

**DIFFERENTIAL EFFECTS OF POLYLYSINE ON  
NADH-LINKED ELECTRON TRANSFER IN CORN ROOT  
PLASMA MEMBRANE VESICLES**

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**ABSTRACT:** The effects of polylysine on the electron transfer from NADH to cytochrome *c* and ferricyanide in corn root plasma membrane vesicles were investigated. The presence of polylysine decreased the reduction rate of cytochrome *c* but slightly stimulated the rate of ferricyanide reduction in a pH 7.5 medium of low ionic strength. An increase in ionic strength eliminated the effects of polylysine on ferricyanide reduction but not the inhibition of cytochrome *c* reduction. Kinetic analyses indicated that in high ionic strength medium, polylysine inhibition appeared to assume a competitive model suggesting its binding to cytochrome *c* reduction site. In low ionic strength medium, a complex inhibition was observed. A study on the inhibitory efficiency of different size polylysines suggested that the reduction site of cytochrome *c* may best fit a polylysine with molecular weight close to 25,000. The results support the notion that the electron transfer from NADH to these two acceptors may involve two

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Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycerol bis-( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

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separated enzymes or two different sites along an electron transfer chain in the plasma membrane.

## INTRODUCTION

The plasma membrane of higher plant cells contains a NADH-linked redox system. Evidence for its existence include, but is not limited to, observed activities of NADH-cytochrome  $c$  reductase (Larsson, 1985) and flavin-NADH dehydrogenase (Ramirez et al., 1984) in purified plasmalemma. Particles enriched with multiple NADH-linked redox activities have been obtained from detergent treated plasma membrane of corn roots (Luster and Buckhout, 1988). Purified plant plasma membrane may, based on differences in surface property, 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). Transmembrane (Askerlund and Larsson, 1991) and external surface specific (Crane et al., 1985) NADH-linked electron transfer reactions are also found in purified plasmalemma. The exact relationship between all these NADH-linked activities is not known. However, the proposal that the plasma membrane contains an electron transfer chain analogous to that found in mitochondria (Møller and Crane, 1990, Luster and Buckhout, 1989) appears to be attractive.

The physiological roles of NADH-linked electron transfer in plasmalemma are yet to be fully explored. 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 maintaining membrane protein integrity by keeping -SH groups in a reduced state (Crane et al., 1985, Møller and Crane, 1990). As described, both cytochrome  $c$  and ferricyanide have been extensively utilized to follow the membrane catalyzed NADH oxidation. While both ferricyanide and cytochrome  $c$  are not physiological electron acceptors of plasma membrane redox activities, information on the characteristics of the reductions is essential for establishing the roles of NADH-linked electron transfer. In present work, we

reported for the first time, that polylysine inhibits the reduction of cytochrome *c* but not ferricyanide. This information together with the ionic strength-dependence of the inhibition, indicate the presence of a negatively charged electron transferring site(s) for cytochrome *c*, but not shared with ferricyanide, on the cytoplasmic face of the plasma membrane.

## MATERIAL AND METHODS

**Isolation of corn root plasma membrane:** The plant materials were prepared at 4°C according to procedures mentioned in our previous report (Tu et al., 1986). The roots of 3-day old corn (Cross Hybrid WF7551) seedlings germinated in the dark were homogenized in a buffer containing 50 mM Hepes pH 7.5, 10% (w/v) glycerol, 0.25 M sucrose, 0.5% (w/v) bovine serum albumin, 2 mM MgSO<sub>4</sub>, 2 mM ATP, 1 mM PMSF, 2 mM EGTA, 5 mM and 5 mM DTT. After filtering the homogenate through cheesecloth, the filtrate was centrifuged at 6,000 *g* for 15 min. The microsomal membranes obtained by centrifuging the supernatant at 96,000 *g* for 40 min, were suspended in 4 mL of homogenization buffer and applied to a discontinuous sucrose gradient of 34 (10 mL) and 42% (10 mL) (w/w). The gradient was centrifuged at 100,000 *g* for 150 min. The plasma membrane was extracted from the interface of 34 and 42% sucrose solutions. As previously described (Hsu et al., 1990), the plasma membrane isolated from the corn hybrid by the procedure contains only minimal contaminations of mitochondria, Golgi, and tonoplast.

