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Decrease of NADH in yeast cells by external ferricyanide reduction

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Ferricyanide reduction catalyzed by vitamin K-3 was accompanied by the decrease in intracellular NAD(P)H concentration of yeast cells, and the rate of ferricyanide reduction depended on intracellular concentration of NADH rather than NADPH. The addition of glucose to the cell suspensions enhanced both ferricyanide reduction and intracellular NADH concentration. The catalytic action of vitamin K-3 on ferricyanide reduction was observed in the presence of NADH and plasma membrane preparations. As the toxic action of vitamin K-3 on cell growth of yeast was enhanced by addition of ferricyanide, ferricyanide reduction catalyzed by vitamin K-3 may inhibit cell growth by decreasing intracellular NADH concentration.

Introduction

It has been known that plasma membrane redox systems of eukaryotic cells are related to several vital functions which are involved in cell growth [1–4] and nutrition uptake [5–9]. In these systems NADH and NADPH are the intracellular reductants, and molecular oxygen and iron compounds are the extracellular oxidants [10]. The properties and orientation of the dehydrogenase enzymes in plasma membranes have also been investigated [11]. Yeast is a simple eukaryote with plasma membrane redox systems [12], and ferricyanide reduction by yeast cells has been found to be greatly promoted by addition of vitamin K_3 which is thought to be the redox mediator for the transmembrane electron transport [13]. However, the reductant for ferricyanide reduction by yeast cells and the ferricyanide reductase have not been identified. NADH is speculated to be the intracellular reductant because of the inhibition of ferricyanide reduction by pyrazole which is the inhibitor of alcohol dehydrogenase generating NADH [12]. We also proposed the involvement of NADH produced by glycolysis because of the promotion of ferricyanide reduction by addition of glucose [13].

In this paper, we show the relationships between ferricyanide reduction and intracellular NAD(P)H concentration, indicating that NADH is the intracellular reductant for ferricyanide reduction catalyzed by vitamin K_3. The effect of glucose on ferricyanide reduction and intracellular NADH concentration is also discussed.

Materials and Methods

Organism and cell growth. The strain IFO 2044 of *Saccharomyces cerevisiae* was used for this study. The cells were grown in flasks on shaker at 28°C to the end of exponential phase. Two different media were used to get the cells with different intracellular NAD(P)H concentration. The composition of these media was as follows: medium (A) composed of 2% glucose/0.4% yeast extract/

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

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1% malt extract; medium (B) composed of 4% glucose/0.5% yeast extract/1% polypeptone/0.5% KH_2PO/0.2% $MgSO_4 \cdot 7H_2O$. Medium (C) composed of 0.67% bacto yeast nitrogen base/1% glucose was used to avoid the contamination of unknown iron compounds for the study on the toxic effects of ferricyanide on cell growth. The harvested cells were washed twice with distilled water, and suspended in 20 mM Hepes (pH 7.0). The amount of yeast cells is expressed as wet weight.

Assay of ferricyanide reduction. This assay was carried out according to the previous method [13].

Assay of intracellular NAD(P)H and NAD(P) concentrations. After ferricyanide reduction the cells were washed twice with distilled water, heated in distilled water at 70°C for 7 min, and centrifuged. The supernatant was used for the determination of NAD(P)H and NAD(P) concentrations according to the method described in Ref. 14.

Preparation of plasma membranes. Yeast protoplasts were obtained as reported in Ref. 15, and plasma membranes were prepared after the pretreatment of the protoplasts with concanavalin A [16].

Assay of cell growth. Yeast cells were grown in 5 ml of media (A), (B) and (C) at 28°C for 24 h. The cells were washed twice with water, and suspended in distilled water. The cell density was determined from the turbidity at 600 nm.

Results

Ferricyanide reduction catalyzed by vitamin K_3 was accompanied by the decrease in intracellular NAD(P)H concentration as shown in Fig. 1. Yeast cells with the high level of intracellular NAD(P)H concentration had the great ferricyanide reductase activity. For example, yeast cells grown in the medium (A) had the activity to reduce ferricyanide at the rate of 1.5 μmol per 20 mg cells per min, and intracellular NAD(P)H concentration was reduced to one-half its initial value after the completion of ferricyanide reduction. However, both ferricyanide reductase activity and intracellular NAD(P)H concentration of the cells grown in the medium (B) were lower than those of the cells grown in the medium (A). These facts

