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Catalytic action of vitamin K_3 on ferricyanide reduction by yeast cells

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Ferricyanide reduction by yeast cells was greatly promoted by preincubation of the cells with vitamin K_3. The reduction was rapid, and the amount reduced was equal to the amount of vitamin K_3 used. The rate of ferricyanide reduction decreased with time. Proton release and ferricyanide reduction in the presence of vitamin K_3 proceeded stoichiometrically at one proton per electron. Vitamin K_3 did not cause proton release in the absence of ferricyanide. These results suggest that vitamin K_3-accepting hydrogen from the plasma membrane redox system releases protons by reducing ferricyanide. Proton release coupled with ferricyanide reduction in the presence of vitamin K_3 was not inhibited by diethylstilbestrol (an inhibitor of plasma membrane ATPase), suggesting independence of the plasma membrane redox system from the ATPase pumping the protons.

Introduction

Plasma membrane-bound redox systems have been detected in eukaryotic cells [1–11]. These redox systems are thought to transfer electrons from intracellular NAD(P)H to extracellular electron acceptors, such as ferricyanide [1–3,5–8], vanadate [4], cytochrome *c* [1–3,6] and oxygen [1–3,6,9–11]. Recent research has shown that the anticancer drug adriamycin inhibits the activity of NADH-ferricyanide reductase of tumor cells [12,13] and that the impermeable adriamycin linked to agarose inhibits the growth of tumor cells [14]. Furthermore, tumor cell growth is known to depend on the concentration of extracellular ferricyanide which is reduced with proton release from tumor cells [12]. These facts suggest that NADH-ferricyanide reductase in the plasma membrane redox system plays an important role in the growth of eukaryotic cells. On the other hand, the rate of ferricyanide reduction by yeast cells [15] is much slower than that by tumor cells [12], and the relationships between proton release and ferricyanide reduction by yeast cells remain to be elucidated.

The present study investigated the catalytic action of the redox mediator for the slow ferricyanide reduction by yeast cells. Both proton release and ferricyanide reduction were found to be greatly promoted by addition of vitamin K_3 and to proceed stoichiometrically. The role of vitamin K_3 in proton release and ferricyanide reduction is discussed.

Materials and Methods

Organism and growth conditions. Saccharomyces cerevisiae strain IFO 2044 was grown aerobically at 30°C in a medium containing 10% malt extract, 4% yeast extract and 20% glucose (pH 5.8). The

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

cells were harvested in the initial stationary phase of growth, washed twice with distilled water, and suspended in 20 mM Hepes (pH 7.0), in order to obtain stable continuous proton release from the yeast cells at neutral pH range. The amount of yeast cells is expressed as the wet weight.

Measurement of ferricyanide reduction. The rate of ferricyanide reduction by yeast cells at 35°C was determined by recording the decrease in the absorbance at 400 nm with reference to that at 500 nm. The millimolar extinction coefficient was 1.0 [12,15].

Measurement of pH change in yeast cell suspension. The reaction mixture was stirred at 35°C, and the pH change was detected with an electrode attached to a sensitive pH meter and recorder. The lower limit of the detectable pH change was 0.005, which was enough to detect the increment of 0.04 mM proton in the presence of 20 mM Hepes (pH 7.0) and 12 mg/ml yeast cells. The amount of protons released was calculated from the titration curve prepared with 0.1 M HCl. Dissolved CO_2 was removed by bubbling nitrogen through the reaction mixture to prevent additional proton production from CO_2 produced by the yeast cells.

Measurement of glucose uptake by yeast cells. After the yeast cells had been pre-incubated with or without chemical modification reagents for 5 min at 35°C, the reaction was started by adding 2.5 mM glucose. The reaction mixture was incubated for 5 min at 35°C and then centrifuged. The residual concentration of glucose in the supernatant was determined by enzymatic analysis [16].

Chemicals. Diethylstilbestrol and calcium ionophore A23187 were obtained from Sigma and prepared in methanol. Enzymes for enzymatic analysis were obtained from Boehringer Mannheim. All other reagents were of analytical grade.

