Effects of Vanadate on the Molybdoproteins Xanthine Oxidase and Nitrate Reductase: Kinetic Evidence for Multiple Site Interaction

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The inhibition of the activity of xanthine oxidase by vanadate was strikingly similar to vanadate inhibition of another molybdoprotein nitrate reductase. Although the main catalytic activity of both enzymes was inhibited, the partial NADH oxidase activity associated with these enzymes was stimulated several fold. It appears that the metal ion binds at multiple site in both enzymes. In the absence of any enzymes a combination of vanadium (V) and molybdenum (V) in air was found to oxide NADH rapidly.

Introduction

There have been a number of recent reports on the inhibition by vanadate of various enzymes involved in phosphate transfer or in hydrolysis of the pyrophosphate bond [1–3], especially the membrane bound ATPase [4, 5]. It has also been shown that vanadate forms an inactive complex with algal nitrate reductase, a molybdoprotein [6]. The inhibition was shown to be completely reversible by treatment with ethylenediamine tetraacetate (EDTA) and ferricyanide.

The present report describes further studies of the effect of vanadate on nitrate reductase and on another molybdoprotein xanthine oxidase. Both xanthine oxidase and nitrate reductase are flavo-molybdoproteins, but they contain different forms of iron. Xanthine oxidase catalyses the oxidation of xanthine or hypoxanthine with oxygen as the electron acceptor, while nitrate reductase catalyses the reduction of nitrate with reduced pyridine nucleotide as the electron donor. In xanthine oxidase, the substrates xanthine and hypoxanthine have been shown to interact at the molybdenum site [7, 8] and the sequence of electron transfer through other redox centers in the enzyme is thought to occur as shown in Eqn (1):

$$\text{xanthine} \rightarrow \text{Mo(VI)} \rightarrow \text{Fe(III)/S} \rightarrow \text{FAD} \rightarrow \text{O}_2 \quad (1)$$
$$\uparrow$$
$$\text{NADH}$$

In contrast, molybdenum in nitrate reductase is the site of reduction. That is, it forms the terminal of the electron transport chain in the enzyme as shown in Eqn (2):

$$\text{NADH} \rightarrow \text{FAD} \rightarrow \text{cyt}_{b\,557} \rightarrow \text{Mo(VI)} \rightarrow \text{NO}_3^- . \quad (2)$$

Materials and Methods

The following reagents were used: xanthine oxidase from buttermilk (Grade III Sigma 1.2 U/mg), $NaVO_3$, $MoCl_5$, hypoxanthine and xanthine from Merck, NADH (Grade I) Boehringer.

Xanthine oxidase was assayed in a total volume of 1.55 ml containing 100 µmol of potassium phosphate, pH 7.6, 2.0 µmol hypoxanthine, 10 µl (60 µg protein) xanthine oxidase, and $NaVO_3$ at indicated concentrations. The change in oxygen concentration was monitored with a Clark oxygen electrode (Yellow Springs) in a Gilson Medical Electronics oxygraph (Model KIC-oxy) at 25°. Values of oxygen concentrations in solutions were not corrected for the effect of ionic solutes.

Nitrate reductase from *Chlorella vulgaris* was purified essentially according to the procedure of Solomonson [9]. The enzyme used in these studies had a specific activity in the range of 85–90 units per mg protein. NADH: cytochrome c and NADH: NO_3^- reductase activities were assayed at 20° as previously described [10].

A sample of Ferredoxin: $NADP^+$ reductase purified from spinach according to the procedure of Gewitz and Völker [11] was a gift from H.-S. Gewitz. The NADH or NADPH oxidation was

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followed at 20° either with the oxygraph or spectrophotometrically at 340 nm with a Zeiss DM4 recording spectrophotometer. In the latter case, appropriate solutions containing metal ions were used as reference blanks.

H_2O_2 was determined with *o*-dianisidine and peroxidase as described by Bernt and Bergmeyer [12].

For anaerobic reactions, all solutions were prepared from distilled water that was boiled, purged with argon (99.9%) and cooled under this atmosphere. Solutions were stored and used under argon atmosphere.

