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One-Electron Reduction of Vanadium(V) by Flavoenzymes/NADPH[1]

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The biochemical mechanism underlying vanadate-stimulated NAD(P)H oxidation is controversial. Some reports favor an exclusive role for a superoxide (O_2^-)-mediated radical chain reaction, while others cite data that suggest a contribution from O_2^--independent enzymatic pathways. We recently reported that a vanadium(IV) species accumulates over a period of about 30 min in phosphate-buffer mixtures of vanadate, NAD(P)H, and a flavoenzyme such as glutathione reductase, lipoyl dehydrogenase, or ferredoxin-NADP$^+$ oxidoreductase. The concentration of this vanadium(IV) species was found to depend critically on the simultaneous presence of the enzyme and NAD(P)H, but not on superoxide dismutase, or a nitrogen atmosphere. It was thus concluded that the flavoenzyme/NAD(P)H system acts as a vanadate reductase. However, a subsequent report put forth an alternative hypothesis in which the accumulation of this vanadium(IV) species is ascribed to *direct* reduction of vanadate by NAD(P)H itself, starting when buffer-dissolved molecular O_2 and H_2O_2 have been depleted. We have reexamined our earlier data, and carried out new measurements to evaluate the effect of dissolved oxygen and related factors on the kinetics of vanadium(IV) generation in vanadate/NAD(P)H/flavoenzyme mixtures. The new data support our earlier suggestion that the above-mentioned flavoenzymes can indeed act as NAD(P)H-dependent vanadate reductases. © 1993 Academic Press, Inc.

It has been known for more than a decade that vanadium(V) stimulates the oxidation of NADH by plasma membranes (1) but the underlying mechanism is still not fully understood. This effect has been attributed to a membrane-associated NADH-dependent vanadium(V) oxidoreductase (1). Subsequent studies suggest that vanadium(V) can accept an electron from flavoprotein dehydrogenase that oxidizes NADH (2). The phenomenon of NAD(P)H oxidation coupled to vanadium(V) reduction has also been demonstrated in other biological systems (3–6). A recent electron spin resonance (ESR) study from our laboratory reported on the accumulation of vanadium(IV) during the reduction of vanadate (vanadium(V)) by the flavoenzymes glutathione reductase (GSSG-R),[4] lipoyl dehydrogenase, and ferredoxin–NADP$^+$ oxidoreductase in the presence of NAD(P)H (7). It was concluded therein (7) that these flavoenzymes function as NAD(P)H-dependent vanadium(V) reductases. More recently, however, Liochev and Fridovich (8) have argued that the ESR data (7) do not demonstrate that the investigated flavoenzymes act as vanadate reductases. They suggest an alternative mechanism in which the flavoenzyme simply serves as a generator of superoxide (O_2^-) radical, which acts as a propagator of the radical chain reaction leading to NAD(P)H oxidation (9, 10), as summarized below:

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

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

$$V(IV)—OOH + H^+ \rightarrow V(V) + H_2O_2 \quad [c]$$

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

By adding [a]–[d], the net reaction becomes

$$NAD(P)H + O_2 + H^+ \rightarrow NAD(P)^+ + H_2O_2 \quad [e]$$

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[4] Abbreviations used: GSSG-R, glutathione reductase; DMPO, 5,5-dimethyl-l-pyrroline-*N*-oxide; DTPA, diethylenetriaminepentaacetic acid.

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According to this mechanism, vanadium(V) functions as a catalyst and not a substrate for the enzymes: there is no net change in the vanadium oxidation state, molecular oxygen being the electron acceptor in the NAD(P)H oxidation step. This mechanism, therefore, predicts no significant accumulation for any vanadium(IV) species, in contrast to the above-mentioned ESR results (7).

In order to explain our observed vanadium(IV) ESR signals from the reduction of vanadium(V) by the flavoenzymes/NAD(P)H system (7), the following hypothesis was proposed (8). Since the ESR measurements were made utilizing long- and narrow-bore quartz tubes, fore atmospheric O_2 had almost no access to the bulk of the reaction volume. The observed formation of vanadium(IV) must, therefore, have been a result of the exhaustion of dissolved O_2 and of any accumulated H_2O_2, followed by direct reduction of vanadium(V) by NAD(P)H, independent of enzyme involvement (8). It was also stressed that it is not possible to detect accumulation of vanadium(IV) until both O_2 and H_2O_2 are exhausted (8), because vanadium(IV) rapidly autoxidizes in phosphate-buffered solutions to generate vanadium(V) and O_2^- (8–10) according to the reaction

$$V(IV) + O_2 \rightarrow V(V) + O_2^- \qquad [f]$$

As summarized below, our new measurements do not support this model but are in agreement with our earlier contention that flavoenzymes such as GSSG-R can act as NAD(P)H-dependent vanadate reductases (7).

