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Superoxide-Independent Reduction of Vanadate by Rat Liver Microsomes/NAD(P)H: Vanadate Reductase Activity[1]

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It has been reported that vanadate-stimulated oxidation of NAD(P)H by microsomal systems can proceed anaerobically, in contrast to the general notion that the oxidation proceeds exclusively by an O_2^--dependent free radical chain mechanism. The current study indicates that microsomal systems are endowed with a vanadate-reductase property, involving a NAD(P)H-dependent electron transport cytochrome P450 system. Our ESR measurements demonstrated the formation of a vanadium(IV) species in a mixture containing vanadate, rat liver microsomes, and NAD(P)H. This vanadium(IV) species was identified as the vanadyl ion (VO^{2+}) by comparison with the ESR spectrum of $VOSO_4$. The initial rate of vanadium(IV) formation depends linearly on the concentration of microsomes. The Michaelis–Menten constants were found to be: k_m = 1.25 mM and V_{max} = 0.066 μmol (min)$^{-1}$ (mg microsomes)$^{-1}$, respectively. Pretreatment of the microsomes with carbon monoxide or $K_3Fe(CN)_6$ reduced vanadium(IV) generation, suggesting that the NAD(P)H-dependent electron transport cytochrome P450 system plays a significant role in the microsomal reduction of vanadate. Measurements under argon or in the presence of superoxide dismutase caused only minor (less than 10%) reductions in vanadium(IV) generation. The VO^{2+} species was also detected in NAD(P)H oxidation by fructose plus vanadate, a reaction known to proceed via an O_2^--mediated chain mechanism. However, the amount of vanadium(IV) generated by this reaction was an order of magnitude smaller than that by the microsomal system and was inhibitable by superoxide dismutase, affirming the conclusion that the microsomal/NAD(P)H system is endowed with the (O_2^--independent) vanadium(V) reductase property. © 1992 Academic Press, Inc.

While it is known that vanadate and related compounds of pentavalent vanadium (V(V)) exert potent toxic effects on a wide variety of biological systems, the underlying biochemical mechanism is still not fully understood (1–3). One of the pathways is thought to involve the oxidation of NAD(P)H. Thus, considerable effort has been devoted to finding the mechanism of NAD(P)H oxidation by V(V) (1–15). Erdmann *et al.* (5) first noted that vanadate stimulated the oxidation of NADH by plasma membranes and attributed this effect to a membrane-containing NAD(P)H-dependent V(V) reductase. Later studies by Liochev and Fridovich indicated that endogenous superoxide (O_2^-) plays a central role in this reaction (4, 12, 13). They proposed that NAD(P)H dehydrogenases or oxidases produce O_2^-, which causes V(V) to stimulate NAD(P)H oxidation via the following free radical chain mechanism (4, 12, 13):

$$V(V) + O_2^- \rightarrow V(IV) - OO^\cdot \quad [1]$$

$$V(IV) - OO^\cdot + NAD(P)H \rightarrow V(IV) - OOH + NAD(P)^\cdot \quad [2]$$

$$V(IV) - OOH + H^+ \rightarrow V(V) + H_2O_2 \quad [3]$$

$$NAD(P)^\cdot + O_2 \rightarrow NAD(P)^+ + O_2^- \quad [4]$$

By adding Eqs [1]–[4], the net reaction becomes

$$NAD(P)H + O_2 + H^+ \xrightarrow{V(V)} NAD(P)^+ + H_2O_2. \quad [5]$$

In this model, molecular oxygen acts as the electron acceptor, and V(V) functions as a catalyst and not as a substrate for NAD(P)H dehydrogenases or oxidases. This model also obviates the need to postulate the presence of any V(V)-dependent NAD(P)H oxidase or NAD(P)H-dependent V(V) reductase (4).