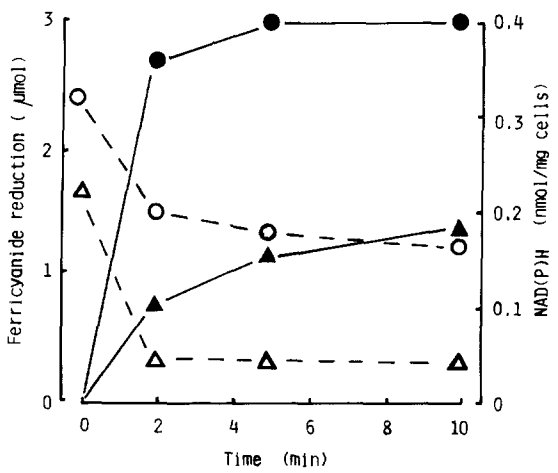

Fig. 1. Effect of ferricyanide reduction on the intracellular NAD(P)H concentration. The reaction mixture contained 3 mM ferricyanide/20 mM Hepes (pH 7.0)/20 mg/ml yeast cells in 1 ml, and was pre-incubated at 35°C for 10 min. The reaction was started by addition of 0.3 mM vitamin K_3. Circular and triangular symbols represent the cells grown in the medium (A) and (B), respectively. Open and closed symbols represent the intracellular NAD(P)H concentration and ferricyanide reduction, respectively.

suggest that yeast cells grown in the medium (A) have enough activity to keep the high level of NAD(P)H concentration during ferricyanide reduction. Yeast cells grown in the medium (A) had the activity to reduce 5 μmol ferricyanide per 20 mg cells within 30 min, and ferricyanide reduction by the cells grown in the medium (B) was limited to 1.5 μmol ferricyanide per 20 mg cells as shown in Fig. 2. The addition of glucose enhanced the ferricyanide reductase activity of yeast cells grown in the medium (B) (Fig. 2). Total pyridine nucleotide pool of the cells was not affected by ferricyanide reduction, because a corresponding rise in NAD(P) was observed during ferricyanide reduction (not shown here). For example, the total amount of the oxidized and reduced pyridine nucleotide of the cells grown in the medium (A) was constant, and was about 0.43 nmol per mg cells during ferricyanide reduction. When vitamin K_3 acting as electron carrier was absent in the reaction mixture, NAD(P)H concentration was not affected in both the presence and the absence of ferricyanide. These facts indicate that ferricyanide reduction catalyzed by vitamin K_3 is required for

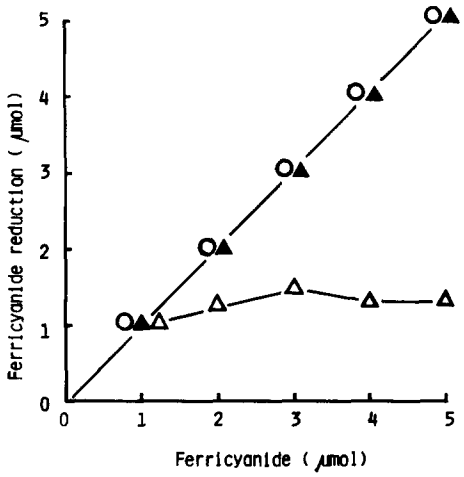

Fig. 2. Difference in ferricyanide reductase activities of yeast cells grown in the medium (A) and (B). Reaction conditions were the same as those shown in Fig. 1, except ferricyanide concentration indicated on abscissa. Reaction time was 30 min. Circular and triangular symbols represent the cells grown in medium (A) and (B), respectively. Closed symbols represent the system with 0.1 M glucose and the cells grown in the medium (B).

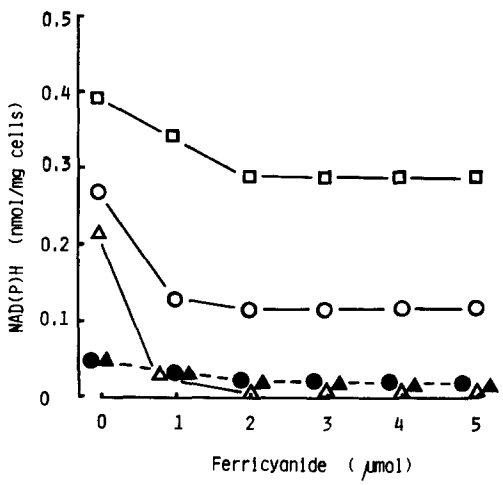

Fig. 3. Intracellular NAD(PH) concentration after ferricyanide reduction. Reaction conditions were the same as those shown in Fig. 1, except fericyanide concentration shown on abscissa. Reaction time was 30 min. Open and closed symbols represent the concentrations of NADH and NADPH, respectively. Circular and triangular symbols represent the cells grown in the medium (A) and (B), respectively. Symbol (□) represents the system with 0.1 M glucose and the cells grown in the medium (B).

oxidation of NAD(P)H in yeast cells.

