

EFFECTS OF DIVALENT CATIONS ON NADH-LINKED ELECTRON TRANSFER IN CORN ROOT PLASMA MEMBRANE**Shu-I Tu¹, Deidre Patterson, David Braver, and An-Fei Hsu***U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center 600 E. Mermaid Lane, Wyndmoor, PA 19118²*

ABSTRACT: The effects of calcium (Ca^{2+}), cadmium (Cd^{2+}), and copper (Cu^{2+}) cations on NADH-linked electron transfer in corn root plasma membrane vesicles were investigated. The reduction of both cytochrome c and ferricyanide were slightly stimulated by Ca^{2+} but not significantly affected by Cd^{2+} . However, Cd^{2+} induced a redox-linked increase in light scattering suggesting an increase in the size/volume of the vesicles. The presence of micromolar levels of Cu^{2+} decreased the reduction rates of both cytochrome c and ferricyanide. However, in contrast to ferricyanide reduction, Cu inhibition to the cytochrome c reduction was more effective and was less sensitive to ionic strength. Copper inhibition changed the Michaelis-Menten dependence of the ferricyanide reduction but not that of cytochrome c . These results suggest that the reduction of cytochrome c and ferricyanide must occur at different membrane sites.

Abbreviations:

BSA, bovine serum albumin; BTP, bis-tris propane; DOC, deoxycholate; DTT, DL-dithiothreitol; EGTA, ethylene glycerol bis-(β -amino ethyl ether) N,N,N',N'- tetraacetic acid; Hepes, N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid); Mes, 2-(N-morpholino) ethane sulfonic acid; PMSF, phenylmethylsulfonyl fluoride; and TCA, trichloroacetic acid.

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

INTRODUCTION

The plasma membrane of higher plant cells is known to contain a NADH-supported redox system which may contain many membrane components exhibiting activities of NADH-cytochrome c reductase (Larsson, 1985), flavin-NADH dehydrogenase (Ramirez et al. 1984), and b-type of cytochrome (Leong et al., 1981). Particles contain reduction activities linked to NADH oxidation have been obtained from corn root plasma membrane (Luster and Buckhout, 1988). A 27 kD, FMN-activated, electron transfer protein has been identified and isolated from corn plasma membrane (Luster and Buckhout, 1989). Based on the availability of right-side-out and inside-out plasma membrane vesicles (Larsson et al., 1988), the oxidation site of NADH and the reduction sites of cytochrome c and ferricyanide are assigned to the cytoplasmic face of the membrane (Askerlund et al., 1988). However, NADH-linked transmembrane electron transfer (Askerlund and Larsson, 1991) and *in vivo* electron transfer from NADH to ferricyanide at the external surface of the plasma membrane (Crane et al., 1985) are reported also.

The exact physiological roles of the NADH-linked electron transfer in plasmalemma are yet to be determined. Possibly, the process may be involved in the reduction of Fe^{3+} to Fe^{2+} for uptake (Møller and Crane, 1990), hormonal regulation of cell growth, and maintaining membrane -SH groups in reduced state (Crane et al., 1985; Møller and Crane, 1990). While both cytochrome c and ferricyanide have been extensively used to investigate the NADH-linked electron transfer, only limited information is available to delineate the relationship between the reduction of these two artificial electron acceptors. The detailed kinetic analysis on the reduction of cytochrome c and ferricyanide (Tu et al., 1993) and the differential effects of polylysine to the reduction of these two acceptors (Tu et al., 1994), suggest that the reduction of cytochrome c and ferricyanide should occur at different membrane sites. In present study, the effects of divalent cations on NADH-linked electron transfer were investigated. We found that Ca^{2+} stimulated the electron transfer to cytochrome c but not to ferricyanide. The presence of micromolar levels of Cu^{2+} inhibited both electron transfer processes in media with different ionic strength. However, the cytochrome c reduction was considerably more sensitive than ferricyanide reduction to the presence of Cu^{2+} . These results were used to propose possible models for the electron transfer in corn root plasma membrane.