**Measurements of NADH-linked Electron Transfer:** The reduction of ferricyanide and cytochrome *c* was monitored at 22°C by absorbance decrease at 420 and absorbance increase at 550 nm, respectively. The oxidation of NADH was followed at 340 nm. To quantify the changes, differences in extinction coefficients between reduced and oxidized forms of the substrates were used (18.7 mM<sup>-1</sup> cm<sup>-1</sup> for ferri-ferro cytochrome *c* and -0.93 mM<sup>-1</sup> cm<sup>-1</sup> for ferri-ferricyanide pairs). The time courses of reduction were monitored by a Beckman DU-70 Spectrometer interfaced to an IBM Model 80 personal computer. The

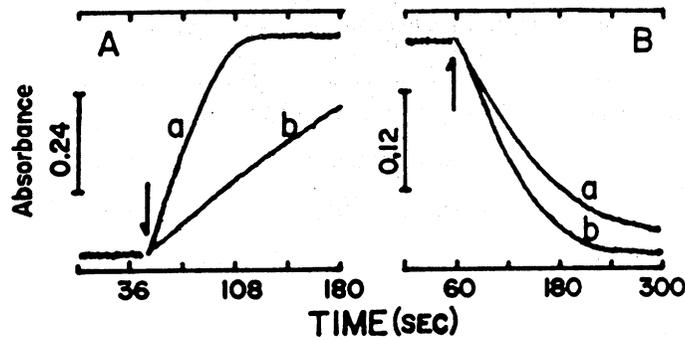
digitized inputs were analyzed on-line to determine the initial rates. Unless specified otherwise, the basal assay medium contained approximately 40  $\mu\text{g}/\text{mL}$  of purified plasma membrane vesicles, 20 mM Hepes, pH 7.5 and supplemented with either 250 mM sucrose and 20 mM KCl (low ionic strength medium) or 170 mM KCl (high ionic strength medium). After incubating the substrates in the basal medium with indicated amounts of polyelectrolyte for 10 min, 0.25 mM NADH was added to initiate the reductions.

**Determination of Kinetic Parameters:** The initial rates of electron transfer reactions were further analyzed according to the simple Michaelis-Menten kinetic model to determine the parameters of  $K_m$  and  $V_{max}$ . The linearity of the double reciprocal plots was better than 99% under employed experimental conditions.

**Others:** The protein content of the membrane was determined by a modified Lowry method after precipitation by trichloroacetic acid in the presence of deoxycholate (Brauer and Tu, 1991). Polylysine, polyaspartic acid, and cytochrome  $c$  were from Sigma Chemical Co. All other chemicals used were of analytic grades.

## RESULTS AND DISCUSSION

**NADH-linked Reduction of Cytochrome  $c$  and Ferricyanide:** The plasma membrane vesicles used in this study contained a minimal contamination of activities of other subcellular membranes (Hsu et al., 1990). It has been shown that a great majority of NADH-linked reduction activities occur on the cytoplasmic (inside) surface of the plasma membrane (Askerlund et al., 1988, Askerlund and Larsson, 1991) which may assume either right-side-out or inside-out orientation in isolated vesicles. Since ferricyanide, cytochrome  $c$ , and polylysine are not membrane permeable, the observed effects described in this study should relate to inside-out vesicles and unsealed fragments of the plasma membrane. As shown in Figure 1, the plasma membrane vesicles catalyzed the reduction of ferricyanide and cytochrome  $c$  by NADH. The inclusion of antimycin A minimized mitochondrial interference in the reduction measurements.



**FIGURE 1.** Effects of Polylysine on NADH-linked Reduction Processes. The reduction of cytochrome  $c$  (Panel A) and ferricyanide (Panel B) catalyzed by the plasma membrane using NADH as the reductant were followed by the time course of absorbance change at 550 (increase) and 420 nm (decrease), respectively. The membrane vesicles (90  $\mu\text{g}$  protein) in 1.6 mL of the low ionic strength medium (defined in Material and Methods) containing 20  $\mu\text{g}$  of antimycin A, and either 32  $\mu\text{M}$  of cytochrome  $c$  or 0.25 mM of ferricyanide were allowed to incubate for 15 min at 22°C. before taking measurement. The reduction was initiated by the addition of 0.5 mM of NADH at the time indicated by arrow. Traces labeled as "a" and "b" were obtained by including 0 and 25  $\mu\text{g}/\text{mL}$  of polylysine (MW 9,000) at incubation stage, respectively.

*Effects of Polyelectrolyte on the Reduction Processes:* At physiological pH, cytochrome  $c$  ( $pI = 10.6$ ) carries net positive charges but ferricyanide remains negatively charged. The Michaelis-Menten constants ( $K_m$ ) for ferricyanide and cytochrome  $c$  reduction have been determined to be quite different (Askerlund et al., 1988) for plasma membrane from red beet leaves. It was reported that p-chloro-mercuri phenyl sulfonate, a non-specific -SH reagent reduced the ferricyanide reduction catalyzed by a 27 kD protein isolated from corn root plasma membrane (Luster and Buckhout, 1989). Yet, these described information is of limited value to assess whether the same site is involved for the reduction of both substrates.