MATERIALS AND METHODS

Phosphate buffer (pH 7.2), $VOSO_4$ (as a source of vanadium(IV)), and sodium vanadate ($NaVO_3$) (as a source of vanadium(V)) were purchased from Fisher. GSSG-R from bovine intestinal mucosa and NADPH were purchased from Sigma. 1,1-Diphenyl-2-picrylhydrazyl (an ESR g-value and concentration standard) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) were purchased from Aldrich. Diethylenetriaminepentaacetic acid (DTPA), xanthine, and xanthine oxidase (from bovine liver) were purchased from Sigma. Chelex-100 chelating resin was purchased from Bio-Rad. The phosphate buffer was treated with Chelex-100 to remove possible transition metal ion contaminants. DMPO solution was purified using activated charcoal until free radical impurities disappeared as verified by ESR spectroscopy.

ESR measurements were made with a Varian E3 ESR spectrometer utilizing a quartz flat cell (purchased from Wilmad). Hyperfine splittings were measured directly from magnetic field separations. The concentrations given in the figure legends were the final concentrations. All experiments were carried out at room temperature essentially as reported earlier (7).

RESULTS

(a) Does O_2 Play a Significant Role in the Generation of Vanadium(IV) in a Mixture of Vanadium(V), GSSG-R, and NADPH?

Figure 1a shows a typical ESR spectrum obtained at 32 min after mixing 2.5 mM $NaVO_3$, 2.5 mM NADPH, and 15 units/ml GSSG-R in a phosphate buffer (pH 7.2).

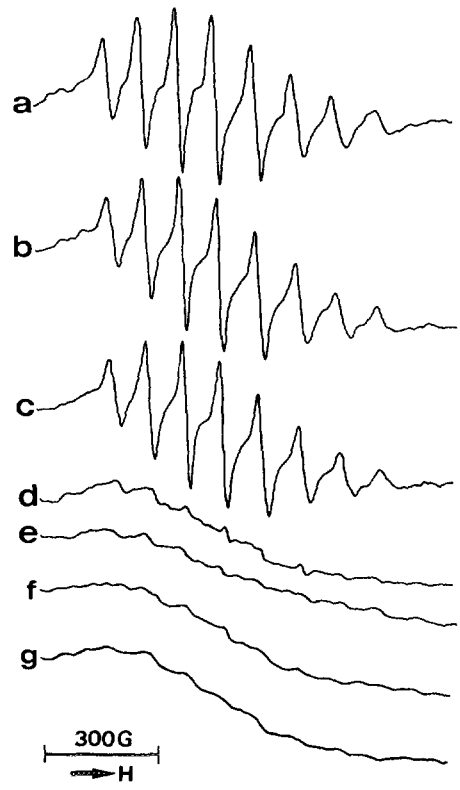

FIG. 1. (a) ESR spectrum recorded 35 min after mixing in a phosphate buffer solution (pH 7.2, 100 mM) of 2.5 mM $NaVO_3$, 2.5 mM NADPH, and 15 units/ml GSSG-R. The mixture was transferred to a flat cell immediately after the reactants were mixed in a test tube and kept in the flat cell until measurement. (b) The sample in (a) was removed from the flat cell after ESR measurement and shaken in a test tube under ambient atmosphere for about 2 min and then put back into the flat cell for ESR measurement. (c) Same as (a) but the sample was shaken in a test tube under ambient air for 35 min before transferring to the flat cell for ESR measurement. (d) Same as (a) but without GSSG-R. (e) Same as (c) but without GSSG-R. (f) Same as (a) but without NADPH. (g) Same as (c) but without NADPH. The spectrometer settings were: receiver gain, 2.0×10^5; modulation amplitude, 10 G; scan time, 200 s; magnetic field, 3480 ± 1250 G; time constant, 0.3 s.

For this measurement the reactants were mixed in a test tube in a total final volume of 250 μl and were then transferred to a flat cell for ESR measurement. The spectrum in Fig. 1a exhibits an eight-line hyperfine structure, which is characteristic of the vanadyl (VO^{2+}) ion, a vanadium(IV) species containing a $3d^1$ electron coupled to its nuclear spin (V^{51}, $I = 7/2$, $a_{iso} = 118.7$ G) (11). The time course of vanadium(IV) generation from a mixture containing vanadium(V), NADPH, and GSSG-R in a phosphate buffer (pH 7.2) was in agreement with that reported earlier (7). At 30 min after the reaction initiation, the sample was taken out of the flat cell and shaken in air for about 2 min. The sample was then put back into the flat cell for ESR measurements. Figure 1b shows the spectrum obtained, which is similar to that in Fig. 1a, indicating no significant role for dissolved oxygen.

