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Transmembrane ferricyanide reduction in carrot cells

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Carrot cells (*Daucus carota*) grown in tissue culture are capable of reducing the non-permeable electron acceptor, ferricyanide, with concomitant proton extrusion from the cell. Optimum conditions for transmembrane ferricyanide reduction include a pH of 7.0–7.5 in a medium containing 10 mM each KCl, NaCl and $CaCl_2$. Data are shown to prove that transmembrane ferricyanide reduction is an enzymatic process. It does not depend on the secretion of phenolics from the cell within the time limits of the assay (10 min). The presence of broken cells and cell fragments are excluded on the basis of stimulation or only slight inhibition by mitochondrial inhibitors. However, transmembrane ferricyanide reduction by carrot cells is inhibited about 50% by various glycolysis inhibitors, which are presumed to reduce the internal levels of NADH. Treatment of cells with *p*-diazoniumbenzenesulfonic acid, a non-permeant membrane modifying agent, also inhibits transmembrane ferricyanide reduction more than 90%. The data presented support the existence of a transplasma membrane redox system in carrot cells.

Introduction

Transmembrane ferricyanide reduction occurs in various types of animal cells [1,2], including perfused liver [3–5]. It has also been demonstrated in yeast cells [6], bean roots [7], peanut roots [8], maize roots [9–12], tobacco cells [13], *Escherichia coli* normal and mutant strains [14], *Anacystis nidulans* cells [15] and as a preliminary demonstration in carrot cells [16,17]. The link between transport and plasma membrane redox has also been studied in carrot cells [18]. In this communication we present data for establishing optimum conditions of transmembrane ferricyanide reduction in carrot cells and show that this reaction in whole cells is associated with a plasma membrane redox system and not secretion of phenolics by these cells or exposure of broken cells fragments.

Materials and Methods

Carrot cells were originally obtained from Drs. P.M. Hasegawa and R.A. Bressan of the Purdue University Horticulture Department. They were grown in liquid suspension culture on Murashige and Skoog's medium without agar, obtained from K.C. Biological, Inc., Lenexa, KS 66215 (cat. No. MM-100) and supplemented with vitamins and 2.4-D (4 mg/l). 2,4-D was purchased from the Aldrich Chemical Co.

Cells were harvested 2–10 days after transfer by a 2-min centrifugation in an international table model centrifuge at 1500 rpm. They were rewashed 3 times with a sucrose-salts solution (0.1 M sucrose

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; DABS, *p*-diazoniumbenzenesulfonic acid; FeCN, ferricyanide; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; Mes, 4-morpholineethanesulfonic acid.

with 10 mM NaCl, KCl and $CaCl_2$). After centrifugation, the cells were suspended in about 20 ml sucrose-salts and put on a shaker to keep aerated. Aliquots were removed from this suspension for assays when needed.

Transmembrane ferricyanide reduction by carrot cells was carried out in an Aminco DW-2A spectrophotometer in the dual mode, recording the difference in absorbance between 420 and 500 nm. A standard assay mixture contained cells, 25 mM Tris-Mes (pH 7.0 or 7.5), and sucrose-salts solution to a volume of 1.5 ml. The ferricyanide concentration was 0.4 mM except in Fig. 1. A millimolar extinction coefficient of 1 was used for ferricyanide reduction ($\varepsilon_{420} = 1\ mM^{-1} \cdot cm^{-1}$).

Cytochrome *c* reduction in carrot cells was also measured with the Aminco W2-A spectrophotometer as the difference in absorbance between 550 and 541 nm in the standard buffered sucrose-salts solution as for ferricyanide reduction. The millimolar extinction coefficient for cytochrome *c* was 19.5 ($\varepsilon_{550} = 19.5\ mM^{-1} \cdot cm^{-1}$).