Although the above-summarized O_2^--based model can explain much of the data on vanadate-stimulated

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NAD(P)H oxidation by cellular membranes, some literature data indicate other, possibly O_2^--independent, pathways. For example, in 1986 Patole *et al.* (6) reported that the rate of V(V)-dependent NADH oxidation by rat liver microsomes was decreased, but not abolished, by superoxide dismutase (SOD)[3] or under anaerobic conditions. Later Reif *et al.* (11) reported that the orthovanadate-dependent oxidation of NAD(P)H, catalyzed by rat liver microsomes, microsomal NAD(P)H-cytochrome P450 reductase, and NADH-cytochrome b_5 reductase, occurred in an anaerobic system, implying that O_2^- is not an obligate intermediate in the microsomal/NAD(P)H reduction of V(V). More recently, we have reported some data which indicate that the flavoenzymes glutathione reductase, ferredoxin–$NADP^+$ oxidoreductase, and lipoyl dehydrogenase can function as V(V) reductases in cell free systems, independent of O_2^- involvement (16–18). These observations suggested the possibility that some O_2^--independent V(V) reductase could also be operative in microsomal systems. In the present study, therefore, we utilized ESR spectroscopy as a direct technique to follow V(IV) formation. It is found that V(IV) is indeed generated in the reduction of vanadate by rat liver microsomes, with the V(IV) formation exhibiting typical enzymatic kinetics. Measurements under argon, or utilizing SOD, showed that O_2^- does not play any significant role in the NAD(P)H/microsomal reduction of vanadate. Additional measurements utilizing carbon monoxide and $K_3Fe(CN)_6$ suggest that the electron transport cytochrome P450 system plays an important role in this newly detected vanadate reductase behavior of microsomal/NAD(P)H systems.

MATERIALS AND METHODS

(a) Chemicals. Potassium ferricyanide ($K_3Fe(CN)_6$), phosphate buffer (pH 7.2), and ethanol were purchased from Fisher. Bovine superoxide dismutase and fructose were purchased from Sigma. Vanadyl sulfate ($VOSO_4$), and sodium metavanadate ($NaVO_3$), henceforth to be referred to as V(IV) and V(V), were purchased from Aldrich. All chemicals were used as received.

(b) Vanadium(IV) measurements. Vanadium(IV) generation was measured directly via ESR spectroscopy. All ESR measurements were made utilizing a Varian E3 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured (to 0.1 G) directly from the magnetic field separations. Care was taken to use the same flat cell and to maintain the same orientation of the flat cell in the ESR cavity. Absolute vanadium(IV) concentrations were determined by using vanadyl sulfate ($VOSO_4$) as a standard.

(c) Microsome preparation. Microsomes were prepared from livers of Sprague–Dawley rats. Microsome pellets were isolated from the livers by the differential centrifugation method (19, 20). Washed microsomes were suspended in a phosphate buffer (pH 7.2) solution, stored at 4°C, and used within 2 days. The microsomal protein concentration was measured colorimetrically by the method of Lowry *et al.* (21). Carbon monoxide-treated microsomes were obtained by bubbling carbon monoxide gas (99%) through a microsome solution for about 3 min. Inactivated microsomes were obtained by keeping the microsome-containing glass tube in boiling water for about 5 min.

The reactants were mixed in test tubes with a total final volume of 250 μl. The reaction mixture was then transferred to a flat cell for ESR measurement. The concentrations given in the figure legends are final concentrations. All the experiments were carried out at room temperature except those specifically indicated.

RESULTS

I. Evidence for V(IV) Generation by NAD(P)H/Microsomal Reduction of Vanadate: Comparison with VO^{2+}

Figure 1a shows a typical ESR spectrum obtained from a mixture of 4.8 mM $NaVO_3$, 4 mM NADH and 3.8 mg/ml microsomes in a pH 7.2 phosphate buffer. The spectrum exhibits an eight-line hyperfine structure, which is characteristic of the vanadyl (VO^{2+}) ion, a V(IV) species containing a $3d^1$ electron coupled to its nuclear spin (V^{51} $I = 7/2$, $a_{iso} = 118.7$ G) (22). V(V), with no unpaired d electrons, exhibits no ESR signal. To verify the signal assignment, we recorded the ESR spectrum of 4 mM $VOSO_4$ in the presence of NADH/microsomes. As may be seen in Fig. 1b, the lineshapes and splittings (a_{iso} = 119.0 G) of the obtained spectrum were very close to those of Fig. 1a, implying that the V(IV) species generated from $NaVO_3$ by the microsomal/NADH system is basically the vanadyl (VO^{2+}) moiety.