Results and Discussion

Inhibition of xanthine oxidase by vanadate

The inhibition of nitrate reductase by vanadate ($NaVO_3$) has been described previously [6]. Vanadate was also found to inhibit the oxidation of hypoxanthine or xanthine by xanthine oxidase. Fig. 1a shows the development of inhibition with time during the oxidation of hypoxanthine. As noted earlier for nitrate reductase [6], the inhibition increased progressively with time. This suggests that the inhibition was established during the turnover of the enzyme. In analogy with nitrate reductase, it is probably the molybdenum (Mo) reduced form of the enzyme which binds vanadate. There was little extra inhibitory effect if the enzyme was preincubated with vanadate alone (up to 1 mM). This is as expected, since xanthine or hypoxanthine is necessary for reduction of the Mo. There was no inhibition by preincubation, however, with NADH and vanadate even under anaerobic conditions. Fig. 1b shows the effect of vanadate concentration on xanthine oxidase activity. At 0.75 mM vanadate, about 85% inhibition was observed. A much lower concentration of vanadate (0.16 mM) sufficed to produce a similar inhibition of nitrate reductase from *Chlorella vulgaris* [6].

EDTA, which has been shown to form a 1:1 complex with vanadate anion [13], completely protected the xanthine oxidase from vanadate inhibition, when the chelating agent was added before vanadate (Table I). When the chelating agent was added after the vanadate inhibition was established, there was a rapid reactivation of the enzyme to give about 65–70% of the initial control activity (Table I). Thus, as for nitrate reductase, we conclude

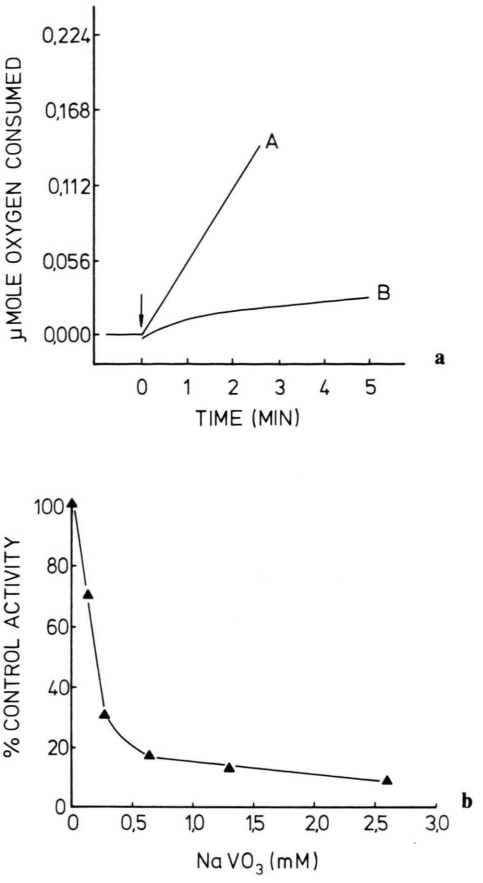

Fig. 1. a) Effect of vanadate on xanthine oxidase. Assay was carried out as described under Materials and Methods. The rate of oxygen consumption (A) in the absence and (B) in the presence of vanadate are given. b) Conditions of assay are the same as under a). The different points represent the activity (% of control) obtained with indicated vanadate concentration after full inhibition had developed.

Table I. Reversibility of vanadate inhibition of xanthine oxidase by EDTA.

Experiment	Additions	Xanthine Oxidase Activity [%]
1	none	100
2	$NaVO_3$ (1.3 mM)	13
3	EDTA (1.6 mM) + $NaVO_3$ (1.3 mM)	95
4	$NaVO_3$ (1.3 mM); EDTA (1.6 mM) added later	65
5	$NaVO_3$ (1.3 mM); EDTA (5.0 mM) added later	65

The assay was carried out as described under Materials and Methods: In experiments 4 and 5 EDTA was added after full inhibition was established.

that part of the metal ion is loosely bound. The recovery in activity after EDTA addition is somewhat higher than in the case of nitrate reductase. In the case of nitrate reductase, complete recovery of activity was obtained by a combined use of EDTA and ferricyanide. Attempts to duplicate this with inactive xanthine oxidase were unsuccessful. It is possible that critical conditions necessary for a full reactivation were not found. In any case, however, the pattern of inhibition by vanadate appeared similar for both enzymes.