DABS treatment of carrot cells was performed as follows: to 5-ml aliquots of carrot cells in 50-ml Erlenmeyer flasks 0.8, 1.6, 2.4 or 3.2 mM diazotized DABS reagent [19] was added and the cells were put on the shaker for 15 min. At the end of the incubation period, an appropriate amount of histidine quencher was added to stop the reaction. Then the treated cells were rewashed with fresh sucrose-salts solution and tested for ferricyanide reduction as described above.

Transmembrane ferricyanide reduction rates are expressed as μmol ferricyanide reduced/g dry wt. per min. The dry weight of cells was determined after drying in a 100°C oven overnight.

Results

Transmembrane ferricyanide reduction in carrot cells increases with ferricyanide concentration up to 200 μM ferricyanide (Fig. 1). The optimum pH for the reaction is between pH 7 and 8 (Fig. 2). Activity is generally lower in young cells (1–3 days) and slowly declines after day 8, as active growth stops. Transmembrane ferricyanide reduction is proportional to the amount of cells present in the reaction mixture (Fig. 3), as determined from dry weight determinations.

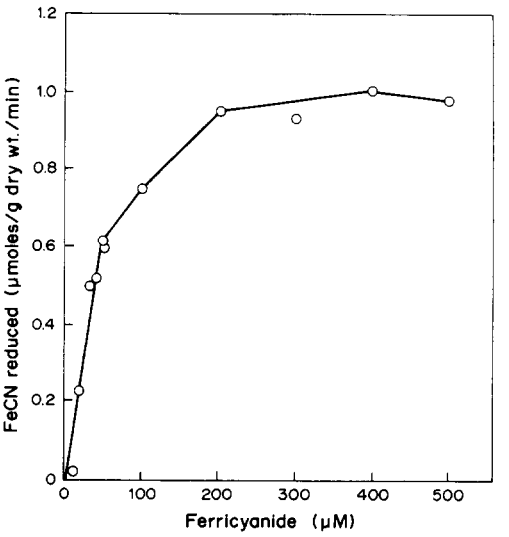

Fig. 1. Variation of transmembrane ferricyanide reduction rates by carrot cells as a function of ferricyanide concentration. The reaction mixture contained carrot cells, 25 mM Tris-Mes (pH 7.5), sucrose-salts solution to 1.5 ml vol. and ferricyanide in the concentrations indicated.

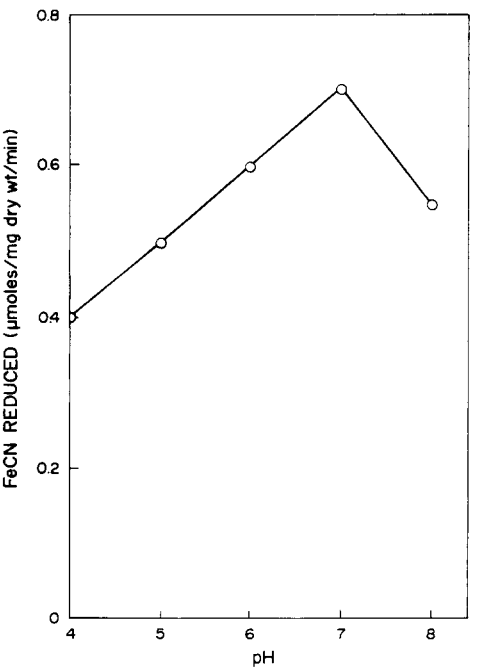

Fig. 2. Variation of transmembrane ferricyanide reduction rates by carrot cells as a function of pH. Reaction conditions as in Fig. 1, except that pH values varied from 4 to 8. The ferricyanide concentration was 0.4 mM.

