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Characterization of Vanadate-Dependent NADH Oxidation Stimulated by *Saccharomyces cerevisiae* Plasma Membranes

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Plasma membrane-stimulated vanadate-dependent NADH oxidation has been characterized in *Saccharomyces cerevisiae*. This activity is specific for vanadate, because molybdate, a similar metal oxide, did not substitute for vanadate in the reaction. Vanadate-dependent plasma membrane-stimulated NADH oxidation activity was dependent on the concentrations of vanadate, NADH, and NADPH and required functional plasma membranes; no stimulation occurred in the presence of boiled membranes or bovine serum albumin. The dependence of membrane-stimulated vanadate-dependent NADH oxidation was not linearly dependent on added membrane protein. The activity was abolished by the superoxide anion scavenger superoxide dismutase and was stimulated by paraquat and NADPH. These data are consistent with the previously proposed chain reaction for vanadate-dependent NADH oxidation. The role of the plasma membrane appears to be to stimulate superoxide radical formation, which is coupled to NADH oxidation by vanadate. ^{51}V-nuclear magnetic resonance studies are consistent with the hypothesis that a phosphovanadate anhydride is the stimulatory oxyvanadium species in the phosphate buffers used at pHs 5.0 and 7.0. In phosphate buffers, compared with acetate buffers, the single vanadate resonance was shifted upfield at both pH 5.0 and pH 7.0, which is characteristic of the phosphovanadate anhydride. Since the cell contains an excess of phosphate to vanadate, the phosphovanadate anhydride may be involved in membrane-mediated vanadate-dependent NADH oxidation in vivo.

A physiological role for vanadium has not been identified until recently. The discovery of a bacterial alternative nitrogenase (18) and an algal bromoperoxidase (5) which contain vanadium at their active sites indicates that vanadium has an essential role in biology. These enzymes are the only known examples of vanadium functioning as a prosthetic group in cellular redox reactions. To date the role of vanadium in cellular metabolism remains elusive (15, 21).

The vanadate-dependent oxidation of NADH has also been studied in a number of biomembranes. Ramasarma et al. (17) showed that vanadate markedly increases the nonenzymatic oxidation of NADH. This oxidation of NADH in the presence of vanadate was stimulated by the addition of mouse liver plasma membranes, was abolished under anaerobic conditions, and was inhibited by superoxide dismutase, which scavenges superoxide anions. The vanadate-dependent NADH oxidation activity of mouse liver plasma membranes generated hydrogen peroxide.

Liochev and Fridovich have studied the vanadate-dependent oxidation of NADPH by rat liver microsomes and recently reviewed the field (10, 11). Their observations indicated that superoxide production by membrane-associated NADPH oxidases could account for the reported vanadate-dependent oxidation of NAD(P)H. A similar NADH oxidation activity has also been reported in erythrocyte plasma membranes (19), rat liver microsomes (3), and sugar beet microsomal membranes (2). This report on the plasma membrane-stimulated vanadate-dependent NAD(P)H oxidation activity of *Saccharomyces cerevisiae* is the first description of this activity in a microorganism.

Darr and Fridovich (4) had previously examined the nonenzymatic oxidation of NADH by using the xanthine-oxidase reaction as a source of superoxide. They proposed the following mechanism for the nonenzymatic vanadate-dependent oxidation of NADH:

$$V(V) + O_2^- \rightarrow V(IV)\text{-}OO$$
$$V(IV)\text{-}OO + NADH \rightarrow V(IV)\text{-}OOH + NAD\cdot$$
$$V(IV)\text{-}OOH + H^+ \rightarrow V(V) + H_2O_2$$
$$NAD\cdot + O_2 \rightarrow NAD^+ + O_2^-$$

In this reaction, vanadate reacts nonenzymatically with superoxide, producing a species which can oxidize NADH. The products of this reaction are a hydroperoxy vanadium (IV) and an NAD radical. The former could hydrolyze to vanadate plus H_2O_2, whereas the latter would react with O_2, producing NAD^+ and O_2^-. Vanadate and O_2^- are regenerated, providing the opportunity for a chain reaction. The hypothesis that the plasma membrane-stimulated vanadate-dependent NADH oxidation activity observed is due to membrane-stimulated production of the superoxide radical and that this radical is then involved in the chain reaction mechanism described above is consistent with the data reported in the literature (11).

This report describes the characterization of plasma membrane-stimulated, vanadate-dependent NADH oxidation in the fungus *S. cerevisiae*, which is similar to the membrane-stimulated vanadate-dependent NADH oxidation activities previously described. The yeast plasma membrane also couples the production of superoxide to vanadate-stimulated NADH oxidation. ^{51}V-nuclear magnetic resonance (NMR) studies of the reaction mixtures used to study this reaction were undertaken to identify the active oxyvanadium species

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involved in the reaction and implicated a phosphate-vanadate anhydride as the stimulatory form of oxovanadium in phosphate buffers.

MATERIALS AND METHODS

Chemicals. NADH, disodium salt (grade III); NADPH, tetrasodium salt (type I); ATP, disodium salt; sodium orthovanadate; paraquat (PQ; methyl viologen); phenylmethylsulfonyl fluoride; semidine hydrochloride (4-aminodiphenylamine); *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, (HEPES); 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT); Tris; and 2-(*N*-morpholino)ethanesulfonic acid (MES) were obtained from Sigma Chemical Company. Superoxide dismutase was obtained from Boehringer Mannheim Biochemicals. All other chemicals were of reagent-grade quality.