Stimulation of NADH oxidase activity by vanadate

Xanthine oxidase also catalyses the oxidation of NADH by oxygen. It is known that this substrate binds near flavin [14], while purine derivatives bind to the molybdenum center [7]. Surprisingly, the oxidation of NADH, as measured either by the disappearance of NADH or by the consumption of oxygen, was stimulated several fold by vanadate. The rate of oxidation was dependent upon the concentration of the enzyme (Fig. 2a) as well as on the concentration of the metal ion (Fig. 2b). When boiled enzymes was used along with vanadate, there was no NADH oxidation.

Like xanthine oxidase, nitrate reductase also oxidizes NADH [9]. To obtain reasonable NADH oxidase activity requires 100 times more enzyme than is used in the assay of nitrate reductase or diaphorase activity. Though the NADH:NO_3^- reductase activity is inhibited by vanadate, the NADH oxidase activity is stimulated 3–4 fold by the metal ion. At each protein concentration of a homogenous preparation of the enzyme, stimulation was observed (Fig. 3).

In the oxidation of NADH, oxygen is reduced to H_2O_2. Quantitation of the product formed showed that for one mole of NADH oxidized about 0.8–0.9 mol of oxygen was consumed and 0.8 mol of H_2O_2 was formed. It is possible that some of the H_2O_2 formed was decomposed during the reaction.

In the xanthine oxidase reaction, both 1 and 2 electron mechanisms are used for the reduction of oxygen [15, 16]. The superoxide anion generated in the 1 electron reduction mechanism is responsible for the further reduction of cytochrome c. Since vanadate inhibited the oxidation of xanthine and hypoxanthine, it was not unexpected that the cytochrome c reduction was also inhibited in the pres-