II. Evidence for the V(IV) Generation Being Enzymatic

The V(IV) generation progresses linearly as a function of time (Fig. 2a). Inactivated microsomes, prepared by

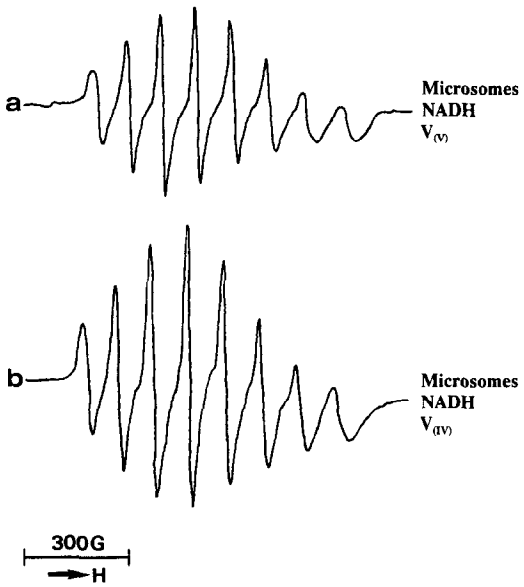

FIG. 1. (a) ESR spectrum recorded 30 min after mixing, in 60 mM phosphate buffer (pH 7.2, 60 mM), 4.8 mM $NaVO_3$, 4 mM NADH, and 3.48 mg/ml microsomes; (b) same as (a) but with 4 mM $VOSO_4$ instead of $NaVO_3$.

[3] Abbreviation used: SOD, superoxide dismutase.

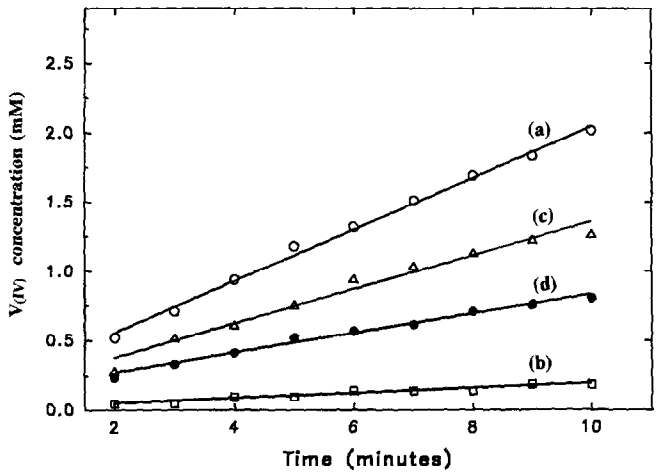

FIG. 2. Time course of vanadium(IV) formation: (a) reaction mixture containing 4.8 mM $NaVO_3$, 4 mM NADH, and 3.48 mg/ml microsomes in 60 mM phosphate buffer (pH 7.2); (b) same as (a) but using microsomes kept in boiling water for 5 min; (c) same as (a) but with 0.5 mM $K_3Fe(CN)_6$ added; (d) same as (a) but using carbon monoxide-pretreated microsomes.

