NADV: A New Cofactor for Alcohol Dehydrogenase from *Thermoanaerobium brockii*

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Cofactor recognition and conversion are important when conducting reactions using alcohol dehydrogenases in enzyme-catalyzed synthesis. In this paper alcohol dehydrogenase from *Thermoanaerobium brockii* is found to accept a solution of NAD and vanadate (presumably 2'-NADV) as a cofactor with a better k_{cat}/K_m ratio than NADP. A combination of ^{51}V NMR spectroscopy and enzyme kinetics were used to determine the Michaelis Menten parameters for this cofactor. Comparisons with previous studies suggest ADH for *T. brockii* may be an enzyme prone to cofactor substitution.

Introduction

The phosphate group in pyrimidine nucleotide cofactors is essential for enzyme recognition. Active cofactor analogs where phosphate or pyrophosphate has been replaced by alternate bifunctional groups have been reported but their activities are often poor and their preparation complex.[1] Most of the synthetically interesting cofactor dependent enzymes are dehydrogenases and oxido-reductases, which perform reactions under mild conditions with high regio and stereospecificity and innocuous side products.[2] Application of dehydrogenases as catalysts such as the alcohol dehydrogenase from *Thermoanaerobium brockii* has proved particularly useful in synthesis of chiral multifunctional compounds.[3] The synthetic usefulness of the enzyme catalysts is dictated by substrate specificity, the nature of the products, and the availability, cost, and stability of both the enzyme and cofactor.[4] Given the expense of cofactors, in situ cofactor regeneration has been developed in larger laboratory- and industrial-scale syntheses. The most convenient cofactor regenerations are those involving ATP/ADP and NAD/NADH. Additional regeneration systems have been developed for NADP(2'-NADP)/NADPH(2'-NADPH), NADH/NAD, NADPH(2'-NADPH)/NADP(2'-NADP), and acetyl-CoA/CoA in practical synthetic schemes.[5] The expense of NADP has, however, prevented the scale-up of NADP-dependent enzyme reactions and awaits methods for circumventing the use of NADP as a cofactor for these enzymes. This work describes a cofactor analog for the NADP dependent alcohol dehydrogenase (ADH).

Various atoms including sulfur, nitrogen, aluminum, arsenic, and vanadium(V) have been used to generate phosphate mimics. Sulfates and nitrates tend to be poor mimics because they have a different charge distribution.[6] Tetrafluoroaluminate and tetrafluoroborate have been found to be good mimics of phosphate in biological systems.[7] Arsentate ($HAsO_4^{2-}$ and $HAsO_4^-$) and vanadate (HVO_4^{2-} and $H_2VO_4^-$) have been found to have electronic and structural characteristics similar to phosphate.[8] Arsenate and vanadate, for example, are more potent inhibitors than phosphate for phosphatases and ATPases.[8] Descriptions of organic phosphate analogs have been less common.[9-11] Organic phosphonates are used most often as analogs for organic phosphates, although difficulties in their preparation combined with low biological activity limit their use.[11] A few reports suggest organic arsenates are excellent analogs for organic phosphates with respect to electronic properties and biological activity.[9] The biological activities of organic arsenates promise well for the activities of organic vanadate. Recent findings with

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glucose 6-phosphate dehydrogenase,[10,12] glycerol 3-phosphate dehydrogenase,[10] and aldolase[12] show that organic vanadates can act as substrate in place off organic phosphates.