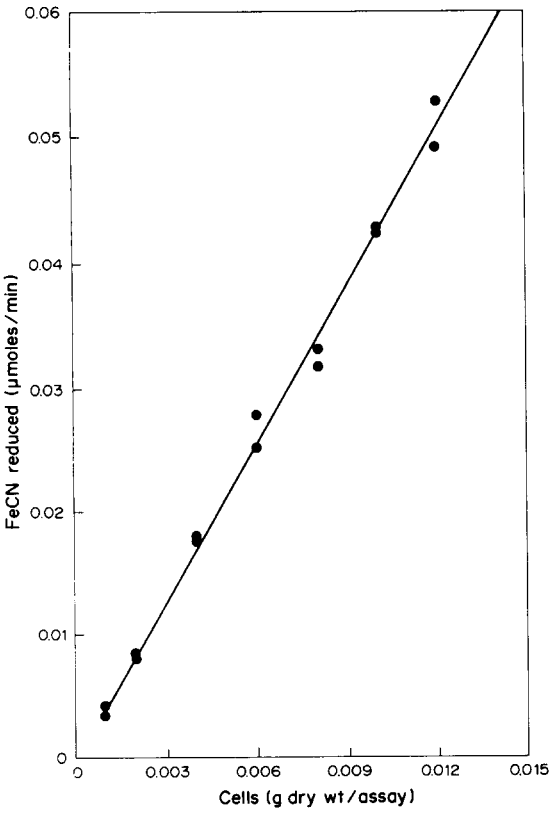

Fig. 3. Variation of transmembrane ferricyanide reduction rates as a function of dry weight of carrot cells. Reaction conditions as in Fig. 1, except that the pH of the reaction mixture was 7 and ferricyanide concentration was 0.4 mM

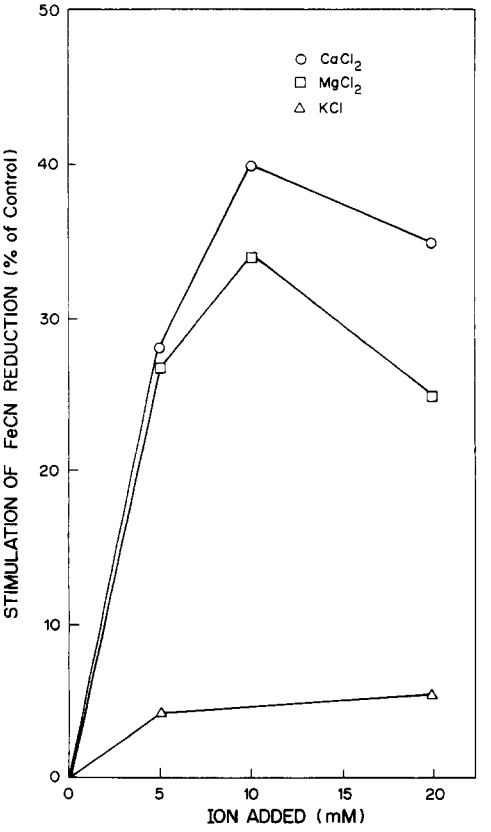

Fig. 4. Stimulation of transmembrane ferricyanide reduction in carrot cells by various ions. The carrot cells were washed and suspended in distilled water. The reaction mixture contained 25 mM Tris-Mes (pH 7.5), 0.4 mM ferricyanide and various ions in the concentrations indicated.

TABLE I

THE EFFECT OF VARIOUS IONS ON TRANSMEMBRANE FERRICYANIDE REDUCTION IN CARROT CELLS

The reaction medium contained carrot cells, 25 mM Tris-Mes (pH 7.5), 0.4 mM ferricyanide and sucrose-salts solution to 1.5 ml volume. + indicates stimulation, − inhibition of rate in relation to control. ± standard deviation.