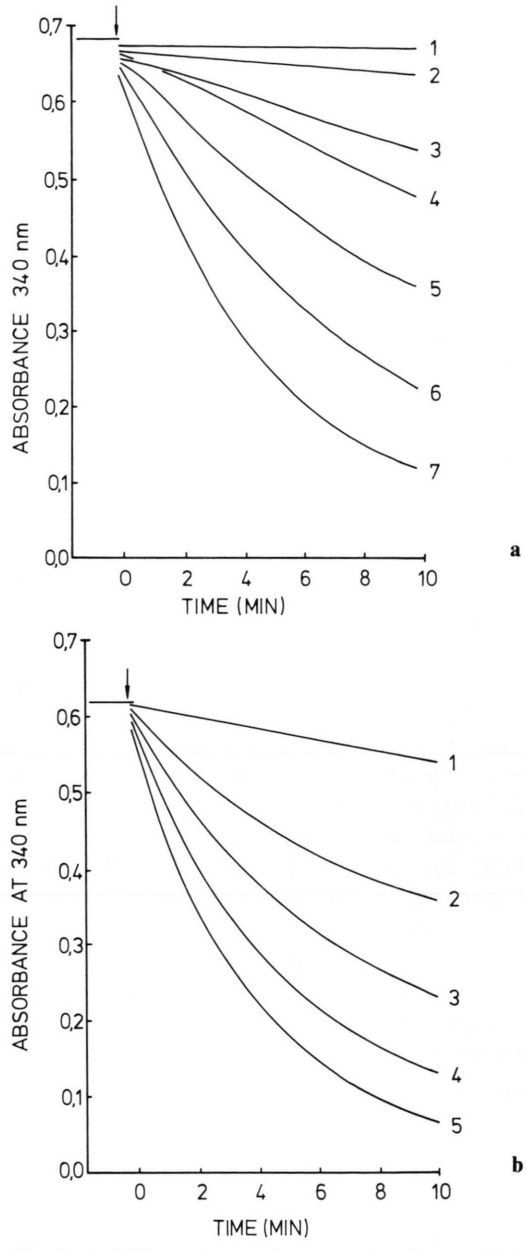

Fig. 2 a) Effect of vanadate on NADH oxidation by varying concentration of xanthine oxidase. Assay was carried out as described in Materials and Methods with 0.26 mM vanadate where indicated. The arrow indicates enzyme addition to start the reaction. (1) $NaVO_3$ only, (2) 75 µg enzyme, (3) 300 µg enzyme, (4) 37.5 µg enzyme plus $NaVO_3$, (5) 75 µg enzyme plus $NaVO_3$, (6) 150 µg enzyme plus $NaVO_3$, (7) 300 µg enzyme plus $NaVO_3$. b) Effect of increasing vanadate concentration on NADH oxidation by xanthine oxidase. Assay mixture was the same as that used in experiments under a). In these experiments 300 µg enzyme and indicated concentration of $NaVO_3$ were used. (1) without $NaVO_3$, (2) 0.026 mM $NaVO_3$, (3) 0.065 mM $NaVO_3$, (4) 0.13 mM $NaVO_3$, (5) 0.26 mM $NaVO_3$.

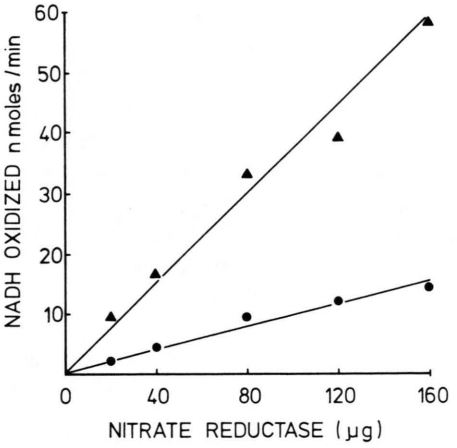

Fig. 3. Stimulation of NADH oxidation by nitrate reductase in the presence of vanadate. Assay was carried out in the absence (●—●) and in the presence of 0.26 mM $NaVO_3$ (▲—▲) as described under Materials and Methods. The initial velocities of the reactions were plotted against enzyme concentration.

Table II. Effect of vanadate on cytochrome c reduction by xanthine oxidase.

Additions	% of control activity	
	Xanthine→Cyt.c	NADH→Cyt.c
(1) None	100	100
(2) $NaVO_3$ (0.065 mM)	64	79
(3) $NaVO_3$ (0.13 mM)	45	57
(4) $NaVO_3$ (0.26 mM)	19	43
(5) $NaVO_3$ (0.65 mM)	0	29

The reduction of cytochrome c was followed at 550 nm. The experimental details are given under Materials and Methods.

ence of these substrates (Table II). But the reduction of cytochrome c was also inhibited when NADH was the reducing substrate (Table II). In the oxidation of NADH, stoichiometric amounts of H_2O_2 were formed. Therefore, the apparent inhibition of the cytochrome c reduction, in the presence of vanadate, is probably due to oxygen preferentially undergoing 2 electron reduction. Dismutation of superoxide anion formed cannot be completely ruled out.

In the oxidation of NADH by xanthine oxidase, oxygen has been shown to accept electrons from the flavin site [14]. Presumably similar events occur in nitrate reductase also. However, in the light of the observation that NADH oxidation proceeds rapidly in the presence of a combination of molybdenum(V) and vanadium(V) (see later), the possibility of oxygen activation occurring at the molybdenum site in the enzyme had to be considered.

To test this, inhibitors which are known to bind at the molybdenum site in the enzymes were used to study their effect on vanadate-stimulated NADH oxidation. Allopurinol, a potent inhibitor of xanthine oxidase, binds at the molybdenum site forming a complex [7, 17]. Such a xanthine oxidase-inhibitor complex showed enhanced NADH oxidation in the presence of vanadate. Cyanide is a potent inhibitor of nitrate reductase and binds at the molybdenum site in the reduced enzyme [18, 19]. The NADH oxidase activity of such a cyanide complex of nitrate reductase was stimulated 3–4 fold by vanadate. In these cases, where the molybdenum center is complexed with inhibitors, it is very unlikely that oxygen activation occurs at the molybdenum site.