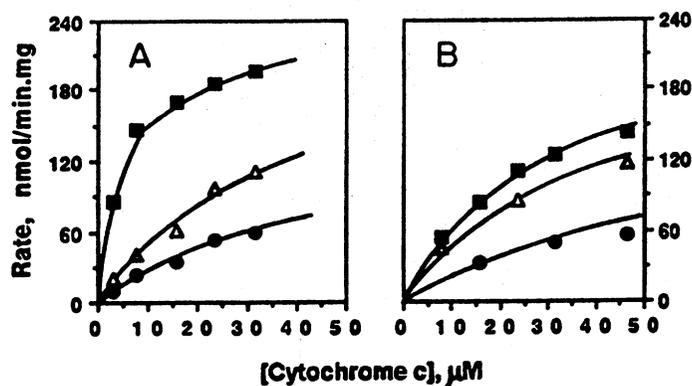
It has been shown that polylysine can inhibit the reduction of externally added cytochrome  $c$  in mitochondria by competing with the same negatively

**TABLE 1. Ionic Strength Influence of Polyelectrolyte Effects.**

Polyelectrolyte added (25 $\mu\text{g/mL}$ )	Relative Initial Reduction Rate			
	<i>Cytochrome c</i>		<i>Ferricyanide</i>	
	<u>Low I</u>	<u>High I</u>	<u>Low I</u>	<u>High I</u>
NONE	104	100 <sup>1</sup>	90	100 <sup>2</sup>
Polylysine	25	36	113	100
Polyaspartate	102	100	68	100

The reduction of cytochrome *c* and ferricyanide by NADH-linked electron transfer in the plasma membrane vesicles were followed as described in Material and Methods. The relative reduction rates were calculated by assigning the rate obtained in high ionic medium without added polyelectrolyte as 100%. These reference rates are 88 and 214 nmol reduced/min.mg protein for cytochrome *c* and ferricyanide, respectively. The values shown represent an average of two independent measurements with an error of  $\pm 5\%$ .

charged binding site (Davies et al., 1964, Racker and Horstman, 1972). As shown in Figure 1, polylysine (ave MW = 9,000) also inhibited NADH-linked reduction of cytochrome *c* by the plasma membrane vesicles in the low ionic strength medium defined in Material and Methods. In contrast, the reduction of ferricyanide was somewhat stimulated in the same medium. When the high ionic strength medium was used, a slightly less of inhibition to cytochrome *c* reduction was observed (Table 1). However, the stimulation of ferricyanide reduction was negated. These results suggest that ferricyanide and cytochrome *c* do not share the same reduction site on the membrane. The site for cytochrome *c* reduction, is most likely negatively charged. The strong electrostatic interaction between cytochrome *c* and its binding site appears to be only slightly affected by the change in ionic strength. On the other hand, being a positive charge carrier,



**FIGURE 2.** Effects of Polylysine on the Initial Rate of NADH-linked Reduction of Cytochrome c. The dependence of initial reduction rate on the concentration of cytochrome c was measured in both media of high (A) and low (B) ionic strength containing different amounts of polylysine (MW 9,000). Rates obtained in the presence of zero, 12.5 µg/mL, or 25.0 µg/mL of polylysine are shown as filled squares, open triangles, or filled circles, respectively.

polylysine may shield some of the negative charges including the phospholipid head groups residing on the surface of the membrane and thus allows a higher local concentration of  $\text{Fe}(\text{CN})_6^{3-}$  near its reduction site on the membrane. An increase in ionic strength minimized the shielding effect and thus negated the stimulation.

The presence of negatively charged polyaspartate slightly inhibited the reduction of ferricyanide but had no effect on the reduction of cytochrome c (Table 1). Unlike polylysine, changes in ionic strength do not appear to have significant effects on the influence of polyaspartate. The details of polyaspartate on NADH-linked electron transfer are currently being investigated.

**Kinetic Origin of Polylysine Inhibition on Cytochrome c Reduction:** The inhibition of cytochrome c reduction by polylysine is observed in media of both high and low ionic strength. The relationship between the initial reduction rate

**TABLE 2. Origin of Polylysine Inhibition on Cytochrome c Reduction.**

Polylysine added ( $\mu\text{g/mL}$ )	Kinetic Parameters			
	$K_m$ ( $\mu\text{M}$ )		$V_{max}$ (nmol/mg.min)	
	<u>Low I</u>	<u>High I</u>	<u>Low I</u>	<u>High I</u>
NONE	23.6	5.7	217	211
12.5	22.5	21.6	172	164
25.0	30.0	50.1	95	160

The standard V verse S plots as shown in Figure 2 were further analyzed by double reciprocal treatment to generate  $K_m$  and  $V_{max}$ . The employed low and high ionic strength media are defined in Material and Methods. The values shown are the average of two determinations (error =  $\pm 10\%$ ).