Results

Ferricyanide reduction promoted by vitamin K_3. Fig. 1 shows that ferricyanide reduction by yeast cells was promoted in the presence of vitamin K_3. Fericyanide (A in Fig. 1) added to a cell suspension pre-incubated with vitamin K_3 was rapidly reduced, with the amount equaling that of the vitamin K_3 used. The initial rate of ferricyanide reduction could not be determined, because it occurred instantaneously. The rate of ferricyanide reduction after this rapid reduction decreased with time. When vitamin K_3 was added to yeast cell suspension pre-incubated with ferricyanide, the initial rate of ferricyanide reduction was about 130 nmol/min per mg. These results suggest that ferricyanide accepts the electron from vitamin K_3 which is reduced by yeast cells.

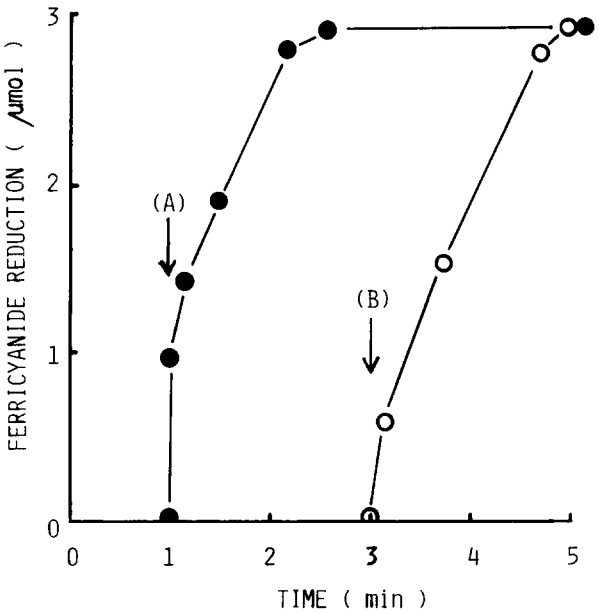

Fig. 1. Ferricyanide reduction promoted by vitamin K_3. The reaction mixture contained 20 mM Hepes (pH 7.0) and 12 mg/ml yeast cells in 3 ml. Arrow (A) indicates the addition of 1 mM ferricyanide to the reaction mixture (●) pre-incubated with 0.33 mM vitamin K_3. Arrow (B) indicates the addition of 0.33 mM vitamin K_3 to the reaction mixture (○) pre-incubated with 1 mM ferricyanide. The initial rate of ferricyanide reduction after (B) was about 130 nmol/min per mg.

Fig. 2 shows that the initial rate of ferricyanide reduction increased with increasing concentration of vitamin K_3. On other hand, vitamin K_1 had no effect on ferricyanide reduction (data not shown).

Proton release coupled with ferricyanide reduction. Addition of both ferricyanide and vitamin K_3 was required to promote the proton release, and the promotion continued until ferricyanide reduction was completed. The initial rate of proton release coupled with ferricyanide reduction after the addition of vitamin K_3 was about 120 nmol/min per mg, and this rate was very similar to the initial rate of ferricyanide reduction promoted by vitamin K_3. Fig. 3 shows that proton release and

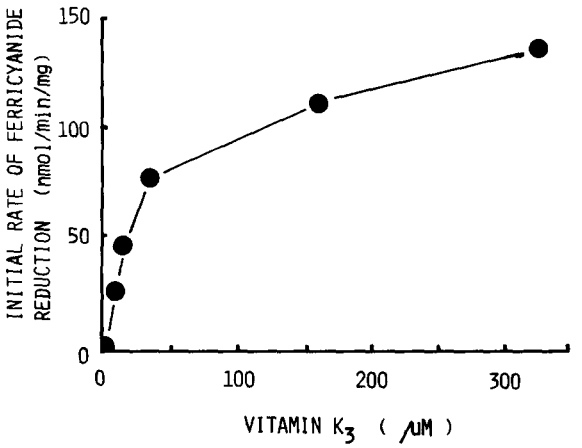

Fig. 2. Dependence of ferricyanide reduction on the concentration of vitamin K_3. The reaction mixture contained 20 mM Hepes (pH 7.0)/1 mM ferricyanide/12 mg per ml yeast cells in 3 ml. The reaction was started by adding vitamin K_3.