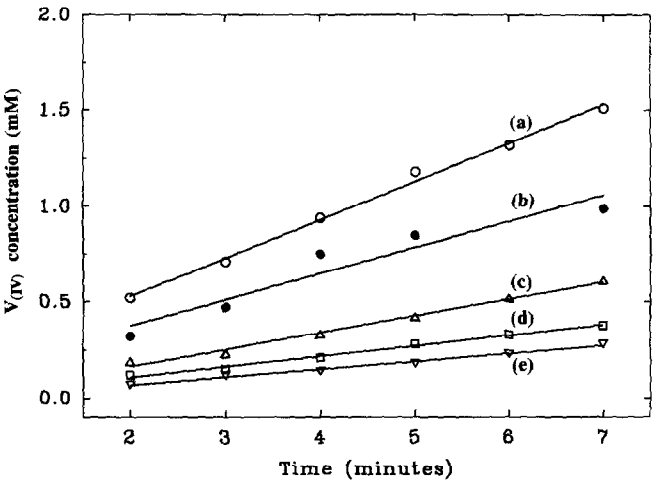

FIG. 4. Effect of microsome concentration on V(IV) generation and its time course. (a) Reaction mixture containing 4.8 mM $NaVO_3$, 4 mM NADH, and 3.48 mg/ml microsomes in 60 mM phosphate buffer (pH 7.2); (b) same as (a) but 1.74 mg/ml microsomes; (c) same as (a) but 0.87 mg/ml microsomes; (d) same as (a) but 0.44 mg/ml microsomes; (e) same as (a) but 0.22 mg/ml microsomes.

keeping them in boiling water for 5 min, did not generate any significant amount of V(IV) (Fig. 2b). V(IV) generation depends also on the presence of NADH as a cofactor, increasing with NADH concentration (Fig. 3a–3d). Omission of NADH eliminates V(IV) generation (Fig. 3d). Replacement of NADH by NADPH causes only about 30% reduction in the amount of V(IV) generation (Fig. 3e). Together, these data indicate that this V(IV) generation is an enzyme-catalyzed reaction. Further support for this interpretation was provided by the following kinetics data.

It is known that enzyme-catalyzed reactions evidence a linear dependence of initial rate upon enzyme concentration, provided that the substrate concentration greatly exceeds the enzyme concentration (4). Figure 4 shows the time course of V(IV) generation as a function of microsome concentration, decreasing order from (a) to (e). The initial rates (i.e., initial slopes of the plots in Fig. 4) are plotted in Fig. 5 as a function of microsome concentration. As may be noted from Fig. 5, the initial rates do increase linearly with microsome concentration.

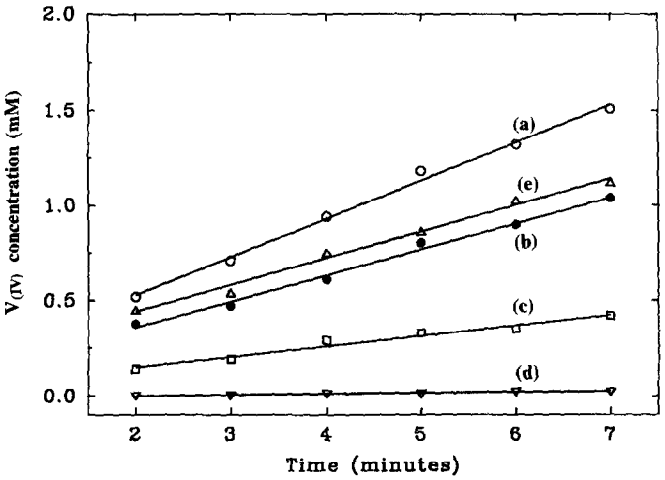

FIG. 3. Time course of the effect of NADH and NADPH concentration on vanadium(IV) generation: (a) reaction mixture containing 4.8 mM $NaVO_3$, 4 mM NADH, and 3.48 mg/ml microsomes in 60 mM phosphate buffer (pH 7.2); (b) same as (a) but 2 mM NADH; (c) same as (a) but 0.5 mM NADH; (d) same as (a) but without NADH; (e) same as (a) but utilizing NADPH (4 mM) instead of NADH. Note the decrease in the initial slope (i.e., initial rate) with increase in the NADH concentration from (a) to (d).