Additionally, ESR measurements were made on reaction mixtures prepared in standard glass tubes that were

stirred every 2 min in ambient air. After 30 min the top portion of the mixture was transferred to a flat cell for ESR measurement. Figure 1c shows a typical spectrum obtained from such samples. The intensity of the spectrum in Fig. 1c is essentially the same as those in Figs. 1a and 1b, demonstrating that dissolved oxygen plays no significant role in the mechanism of vanadium(IV) generation by the GSSG-R/NAD(P)H/vanadate system.

(b) Does a Mixture of NADPH and Vanadium(V) without a Flavoenzyme in Narrow Tubes Generate Vanadium(IV)?

Measurements similar to those described above were carried out without employing GSSG-R. Figure 1d shows the spectrum obtained from a sample which was stored in the ESR flat cell for 30 min prior to measurement. Figure 1e shows the corresponding spectrum from the sample kept in a test tube that was shaken every 2 min during a period of 30 min, and transferred to the flat cell just before measurement. The similarity of the spectra in Figs. 1d and 1e shows that no significant amount of vanadium(IV) is generated in the absence of the enzyme, regardless of whether the sample is stored in a flat cell or in a test tube under ambient air with mechanical shaking for 30 min. Similarly, a mixture containing vanadium(V) and GSSG-R without NADPH did not generate any significant amount of vanadium(IV) (Figs. 1f and 1g).

(c) Does Vanadium(IV) Rapidly Autoxidize in a Phosphate Buffer?

In order to measure the rate of autoxidation of vanadium(IV) in a phosphate buffer (pH 7.2), ESR measurements were made on a phosphate buffer (pH 7.2) containing 2 mM $VOSO_4$ at time intervals of 5, 30, and 45 min. During this period, the $VOSO_4$ solution was mechanically shaken in a test tube under ambient air. As shown in Fig. 2, the vanadium(IV) spectrum exhibited no significant change due to air exposure for at least 45 min. Further, to examine the validity of step [f] (9, 10), we employed the ESR spin trapping methodology (12, 13) with the view of detecting O_2^- generation. As shown in Fig. 3a, a mixture of 4 mM $VOSO_4$ and 100 mM DMPO did not generate any significant ESR spin adduct signal. The broad peak in Fig. 3a is the fourth ($m_I = -\frac{1}{2}$) of the eight hyperfine lines of vanadium(IV). To check the sensitivity our O_2^- detecting system, a mixture of xanthine and xanthine oxidase was used as a source of O_2^- radical. As shown in Fig. 3b, this mixture yielded a spin adduct spectrum with hyperfine splittings of a_N = 14.2 G, a_H^β = 11.2 G, and a_H^γ = 1.3 G. These splittings are typical of the DMPO/O_2^- spin adduct (14), indicating that our detection system was functioning well. This result clearly suggests that the rate of vanadium(IV) autoxidation in a phosphate buffer is so slow that it leads to no observable change in vanadium(IV) concentration over a 30- to 40-min period, thus arguing against the validity of step [f].

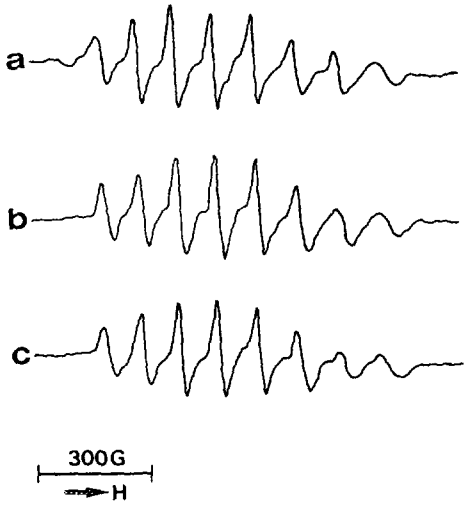

FIG. 2. Time dependence of ESR spectra of vanadium(IV) in a phosphate buffer (pH 7.2, 100 mM). ESR spectra were recorded from 2 mM $VOSO_4$ after aerating the sample for (a) 5 min, (b) 30 min, and (c) 45 min. The sample was stirred in a test tube under ambient air during these time intervals. The spectrometer settings were: receiver gain, 2.0 × 10^5; modulation amplitude, 2G; scan time, 200 s; magnetic field, 3480 ± 1250 G; time constant, 0.3 s.