Aqueous solutions of vanadate in millimolar concentrations contain several vanadate species at neutral pH; these include monomer ($H_2VO_4^-$, HVO_4^{2-}, VO_4^{3-}; abbreviated V_1), dimer ($H_3V_5O_7$, $H_2V_2O_7^{2-}$, $H_2VO_7^{3-}$, $V_2O_7^{4-}$; abbreviated V_2), tetramer ($V_4O_{12}^{4-}$; abbreviated V_4), and pentamer ($V_5O_{15}^{5-}$; abbreviated V_5).[13] These species are in rapid equilibrium and convert in the course of ms, thus preventing isolation.[14] In the presence of thiols or other reducing agents vanadate is reduced, presumably to vanadium(IV).[15] Studies exploring the properties of organic vanadates as organic phosphate analogs need to avoid conditions facilitating redox chemistry. One method to avoid reduction is to add an interacting buffer such as imidazole.[16] In the presence of imidazole, an organic vanadate forms within milliseconds after the mixing of an organic compound containing a hydroxyl group with vanadate in neutral aqueous solutions.[17] The vanadium(V) in these solutions is stable towards reduction for several hours. In this manner, glucose 6-vanadate can be prepared from solutions containing glucose and vanadate.[12] This compound is substrate for glucose 6-phosphate dehydrogenase.[10,12] Studies with various nucleosides and nucleotides suggest vanadate can form vanadium analogs of AMP and ADP as well as other vanadium-based nucleoside/nucleotide derivatives.[19] The reaction between NAD and vanadate monomer (in the form of HVO_4^{2-} or $H_2VO_4^-$) is expected to generate several vanadium–NAD complexes, since each reactive functionality (including hydroxyl groups, the purine base and nicotinamide moity) on NAD can lead to a product.[18] The reaction of vanadate with NAD has been described previously,[18,20] although the reactions of vanadate with nucleosides and nucleotides have been described in greater detail.[19] We will use the abbreviation NADV for the β-nicotinamide adenine dinucleotide 2′-vanadate derivative which corresponds to the biologically active phosphate derivative, NADP. The possibility for generating the vanadium analog of NADP with NAD and vanadate as shown in (1) has intriguing

$$\mathrm{NAD} + \mathrm{HVO_4^{2-}} \overset{K_{eq,NADV}}{\rightleftharpoons} \mathrm{NADV} \qquad (1)$$

implications for the use of enzymes in organic synthesis (Figure 1).

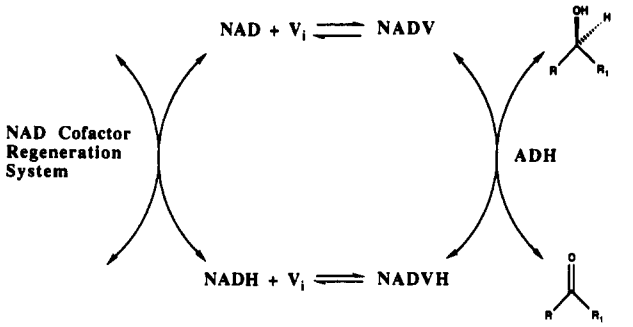

Figure 1. ADH-based reactions using NADV or NADVH as a cofactor.

The activity of NADV, a NADP analog, was examined for ADH isolated from *T. brockii*. This enzyme is thermally stable up to 89 °C and exhibits a high tolerance toward organic solvents. It is of particular synthetic interest because it converts keto substrates to chiral secondary alcohols.[3] NADV forms rapidly after mixing NAD and vanadate in aqueous solutions.[18] The in situ formation of NADV from NAD and vanadate in principle should allow for the use of an NADP-dependent dehydrogenase as a catalyst using NAD as the cofactor. Since NADP is much more expensive and significantly less stable than NAD, formation of NADV is of practical importance. The results presented below suggest that NAD plus vanadate (NADV) is a good cofactor for alcohol dehydrogenase from *T. brockii* and that approaches to modify cofactor specificity such as this may become important in future applications of enzymes in synthesis (Figure 1).

Experimental Methods

General. All reagents were purchased from Aldrich or Sigma unless noted otherwise. Ethanol (200 proof) was purchased from Midwest Grain Products Co. (F = 0.785 g/ml, D = 46.07 g/mol). The solutions of the cofactors NAD and NADP were prepared from solid materials of 95% or 98% purity. Aqueous solutions were prepared with distilled water which was further purified by deionization on an anion-exchange column. Vanadate solutions (2.00 or 50.00 mM) were generated from vanadium pentoxide and 2 equiv of NaOH and stored at 4 °C before use. The concentration of vanadium was verified by measuring the UV adsorption (ϵ 3.55 × 10^3 M^{-1} cm^{-1})[21] at λ = 260 nm at pH 12.5.