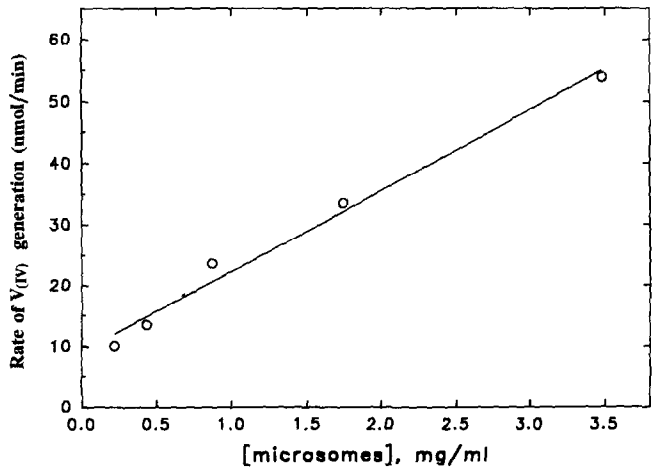

FIG. 5. Effect of microsome concentration on the rate of vanadium(IV) formation.

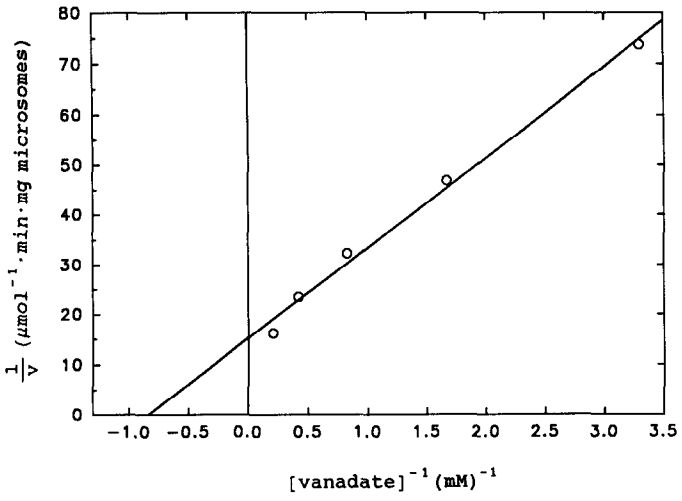

FIG. 6. Determination of the Michaelis–Menton constants (k_m and V_{max}), using the Michaelis–Menton equation, Eq. (6) in the text. The intercept on the abscissa yields ($-1/k_m$) while the ordinate gives ($-1/V_{max}$), with k_m = 1.25 mM and V_{max} = 0.066 μmol (min)$^{-1}$ (mg microsomes)$^{-1}$.

For further analysis, a double-reciprocal plot was made for the initial velocity of V(IV) formation (V_i) as a function of V(V) concentration [S] (Fig. 6) according to the well known Michaelis–Menten equation,

$$\frac{1}{V_i} = \frac{k_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}, \qquad [6]$$

where k_m is the apparent Michaelis constant and V_{max} is the maximal velocity. As may be noted from Fig. 6, the double-reciprocal plot was found to be linear, in conformity with Eq. (6). From the intercept on the abscissa we found that k_m = 1.25 mM, which is well within the range (0.01–10.0 mM) for most enzymatic reactions (23). V_{max} was found to be 0.066 μmol (min)$^{-1}$ (mg microsomes).

III. Cytochrome P450 Involvement in Microsomal Reduction of Vanadate

Two tests indicated the role of NAD(P)H-dependent electron transport cytochrome P450 system in the microsomal reduction of vanadate to V(IV). First, addition of $K_3Fe(CN)_6$, an inhibitor of the electron transport cytochrome P450 system, reduced the rate of V(IV) generation (Fig. 2c). Second, carbon monoxide is known to bind the reduced form of cytochrome P450 and inhibit its enzymatic activity (24, 25). Carbon monoxide pretreatment of the microsomes also caused a substantial reduction in V(IV) generation (Fig. 2d), providing further support for the involvement of the NAD(P)H-dependent electron transport cytochrome P450 system in the mechanism of vanadate reduction.