In this report, the concentration of vanadate in each assay is given as millimolar orthovanadate (VO_4^{3-}) added to the assay, which is equivalent to millimolar monovanadium. In the reaction buffers used, orthovanadate converts to metavanadate. The concentration of vanadate is reported in millimolar monovanadium because the chemical structure of metavanadate in solution at concentrations above 100 μM consists of tetrameric vanadate ($V_4O_{12}^{4-}$) and mixtures of monomeric and dimeric species.

Membrane preparations. Commercial baker's yeast (1 lb [ca. 0.5 kg]) was obtained from Federal Bakers Supply Company. The yeast was washed twice with distilled water and twice with 10 mM Tris hydrochloride–1 mM EDTA, pH 7.5, and left at 4°C overnight. Cells were resuspended in 10 mM Tris hydrochloride–1 mM EDTA and 17% sucrose (wt/vol), pH 7.5, to 75% wet weight. Phenylmethylsulfonyl fluoride was added to 0.2 mM (from a freshly prepared 0.1 M stock solution in ethanol), and the cells were broken in a Dynomill type KDL cell disrupter (Willy Braun, Basel, Switzerland). Membrane fractions were obtained from the cell homogenate by using differential centrifugation. Cell debris, nuclei, and unbroken cells were removed by centrifugation at 8,000 × *g* for 15 min. The supernatant was centrifuged at 50,000 × *g* for 45 min to sediment the crude membrane fraction. The plasma membrane precipitates in this low-speed spin because of aggregation of the membranes. The membrane pellet was resuspended in 10 mM HEPES-KOH–1 mM EDTA–250 mM sucrose–1 mM $MgSO_4$–0.2 mM phenylmethylsulfonyl fluoride, pH 7.5 (resuspension buffer). To obtain microsomal membranes, the supernatant from this step was centrifuged at 60,000 × *g* for 1.5 h. The pellet was resuspended in the resuspension buffer.

The crude membrane fraction was layered over a glycerol block gradient (20 mg of fraction per 15 ml of gradient; 1.5 ml of 10% glycerol; 3 ml (each) of 20, 40, and 60% glycerol; and 1.5 ml of 100% glycerol) and centrifuged at 120,000 × *g* for 16 h to obtain a plasma membrane-enriched pellet. The pellet was resuspended in the resuspension buffer. The band on the gradient above the pellet was the mitochondrion-enriched fraction. Identification of plasma membrane and mitochondria in these fractions was confirmed by using Mg^{2+} ATPase and succinic dehydrogenase assays, respectively.

Assays. (i) Protein. The protein concentrations of plasma membrane fractions were determined by using the commercially prepared Bradford reagent (Bio-Rad) with bovine serum albumin as the standard.

(ii) ATPase. ATP hydrolysis was carried out as previously described by Bowman and Slayman (1). The reaction buffer contained 10 mM MES–5 mM ATP–5 mM $MgCl_2$ (pH 6.0)–25 μg of plasma membrane protein in a volume of 0.6 ml. The assay was run for 10 min in a 30°C water bath, and the reaction was stopped by the addition of 0.1 ml of 25% sodium dodecyl sulfate (SDS). The concentration of free phosphate liberated by ATP hydrolysis was determined by spectroscopic assay (6). The following additions were made to the ATPase assay mixture: 1 N H_2SO_4, 5 mM ammonium molybdate, and 2 ml of semidine reagent (see below) in a volume of 5 ml. The A_{700} and the concentration of phosphate were determined by using a phosphate standard curve from 50 to 500 nmol. Semidine reagent was prepared as follows. Semidine hydrochloride (0.25 g) was wet with a few drops of 95% ethanol. Sodium bisulfite (5 g) was added, and the volume was brought to 500 ml with distilled water. The majority of crystals do not go into solution. The solution was filtered with Whatman filter paper and stored in a dark bottle.

(iii) Succinic dehydrogenase. Succinic dehydrogenase activity was measured by using a modification of the procedure described by Pennington (16). The assay consisted of 45 mM Tris sulfuric acid (pH 7.8)–20 mM EDTA (pH 8.0)–50 mM succinate (pH 7.8)–0.1 mg of INT (from a 1 mg/ml stock solution in 50% ethanol) per ml–15 g of plasma membrane protein in a final volume of 1.2 ml. INT accepts electrons in the oxidation of succinate by succinic dehydrogenase of mitochondrial membranes. The reduction of INT was monitored by the increase in formazan produced ($\varepsilon_{490} = 2.01 \times 10^4\ M^{-1}\ cm^{-1}$).

(iv) NADH oxidation. The assay system consisted of NADH (0.1 mM), potassium phosphate buffer (50 mM, pH 7.0), vanadate (0.25 to 0.35 mM orthovanadate), and various concentrations of plasma membrane protein (17). The total volume was 1.4 ml. NADH oxidation was measured by a decrease in A_{340} by using a Pye-Unicam spectrophotometer at 30°C attached to a Unicam linear recorder. The nanomoles of NADH oxidized per min was calculated from the recorded slope. NADH oxidation activity of less than 0.08 nmol/min was not detectable. The slopes were traced after making baseline adjustments for any change in absorption caused by the additions to the reaction mixture.