Ion	Concn. (mM)	Ferricyanide reduced	
		Rate (μmol/g dry wt. per min)	Stimulation or inhibition (%)
None	–	0.44 ± 0.022	–
$CoCl_2$	1	0.07 ± 0.025	−84
$NiCl_2$	1	0.30	−32
$SnCl_2$	1	0.36	−19
$MnCl_2$	0.1	0.60 ± 0.024	+38
$FeCl_3$	0.03	0.61 ± 0.042	+38

The ferricyanide reduction rate is stimulated by various ions in concentrations from 1–10 mM (Fig. 4). These include NaCl, KCl, $MgCl_2$ and $CaCl_2$, as well as $MnCl_2$ and $FeCl_3$ (Table I). The reaction is severely inhibited by 1 mM $CoCl_2$ (84% inhibition). $NiCl_2$ and $SnCl_2$ give comparatively less inhibition (Table I).

p-Diazoniumbenzenesulfonate is an impermeable reagent which can react with amino or hydroxyl groups on proteins at the membrane surface [17]. DABS treatment of carrot cells (Fig. 5) for 15 min destroys transmembrane ferricyanide reduction completely. This provides evidence that transmembrane ferricyanide reduction requires a protein exposed to the external surface.

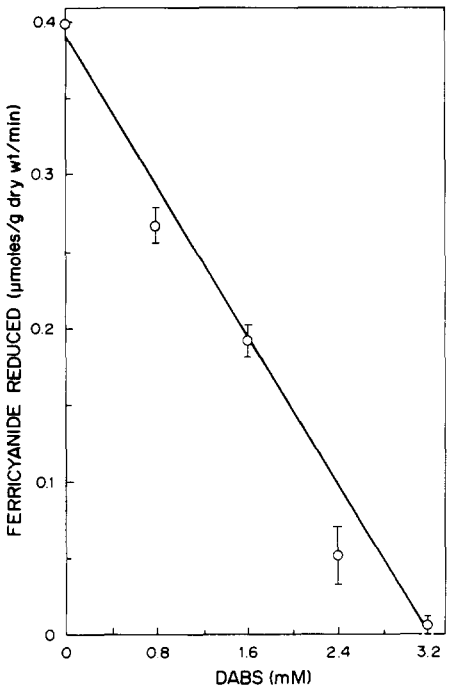

Fig. 5. Transmembrane ferricyanide reduction by carrot cells after DABS treatment. Condition for DABS treatment are described in Materials and Methods, assay conditions as in Fig. 1. The ferricyanide concentration was 0.4 mM. Bars indicate standard deviation for three separate aliquots.

TABLE II

FERRICYANIDE RECOVERY IN SUSPENSION MEDIUM FOLLOWING FERRICYANIDE REDUCTION BY CELLS

Assays contained 0.05 M Tris-Mes-HCl (pH 7.0) and 0.047 M sucrose. A small amount of sodium ascorbate was added to the two buffered sucrose controls to reduce the ferricyanide for subsequent determination of recovery observed with no cells present. 7.02 mg dry wt of cells in 1.5 ml vol. were allowed to reduce ferricyanide for 10 min after which cells were removed by a 4 min spin in a Beckman 152 microfuge. Oxone oxidized all ferrocyanide and the change in absorbance on ascorbate addition was used for measuring total ferricyanide. Data show results as averages of two experiments or standard deviation of three experiments.

Incubation medium	Ferricyanide reduced after 10 min (μmol)	Ferricyanide recovered in supernatant (μmol)	Recovery (%)
Buffer only plus ascorbate	0.207(2)	0.196(2)	95
Buffer plus 7.02 mg cells	$0.188 \pm 4(3)$	$0.175 \pm 2(3)$	93

Discussion

Transmembrane ferricyanide reduction is well established in animal cells, such as porcine erythrocytes [1], neutrophils [2], hepatocytes [3] and perfused liver [4,5]. It has been reported to occur in *E. coli* normal and mutant cells [14] and in yeast [6] and tobacco callus cells [13]. Preliminary reports [16,17] have also demonstrated transplasma membrane ferricyanide reduction in carrot cells. In the present communication it is shown that this process requires oxygen and does not depend on the release of phenolics into the medium within the time limits of the assay (10 min). Over 90% of the ferricyanide can be recovered from the assay medium after removal of the cells and after reoxidation of ferrocyanide with an oxidizing agent, such as H_2O_2 or persulfate. These data prove that transmembrane ferricyanide reduction does not result from uptake of ferricyanide by the cells.