The NADPH oxidase activity of ferredoxin:$NADP^+$ reductase, which has only flavin as the prosthetic group, was also stimulated 2–3 fold by vanadate at all protein concentrations tested (Fig. 4). Therefore it seemed likely that vanadate interacts at or near the flavin moiety. It is also conceivable that a ternary complex involving enzyme, nucleotide and

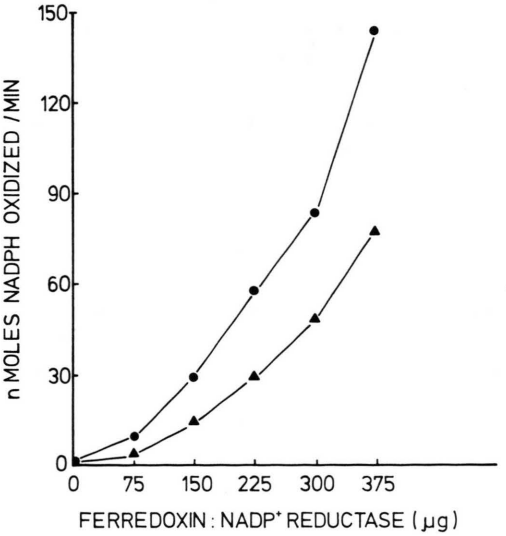

Fig. 4. Stimulation of NADPH oxidation by ferredoxin: $NADP^+$ reductase in the presence of vanadate. In this experiment 0.1 mM NADPH was used. Initial velocities obtained in the absence (▲—▲) and in the presence of 0.26 mM $NaVO_3$ (●—●) were plotted against enzyme concentration.

Table III. Effect of EDTA treatment of vanadate-inhibited nitrate reductase on its ability to oxidize NADH.

Treatment	NADH → cyt. c cyt. c reduced [μmol/min/ml]	NADH → NO_3^- NADH oxid. [μmol/min/ml]	NADH → O_2 NADH oxid. [μmol/min/ml]
None	119.9	32.9	0.036
$NaVO_3$	119.9	4.4	0.072
$NaVO_3$; EDTA later	119.9	13.6	0.036

The incubation mixture contained the following in a total volume of 0.40 ml: 20 μmol of potassium phosphate, pH 7.6; 125 μg of NADH; 190 μg of nitrate reductase; 2 μmol of $NaVO_3$ where indicated. After 15 min at 24 °C aliquots were used testing enzyme activities. Both control and test samples were treated with 5 μmol of EDTA (in 100 μl) and let stand in ice for 30 min. Again aliquots were used for testing various activities. The activities were corrected for the dilution due to EDTA addition.

vanadate is formed and this in some manner stimulates NADH oxidation.

It was shown earlier that vanadate-inhibited reductase could be partially reactivated by EDTA. This indicated that at least part of the vanadate was loosely bound. It was found that the enzyme from which loosely bound metal ions are removed did not show enhanced NADH oxidation (Table III). Thus, it is clear that the loosely bound form of vanadate, which apparently interacts near the flavin moiety is responsible for the stimulation of NADH oxidation. It seems possible that there are different types of loosely bound vanadate.

Although part of the vanadate bound to nitrate reductase (and also to xanthine oxidase) is responsible for stimulation of NADH oxidation, the inhibition of the main activity of the enzyme comes in part probably from firmly bound vanadate. In nitrate reductase, with the firmly bound species, which represents the metal ions remaining in the enzyme after EDTA treatment, still shows 50% of the control activity [6]. Therefore, the complete inhibition must result from a combination of perhaps various types of binding on to the enzyme.

Oxidation of NADH by a combination of vanadium(V) and molybdenum(V)

In the course of attempts to reactivate the vanadium inhibited nitrate reductase with various molybdenum compounds, it became apparent that a combination of vanadium(V) plus molybdenum(V) will rapidly oxidize NADH or NADPH. In the presence of 2-fold excess metal ions, the NADH oxidation proceeded to completion, with a pseudo first order rate constant of $6.9 \times 10^{-2}\ s^{-1}$. In the absence of vanadium(V) there was very negligible oxidation of NADH. On the other hand, when molybdenum(V) was omitted, vanadium(V) caused a slow oxidation of NADH after an initial lag. The $t_{1/2}$ for this reaction, however, was found to be 500 times higher than that observed with the combination of the metal ions. In the oxidation of NADH, a mole of O_2 was consumed per mole of NADH oxidized. There was no oxidation when the system was made anaerobic.