MATERIAL AND METHODS

Plasma Membrane Isolation: The plasma membrane vesicles were isolated at 0 to 4°C from corn roots (*Zea mays* L. cv. W7551, Custom Farm Seed hybrid) as previously described (Brauer et al., 1988). Excised 3-day-old roots were homogenized in a buffer containing 25 mM Mes-BTP pH 7.8, 10% (w/v) glycerol, 0.25 M sucrose, 0.5% (w/v) BSA, 5 mM DTT, 2 mM MgSO₄, 2 mM ATP, 1 mM PMSF, and 2 mM EGTA. After filtering the homogenate through four layers of cheesecloth, the supernatant was subjected to differential centrifugation (6,000 x g for 10 min to remove particulate and 96,000 x g for 40 min to collect microsomal pellets). The microsomes were suspended in the homogenizing buffer and applied to a discontinuous sucrose density gradient of 34 and 42% (w/w). After being centrifuged at 100,000 x g for 150 min, the plasma membrane vesicles were extracted from the interface of 34 and 42% sucrose solution. The protein content of the membrane was determined by a modified Lowry method after DOC solubilization and TCA precipitation (Brauer et al., 1988). As previously described, the plasma membranes isolated from the corn hybrid by discontinuous sucrose gradient were only minimally contaminated by mitochondria, Golgi, and tonoplasts (Hsu et al., 1990).

Measurements of Electron Transfer: NADH-linked reduction of ferricyanide and cytochrome c at 22°C were measured spectroscopically at 420 and 550 nm, respectively. The time courses of the reduction were recorded by a Beckman DU-70 Spectrometer which was interfaced to an IBM PC for on-line data analysis. The difference in extinction coefficients between oxidized and reduced states of added substrates was used to quantitatively analyze the electron transfer processes. The values used (reduced minus oxidized) were 18.7 and -0.93/mM/cm for cytochrome c and ferricyanide, respectively. Two standard solutions of different ionic strength were used for electron transfer measurements. The high ionic strength (HI) solution contained 150 mM KCl and 20 mM Hepes, pH 7.5. The low ionic strength (LI) solution was obtained by replacing the KCl in HI solution with 300 mM sucrose. The plasma membrane vesicles were incubated with divalent cations for 15 min in either the HI or LI medium with the exact composition described in legends of figures. The reduction was initiated by the addition of 0.1 mM NADH. The kinetic parameters of the electron transfer reactions were determined by the simple Michaelis-Menten model using double reciprocal plots.

Chemicals: ATP, BSA, BTP, cytochrome c , DTT, Hepes, Mes, NADH, and PMSF were obtained from Sigma Co. The chemical form of ferricyanide was $K_3Fe(CN)_6$. The divalent cations when used were in the form of $CaCl_2$, $CdCl_2$, and $CuSO_4$. All chemicals and reagents were of analytical grade.

RESULTS

Effects of Ionic Strength and Divalent Cations on Electron Transfer:

The plasma membrane vesicles catalyzed the electron transfer from NADH to cytochrome c and ferricyanide. In a previous study (Tu et al., 1994), we reported that the rates of reduction of these two substrates were relatively insensitive to the change of KCl concentration (between 20 to 170 mM). In this study, we extended the range to zero KCl in the medium and found that the reduction rate of cytochrome c at pH 7.5 was not affected by ionic strength (Table 1, first row). However, the reduction rate of ferricyanide was decreased by about 1/3 in the LI medium. The results implied that the non-specific, electric static interactions associated with the surface of the membrane vesicles may be important in regulating the NADH-linked ferricyanide reduction but have no effect on the cytochrome c reduction.

In the presence of Ca^{2+} , the reduction of cytochrome c became somewhat more sensitive to the change of ionic strength. The significant increase of cytochrome c reduction observed in LI medium could be due to a saturation of non-specific binding of Ca^{2+} to negative charges on the surface of the vesicles and thus minimized the binding of positively charged cytochrome c ($pI > 10$) to those sites. This is equivalent to an increase in the effective substrate concentration for the redox enzyme. The addition of Ca^{2+} did not significantly affect the response of the ferricyanide reduction to the change in ionic strength.