and the concentration of cytochrome c in the presence of different levels of polylysine is shown in Figure 2. The standard V vs S plots clearly indicate that the inhibition increases as the concentration of polylysine increased. When the data were analyzed according to standard double-reciprocal ( $1/v$  vs  $1/S$ ) plots, results shown in Table 2 were obtained. It is clear that the  $K_m$  of cytochrome c reduction, in the absence of polylysine, has a lower value in the medium of high ionic strength. This is consistent with the suggestion that the probable binding of positively charged cytochrome c to the negative charges on the membrane, other than these at the reduction site, may increase the apparent concentration of cytochrome c needed ( $K_m$ ) to reach a half-maximum of reduction rate. As described, this interaction may be minimized by an increase in ionic strength.

The results of Table 2 also indicate that the  $K_m$  value of cytochrome c reduction is increased as the concentration of applied polylysine increased in the medium of high ionic strength but remains relatively insensitive to the presence

**TABLE 3. Size and Relative Inhibitory Efficiency of Polylysine (Polylys) to NADH-linked Cytochrome c Reduction.**

Addition ( $\mu\text{g/mL}$ )	Polymer Properties		Relative Rate (%)
	<u>Molecular Weight</u>	<u>Radius</u> ( $\text{\AA}$ )	
NONE	-----	---	100.0
Lys (25)	146	---	100.0
Polylys (25)	3,900	12.9	77.8
Polylys (25)	9,000	17.0	31.8
Polylys (25)	25,000	24.0	7.7
Polylys (25)	58,000	31.7	6.5
Polylys (25)	132,000	41.7	8.2

The initial cytochrome c reduction rate was determined as described in text. The rate obtained without any addition, 126 nmol/min.mg, was assigned as 100%. The concentration of added lysine (Lys) and polylysines of various average molecular weight was 25  $\mu\text{g/mL}$ , or 0.17 mM of lysine monomer equivalent. The radius of the polymer was calculated from specific volume ( $V = 0.72 \text{ mL/g}$ ), molecular weight (MW), and a spherical consideration ( $\text{Radius} = \{3\text{MW}/4\pi V N_0\}^{1/3}$ ).  $N_0$  is the Avogadro's number.

of polylysine in the medium of low ionic strength. On the other hand, the  $V_{\text{max}}$  is only minimally affected in high ionic strength medium but significantly decreased in low ionic strength medium by polylysine. These results suggest that the polylysine inhibition may be changed from noncompetitive to competitive as the ionic strength of the medium increased. It appears that in addition to the binding to cytochrome c reduction site, the shielding effect of polylysine may also affect the reduction processes noncompetitively by binding to membrane site(s) not directly involved in but indirectly influencing the reduction of cytochrome c at its reduction site.

***Molecular Size and Inhibitory Potency of Polylysine:*** Although the results of Figure 2 and Table 2 indicate a rather complex pattern for the inhibition, it is obvious a strong interaction between polylysine and the membrane must be involved. Because the presence of simple lysine exhibits no effects on the reduction of cytochrome  $c$  (Table 3), it seems that a simultaneous binding to a group of negative charges in a membrane domain is required to reach the inhibition. Thus, an investigation on the relationship between the size and the inhibitory efficiency of polylysine was conducted.

As shown in Table 3, the inhibition of cytochrome  $c$  reduction appears to approach a maximum with the molecular weight of applied polylysine close to 25,000. It has been shown that polylysine acquires a random-coil conformation in solution of near neutral pH (Greenfield and Fasman, 1969), the radius of polylysine coils may be estimated from the specific volume (0.72 mL/g) and a spherical approximation. Thus, polylysine with MW as 3,900 would have a radius of 12.9 Å or a cross section area of 523 Å<sup>2</sup>. The radius and cross-section area of polylysine with MW as 25,000 would then be 24.0 Å and 1,810 Å<sup>2</sup>, respectively. The study suggests that a simultaneous binding of negative charges localized in a membrane domain which can best fit with polylysine having a molecular weight close to 25,000, would yield the maximum inhibition. However, the possibilities of other hydrophobic or hydrophilic effects may not be excluded by obtained data.

## CONCLUSION

In present study, we demonstrated that polylysine exerts an ionic strength dependent inhibition to the reduction of cytochrome  $c$  but not ferricyanide by NADH-linked electron transfer in purified corn root plasma membrane. This differential effect clearly indicates that the reduction of cytochrome  $c$  and ferricyanide must occur at two different regions of the plasma membrane. This requirement may be satisfied by having two independent but NADH-linked reductases for the acceptors. Alternatively, cytochrome  $c$  and ferricyanide may interact with a NADH-linked electron transfer chain at two different sites.

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