Total NADH oxidation activity is the activity measured in the presence of both vanadate and protein, while nonenzymatic activity is NADH oxidation in the presence of vanadate only. Plasma membrane-stimulated vanadate-dependent NADH oxidation is calculated as total activity minus nonenzymatic activity. Total vanadate-dependent NADH oxidation and membrane-stimulated vanadate-dependent NADH oxidation were determined in separate assays.

Oxygen uptake. The uptake of oxygen was measured by using a YS1 5331 Clark-type O_2 electrode and model 53 O_2 monitor.

^{51}V-NMR. Spectra were obtained at 71 MHz on a JEOL FX 270 NMR Fourier transformation spectrometer at 30°C in 10-mm tubes as previously described (22). Free induction decays were collected using single 34° pulse widths with a 50-μs deadtime. Sweep widths of 50 kHz were used. A line-broadening factor of 50 Hz was applied to all spectra before Fourier transformation to the frequency domain. Generally, 5,000 scans with 2,000 data points were collected at a 10-ms repetition rate for each spectrum. All spectra to be compared are shown with the same gain and phasing constants. The positions of ortho- and metavanadate resonances were determined (see Fig. 7) (9). ^{51}V-NMR chemical shifts are reported relative to $VOCl_3$. Because we are interested in the spectra between −450 to −600 ppm, all of our chemical

TABLE 1. Characterization of glycerol gradient-purified plasma membrane-enriched fractions[a]

Enriched fraction	NADH oxidation[b] (nmol/min)			ATPase (μmol/min/mg) at pH:			Succinic dehydrogenase (μmol/min/μg)
	−V	+V	V-Dep	6.0	9.0	6.0/9.0	
Mitochondria	4.74	4.5	None	0.39	0.33	1.2	0.60
Plasma membrane	≤0.16	2.72	≥2.56	1.39	0.13	10.7	0.24

[a] Membrane fractions were isolated and enzymatic assays were performed as described in Materials and Methods. Each assay value is the average of two determinations within 15% of each other.

[b] NADH oxidation assays were done by using 2 μg of protein and 0.35 mM vanadate (V). Non-protein-dependent vanadate-stimulated NADH oxidation activity (0.60 nmol/min) was subtracted from the +V samples. Vanadate-dependent activity (V-Dep) is +V activity minus −V activity.

shifts are calculated relative to orthovanadate, which has been assigned a value of −541 ppm.

RESULTS

Identification of plasma membrane-stimulated vanadate-dependent NADH oxidation in *S. cerevisiae*. A plasma membrane-enriched fraction was obtained from *S. cerevisiae* after passage of the crude membrane fraction over a glycerol gradient. Marker enzymes were used to identify the partially purified plasma membrane and mitochondrial membrane fraction (Table 1). Succinic dehydrogenase and Mg^{2+} (pH 9.0) ATPase activity were markers for the mitochondrial membrane, while Mg^{2+} (pH 6.0) ATPase activity was a marker for the plasma membrane. The greater the ratio of pH 6.0 to pH 9.0 ATPase activity, the more highly purified is the plasma membrane (20). The mitochondrial fraction and plasma membrane fractions were enriched in the appropriate marker enzymes. The plasma membrane-enriched fraction contained vanadate-dependent NADH oxidation activity and lacked vanadate-independent activity. The mitochondria-enriched fraction contained high levels of vanadate-independent NADH oxidation activity, most likely due to mitochondrial NADH dehydrogenases (Table 1). There was no significant vanadate-dependent NADH oxidation activity in the mitochondrial membrane fractions. All further assays used the plasma membrane-enriched fraction.

The oxidation of NADH in the presence and absence of both plasma membranes and vanadate was determined. The rate of NADH oxidation in the presence of vanadate (Fig. 1, tracing 1 and the first part of tracing 2) was increased approximately threefold on addition of yeast plasma membranes (Fig. 1, tracing 2). The rate of NADH oxidation in the presence of 20 μg of yeast plasma membranes alone was approximately 0.54 nmol of NADH oxidized per min (Fig. 1, tracing 3 and the first part of tracing 4), and this activity was stimulated approximately sevenfold by the addition of vanadate (Fig. 1, tracing 4). Note that the tracing for plasma membrane-stimulated vanadate-dependent NADH activity is slightly curved with greater activity observed at early times. To enable accurate slopes to be obtained over a 2- to 3-min period, further experiments were done using 5 to 10 times less protein.

Characterization of plasma membrane-stimulated vanadate-dependent NADH oxidation in *S. cerevisiae*. Vanadate-dependent NADH oxidation activity was determined at pH values between 5.0 and 8.0. The total vanadate-dependent NADH oxidation activity in the presence of 4 μg of plasma membranes reached a maximum value of 21.0 nmol/min at pH 5.0 (Fig. 2A). At this pH, the nonenzymatic rate of NADH oxidation of 23.0 nmol/min was approximately equal to the total rate. The membrane-stimulated vanadate-dependent NADH oxidation rate was obtained by subtraction of the nonenzymatic activity from the total activity shown in Fig. 2A (Fig. 2B). This activity was greatest at pHs 6.5 and