Fig. 3 shows intracellular concentrations of NADH and NADPH after 30-min preincubation of yeast cells with ferricyanide and vitamin K_3. Intracellular NADH concentration of yeast cells grown in the medium (A) was reduced to one-half its initial concentration by addition of 1–5 μmol of ferricyanide per 20 mg cells, but that of the cells grown in the medium (B) was reduced to about 5% of the initial concentration. Intracellular NADH concentration of yeast cells grown in the medium (B) was increased about 20-fold by addition of glucose in the presence of vitamin K_3 and 1–5 μmol of ferricyanide per 20 mg cells. On the other hand, there was no difference in intracellular NADPH concentration between yeast cells grown in the medium (A) and (B). Figs. 2 and 3 suggest that addition of glucose enhances ferricyanide reduction by inducing the production of NADH by glycolysis, and that ferricyanide reduction by yeast cells depends on intracellular NADH concentration.

Fig. 4 shows the participation of NADH in ferricyanide reduction by plasma membrane pre-

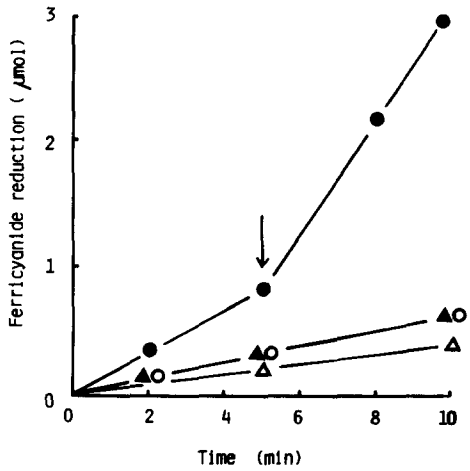

Fig. 4. Ferricyanide reductase activities of plasma membrane preparations. Reaction mixture contained 150 μg protein/1 mM ferricyanide/20 mM Hepes (pH 7.0) in 3 ml. The reaction was started by addition of 1 mM NADH or NADPH, and the temperature was kept at 35°C. Open and closed symbols represent the systems containing cytosolic preparations and plasma membrane preparations, respectively. Circular and triangular symbols represent the system with 1 mM NADH and that with 1 mM NADPH, respectively. Arrow indicates the addition of 0.3 mM vitamin K_3.

parations. Ferricyanide reduction in the presence of NADH proceeded faster than that in the presence of NADPH, and was promoted by addition of vitamin K_3. Ferricyanide reduction by cytosol preparations was not promoted by addition of vitamin K_3 in the presence of NADH or NADPH. These facts show that vitamin K_3-catalyzed NADH-ferricyanide reductase is present in plasma membranes.

Cell growth of yeast cells was inhibited by vitamin K_3 as shown in Fig. 5. Yeast cells were resistant to the inhibitory effect in the medium (A). The resistance of the cells to the toxicity of vitamin K_3 may depend on the activity of the cells to keep the high level of intracellular NADH concentration, because the cells grown in the medium (A) had higher level of intracellular NADH concentration than the cells grown in the medium (B). The toxicity of vitamin K_3 was enhanced in the presence of ferricyanide as shown in Fig. 6, suggesting that ferricyanide enhances the regeneration of the oxidized vitamin K_3 which can be reduced by the cells.