Alcohol dehydrogenase from *T. brockii* [E.C. 1.1.1.2] is commercially available as a lyophilized powder. It loses 30% activity in 6 days once dissolved in TAPS buffer containing 1% bovine serum albumin at pH 9.0. The results reported in this work were carried out on solutions less than 1 day old.

Kinetic Measurements. Rates of oxidation were measured in continuous assays at 25 °C and at 340 nm on a Lambda 4B Perkin-Elmer doublebeam spectrophotometer equipped with a constant temperature cell. The assay solutions were incubated for 2 min before the enzyme was added to initiate the reaction. The rates were taken from the initial linear section of the absorbance vs time plot; the reaction was monitored from 5 to 15 min. Doubling the concentration of ADH doubled the rate of oxidation.

Speciation Analysis. At millimolar total vanadate concentration the vanadate oligomers are in equilibrium as shown by eqs 2–5.[13] Accordingly, the concentration of each species can be calculated if the concentration of one species is measured. At pH 9.0 the chemical shifts of V_1, V_2, V_4, and V_5 are −538, −562, −573, and −580 ppm, respectively. At low vanadate concentrations V_1 is the major species in solution; below 1 mM total vanadate the concentrations of oligomers are essentially nonexistent. At

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higher vanadate concentrations V_4 becomes the major species in solution with more than 50% of the vanadium in this form. At pH 9.0 and 10 mM solutions of vanadate small changes in pH (±0.05) will significantly effect the V_4 concentration. Analogous

$$2V_1 \overset{K_{12}}{\rightleftarrows} V_2 \quad (2)$$

$$4V_1 \overset{K_{14}}{\rightleftarrows} V_4 \quad (3)$$

$$5V_1 \overset{K_{15}}{\rightleftarrows} V_5 \quad (4)$$

$$2V_2 \overset{K_{24}}{\rightleftarrows} V_4 \quad (5)$$

pH changes do not effect speciation at both lower and higher pH or at low total vanadate concentrations in a similar manner. The analysis of speciation at pH 9.0 should include generation of speciation curves, so that small pH changes do not contribute to erroneous H^+-dependent equilibrium constants which would complicate the analysis of complexes formed between vanadate and NAD. The formation of ethyl vanadate esters is small but measurable and thus is accounted for in this analysis. The H^+-dependent equilibrium constants at pH 9.0, 50 mM TAPS, 200 mM KCl, and 1.0 M ethanol are $K_{12} = 60\ M^{-1}$, $K_{14} = 7.1 \times 10^6\ M^{-3}$, $K_{24} = 2.0 \times 10^3\ M^{-1}$, and $K_{15} = 2.1 \times 10^8\ M^{-4}$.

^{51}V NMR Spectroscopy. Spectra of solutions were recorded both at 79.4 MHz on a ^1H ACE-300 Bruker Spectrometer (7.0 T) and at 131 MHz on a ^1H AM-500 Bruker spectrometer (11.7 T). Typical spectral parameters include a sweep width of 40 000 Hz, a 60° pulse angle, and an accumulation time of 0.07 s with no relaxation delay. The spectra were measured using an external lock. The T_1's of the vanadate oligomers varied from 8 to 15 ms, and accordingly no changes were observed in the integrations by using relaxation delays. The chemical shifts were reported relative to the external reference $VOCl_3$ (0 ppm).

Data Analysis. Analysis of the kinetic data was carried out using several programs including Cricket Graph, Statworks, Madak, and Wing 2. Eisenthal and Cornish-Bowden plots were used to determine K_m and V_{max} whereas Lineweaver-Burk plots and slope replots were used for illustration and for determination of K_i's. The uncertainties were calculated using Statworks and were derived from the inherent variance in the data using standard methods.

Specific Enzyme Assay.[22] Vanadate Inhibition in ADH Assay: Varying Ethanol Concentrations. The rate measurements were carried out using 0.040–0.30 M ethanol, 0.30 mM NADP, 50 mM TAPS, 200 mM KCl, 5.0 mM semicarbazide at pH 9.0, 25 °C, and 0.0–5.0 mM vanadate in the presence of 0.06 mg (~0.4 U) of ADH. The assay was observed for 2 min.