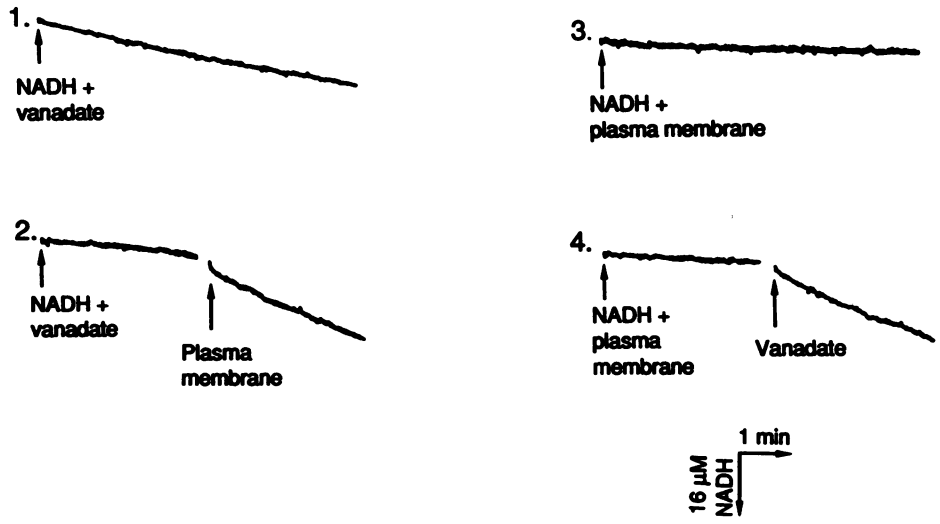

FIG. 1. The NADH oxidation activity of *S. cerevisiae* plasma membranes. NADH oxidation was measured in the presence and absence of 0.25 mM vanadate or 20 μg of plasma membrane protein under aerobic conditions in the presence of 0.35 mM NADH. The order of addition is indicated in the tracings.

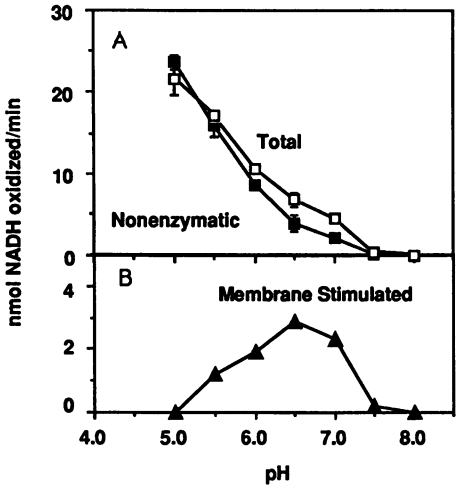

FIG. 2. The pH dependence of plasma membrane-stimulated NADH oxidation in *S. cerevisiae*. NADH oxidation was measured in 50 mM phosphate buffer containing 0.35 mM vanadate and 0.2 mM NADH at the indicated pH values. Each point was done in triplicate and is graphed as the mean ± standard error of the mean. If error bars are not present they are within the symbol. (A) For total NADH oxidation (□), 4 μg of plasma membrane protein was used. For nonenzymatic activity (■), no protein was added. (B) Membrane-stimulated vanadate-dependent NADH oxidation activity (▲) is shown as the difference between total and nonenzymatic activity calculated from the data in panel A.

7.0, with values of 2.8 and 2.3 nmol/min, respectively. Further assays were done at pH 7.0.

The effects of the concentration of vanadate and NADH on the vanadate-dependent NADH oxidation activity were determined. The stimulation of NADH oxidation by plasma membranes was dependent on vanadate concentration (Fig. 3). The maximum rate of membrane-stimulated activity occurred at 0.35 mM vanadate with 2.2 nmol of NADH

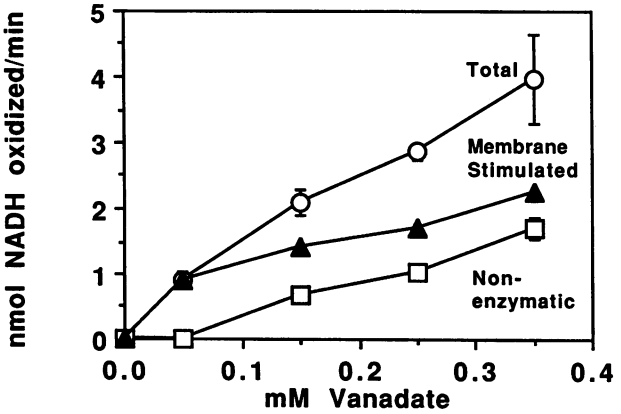

FIG. 3. The vanadate dependence of plasma membrane-stimulated NADH oxidation in *S. cerevisiae*. NADH oxidation was measured in the presence of concentrations of vanadate from 0.05 to 0.35 mM and in the presence of 0.2 mM NADH. Each point is graphed as the mean ± standard error of the mean (n = 6). If error bars are not visible they are within the symbol. For nonenzymatic activity (□), no further additions were made. For total activity (○), 2 μg of plasma membrane protein was added. Plasma membrane-stimulated vanadate-dependent NADH oxidation activity (▲) is calculated as total minus nonenzymatic activity.