The presence of Cd^{2+} caused a rather complex response from the membrane vesicles. The details of the observations are described in the subsequent sections of the report. The presence of Cu^{2+} profoundly affected the electron transfer processes. As shown in Table 1, 0.2 mM Cu^{2+} almost completely abolished cytochrome c reduction. The same concentration of Cu^{2+} also reduced ferricyanide reduction by 80% in both HI and LI media. The insensitivity to ionic strength suggests that the ionic interaction of Cu^{2+} with the membrane, if any, must be very strong.

TABLE 1. Effects of Divalent Cations on Rates of NADH-linked Electron Transfer.

Cation (0.2 mM)	Cytochrome <i>c</i> (nmol/min/mg)		Ferricyanide (nmol/min/mg)	
	HI medium	LI medium	HI medium	LI medium
none	247	242	1460	1114
Ca ²⁺	282	322	1504	1042
Cd ²⁺	-a-	-a-	-b-	-b-
Cu ²⁺	0.50	0.73	307	256

To determine the NADH-linked electron transfer activities, purified corn root plasma membrane vesicles containing 150 to 200 µg of protein were incubated with either 20 µM cytochrome *c* or 0.2 mM ferricyanide in a volume of 2.0 mL of HI or LI medium with indicated divalent cations. The incubation media also contained 15 to 20 µg of antimycin A to inhibit NADH consuming reactions of mitochondrial origin. The reaction was started by the addition of 0.2 mM NADH. The initial rates of the reactions were calculated as described in text. The data shown represented an average of 3 measurements with an error of ±5%.

- a The inclusion of Cd²⁺ caused a redox-induced light scattering increase of the vesicle solution. This increase complicated the determination of cytochrome *c* reduction from the absorbance increase at 550 nm.
- b The redox-induced light-scattering increase associated with the presence of Cd²⁺ prevented the determination of ferricyanide reduction from expected absorbance decrease at 420 nm. Details to overcome this difficulty was discussed in text.

Effects of Cadmium on the Membrane Vesicles and Electron Transfer: As described in Table 1, the presence of Cd²⁺ turned the solution milky and increased the absorbance at 420 nm after the addition of NADH (rather than showing a decrease as the reduction of ferricyanide occurred). Visual examination confirmed the disappearance of the yellowish color from the solution associated with the reduction of ferricyanide. Thus, the absorbance increase reflected an increase in the light scattering suggesting an enlargement in the size/volume of the vesicles.

The exact effects of Cd²⁺ to the plasma membrane were then evaluated as follows. In the absence of Cd²⁺ the reduction of ferricyanide catalyzed by the

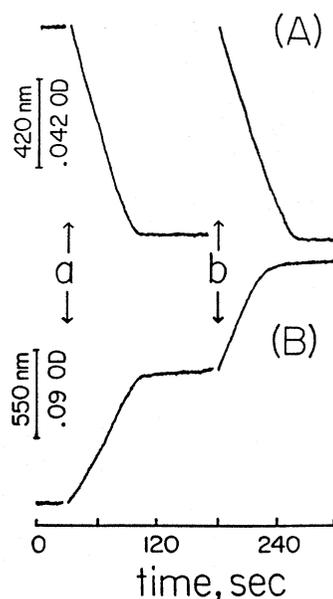


FIGURE 1. (A) Reduction of Ferricyanide by NADH-linked Electron Transfer. The plasma membrane vesicles were incubated with 0.1 mM of ferricyanide in the HI medium as described in text. At time "a", 0.1 mM of NADH was added to initiate the reduction. After the optical density (OD) at 420 nm decreased to a plateau, additional 0.1 mM of ferricyanide was added at time "b" to consume the residual NADH. (B) Redox-linked and Cd-induced Light Scattering Change of the Vesicles. The membrane vesicles were treated as described in (A) except the inclusion of 0.2 mM Cd^{2+} during incubation with ferricyanide.