In experiments designed to show the dependence of the NADH oxidation on the concentration of each of the metal ions, it was found that stoichiometric amounts of each of the metal ions are required for complete and rapid oxidation of NADH (Fig. 5, curve A). When the concentration of

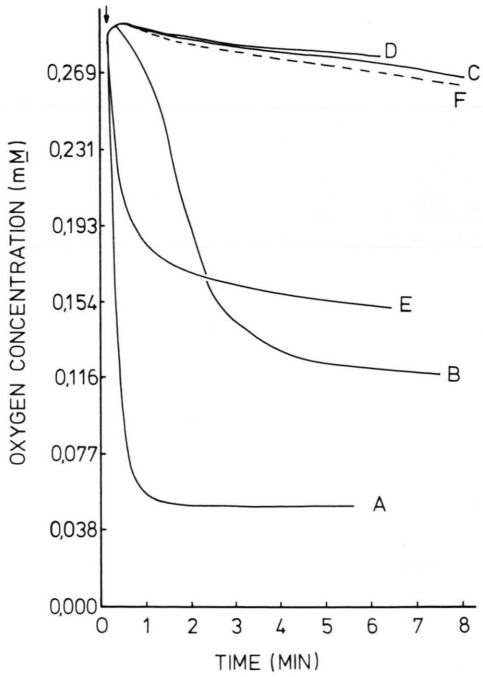

Fig. 5. Oxidation of NADH by a combination of $NaVO_3$ and $MoCl_5$. In this experiment 0.26 mM NADH was used with the indicated metal ion concentrations. The reaction was started by addition of $MoCl_5$ (in ethanol), where it is included. The oxygen consumption was monitored as described under Materials and Methods. (A) 0.26 mM $NaVO_3$ + 0.26 mM $MoCl_5$, (B) 0.065 mM $NaVO_3$ + 0.26 mM $MoCl_5$, (C) 0.026 mM $NaVO_3$ + 0.26 mM $MoCl_5$, (D) 0.26 mM $MoCl_5$ only, (E) 0.26 mM $NaVO_3$ + 0.026 mM $MoCl_5$, (F) 0.26 mM $NaVO_3$ only.

vanadium(V) was fixed at 0.26 mM, which is equivalent to that of NADH, and the molybdenum concentration lowered to 0.026 mM, the initial rate was slightly slower but still about 50% of the NADH was oxidized (Fig. 5, curve E). On the other hand, when the molybdenum(V) concentration was fixed at 0.26 mM and vanadium(V) lowered to 0.065 mM, the initial velocity decreased considerably, though still about 70% of the NADH was eventually oxidized (Fig. 5, curve B). When the concentration of vanadium(V) was lowered to 0.026 mM, there was very little NADH oxidation (Fig. 5, curve C). These results suggest a strong dependence of the reaction on vanadate concentration.

NADPH is also oxidized by these metal ions when they are present together. The oxidized $NADP^+$ could be re-reduced by using an excess of both glucose-6-phosphate and glucose-6-phosphate dehydrogenase. The recovery was about 80–85% showing that most of the cofactor remains intact during this reaction. Also added ATP could be quantitatively recovered suggesting that there is no hydrolysis of phosphate ester coupled to this electron transfer process.

Since oxidation of NADH involves 2 electrons, consumption of a mole equivalent of oxygen should yield H_2O_2. However, attempts to detect H_2O_2 formation were unsuccessful. Addition of H_2O_2, in the quantity expected to be formed in the reaction, to a mixture of vanadium(V) plus molybdenum(V) or to just molybdenum(V) alone, resulted in rapid disappearance of H_2O_2 without evolution of oxygen. It seems likely that any H_2O_2 generated during NADH oxidation would react similarly to oxidize metal ions.

The observation that NADH oxidation proceeds rapidly in the presence of a combination of vanadium(V) and molybdenum(V), may be taken as evidence for the interaction of these metal ions. In nitrate reductase and xanthine oxidase, the reduction of the enzymes with substrates generates EPR signals, which are characteristic of molybdenum(V) species [7, 19, 20]. Interestingly, vanadium(V) interacts only with the reduced form of nitrate reductase [6]. This apparently is true for xanthine oxidase as well. Taken together, these results point to the possible interaction of vanadium(V) with molybdenum(V) in the enzyme. The foregoing conclusions are tentative and await further confirmation, especially from EPR studies.

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