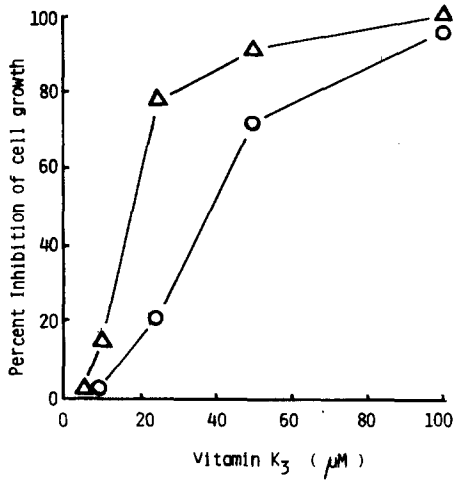

Fig. 5. Toxicity of vitamin K_3 on cells grown in different media. The initial cell density was $7.5 \cdot 10^4$ cells/ml. Cell growth was determined after 24-h incubation in the medium containing various amount of vitamin K_3 shown on abscissa. The cell density in the vitamin K_3-free system was $1.8 \cdot 10^7$ cells/ml after 24-h incubation in the medium (A) and (B). Circular and triangular symbols represent the cells grown in the medium (A) and (B), respectively.

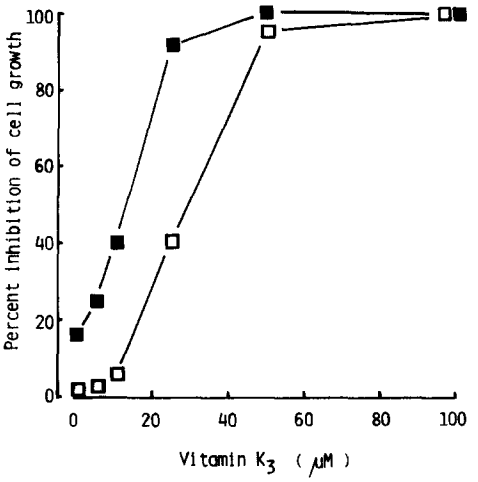

Fig. 6. Effect of ferricyanide on the toxicity of vitamin K_3. The conditions for cell growth were the same as those described in Fig. 5, except the use of the medium (C). The cell density in the system without both vitamin K_3 and ferricyanide was $1.7 \cdot 10^7$ cells/ml after 24-h incubation. Open and closed symbols represent the system without and with 1 mM ferricyanide, respectively.

Discussion

The transmembrane redox systems are important in control of cell growth [1–4], because the inhibition of the plasma membrane redox systems causes the inhibition of cell growth [2,17,18]. The external oxidants such as ferricyanide [1], hexamine ruthenium III [4] and indigo tetrasulfonate [4] stimulate cell growth by keeping the redox components in the oxidized state in the plasma membranes [1]. Proton release from the cells is promoted by plasma membrane redox systems, and an increase in the cytosolic pH is related to stimulation of mitosis [11]. However, excessive transmembrane electron transport and proton release may inhibit cell growth by breaking the equilibrium of the redox state among the plasma membrane redox components. In fact, vitamin K_3 acting as redox mediator exerted toxic action (Fig. 5), and its toxicity was enhanced in the presence of ferricyanide (Fig. 6). Ferricyanide reduction catalyzed by vitamin K_3 induced the decrease in intracellular NAD(P)H concentration. These facts indicate that external oxidants such as vitamin K_3 and ferricyanide exert the toxicity to

yeast cells by inducing excessive oxidation of intracellular reductants. The resistance of the cells against the excessive oxidation of intracellular reductants may depend on the their activities to supply NAD(P)H from the intracellular metabolites. For example, the cells grown in the medium (A) was resistant to the toxicity of vitamin K_3, and had the activity to keep the high level of intracellular NAD(P)H concentration. On the other hand, the cells grown in the medium (B) was sensitive to the toxicity of vitamin K_3, and the intracellular NAD(P)H concentration decreased remarkably during ferricyanide reduction. As the addition of glucose to the cell suspensions increased intracellular NADH concentration, glycolytic system might supply NADH (Fig. 3). In order to clarify the mechanism for the supplement of NADH in yeast cells, we have been studying on the metbolites involved in the generation of NADH during ferricyanide reduction.

Here it is demonstrated that ferricyanide reduction by yeast cells depends on the intracellular concentration of NADH rather than NADPH (Fig. 3). Ferricyanide reduction in the system with plasma membrane preparations also required NADH rather than NADPH, and was promoted by vitamin K_3 in the presence of NADH (Fig. 4). These facts suggest that NADH acts as the intracellular reductant for vitamin K_3-catalyzed ferricyanide reduction by yeast cells. Recently, Navas et al. [19] reported a decrease in intracellular NADH concentration of HeLa cells in the presence of ferricyanide and provided evidence that NADH is the intracellular reductant for the transmembrane redox system of these cells. It is expected that external oxidants and redox mediators can control cell growth by oxidizing the intracellular reductants via plasma membrane redox systems.

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