ferricyanide reduction in the presence of vitamin K_3 proceed stoichiometrically at the ratio of one proton per electron. The stoichiometrical relationships suggest that vitamin K_3 accepting hydrogen

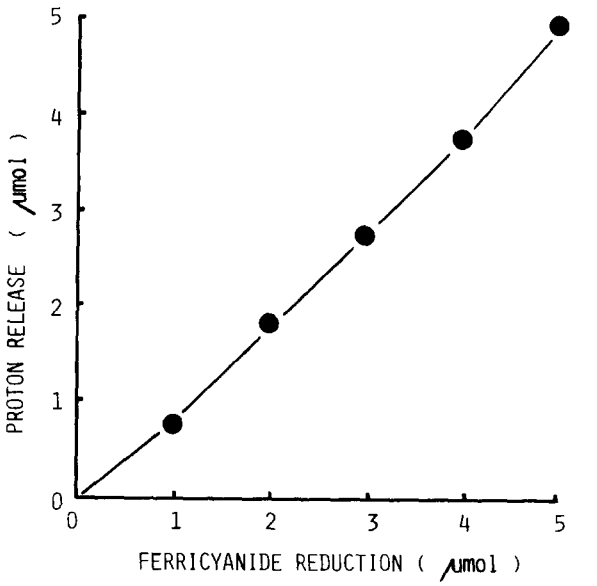

Fig. 3. Stoichiometricaly relationships between proton release and ferricyanide reduction promoted by vitamin K_3. The reaction mixture contained 20 mM Hepes (pH 7.0)/0–5 mM ferricyanide/0.33 mm vitamin K_3/12 mg per ml yeast cells in 3 ml. The reaction was started by adding vitamin K_3, and the amounts of the released protons and the reduced ferricyanide were determined within 3 min.

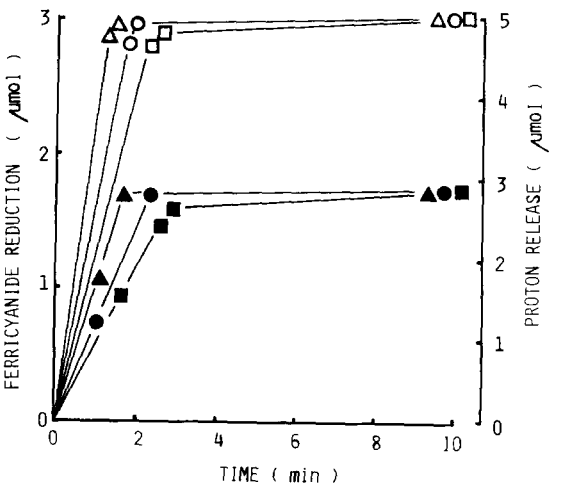

Fig. 4. Effects of chemical modification reagents on ferricyanide reduction and proton release promoted by vitamin K_3. The reaction mixture containing 20 mM Hepes (pH 7.0)/1 mM ferricyanide/0.33 mM vitamin K_3/12 mg per ml yeast cells in 3 ml was pre-incubated with chemical modification reagents for 5 min at 35°C, then the reaction was started by adding vitamin K_3. Circles, no addition; squares, 5 mM *N*-ethylmaleimide; triangles, 5 mM diethylsuberimide. Open and closed symbols represent ferricyanide reduction and proton release, respectively.