plasma membrane maintained a nearly constant rate until the exhaustion of added NADH (Fig. 1A). The swelling of the membrane vesicles, caused by Cd^{2+} , can be monitored at 550 nm where the complication of ferricyanide reduction may be avoided. An examination of Figure 1B indicated that the light scattering increase associated with Cd^{2+} was related to the initiation of redox events. This increase in light scattering appeared to terminate at the time when ferricyanide reduction was near completion (comparing Figures 1A and 1B). It should be mentioned that the 0.1 mM NADH added at time "a" was sufficient to reduce both the 0.1 mM ferricyanide present during the incubation and the 0.1 mM ferricyanide added at

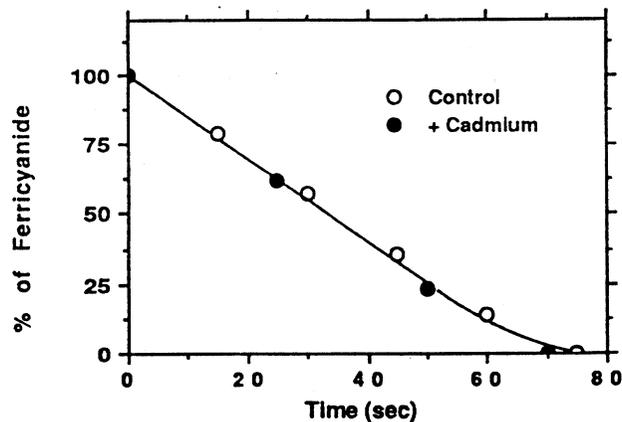


FIGURE 2. Reduction of Ferricyanide in the Presence of Cd^{2+} . The membrane vesicles were incubated with ferricyanide in the presence of 0.2 mM Cd^{2+} as described in Figure 1B. Aliquots of the vesicle mixture were withdrawn at indicated time after the addition of NADH and rapidly injected into equal volumes of 10% TCA to quench all membrane activities. The solution was then centrifuged at 10,000 g for 10 min. The absorption spectrum of the supernatant was recorded and the absorbance at 420 nm was used to calculate the percentage of unreacted ferricyanide. The 100% point was obtained by injecting ferricyanide incubated vesicles into the 10% TCA first before the addition of NADH.

time "b". The extent of induced light scattering increase of the vesicles under the redox conditions mentioned in Figure 1B, was affected by the concentration of Cd^{2+} added (data not shown).

To quantitatively test the effect of Cd^{2+} on NADH-linked electron transfer, a rapid acid quench method described in Figure 2 was developed. An injection of the vesicle suspension (with or without Cd^{2+}) undergoing active ferricyanide reduction to a concentrated TCA solution, rapidly quenched all enzymatic reactions and precipitated the membrane proteins. The denatured membrane could then be removed by centrifugation and the residual ferricyanide in the supernatant was measured. As shown, no difference in ferricyanide reduction rate was found between samples with and without Cd^{2+} . The exact origin of the observed vesicle size/volume increase which appears to relate to NADH-linked electron transfer and the presence of Cd^{2+} remains to be established.

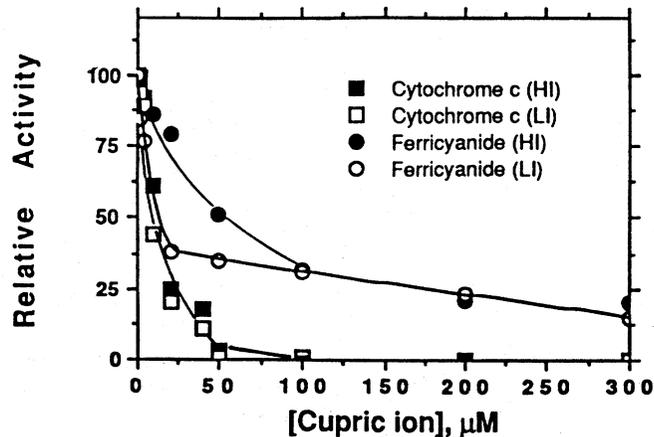


FIGURE 3. Inhibition of NADH-linked Electron Transfer by Cu^{2+} . The plasma membrane vesicles were allowed to incubate with $25 \mu\text{M}$ of cytochrome c or $100 \mu\text{M}$ ferricyanide and indicated concentrations of Cu^{2+} for 10 min in either HI or LI medium at room temperature. The reduction was then initiated by the addition of NADH. The initial rates of absorbance change at 550 nm or 420 nm were then determined. The rate found in the absence of Cu^{2+} was assigned as 100. While not shown, the inclusion of 0.2 mM Ca^{2+} did not change the Cu^{2+} inhibition.