IV. Comparison with an O_2^--Involving System

In order to investigate the role of O_2^- in V(IV) generation, we examined the V(IV) formation in the reaction between NADH and V(IV) plus fructose, and its possible inhibition by SOD. As shown by Liochev and Fridovich (19), V(V) stimulates NAD(P)H oxidation in the presence of sugars such as fructose with the reaction being mediated by O_2^-. We therefore compared V(IV) generation in this reaction with that from the NAD(P)H/microsomal system. For ease of comparison, the ESR spectrum of V(IV) generated from the microsomal system is shown in Fig. 7a. Figure 7b shows that a mixture of V(V), fructose, and NADH does generate V(IV), but about an order of magnitude lower in intensity. The similarity of the lineshape and hyperfine structure of the spectrum in Fig. 7b to those obtained using $VOSO_4$ (Fig. 1b) indicates that in the case of fructose the detected V(IV) species is also

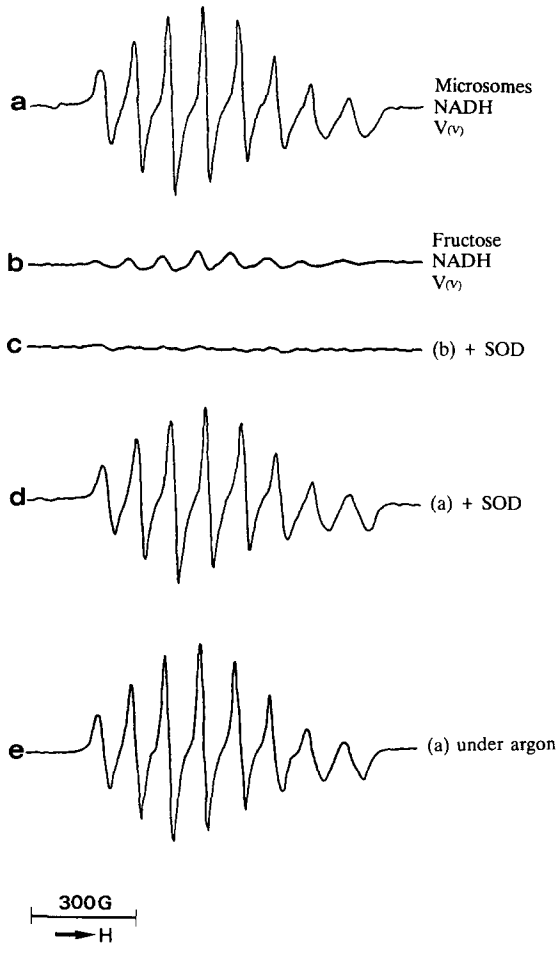

FIG. 7. (a) ESR spectrum recorded 30 min after mixing, in 60 mM phosphate buffer (pH 7.2, 60 mM), 4.8 mM $NaVO_3$, 4 mM NADH, and 3.48 mg/ml microsomes; (b) same as (a) but reaction mixture containing 4.8 mM $NaVO_3$, 4 mM NADH, and 40 mM fructose in 60 mM phosphate buffer (pH 7.2); (c) same as (b) but with 15 units/ml SOD added; (d) same as (a) but with 100 units/ml SOD added; (e) same as (a) but under anaerobic (argon) atmosphere.

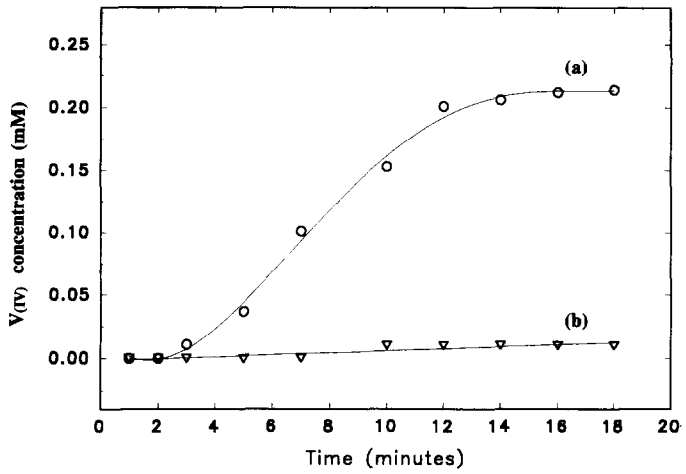

FIG. 8. Time course of vanadium(IV) generation from: (a) a mixture containing 4.8 mM $NaVO_3$, 4 mM NADH, and 40 mM fructose in 60 mM phosphate buffer (pH 7.2); (b) same as (a) but with 15 units/ml SOD added.