To establish that the ferricyanide reduction is not caused by cell breakage, we have determined the release of lactate dehydrogenase into the medium during cell incubation. After 8 min incubation, the lactate dehydrogenase in the medium is less than 0.7% of the amount present after homogenization of the cells. The lack of cytochrome *c* reduction is also consistent with no cell breakage, since thiols released by broken cells would reduce cytochrome *c*.

If mitochondria were released from broken cells, they could cause an antimycin a or a heptylhydroxyquinoline *N*-oxide-sensitive reduction of ferricyanide. Very little inhibition is observed (Table III). The lack of ferrocyanide oxidation also shows that mitochondria are not released from the cells.

The lack of cytochrome *c* reduction also indicates that the external ferricyanide reduction is not caused by superoxide production and release as observed in leucocytes [21]. Retention of part of the activity under an argon atmosphere also shows that superoxide production is not the mechanism. Partial inhibition under argon can indicate that full oxidative metabolism is necessary for proper substrate supply for ferricyanide reduction. Since cyanide does not inhibit as in yeast [6], it appears that the argon effect may not be based on inhibi-

TABLE III

THE EFFECT OF MITOCHONDRIAL AND GLYCOLYSIS INHIBITORS ON TRANSMEMBRANE FERRICYANIDE REDUCTION BY CARROT CELLS

The reaction medium contained carrot cells, 25 mM Tris-Mes (pH 7.5), 0.4 mM ferricyanide and sucrose-salts solution to 1.5 ml vol.

Inhibitor	Concn. (μM)	Ferricyanide reduced	
		Rate (μmol/g dry wt. per min)	Stimulation or inhibition (%)
None	–	0.32 ± 0.013	–
Antimycin a	1	0.29	−11.4
KCN	1 mM	0.44	+36.0
Sodium azide	500	0.26	−18.0
HOQNO	10	0.30	−5.3
PCMB	10	0.28	−12.1
PCMBS	10	0.32	−1.8
Sodium arsenite	20 mM	0.14	−58.0
Iodoacetate	3.3 mM	0.18	−45.4
Malic hydrazide	6.7 mM	0.15	−54.6

+ indicates stimulation, − inhibition of rate in relation to control. ± standard deviation. HOQNO, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide; PCMB, *p*-chloromercuribenzoic acid; PCMBS, *p*-chloromercuribenzenesulfonic acid.

tion of mitochondrial oxidations.

The source of electrons for transmembrane ferricyanide reduction may be internal NADH. Therefore, glycolysis inhibitors, which diminish NADH inside the cell, are expected to inhibit transmembrane ferricyanide reduction. The glycolysis inhibitors iodoacetate, malic hydrazide or sodium arsenite give 50% inhibition, indicating that a decrease in internal NADH can decrease the transmembrane redox activity (Table III).

To show that modification of the external cell surface can also affect transmembrane ferricyanide reduction, we have used DABS treatment on carrot cells. A 15 min incubation with 3.2 mM DABS gives over 90% inhibition of transmembrane ferricyanide reduction (Fig. 5). DABS is known to modify histidine or tyrosine residues on proteins [22,23] converting them to mono- or diazo derivatives. Inhibition of transmembrane ferricyanide reduction by DABS indicates the involvement of an external site in the process of plasma membrane electron transport, the modification of which appears crucial for activity.

The reducing equivalents, from NADH or NADPH [24,25] in the cell, travel through the plasma membrane via an electron transport chain, which is also associated with a proton transferring ATPase [18,26]. The data on transmembrane ferricyanide reduction by carrot cells support such a hypothesis. Preliminary studies on proton extrusion concomitant with ferricyanide reduction suggest that the plasma membrane redox system is similar to that of chloroplasts or other organelles [16,27,28] and may energize membrane functions.

Acknowledgements

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