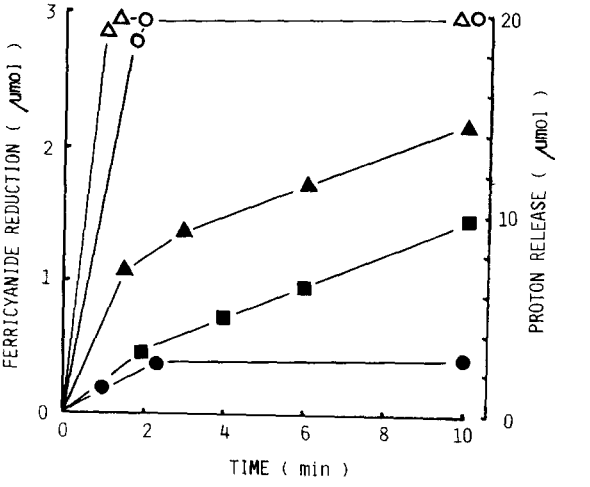

Fig. 5. Effect of glucose on ferricyanide reduction and proton release promoted by vitamin K_3. The reaction mixture contained 20 mM Hepes (pH 7.0)/12 mg per ml yeast cells in 3 ml. The reaction was started by adding vitamin K_3 or glucose. Circles, 1 mM ferricyanide/0.33 mM vitamin K_3; triangles, 1 mM ferricyanide/0.33 mM vitamin K_3/50 mM glucose; ■, 50 mM glucose. Open and closed symbols represent ferricyanide reduction and proton release, respectively.

TABLE I

EFFECT OF DIETHYLSTILBESTROL ON PROTON RELEASE

The reaction mixture contained 20 mM Hepes (pH 7.0)/12 mg per ml yeast cells in 3 ml. The amount of the released protons induced by 50 mM glucose and by 1 mM ferricyanide and 0.33 mM vitamin K_3 were 1.8 μmol and 1.5 μmol for 1 min, respectively. Measurements were made after 5 min preincubation of yeast cells with diethylstilbestrol at 35°C. Data represent mean ± S.E. for three determinations.

Diethylstilbestrol (μg/ml)	Inhibition	
	system with glucose (%)	system with ferricyanide and vitamin K_3 (%)
0	0±2	0±4
17	15±3	0±4
65	75±5	0±5

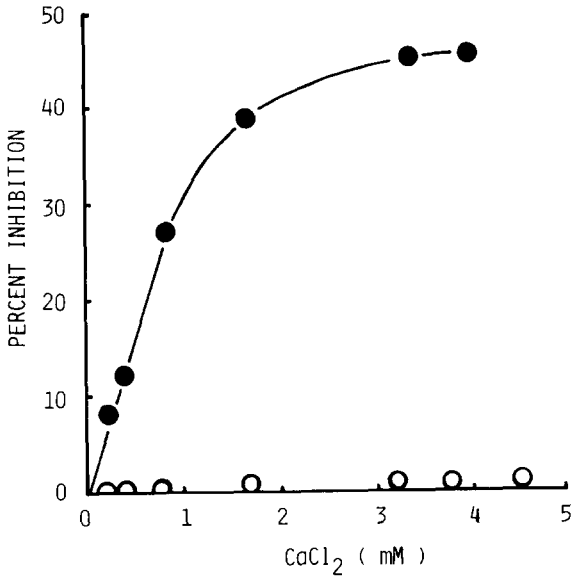

Fig. 6. Effect of calcium on proton release induced by glucose or ferricyanide reduction. The reaction mixture contained 20 mM Hepes (pH 7.0)/12 mg per ml yeast cells in 3 ml. Calcium at the concentrations shown on the abscissa was pre-incubated for 5 min at 35°C, and the reaction was started by adding vitamin K_3 or glucose. Open symbols represent proton release in the presence of 1 mM ferricyanide/0.33 mM vitamin K_3; the total amount of the released protons without calcium was 1.5 μmol for 1 min. Closed symbols represent proton release in the presence of 50 mM glucose; the total amount of the released proton without calcium was 1.8 μmol for 1 min.

from the plasma membrane redox system releases the protons by reducing ferricyanide.

Fig. 4 shows that proton release and ferricyanide reduction catalyzed by vitamin K_3 are little affected by 5 min preincubation of the yeast cells with chemical modification reagents.