the vanadyl VO^{2+} ion. However, the V(IV) generation in this reaction did not increase linearly with time (Fig. 8a), in conformity with a free radical-mediated reaction (4, 19). As expected (19), the V(IV) generation became nondetectable when SOD was added (Figs. 7c and 8b). In contrast, for the microsomal system, addition of SOD or measurements under argon did not significantly alter V(IV) generation (Figs. 7d and 7e), showing that NAD(P)H/microsomes have the function of V(V) reductase, without any significant O_2^- involvement.

DISCUSSION

The overall goal of this study was to search for an O_2^--independent pathway in the reduction of vanadate by rat liver microsomes in the presence of NAD(P)H. Our strategy was to monitor the one-electron reduction of vanadate by measuring V(IV) directly via ESR spectroscopy, in contrast to most earlier studies wherein the focus was on monitoring NAD(P)H oxidation (4–15, 19). We find that the V(IV) generation progresses linearly as a function of time, suggesting the mechanism to be enzyme-catalyzed, rather than a free radical-mediated chain reaction (4). The rate of V(IV) generation is found to increase linearly with increase in microsome concentration, also as expected for an enzymatic process. The V(IV) formation exhibited Michaelis–Menten (enzymatic) kinetics. Measurements utilizing SOD, fructose, or under argon atmosphere demonstrated that O_2^- was not significantly involved in the V(IV) generation. Utilization of carbon monoxide or $K_3Fe(CN)_6$ clearly indicated an important role for the NAD(P)H-dependent cytochrome P450 electron transport system in the NAD(P)H/microsomal reduction of V(V).

Since microsomal enzymes have the capability to generate O_2^-, it was considered that these enzymes might cause the V(V)-dependent NAD(P)H oxidation via the O_2^- radical-mediated chain reaction. However, as discussed in the results section, addition of SOD to the reaction mixture or carrying out measurements under anaerobic conditions did not significantly alter the extent of vanadate reduction. Thus our data do not support the hypothesis that the V(V)-stimulated NAD(P)H oxidation by microsomal enzymes is due to O_2^- produced by membrane-associated NAD(P)H oxidase, followed by the free radical chain oxidation of NAD(P)H by O_2^- plus V(V). The data presented in this paper indicate that the NAD(P)H-dependent V(V) reductases or V(V)-dependent NAD(P)H oxidases also contribute V(V)-dependent NAD(P)H oxidation, independent of O_2^- involvement.

The possible coexistence of the above two mechanisms, the free radical-mediated chain reaction (Eqs. [1]–[6]) (4, 12, 13) and the enzyme-catalyzed reaction, might explain an earlier observation that the rate of V(IV)-dependent NADH oxidation by rat liver microsomes was decreased, but not abolished, in the presence of SOD or under anaerobic conditions (6, 11), as well as vanadate-stimulated NAD(P)H oxidation by rat liver microsomes and microsomal NAD(P)H-dependent cytochrome b_5 reductase.

In conclusion, the present study demonstrates that (i) V(IV) is generated in the microsomal reduction of V(V) in the presence of NAD(P)H; (ii) this V(IV) species is likely the vanadyl ion, VO^{2+}; (iii) the NAD(P)H-dependent electron transport cytochrome P450 system is involved in the V(IV) generation; (iv) the V(IV) formation exhibits typical enzymatic kinetics; (v) O_2^- radical is not significantly involved in the V(IV) generation; (vi) the microsomal system is endowed with the (O_2^--independent) V(V) reductase or V(V)-dependent NAD(P)H oxidase. The present study thus reports on a new vanadate reductase property for the microsomal system, and, therefore, also a biochemical pathway for cellular redox reactions of vanadate.

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