptors.

Determination of kinetic parameters—The electron transfer kinetics were analyzed according to simple Michaelis-Menten model. For NADH related parameters (*K_m* and *V_{max}*), the concentration of the acceptor (ferricyanide or cytochrome *c*) was kept as excess but constant. For acceptor related parameters, the concentration of NADH was maintained as excess. The linearity of the double reciprocal plots was usually better than 99% under employed experimental conditions.

Other chemicals and reagents—All chemicals and reagents used were of analytic grades available commercially.

Results and Discussion

NADH-linked reduction of cytochrome *c* and ferri-

cyanide—Isolated plasma membrane vesicles assume either right-side-out or inside-out orientation. It has been reported that essentially all NADH-linked reduction activities should occur on the inside surface of the plasma membrane (Askerlund et al. 1988, Askerlund and Larsson 1991). Since both ferricyanide and cytochrome *c* are not membrane permeable, it is assumed that inside-out vesicles and unsealed membrane fragments would exhibit NADH-linked reduction activities. The pH dependence of NADH-linked reduction of cytochrome *c* and ferricyanide, catalyzed by isolated plasma membrane, is shown in Fig. 1. The reduction of ferricyanide appeared to have two pH optima centering around pH 7.5. In a relative sense, the reduction rate of ferricyanide increased more than that of cytochrome *c* as the pH was increased from 5.0 to 7.0. Between pH 6.5 to 8.5, the reduction rate of cytochrome *c* exhibited little sensitivity to pH change. For physiological considerations, a cytoplasmic pH value of 7.5 was chosen to conduct the rest of the study.

Kinetic properties of the electron transfer processes—The redox reactions of NADH-cytochrome *c* and NADH-ferricyanide catalyzed by the plasma membrane appeared to follow Michaelis-Menten kinetics. At pH 7.5, the apparent Michaelis constant (*K_m*) for NADH oxidation showed a significant difference between using cytochrome *c* or ferricyanide as the oxidant (Table 1). The apparent affinity for NADH was much stronger with cytochrome *c* as elec-

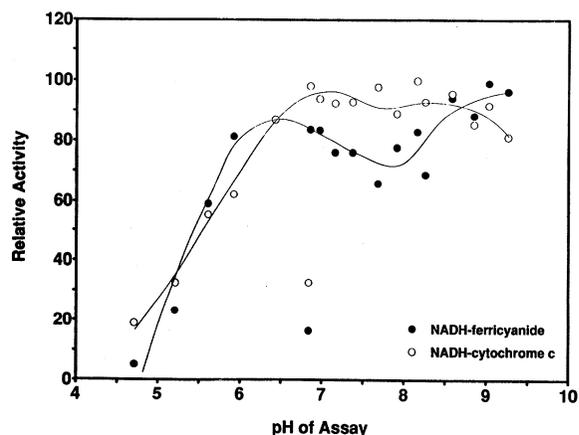


Fig. 1 pH Dependence of NADH-linked electron transfer processes. The rates of NADH-linked reduction of cytochrome *c* and ferricyanide were measured at different pH. The assay media contained 100 mM KCl and 10 mM different buffers (pH 4 to 7, MES, and pH 7 to 9.3, HEPES). The applied concentrations of plasma membrane, NADH, cytochrome *c*, and ferricyanide were 38 μg ml⁻¹, 30 μM, and 120 μM, respectively. To calculate the relative rates, the highest activities obtained for the reduction of cytochrome *c* and ferricyanide in tested pH range are arbitrarily assigned as 100% which are 556 and 562 nmol mg⁻¹ min⁻¹ for cytochrome *c* and ferricyanide reductions, respectively.

Table 1 Kinetic parameters of NADH-linked electron transfer processes

	NADH oxidation		Ferricyanide reduction	Cytochrome <i>c</i> reduction
	A	B		
K_m (μM)	160	27	56	3.8
V_{\max} ($\text{nmol min}^{-1} \text{mg}^{-1}$)	300	330	590	640

The kinetic parameters were determined in the medium of pH 7.5 as described. For NADH oxidation, values in columns **A** and **B** were obtained by using ferricyanide and cytochrome *c* as electron acceptors, respectively. The values represent averages of 3 experiments with errors as $\pm 10\%$.

tron acceptor. With NADH as the electron donor, the membrane also showed considerably higher affinity for cytochrome *c* than ferricyanide. Since the kinetic parameters of the redox pair NADH-ferricyanide are different from those of the NADH-cytochrome *c* pair, the involvement of the same enzyme system for both pairs seems less likely. For this model to be operative, the K_m value for NADH should not be significantly affected by the electron acceptor used. A similar conclusion was also advanced for the plasma membrane of red beet leaves (Askerlund et al. 1988). It should be mentioned that, with the exception of cytochrome *c*, the K_m values for NADH and ferricyanide in corn root plasma membrane vesicles mentioned in Table 1 are about 4 to 5 times higher than these found in the plasma membrane vesicles of red beet leaves (Askerlund et al. 1988). The K_m values for cytochrome *c* in both membrane systems are comparable. Difference in the choice of plant materials and assay conditions may contribute to this discrepancy.