Effects of glucose and ATPase inhibitor. Proton release induced by glucose has been observed in suspensions of yeast cells of different strains [17–19], suggesting that proton release requires metabolic energy. Fig. 5 shows that addition of glucose enhances both proton release and ferricyanide reduction catalyzed by vitamin K_3, and that the rate of proton release after the completion of ferricyanide reduction is the same as that of proton release induced by glucose alone. A reductant such as NADH produced by glycolysis may be used for ferricyanide reduction catalyzed by vitamin K_3.

Table I shows that proton release induced by glucose is inhibited by adding diethylstilbestrol which is an inhibitor of plasma membrane ATPase of yeast [17]. However, this compound did not affect proton release coupled with ferricyanide reduction. These results suggest that proton release coupled with ferricyanide reduction in the presence of vitamin K_3 is independent of proton release controlled by plasma membrane ATPase.

TABLE II

EFFECTS OF CALCIUM AND CHEMICAL MODIFICATION REAGENTS ON GLUCOSE UPTAKE AND PROTON RELEASE INDUCED BY GLUCOSE

The reaction mixture contained 20 mM Hepes (pH 7.0)/12 mg per ml yeast cells in 3 ml. The conditions for the assay of glucose uptake are described in Materials and Methods. The amount of glucose uptake in the presence of 2.5 mM glucose was 4 μmol for 5 min. The amount of proton release in the presence of 50 mM glucose was 1.8 μmol for 1 min. Data represent mean ± for four determinations.

Addition	Inhibition	
	proton release (%)	glucose uptake (%)
None	0±2	0±2
4 mM $CaCl_2$	45±4	45±5
5 mM *N*-ethylmaleimide	100±2	100±3
5 mM *N,N'*-(1,2-phenylene)-bismaleimide	46±4	29±2
5 mM diethylsuberimide	40±5	28±2

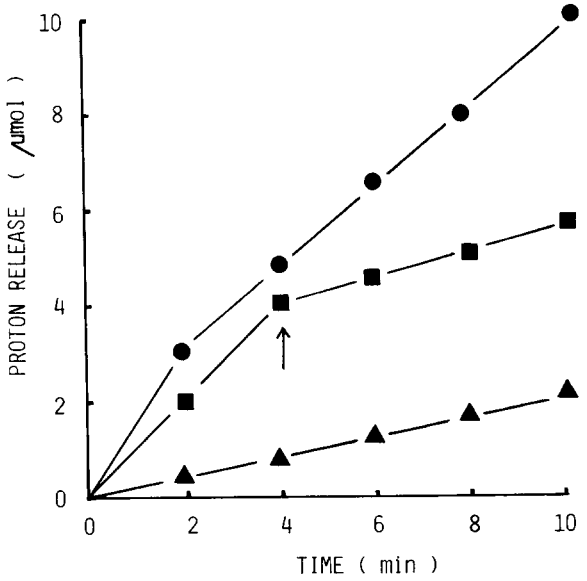

Fig. 7. Effects of calcium and calcium ionophore on proton release induced by glucose. The reaction mixture contained 20 mM Hepes (pH 7.0)/50 mM glucose/12 mg per ml yeast cells in 3 ml. Calcium and calcium ionophore A23187 were pre-incubated for 5 min at 35°C, and the reaction was started by adding glucose. The arrow indicates the addition of 1.7 mM $CaCl_2$. ●, no addition; ■, 32 μM A23187; ▲, 1.7 mM $CaCl_2$ and 32 μM A23187.

Effects of calcium and chemical modification reagents. $CaCl_2$ had no effect on proton release coupled with ferricyanide reduction in the presence of vitamin K_3, but inhibited proton release induced by glucose as shown in Fig. 6. This latter inhibitory effect was enhanced in the presence of calcium ionophore A23187 as shown in Fig. 7. The inhibition of glucose uptake by $CaCl_2$ may decrease proton release induced by glucose, because $CaCl_2$ inhibition of proton release was the same as that of glucose uptake as shown in Table II.