Vanadate Inhibition in ADH Assay: Varying NADP Concentration. The rate measurements were carried out using 0.015–0.20 mM NADP, 1.0 M ethanol, 50 mM TAPS, 200 mM KCl, 5.0 mM semicarbazide at pH 9.0, 25 °C, and 0.0–5.0 mM vanadate and in the presence of 0.06 mg (~0.4 U) of ADH. The assay was observed for 2 min.

NADV Activity of ADH. Rates were measured in assay solutions containing from 0 to 10.0 mM NAD, 0.60 M ethanol, 50 mM TAPS, 200 mM KCl, 5.0 mM semicarbazide at pH 9.0, 25 °C, and 0.0–3.0 mM vanadate (containing 0–1.5 mM monomeric vanadate) in the presence of 0.68 mg (4.1 U) of ADH. The assay was observed for 20 min.

Results and Discussion

ADH Dehydrogenase Assay. Vanadate interacts with buffers and other assay components. Mildly reducing compounds form complexes with vanadate which may reduce the vanadium and oxidize the ligand.[12,13,15,16] Past studies showed the effects of reducing aldehydes, ketones, and buffers can be minimized appropriately during the experiment at hand.[12,13,15,16] In the presence of vanadate, the alcohol dehydrogenase assay is best determined in direction of oxidation in the presence of 5.0 mM semicarbazide.[15b] Semicarbazide forms a semicarbazone with a carbonyl compound, and in the event the reaction is not favored, semicarbazone formation will remove the carbonyl group from the reaction mixture. Although these precautions may not be as crucial for ethanol as substrate, they are for other reducing products. We have been using TAPS at pH 9.0 with a fairly high ionic strength to maintain constant vanadate equilibria.[15b] Further discussions concerning choice of buffer in studies with vanadate have been described in detail elsewhere.[13c,16]

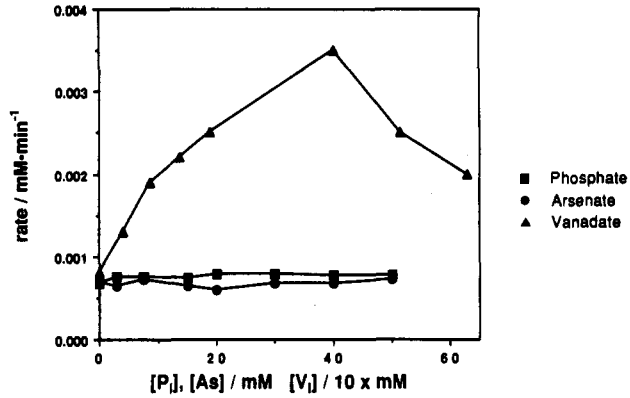

Figure 2. Rate of ADH-catalyzed oxidation of ethanol as a function of vanadate, phosphate, and arsenate. The rates were measured at 0.125 M ethanol, 0.30 mM NADP, 50 mM TAPS, 200 mM KCl, and 5 mM semicarbazide at pH 9.0 and 25 °C, and the oxoanions are represented as vanadate (▲), phosphate (■), and arsenate (●).

The K_m ("$K_{m\ app}$") of ethanol, measured in the presence of 0.30 mM NADP, was 50 (±10) mM, and the V_{max} ("$V_{max\ app}$") was 0.04 (±0.02) mM/minu. The K_m ("$K_{m\ app}$") for NADP, measured in the presence of 1.0 M ethanol, was 0.029 (±0.002) mM, and the V_{max} ("$V_{max\ app}$") was 0.018 (±0.001) mM/min. These results deviate somewhat from those reported in the literature determined under different conditions.[22]

ADH-Facilitated Oxidation of Ethanol Using NAD–Oxoanion Mixtures. The catalytic activity of a mixture of NAD and a particular oxoanion with ADH was examined using ethanol as a substrate. Oxoanions, including phosphate, arsenate, and vanadate, were added to assay solutions containing TAPS buffer, KCl, semicarbazide, ethanol, NAD, and ADH at pH 9.0 and 25 °C. Solutions containing no vanadate showed very low rates, whereas the rates increased 6-fold by addition of 1.0 mM vanadate. Rate enhancements were not observed when adding up to 10-fold higher concentrations of phosphate or arsenate. The rates of ethanol oxidation as a function of vanadate, phosphate and arsenate concentrations are shown for comparison in Figure 2. Since esterifications with arsenate are significantly slower, a solution containing 0.30 mM NAD was incubated with 60 mM arsenate for 48 h without observation of the rate enhancements obtained with vanadate. No significant changes in the rates were observed in these experiments analogous to those shown in Figure 2.