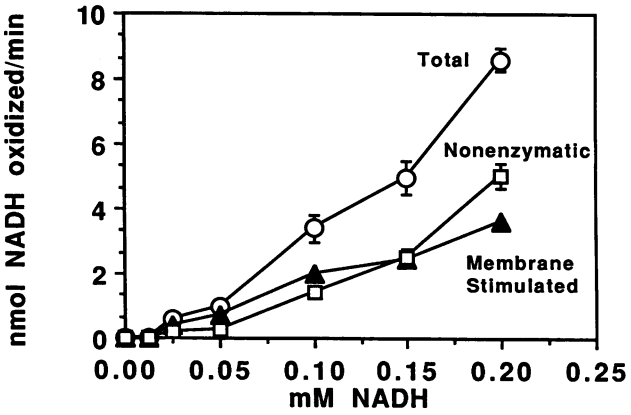

FIG. 4. The NADH dependence of plasma membrane-stimulated vanadate-dependent NADH oxidation in *S. cerevisiae*. NADH oxidation activity was measured in the presence of 0.25 mM vanadate at the indicated concentrations of NADH from 0.025 to 0.2 mM. Data are presented as the mean ± standard error of the mean (n = 4). If error bars are not visible they are within the symbol. For nonenzymatic activity (□), no further additions were made. For total activity (○), 2 μg of protein was added. Plasma membrane-stimulated vanadate-dependent NADH oxidation is calculated as total minus nonenzymatic activity (▲).

oxidized per min (Fig. 3; data not shown). In addition, when equal amounts of molybdate were substituted for vanadate, no stimulation of membrane-mediated vanadate-dependent NADH oxidation activity was observed (data not shown).

An increase in the concentration of NADH resulted in an increase in NADH oxidation activity (Fig. 4). In these experiments the membrane-stimulated vanadate-dependent rate of NADH oxidation was 3.6 nmol of NADH oxidized per min in the presence of 0.2 mM NADH. When equivalent amounts of NADPH were substituted for NADH, the rates of plasma membrane-stimulated vanadate-dependent NADPH oxidation were similar, with 2.4 nmol of NADPH oxidized per min in the presence of 0.2 mM NADPH (data not shown).

We have observed that NADH oxidation activity is a saturating function of plasma membrane protein concentration rather than a linear function (data not shown). These results are similar to those reported by Liochev and Fridovich (10, 11) for experiments using rat liver microsomes. For this reason all of our data are reported as nanomoles of NADH oxidized per minute with the amount of plasma membrane present indicated. Plasma membrane-stimulated vanadate-dependent NADH oxidation was not observed when plasma membranes had been boiled for 5 min. Also, there was no increase in NADH oxidation when bovine serum albumin was substituted for plasma membrane protein.

The consumption of oxygen during plasma membrane-stimulated vanadate-dependent NADH oxidation in *S. cerevisiae*. The requirement of the vanadate-dependent NADH oxidation activity of yeast plasma membranes for molecular oxygen has been previously demonstrated in this laboratory. Both vanadate-dependent nonenzymatic and plasma membrane-stimulated NADH oxidation activity were absent in a nitrogen atmosphere, but NADH oxidation resumed when oxygen was added (13). The consumption of oxygen during the oxidation of NADH in the presence and absence of membrane protein was determined (Fig. 5). The nonenzymatic rate of molecular oxygen (O_2) consumption increased

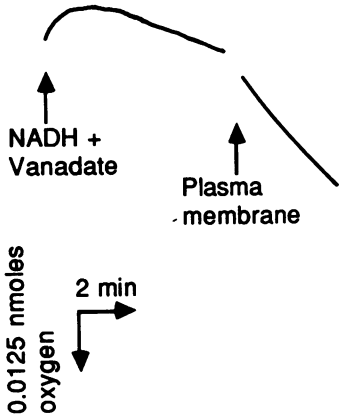

FIG. 5. O_2 consumption during NADH oxidation by *S. cerevisiae* plasma membranes. The consumption of oxygen during NADH oxidation activity was measured in the presence and absence of 20 μg of plasma membrane protein. The assay was done in duplicate using 0.25 mM vanadate and 0.2 mM NADH.

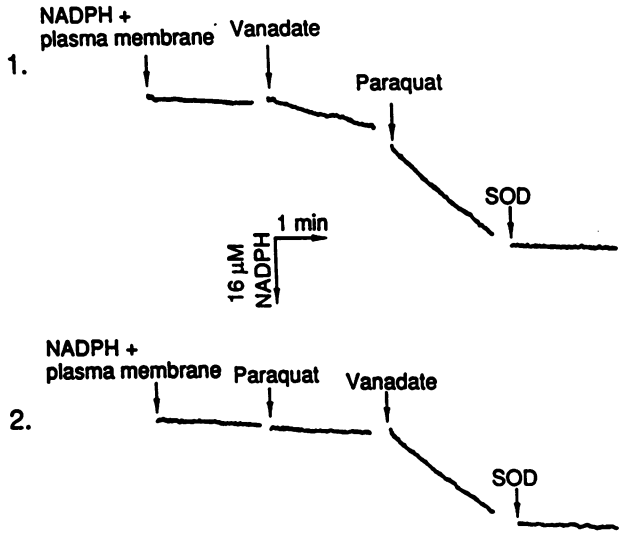

FIG. 6. The effect of PQ on plasma membrane-stimulated vanadate-dependent NADH oxidation in *S. cerevisiae*. For each assay, 0.25 mM vanadate, 10 μg of plasma membrane protein, 0.5 mM PQ, 0.1 mM NADPH, and 10 ng of superoxide dismutase per ml were used. SOD, Superoxide dismutase.

fourfold when plasma membrane protein was added. The average vanadate-dependent membrane-stimulated rate of oxygen consumption during vanadate-dependent oxidation of NADH was 11.4 nmol/min.