Potency of Copper in Inhibiting the Electron Transfer: Among the three divalent cations tested, only Cu^{2+} exhibited inhibitory effects to NADH-linked electron transfer processes. The potency of Cu^{2+} to the transfer processes is shown in Figure 3. The inhibition of cytochrome c reduction reaches 100% with applied Cu^{2+} approaching $50 \mu\text{M}$. The inhibition appears to be independent of the ionic strength of the medium. Under the present experimental conditions, the I_{50} of Cu^{2+} to NADH-cytochrome c electron transfer is about $5 \mu\text{M}$. The inhibition to the ferricyanide reduction, on the other hand, appears to reach 80% with Cu^{2+} concentration exceeding $200 \mu\text{M}$. The inhibition to the ferricyanide reduction seems to be sensitive to ionic strength with applied Cu^{2+} lower than $75 \mu\text{M}$. An increase in ionic strength reduced the extent of inhibition. It should be noted that the presence of Ca^{2+} did not change the response of the cytochrome c reduction to Cu^{2+} (see legend of Fig. 3).

Effects of Copper on Kinetic Parameters of Electron Transfer: The results of Figure 3 indicated that Cu^{2+} ions affected the cytochrome c and ferricyanide reduction processes differently. To further define the nature of this difference, experiments described in Figures 4 and 5 were performed. Keeping the Cu^{2+} concentration as constant but varying the concentration of electron acceptors (cytochrome c or ferricyanide), the change in the inhibition was followed. As shown in Figure 4, the ferricyanide reduction, in the absence of Cu^{2+} , appeared to follow the simple Michaelis-Menten kinetics as previously described (Tu et al., 1993). The presence of Cu^{2+} changed the simple saturation V versus S relationship to a sigmoidal relationship (Fig. 4A). This changed the linear double reciprocal line to the concave upward curves (Fig. 4B). It appears that an increase in ferricyanide concentration may relieve part of the inhibition.

However, for cytochrome c reduction, the presence of Cu^{2+} did not change the simple saturation dependence (Fig. 5A) and the linear double-reciprocal relationship (Fig. 5B) of the reaction kinetics. As shown in Figure 4, the inhibition caused by $10\ \mu\text{M}$ of Cu^{2+} appears to be "competitive". With Cu^{2+} concentration increased to $20\ \mu\text{M}$, complex inhibition pattern emerged. Nevertheless, unlike the reduction of ferricyanide, the Michaelis-Menten kinetics seems to be adequate to describe the Cu-inhibition on the reduction of cytochrome c .

DISCUSSION

Based on the effects to NADH-linked electron transfer reactions in the plasma membrane vesicles of corn roots, there are at least three different types of divalent cations. The first type, like Ca^{2+} , slightly enhances the reduction rate of cytochrome c but not that of ferricyanide by NADH. Its presence does not induce any detectable size/volume changes of the vesicles. Calcium binding to negatively charged phospholipids is known to have a general stabilizing effect on the structure of membrane (McLaughlin, 1982). These changes, if occur in the plasma membrane vesicles, do not appear to profoundly affect the redox activities. The presence of Ca^{2+} has a minimal effect on the sensitivity of the redox reactions or their induced volume/size changes of the membrane vesicles. Thus, the plasma membrane must contain unique binding sites for Cd^{2+} and Cu^{2+} . These binding sites are either inaccessible to Ca^{2+} or have a relatively low affinity to Ca^{2+} . The second type, like Cd^{2+} , has no effect on the electron transfer activity but induces a redox-related size/volume increase of the vesicles. The physiological significance of this effect remains to be established.