A series of controls was carried out to determine the origin of the observed activity. Activity in assays con-

(22) For various sources see refs 2 and 3.

taining only chemicals were found to be insignificant with and without oxoanions, confirming the expected role of the enzyme as catalyst. The necessity of the cofactor in the reaction was illustrated in assay with enzymes and substrates with and without oxoanions (but neither NAD nor NADP) because no significant change in the absorbance was apparent. In a similar manner, no activity was observed in assays containing NADP and enzymes but no substrate with and without oxoanions. These observations eliminate the possibilities that other vanadium derivatives in the assay, such as, for example, the ethyl vanadate or the vanadate–TABS complex, could act as cofactor or substrate.

In aqueous solutions vanadate forms vanadate esters on a ms time scale with organic compounds containing hydroxyl groups.[17] In order to determine the affinity of ADH for the presumed NADV, the equilibrium constant for the reactions between NAD and vanadate under the assay conditions needs to be determined. First, however, the effect of vanadate on the ADH reaction must be addressed since vanadate anions previously have been found to inhibit dehydrogenases[15b] and such interactions could potentially change the analysis required for determination of K_m and k_{cat} for NADV.

The Inhibitory Interaction of Oxoanions with ADH. The interaction of oxoanions with ADH was first examined by measuring the rate of ethanol (0.60 M) oxidation in the presence of NADP (0.30 mM) and increasing concentrations of oxoanions. Vanadate showed inhibition at 3 mM total vanadate, whereas arsenate and phosphate did not inhibit in this concentration range. These studies show that anionic vanadate inhibits alcohol dehydrogenase in contrast to vanadate in combination with NAD, which enhances the ethanol oxidation. Rate measurements of assay solutions containing 0.30 mM NADP and various concentrations of ethanol (from 0.050 to 0.30 M) at fixed vanadate concentrations were conducted. Plotting the results in the form of a Lineweaver–Burk plot showed that vanadate is a competitive inhibitor (data not shown). An analogous series of studies show that vanadate inhibits ADH when NADP is varied with a mixed inhibition pattern (data not shown).

As previously stated, aqueous solutions with millimolar of vanadate contain V_1, V_2, and V_5.[13,14] The concentrations of these species depend on the total vanadate concentration, pH, and ionic strength. If a vanadate oligomer(s) inhibited the ADH reaction, the substrate activity of NADV can conveniently be measured in solutions where concentrations of vanadate oligomers are extremely low and their contribution to inhibition can be ignored.[15b,23] Alternatively, the inhibition by other vanadate derivatives can be considered. Specifically, we will also discuss the possibilities that vanadate derivatives of either substrates or cofactors inhibited the ADH-catalyzed reaction.

Lineweaver–Burk slopes (at varying ethanol concentrations) were plotted as a function of V_1 concentration, and a linear relationship was observed from 0 to 1 mM vanadate (Figure 3a). The linear correlation with V_1 with a non-zero slope suggest V_1 (or a species proportional to the V_1 concentration) inhibits ADH. The inhibition constant (K_{i1}) is defined in (6) for V_1. Using the above

$$\text{slope} = \frac{K_m}{V_{max}}\left(1 + \frac{[V_1]}{K_{i1}}\right) \qquad (6)$$