Effects of polylysine, polyaspartate, and FMN on NADH-linked electron transfer—The kinetic analyses of Table 1 suggest that the electron transfer from NADH to cytochrome *c* and ferricyanide do not appear to share the identical pathway. This suggestion was further tested by the effects of some polyelectrolytes and FMN on the reduction processes. Polylysine has been shown to inhibit the reduction of externally added cytochrome *c* in mitochondria by competing with the same negatively charged membrane binding site (Davies et al. 1964, Racker and Horstman 1972). The addition of polylysine to the membrane vesicles resulted in a significant inhibition to the reduction of cytochrome *c* by NADH (Table 2). However, the reduction of ferricyanide was stimulated. Upon addition of polyaspartate, the reduction of cytochrome *c* was not affected but ferricyanide reduction was inhibited. The observed polylysine stimulation of ferricyanide reduction may be a result of neutralizing the negative charges on the membrane surface. The inhibition of ferricyanide by polyaspartate would suggest that the binding site of this electron acceptor may be positively charged. The effects of polylysine on the oxidation of NADH by the receptors are

shown in Fig. 2. The consumption of NADH by cytochrome *c* was inhibited by preincubating the vesicles with polylysine. Subsequently added ferricyanide caused the oxidation of NADH to resume to the same rate as that obtained in the absence of cytochrome *c*. In agreement with the stimulation of ferricyanide reduction (Table 2), the presence of polylysine also increased the rate of oxidation of NADH by ferricyanide.

It has been reported that the addition of flavin mononucleotide (FMN) to an isolated 27 kDa, NADH (NADPH)-linked electron transport protein from corn root plasma membrane substantially increases its ability to catalyze the reduction of ferricyanide (Luster and Buckhout 1989). This result was used to suggest that the electron transport protein may contain a tightly bound but non-covalently associated FMN which may be directly in-

Table 2 Effects of polyelectrolytes on NADH-linked reduction^a

Additions	Relative reduction rates ^b	
	Cytochrome <i>c</i>	Ferricyanide
None	100	100
Polylysine $\mu\text{g/ml}^{-1}$	12.5	108
	25.0	129
	58.8	158
Polyaspartate $\mu\text{g/ml}^{-1}$	25.0	75
FMN (μM)	5	103
	50	156

^a The NADH reduction of cytochrome *c* and ferricyanide catalyzed by purified corn root plasma membrane at pH 7.5 were followed as described in Figure 1. The membrane vesicles were incubated with polyelectrolytes or FMN for 10 min before the addition of NADH. The mean MW of polylysine and polyaspartate are 8,000 and 12,000 daltons, respectively.

^b The relative reduction rate was calculated by assigning the initial rates obtained without additions as 100. The values listed represented an average of two experiments with an error of no greater than $\pm 2\%$.

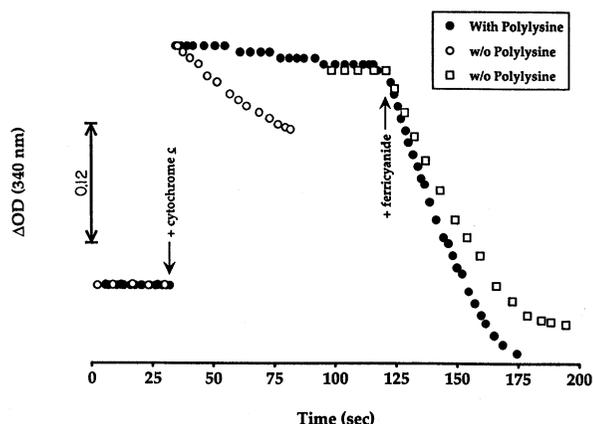


Fig. 2 Effects of polylysine on NADH oxidation. Plasma membrane vesicles (160 μg of protein) in 1.7 ml of the pH 7.5 assay solution, were incubated with 235 μM NADH in the presence of antimycin-A (0.2 μg per μg of membrane protein) and with 58.8 $\mu\text{g ml}^{-1}$ polylysine for 10 min at 22°C. NADH oxidation, measured by absorbance decrease at 340 nm (shown as filled circles), was then initiated by the addition of 29.4 μM cytochrome *c* which caused a constant up shift in the absorbance at 340 nm. At the indicated time, 118 μM of ferricyanide was then added to the solution and a rapid decrease in absorbance was observed. The antimycin-A-insensitive NADH consumptions in the absence of polylysine induced by cytochrome *c* and ferricyanide are shown for comparison (open circles and squares, respectively). It should be noted that in the presence of polylysine, the same stimulation of ferricyanide-induced NADH oxidation was observed with or without a prior addition of cytochrome *c*.