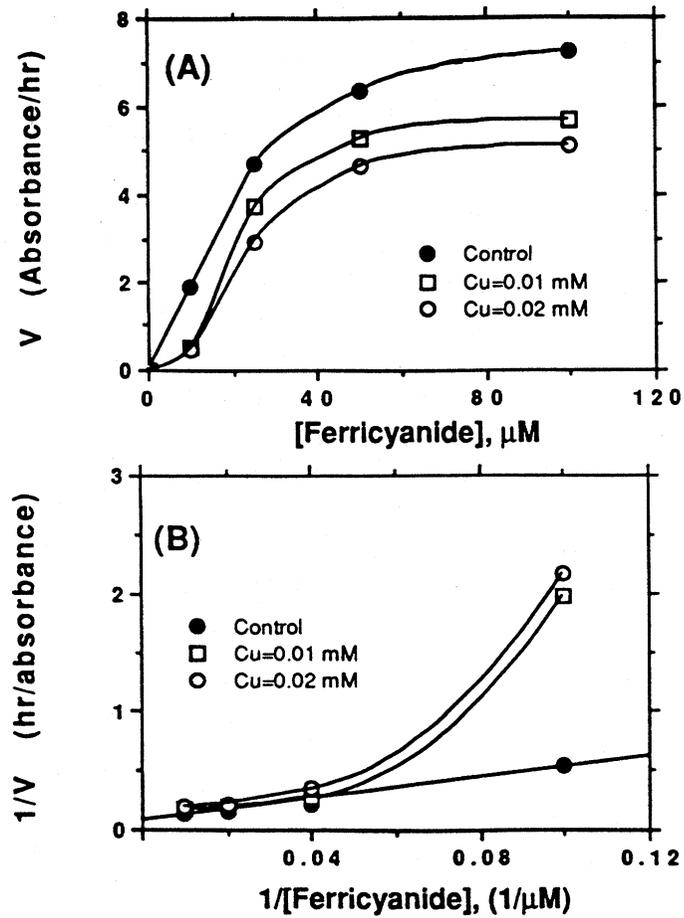


FIGURE 4. Kinetic Analysis of Cu^{2+} Inhibition on Ferricyanide Reduction. (A) The plasma membrane vesicles in HI medium were incubated with indicated concentrations of cytochrome c in the presence of a fixed concentration of Cu^{2+} before the addition of $100 \mu\text{M}$ of NADH. The initial rate of absorbance change at 420 nm was then determined. (B) Double Reciprocal Plot. The data in (A) were transformed to test the linearity of the double reciprocal relationship. The control data showed a linearity of better than 99%.