data K_{i1} for the monomer was calculated to 1.1 (±0.2) mM

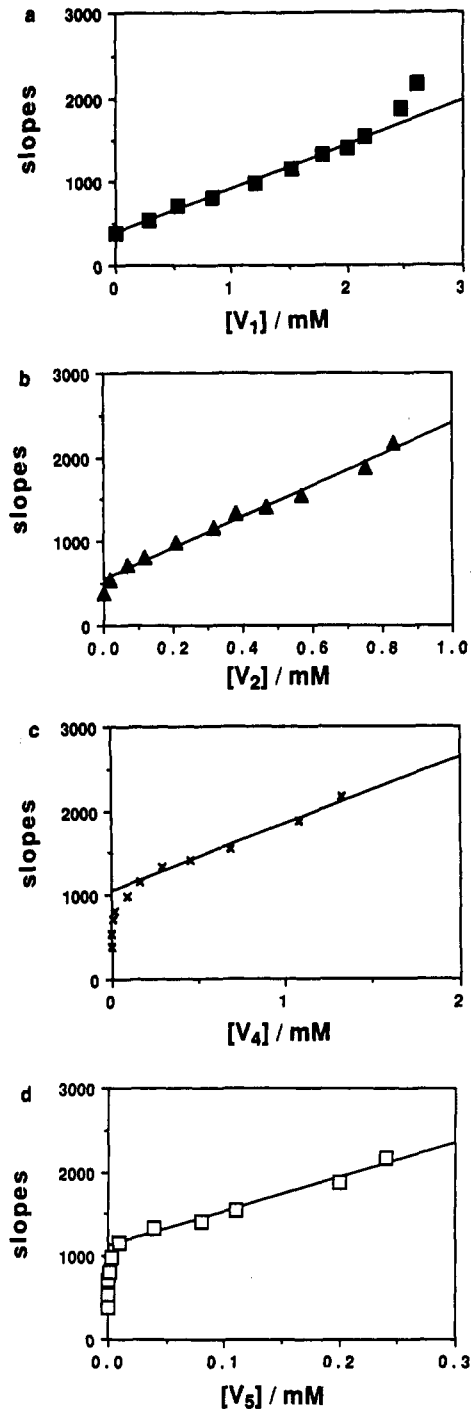

Figure 3. Lineweaver–Burk slopes are plotted as a function of vanadate oligomers; $[V_1]$ ■ (a), $[V_2]$ ▲ (b), V_4 × (c), and V_5 □ (d). The rates were measured from 0.0 to 0.60 M ethanol, 0.30 mM NADP, 50 mM TAPS, 200 mM KCl, and 5 mM semicarbazide at pH 9.0 and 25 °C.

with varying EtOH concentration and 7.7 (±0.4) mM with varying NADP concentration.

Alternative explanations for the observed inhibition by V_1 can be suggested, and one of these involves a complex between V_1 and NADP. If the inhibiting species proportional to the V_1 concentration were derived from a NADP complex, they are likely to interact with ADH as an NADP analog. Such a complex would therefore be expected to be a competitive inhibitor of NADP. The observed inhibition pattern is mixed against NADP and competitive against EtOH, inconsistent with these expectations. Vanadium–NADP complexes are indeed likely to form under

the assay condition. The question whether such complexes interfere with the measured rates of conversion is dependent on the concentration of the vanadium–NADP complexes formed. Since excess of NADP is used in these assays (10-fold excess of $K_{m,NADP}$; 0.30 mM), less than 10% of the NADP will form complexes in solution with 1 mM vanadate (assuming complexes approximate those reported previously for NAD,[18] nucleosides,[19] and nucleotides).[19] The formation of vanadium–NADP complexes are therefore not likely to significantly change the NADP concentration. The possibility that an ethyl vanadate derivative is the inhibitor would be unexpected because the substrate is neutral and ethyl vanadate is charged. We conclude that the observed inhibition proportional to the V_1 concentration is likely to come from the direct interaction between V_1 and ADH.