involved in the reduction of ferricyanide. This protein also appears to contain a minimal NADH-linked cytochrome *c* reduction activity. It is not clear from the report (Luster and Buckhout 1989) whether the low cytochrome *c* reduction activity of isolated protein can be stimulated by FMN incubation. We have tested the effects of incubating purified plasma membrane vesicles with FMN (0 to 50 μM) in this study. An increase in the activity of NADH-linked reduction of ferricyanide was observed (Table 2). However, the reduction activity of cytochrome *c* was not significantly affected by the addition of FMN. Consistent with the observation in purified protein work (Luster and Buckhout 1989), our membrane study also suggest the possible involvement of a flavo-protein in the reduction of ferricyanide.

Relationship between ferricyanide and cytochrome *c* reduction—The differential effects described in Fig. 2 and Table 2 appear to confirm the notion that the two reduction processes do not occur at the same site. To gain further insight on the relationship between the two processes, we compared the time course of NADH oxidation by ferricyanide and cytochrome *c* alone to that obtained in the pres-

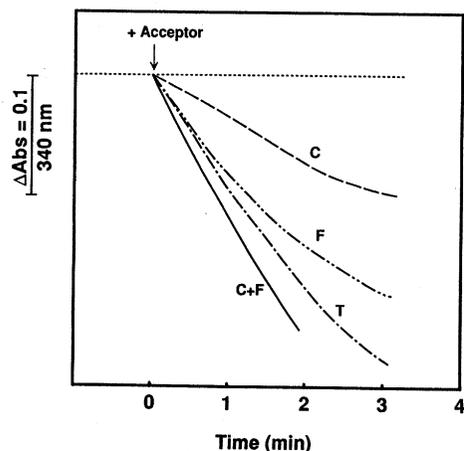


Fig. 3 Kinetics of NADH consumption. Assay medium containing 45 μg plasma membrane protein ml^{-1} was allowed to incubate with 20 μM cytochrome *c* or 60 μM ferricyanide, or both, for 10 min before the addition of 125 μM NADH. The catalyzed oxidation of NADH, measured as absorbance decrease at 340 nm, caused by ferricyanide, cytochrome *c*, and both, is shown as traces F, C, and T, respectively. In the absence of acceptors, the absorbance at 340 nm remained constant. The arithmetic sum of F and C is shown as trace (C+F).

ence of both (Fig. 3). The sum of the two traces was then constructed as trace (C+F) and used to compare with the oxidation kinetics of NADH caused by the simultaneous presence of both ferricyanide and cytochrome *c*. The initial rates of NADH oxidation obtained in the presence of ferricyanide (R_f), cytochrome *c* (R_c), and both (R_t), were 262, 112, and $281 \pm 5\%$ nmoles $\text{min}^{-1} \text{mg}^{-1}$, respectively. After the initial rapid decrease of absorbance at 340 nm, the rate of NADH oxidation, obtained with the presence of both acceptors, gradually decreased to approximately the same level obtained with cytochrome *c* as the only acceptor (data not shown in Fig. 3). The reduction of ferricyanide and cytochrome *c* may involve two independent enzyme systems which for convenience will be designed as E_f and E_c respectively. The total NADH oxidation rate, R_t , would equal to the sum of R_f and R_c if there were no interactions between added acceptors. However, being a strong oxidant, ferricyanide may instantaneously re-oxidize ferrocytochrome *c* generated from E_c . Since the concentration of oxidized cytochrome *c* would remain unchanged until the exhaustion of ferricyanide, the oxidation of NADH through E_c should proceed at a rate as R_c . On the other hand, the reduction of ferricyanide by NADH through E_f would be slowed because ferricyanide could be indirectly reduced by E_c . Thus, because of this complication, independent model predicts that:

$$R_t < R_f + R_c$$

when both ferricyanide and cytochrome *c* are present. The limit of the decrease in initial rate may be estimated from the concentrations of ferricyanide, S_f , and cytochrome *c*, and S_c , by Michaelis-Menten equation as:

$$R_f'/R_f = [1 + K_m/S_f] / [1 + K_m/(S_f - S_c)]$$

in which R_f' is the least possible rate of NADH oxidation through E_f by assuming that nearly all the cytochrome *c* is reduced at time zero. The value of K_m for E_f has been determined to be $56 \mu\text{M}$ (Table 1). Using the experiments of Figure 3 as an example, the R_f' value is estimated as $0.81 R_f$ or the initial NADH oxidation rate should not be lower than $(R_c + 0.81 R_f)$ or $324 \text{ nmol min}^{-1} \text{ mg}^{-1}$. This predicted value is still considerably higher than observed rate of $281 \text{ nmol min}^{-1} \text{ mg}^{-1}$. Similar results were observed with varied values of S_f and S_c . Thus, the idea that two totally independent systems are involved in the reduction of ferricyanide and cytochrome *c* appears to be unlikely.

As shown in Fig. 3, the kinetic trace of NADH oxida-

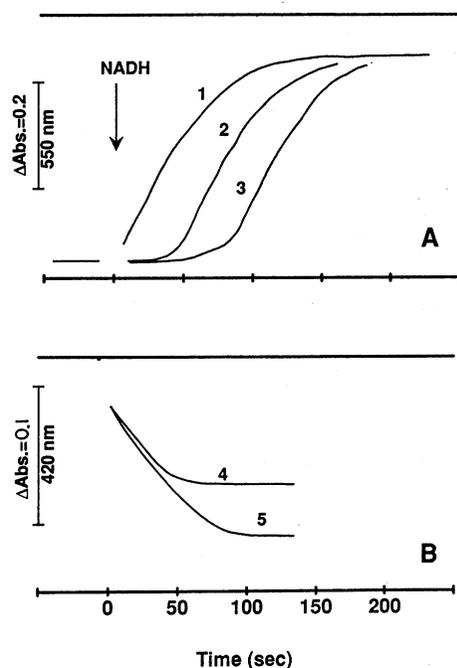


Fig. 4 Delayed reduction of cytochrome *c* by the presence of ferricyanide. (A) Kinetics of cytochrome *c* reduction. Solutions containing $27 \mu\text{g ml}^{-1}$ plasma membrane and $20 \mu\text{M}$ cytochrome *c* were incubated with various concentration of ferricyanide before the addition of $250 \mu\text{M}$ NADH. The reduction of cytochrome *c* was followed by the absorbance increase at 550 nm. The concentrations of ferricyanide used were 0, 90, and $180 \mu\text{M}$ for traces 1, 2, and 3, respectively. (B) ferricyanide reduction in the absence of cytochrome *c*. The reduction kinetics of ferricyanide in the solutions (cytochrome *c* deleted) used in generating traces 2 and 3 were shown as traces 4 and 5, respectively.

tion with the presence of both acceptors changed from one similar to ferricyanide reduction to one similar to cytochrome *c* reduction. In the presence of an excess of NADH, the reduction of cytochrome *c* was delayed by the presence of ferricyanide (Fig. 4A). The time lag before the resumption of the normal reduction of cytochrome *c* increased as the concentration of ferricyanide was increased. Furthermore, the kinetics of ferricyanide reduction obtained in solutions similar to that of Fig. 4A but omitting cytochrome *c* indicated that ferricyanide was readily reduced during the lag periods (Fig. 4B). The observed delay might be due to a non-enzymatic oxidation of reduced cytochrome *c* by ferricyanide. This implies that cytochrome *c* may be reduced prior to ferricyanide. If this were the case then the initial NADH oxidation rate (R_i) described in Fig. 3 would be closer to R_c rather than R_f .

Conclusion

The relationship between plasma membrane catalyzed reductions of ferricyanide and cytochrome *c* by NADH was investigated. Based on the different enzyme kinetic parameters (Table 1), it may be suggested that ferricyanide and cytochrome *c* do not share the same reducing site in the membrane. This suggestion is confirmed by the differential responses of the two reduction processes to the presence of polyelectrolytes and FMN (Table 2 and Fig. 2). By taking the non-enzymatic interaction between ferricyanide and cytochrome *c* into account, the consumption rate of NADH is still considerably less than that predicted from a model involving two totally independent membrane redox systems (Fig. 3). This claim is also supported by ferricyanide-induced delay in the reduction of cytochrome *c* (Fig. 4). Assuming that ferricyanide reduction occurs primarily through a FMN-containing protein (Luster and Buckhout 1989), then additional cofactors must be involved in transferring electrons from this protein to cytochrome *c*.

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