NADH oxidation studies were done on the same day to determine the ratio of NADH oxidized to oxygen consumed. In experiments using the same conditions as reported in the legend to Fig. 5, the plasma membrane-stimulated NADH oxidation rate was 5.1 nmol/min, for a ratio of NADH oxidized to O_2 consumed of 0.5. However, the curves obtained under these conditions (cf. Fig. 1 and 5) are not linear for the first 2 min, making it difficult to calculate the slopes. Therefore, both plasma membrane-stimulated oxygen consumption and NADH oxidation were determined under slower reaction conditions during which the curves were linear for the first 2 to 3 min. By using 10 μg of protein, 0.25 mM vanadate, and 0.2 mM NADH in 50 mM phosphate buffer (pH 7.0), plasma membrane-mediated vanadate-dependent oxygen consumption was 5.5 nmol/min and NADH oxidation was 6.1 nmol/min. By using 20 μg of protein, 0.25 mM vanadate, and 0.1 mM NADH in 50 mM phosphate buffer (pH 7.0), oxygen consumption was 3.4 nmol/min and NADH oxidation was 2.9 nmol/min. The ratios of plasma membrane-stimulated vanadate-dependent NADH oxidized to oxygen consumed in the experiments described above were 1.1 and 0.85, respectively. These data are consistent with the expected 1:1 ratio of NADH oxidized to O_2 consumed expected in the proposed scheme (cf. Introduction).

The effects of superoxide dismutase and PQ on plasma membrane-stimulated vanadate-dependent NADH oxidation in *S. cerevisiae*. Superoxide dismutase, which scavenges superoxide anions, strongly inhibited the reaction and abolished membrane-stimulated NADH oxidation activity by 10 ng of superoxide dismutase per ml. Increasing the concentration of superoxide dismutase inhibited all NADH oxidation activity. Manganese (1 μM), another scavenger of superoxide anions, also inhibited this activity (data not shown).

The involvement of the superoxide anion in the vanadate-dependent NADH oxidation activity of yeast plasma membranes was also demonstrated by using PQ (14). PQ (methyl viologen; PQ^{2+}) is reduced to PQ^+ in an NADPH-dependent reaction. The reoxidation of PQ^+ to PQ^{2+} by molecular oxygen generates the superoxide anion. Because the removal of O_2^- by superoxide dismutase abolished vanadate-dependent NADH oxidation by yeast plasma membranes, an increase in the concentration of O_2^- by PQ^{2+} addition should enhance NADPH oxidation activity. This hypothesis is confirmed by the data shown in Fig. 6. There was a substantial (nearly fourfold) increase in NADPH oxidation in the presence of vanadate and plasma membrane protein when PQ^{2+} was added to the reaction. The oxidation of NADPH was abolished when superoxide dismutase was added.

Identification of the form of vanadate which stimulates vanadate-dependent NADH oxidation. The exact oxyvanadium species required for vanadate-dependent NADH oxidation activity has not been determined. It has been suggested that the high rates of vanadate-dependent NADH oxidation activity observed in pH 5.0 buffer are due to the formation of decavanadate (2, 17). Both orthovanadate and decavanadate have been suggested as the vanadate compound responsible for stimulation of NADH oxidation at pH 7.0 (3, 10). It was therefore of interest to identify the vanadate compound(s) present in pH 5.0 and 7.0 reaction buffers. ^{51}V-NMR spectroscopy was used to characterize the form of vanadate responsible for stimulation of the vanadate-dependent NADH oxidation activity in the presence and absence of membrane protein. Because the presence of protein did not change the ^{51}V-NMR spectra obtained by using the various buffers, all of the following spectra are reported in the absence of protein.

Because phosphate is known to interact with vanadate, another buffer system in which to study vanadate-dependent NADH oxidation was sought. Vanadate-dependent NADH oxidation activity was measured in the presence of various reaction buffers (50 mM) at pH 7.0. The membrane-stimulated oxidation of NADH was inhibited in several Tris buffers (hydrochloride, succinate, and acetate) as well as in HEPES and MES (data not shown). The addition of 50 mM phosphate buffer to assays carried out in the presence of HEPES or MES did not prevent the inhibition. With careful