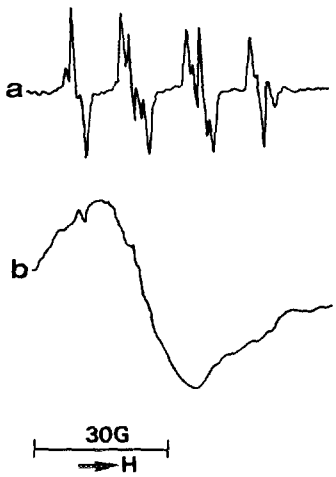

FIG. 3. (a) ESR spectrum recorded 2 min after mixing in a phosphate buffer solution (pH 7.2, 100 mM) of 1 mM xanthine, 0.05 unit/ml xanthine oxidase, 0.15 mM DTPA, and 150 mM DMPO. (b) ESR spectrum recorded 2 min after mixing in a phosphate buffer (pH 7.2, 100 mM) of 4 mM $VOSO_4$ and 150 mM DMPO. The spectrometer settings were: receiver gain, 2.0 × 10^5; modulation amplitude, 0.5 G; scan time, 200 s; field, 3480 ± 100 G; time constant, 0.3 s.

DISCUSSION

The present investigation was undertaken with the view of examining the following questions:

(a) Does vanadium(IV) form in a reaction mixture containing vanadate, GSSG-R, and NADPH under ambient air?

(b) Does a mixture of NADPH and vanadate without the flavoenzyme generate vanadium(IV) under aerobic or anaerobic conditions?

(c) Does vanadium(IV) rapidly autoxidize in a phosphate-buffered solution so that it cannot be detected within a few minutes of air exposure?

The previous section summarizes our new measurements addressing these questions in the same order. While this report presents our data for only GSSG-R, the other two flavoenzymes (lipoyl dehydrogenase and ferrodoxin–$NADP^+$ oxidoreductase) exhibit similar vanadium(V) reductase activity. The present study thus establishes the following results. (a) All three flavoenzymes are capable of reducing vanadate to generate vanadium(IV) in the presence of NAD(P)H under ambient air. (b) Without the enzyme, NAD(P)H produces little, if any, vanadium(IV) ESR detectable species over a period of 45 min. (c) In phosphate buffer, vanadium(IV) autoxidation rate is so slow that it causes no significant change in vanadium(IV) concentration over a 40-min period as measured by ESR.

The conclusion that there are O_2^--independent enzymatic pathways for vanadate reduction/NADH oxidation is supported by several earlier reports (3–6, 15–22). In particular, it has been reported that microsomal enzymes can directly reduce vanadium(V) to vanadium(IV) in the presence of NAD(P)H (17). Vanadium(V) causes efficient NADH oxidation in intact plant mitochondria and sonicated rat liver mitochondria (18, 19). This process was found to be insensitive to rotenone, antimycin A, and cyanide (19). The lack of inhibition by rotenone, which blocks the respiratory chain at the site before ubiquinone, excluded the possibility of O_2^- involvement (20). Similarly, it has been reported that superoxide dismutase does not inhibit completely the reduction of vanadium(V) by NAD(P)H in the presence of rat lung (21) or liver microsomes (15–17), as well as human term placental microsomes (22).

The O_2^--dependent mechanism (outlined in [a]–[d]) cannot explain the observed *accumulation* of vanadium(IV)-containing species in a mixture of vanadium(V), GSSG-R, and NADPH. Liochev and Fridovich (8) attribute the detection of vanadium(IV) in our earlier study (7) to an artifact of our ESR measurement procedure: use of long, narrow ESR tubes, and exhaustion of dissolved O_2 and of any accumulated H_2O_2 followed by *direct* reduction of vanadium(V) by NAD(P)H, independent of any enzyme involvement (8). The results obtained in the present study disagree with this proposal. Instead, they demonstrate that a mixture of vanadium(V), GSSG-R, and NAD(P)H in phosphate-buffered solutions does generate vanadium(IV) under ambient air, that the enzyme has a crucial role in the vanadium(V) reduction process, and that O_2^- has relatively minor, if any, role in the amount of the vanadium(IV) species generated.

In conclusion, this investigation supports our earlier suggestion (7) that flavoenzymes such as GSSG-R can function as NAD(P)H-dependent vanadium(V) reductases. We note, however, that any direct relationship of this result to the overall scheme of the vanadate-stimulated NAD(P)H oxidation process has not been discussed here since this issue was not the focus of our earlier study (7) to which this communication is addressed.

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