Both glucose uptake and proton release induced by glucose were inhibited by 5-min preincubation of the yeast cells with the chemical modification reagents as shown in Table II. The results of Fig. 4 and Table II indicate that proton release induced by glucose is more sensitive to chemical modification of the plasma membrane than that coupled with ferricyanide reduction.

Discussion

Eukaryotic cells have specific electron acceptors for NADH-dehydrogenase in the plasma membrane. For example, human erythrocytes reduce ferricyanide rather than cytochrome *c* [20], vanadate, methoglobin and monohydroascorbate [21], and calf cardiac muscle reduces vanadate [21]. However, yeast cells have poor activity for ferricyanide reduction [15], and the specific electron acceptors for the yeast plasma membrane redox system have not been identified. The present results show that ferricyanide reduction by yeast cells could be greatly promoted by the addition of vitamin K_3. Tetramethyl *p*-benzoquinone was also a good redox mediator, but vitamin K_1 and 2-hydroxy-1,4-naphthoquinone had no effect on proton release coupled with ferricyanide reduction. Interestingly, vitamin K_1 has no catalytic action in spite of the fact that it is a homolog of vitamin K_3. The phytyl group of vitamin K_1 may inhibit the contact with the terminal reductant in the plasma membrane redox system.

Ferricyanide reduction upon addition of ferricyanide to yeast cells pre-incubated with vitamin K_3 was faster than that upon addition of vitamin K_3 to yeast cells pre-incubated with ferricyanide. Proton release coupled with ferricyanide reduction in the presence of vitamin K_3 proceeded stoichiometrically at the ratio of one proton per electron. These findings suggest that vitamin K_3 accepting hydrogen from the plasma membrane redox system releases the protons by reducing ferricyanide as shown in Fig. 8. If vitamin K_3 accepts the electron from the plasma membrane redox system,

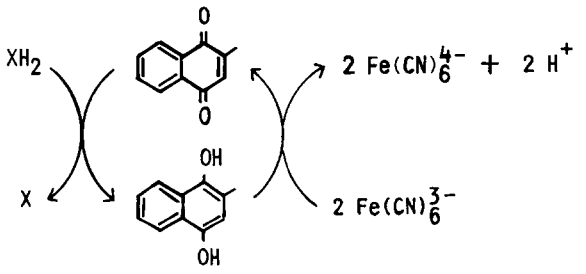

Fig. 8. Proposed mechanism for proton release and ferricyanide reduction promoted by vitamin K_3, XH_2 represents the terminal reductant of the plasma membrane redox system.

the semiquinone produced does not have the ability to release the protons.

NADH and NADPH are electron donors for ferricyanide reduction by the plasma membrane redox system of eukaryotic cells [21]. However, the intracellular electron donor of the yeast plasma membrane redox system has not been identified. It has been proposed that NADH is the intracellular reductant for ferricyanide reduction by yeast cells, because ferricyanide reduction by yeast cells is inhibited by the addition of pyrazole which is an inhibitor of ethanol dehydrogenase generating NADH [15]. As the addition of glucose enhanced the reduction of ferricyanide in the presence of vitamin K_3, NADH produced by glycolysis may also act as a reductant of the plasma membrane redox system. Glucose may be the common energy source supplying NADH and ATP for the proton release which is controlled by the redox system and ATPase of plasma membrane.

Adriamycin inhibits the activity of NADH-ferricyanide reductase [12,13], and impermeable adriamycin linked to agarose is cytotoxic to tumor cells [14]. Adriamycin seems to exert its cytotoxic action by inhibiting NADH-ferricyanide reductase at the surface of tumor cells. The growth of tumor cells depends on the concentration of extracellular ferricyanide [12], and the plasma membrane redox system is thought to be coupled with the active transport of nutrients such as amino acids [23,24]. These findings suggest that reduction of the extracellular electron acceptor by the plasma membrane redox system affects cell growth.

We concluded that extracellular redox mediators such as vitamin K_3 promote the transmembrane electron flow in the plasma membrane which may be coupled with nutrient uptake.

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