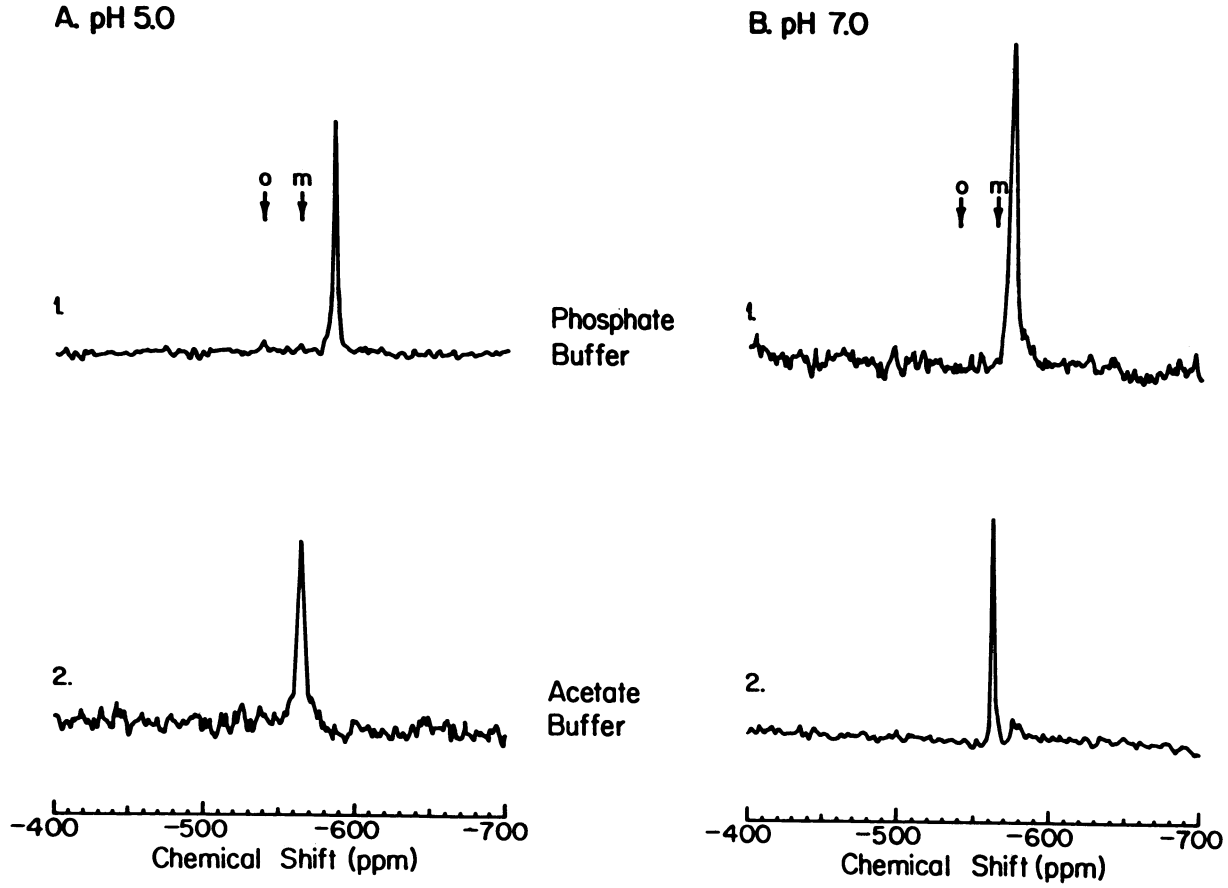

FIG. 7. Identification of the vanadate species responsible for the stimulation of NADH oxidation by ^{51}V-NMR. ^{51}V-NMR spectra were obtained by using the basic procedure as described in Materials and Methods. For each spectrum 0.35 mM orthovanadate was added to the buffer. The arrows indicate the position of the ^{51}V-NMR resonances for sodium orthovanadate (o; −541 ppm) and sodium metavanadate (m; −563 ppm), both in water. (A) pH 5.0 ^{51}V-NMR spectra of vanadate in 50 mM phosphate buffer (tracing 1) and 50 mM acetate buffer (tracing 2). (B) pH 7.0 ^{51}V-NMR spectra of vanadate in 50 mM phosphate buffer (tracing 1) and 50 mM acetate buffer (tracing 2).

attention to pH, membrane-mediated vanadate-dependent NADH oxidation activity (20 to 60% of that observed in phosphate buffer) was not present reproducibly in 50 mM sodium acetate buffer at pH 7.0. Because of the inability of acetate to buffer the highly alkaline orthovanadate solution and the rapid rise in the nonenzymatic vanadate-dependent NADH oxidation activity as the pH is lowered, acetate buffers were not suitable for quantitation studies.

^{51}V-NMR spectra were obtained of 0.35 mM orthovanadate in 50 mM phosphate buffer at pHs 5.0 and 7.0. Phosphate buffer is usually used to study vanadate-dependent NADH oxidation, and membrane-mediated activity is found at pH 7.0 but not at pH 5.0 (Fig. 2). As a control, ^{51}V-NMR spectra of 0.35 mM orthovanadate in 50 mM acetate buffer and HEPES buffer at the same pH values were also obtained. The ^{51}V-NMR spectra are shown in Fig. 7. In each spectrum only one ^{51}V-NMR resonance was observed. The chemical shift of 0.35 mM orthovanadate in water was defined as −541 ppm. The same concentration of metavanadate in water showed a chemical shift of −564 ppm. The positions of these compounds along the chemical shift scale depend on a number of factors; therefore, the parts per million reported in our system may vary slightly from other reported values (9). Orthovanadate (0.35 mM) in 50 mM phosphate buffer at pH 5.0 had a chemical shift of −583 ppm (Fig. 7A). In 50 mM acetate buffer the chemical shift of the vanadium resonance was −563 ppm, characteristic of a metavanadate resonance. The results were the same in HEPES buffer as in acetate (data not shown). Note that at pH 5.0 in these conditions, the characteristic three-resonance ^{51}V-NMR spectrum of decavanadate was not observed. In 50 mM phosphate buffer at pH 7.0, the chemical shift of the single vanadium resonance was −573 ppm (Fig. 7B). The chemical shift of the resonance was −563 ppm in acetate buffer and in HEPES buffer (data not shown).

The ^{51}V-NMR data obtained are consistent with the hypothesis that a phosphate-vanadate anhydride is the oxyvanadium species that stimulates NADH oxidation in phosphate buffers in the presence and absence of protein. The ^{51}V-NMR vanadium resonances in phosphate buffer at pHs 5.0 and 7.0 were shifted upfield by 19 and 9 ppm, respectively. These results are consistent with those obtained by Gresser et al. (8), who reported an upfield chemical shift of 7 ppm for 1 mM vanadate in 50 mM phosphate buffer at pH 8.0. We hypothesize that the upfield chemical shift that we observe is due to the formation of a phosphate-vanadate anhydride and that this is the active form of vanadate for stimulation of the vanadate-dependent NADH oxidation activity in phosphate buffer. When PP_i was substituted for vanadate in the reaction (to replace the putative phosphate-vanadate anhydride), NADH oxidation activity was not detected (data not shown).