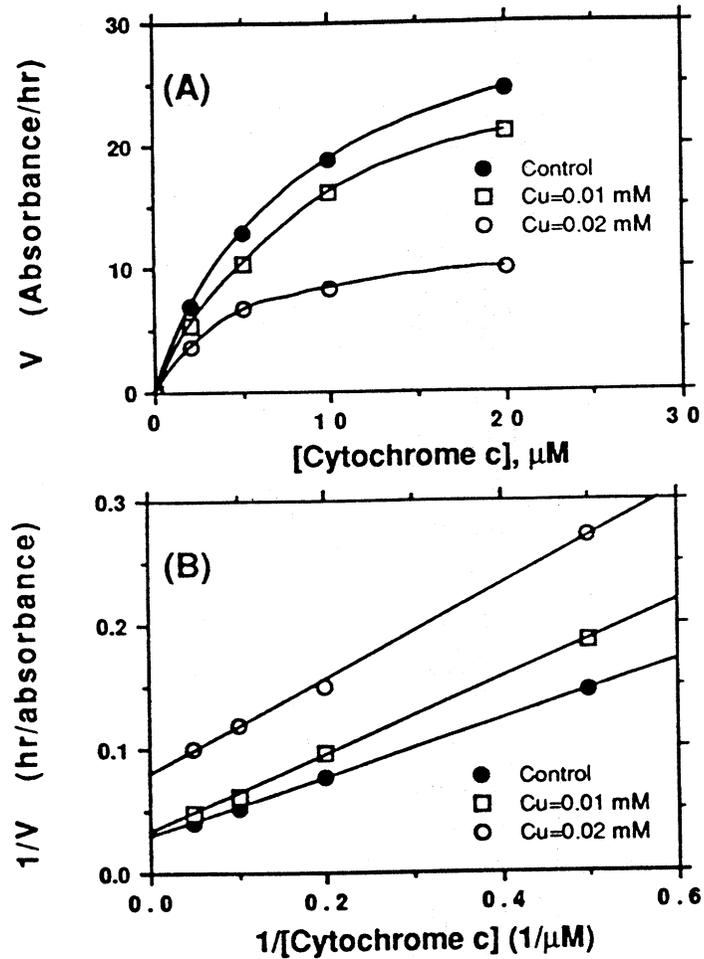


FIGURE 5. Kinetic Analysis of Cu^{2+} Inhibition on Cytochrome c Reduction. (A) The plasma membrane vesicles were treated in the same manner as described in Figure 4A except replacing ferricyanide by cytochrome c and reduction monitored at 550 nm. (B) Double Reciprocal Plot. The data shown in (A) were replotted to determine the fitness of the simple Michaelis-Menten kinetics. The linearity of the lines was better than 99.9%.

The third type, like Cu^{2+} , strongly inhibits NADH-linked electron transfer reactions. In a previous study (Loper et al., 1993), aluminum (Al^{3+}) was identified as an inhibitor to NADH-linked electron transfer in corn root plasma membrane. However, to reach a 50% inhibition of ferricyanide reduction, the needed concentration of Al^{3+} was greater than 400 μM . In contrast, the I_{50} of Cu^{2+} to the electron transfer reactions described in this study was between 5 to 10 μM .

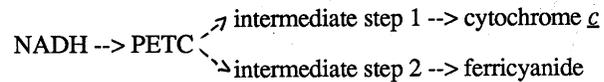
Unlike the inhibition to the ferricyanide reduction, the inhibitory effect of Cu^{2+} to the electron transfer from NADH to cytochrome c is insensitive to the change of ionic strength of the medium. With Cu^{2+} less than 10 μM , the inhibition to the cytochrome c reduction appears to assume a "competitive" mechanism. These results indicate that at least part of the strong Cu^{2+} binding may occur at the same site(s) for cytochrome c . With 20 μM of Cu^{2+} , the inhibition pattern becomes complex suggesting further binding may also occur at site(s) which are indirectly involved in the regulation of the cytochrome c reduction. Nevertheless, the general Michaelis-Menten kinetics is adequate to describe the effects of Cu^{2+} . In contrast, the presence of Cu^{2+} changes the reaction kinetics for ferricyanide reduction from the simple Michaelis-Menten model to a complex scheme involving cooperativity.

The sigmoidal dependence of reduction rate on ferricyanide concentration caused by the presence of Cu^{2+} indicates that an increase in electron transfer activity may decrease the inhibitory effect of Cu^{2+} . It is possible that the activation of electron flow from NADH to ferricyanide may somehow decrease the binding of Cu^{2+} to the site(s) which are directly or indirectly involved in the regulation of the electron flow. Since the similar change was not observed for NADH-linked electron transfer to cytochrome c , the reduction sites for the two electron transfer processes described in this study should locate at two different regions in the plasma membrane.

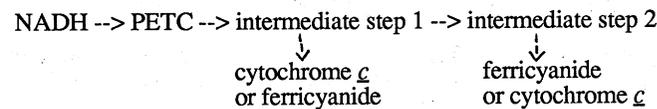
As described in our previous report (Tu et al., 1993), a common primary electron transfer carrier (PETC) must accept reducing power from NADH and then passes the electrons to cytochrome c and ferricyanide. The Cu^{2+} inhibition described in this report and the polylysine effects described elsewhere (Tu et al., 1994) strongly suggest that two different electron transfer intermediate steps are involved in moving electrons from the PETC to the two studied acceptors. The two intermediates may assume either a parallel or a serial relationship in electron

transfer pathway as shown in the following diagram where arrows indicate the direction of electron flow:

Parallel Pathway



Serial Pathway



Additional information is needed to decide which one of these two alternatives is operational in the plasma membrane.

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