DISCUSSION

A vanadate-dependent NADH oxidation activity exists in a number of plant and animal membranes (2–4, 11, 19). The present study demonstrates the existence of this vanadate-dependent activity in plasma membranes isolated from the yeast *S. cerevisiae*. In addition, a phosphate-vanadate anhydride is proposed as the oxyvanadium species responsible for vanadate-dependent NADH oxidation, both in cells and in the phosphate buffer usually used for this assay.

S. cerevisiae plasma membrane-stimulated vanadate-dependent NADH oxidation is similar to that reported for other membranes. The literature in this field is fraught with inaccuracies, and the recent review by Liochev and Fridovich (11) is recommended for putting previous work in the proper perspective. Yeast plasma membranes alone showed a poor rate of NADH oxidation which was stimulated sevenfold in the presence of vanadate. The nonenzymatic oxidation of NADH in the presence of vanadate was increased threefold when yeast plasma membranes were added. The NADH oxidation stimulated by the plasma membranes in the presence of vanadate depends on a functional plasma membrane, since no activity was observed in the presence of boiled membranes or bovine serum albumin. When NADPH was substituted for NADH in the reaction, oxidation of NADPH did occur in the presence of vanadate. The stimulation of NADH oxidation in yeast plasma membranes appeared to be specific for vanadate, since the addition of molybdate caused no detectable stimulation. As reported by other investigators, the plasma membrane-stimulated vanadate-dependent NADH oxidation activity was not a linear function of plasma membrane protein concentration.

The studies reported here show that vanadate-dependent NADH oxidation activity of yeast plasma membranes was accompanied by oxygen uptake, indicating that oxygen is the terminal electron acceptor in the reaction. The involvement of the superoxide anion in the mechanism of NADH oxidation by yeast plasma membranes was also demonstrated. Superoxide dismutase and manganese, which both scavenge superoxide anions, inhibited the vanadate-dependent NADH oxidation activity. The addition of PQ, which produces superoxide in an NADPH-dependent reaction, stimulated the activity. These results indicate that vanadate-dependent NAD(P)H oxidation in yeast plasma membranes occurs by a nonenzymatic process. Specifically, vanadate is likely to couple the production of superoxide by yeast plasma membranes to NAD(P)H oxidation. The generation of superoxide presumably occurs via a membrane-bound NAD(P)H oxidase.

The formation of a phosphate-vanadate anhydride in 50 mM phosphate buffer has been demonstrated by Gresser et al. (8). The formation of mixed phosphate-vanadate anhydrides inhibits myosin ATPase in vitro (7). It is reasonable to speculate that phosphate-vanadate anhydride formation in vivo may affect biological systems despite the low concentration of vanadate in living cells.

The high vanadate-dependent NADH oxidation activity observed in the absence of membrane protein at pH 5.0 suggested that the active species of vanadate may be generated on acidification. Acidic conditions generate the vanadate polymer, decavanadate. It has been postulated (2) that small amounts of decavanadate may form at a low pH, thereby stimulating vanadate-dependent NADH oxidation activity. By using ^{51}V-NMR, we have demonstrated an upfield chemical shift of one vanadate resonance at both pH 5.0 and pH 7.0 in phosphate buffers, which is characteristic of phosphate-vanadate anhydrides (8). At no time did we see the characteristic ^{51}V-NMR spectrum of decavanadate. The chemical shift of the ^{51}V-NMR resonance of the phosphate-vanadate anhydride was pH dependent, as demonstrated by the change in chemical shift at the two pH values. When PP_i was substituted for the phosphate-vanadate anhydride in the reaction, NADH oxidation activity was not detected.

Our ^{51}V-NMR results and literature analysis support the conclusion of Liochev and Fridovich (11) that monomeric vanadate and not decavanadate is the major stimulatory oxovanadium species involved in the membrane-stimulated vanadate-dependent NAD(P)H oxidation activity. We have been unsuccessful in showing an absolute requirement for phosphate, as were other workers (11). In addition, our magnetic resonance studies are consistent with the hypothesis that the both the protein-stimulated and the protein-independent vanadate-dependent NADH oxidation activity studied in phosphate buffers are stimulated by a phosphate-vanadate anhydride.

In summary, the yeast plasma membrane stimulated vanadate-dependent NADH oxidation activity in a manner similar to other membrane systems. The stimulatory oxovanadium species in phosphate buffer may be a phosphate-vanadate anhydride. It is reasonable to expect that such anhydrides would form in cells which have a much higher concentration of phosphate (in millimolar) than of vanadium (in micromolar). The role of this plasma membrane-stimulated vanadate-dependent NADH oxidation in cellular metabolism remains elusive at the present time. We have previously demonstrated that yeast plasma membrane-stimulated vanadate-dependent NADH oxidation, or any other oxidative process, is not the primary mediator of vanadate toxicity in *S. cerevisiae* (12). Further studies are required to ascertain whether membrane-mediated superoxide production is actually coupled to nonenzymatic vanadate-dependent NADH oxidation in any physiological process.

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