Since the linear relationship in the slope replot shown in Figure 3a does not continue above 1 mM total vanadate and 0.9 mM vanadate monomer, V_1 is not the only inhibitor in this system. At higher vanadate concentrations the oligomers begin to form and the increased inhibition suggests at least one of the oligomers is inhibiting the ADH reaction. To determine which of the vanadate oligomers interacts with ADH, the Lineweaver–Burk slopes were plotted as a function of V_2, V_4, and V_5 concentrations (Figure 3a–d). The Lineweaver–Burk slope exhibits an apparent linear relationship with V_4 (Figure 3c) at higher V_4 concentrations. Since inhibition is observed at low vanadate concentrations where no V_4 is present in solution, *both* V_1 and V_4 are likely to be inhibitors. The inhibition constants must therefore be determined from a kinetic scheme where two inhibitors are acting simultaneously on ADH. The kinetics follow the relationship shown in (7). The K_i's were calculated to 1.1 (±0.2) mM for monomer and 1.0 (±0.3) mM V-atoms for tetramer when ethanol is varied.

$$\text{slope} = \frac{K_m}{V_{max}}\left(1 + \frac{[V_1]}{K_{i1}} + \frac{[V_4]}{K_{i4}}\right) \qquad (7)$$

The relationship of the Lineweaver–Burk slopes with V_2 appears to be nonlinear (sigmoidal) at both low and high vanadate concentrations (Figure 3c). The relationship of Lineweaver–Burk slopes with V_5 are also non-linear (sigmoidal), suggesting neither V_2 or V_5 can be the exclusive inhibitors. The possibility of V_1 and V_2 or V_1 and V_5 or permutations of three or four species as inhibitors was also examined. Any fits with three or four vanadium species can be eliminated because such fits would yield a set of inhibition constants that are physically impossible (one of the inhibition constants would have to be negative). Using multiple regression analysis the best fit was obtained with V_1 and V_4; however, the perturbations of V_1 and V_2 or V_1 and V_5 have fits which were consistent with the observed inhibition. Further experimentation was desirable to confirm the possibility that V_1 and V_4 are the two vanadium species most consistent with the observed inhibition.

The vanadate oligomer species changes significantly with pH so we examined the inhibition of a solution containing 3.0 mM total vanadate at lower pH. The rate of ethanol oxidation with and without vanadate in the assay was measured from pH 7–9 in two different buffers, Tris and TAPS, depending on the pH. As shown in Figure 4, the

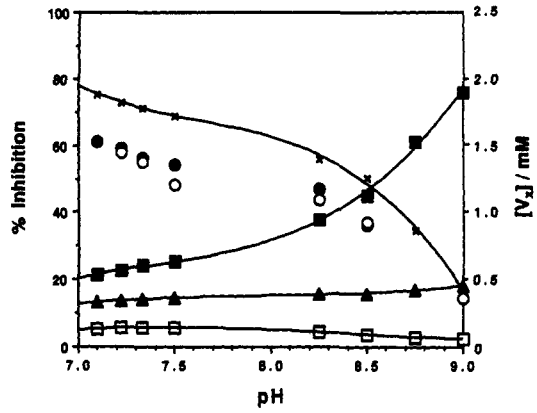

Figure 4. Rate of ADH-catalyzed oxidation of ethanol is shown as a function of pH in the presence of 0.40 M ethanol, 0.060 mM NADP, 200 mM KCl, and 5 mM semicarbazide at 25 °C and 50 mM Tris (●) or 50 mM TAPS (○). The V_1 (■), V_2 (▲), and V_4 (×), and V_5 (□) concentrations are indicated in mM vanadium atoms and were measured by ^{51}V NMR spectroscopy.

observed inhibition decreases as pH increases, suggesting that the major vanadate species responsible for the inhibition is the one that exhibits similar pH-dependent changes in concentration. At pH 7.0 and 3.0 mM the major vanadate species is V_4, whereas at pH 9.0 the major vanadate species is V_1 in the presence of buffer, semicarbazide, and 200 mM KCl. These studies support the conclusion that V_4 in the form of $V_2O_{12}^{4-}$ (major species in this pH range)[13b] is the inhibitory vanadate species in addition to V_1. The conclusions based on the enzyme studies conducted at pH 9.0 are in accord with the pH dependence of the inhibition.

The possibility that V_2 is an inhibitor of ADH appears to be less likely because the V_2 concentration decreases as the observed inhibition increases. The combination V_1 and V_2 as a reasonable alternative to the combination V_1 and V_4 should then be less favorable. In the past it has been shown that V_2 is a more potent inhibitor at low pH for glycerol 3-phosphate dehydrogenase, apparently because the diprotonated form of V_2 ($H_2VO_7^{2-}$) is a 10-fold better inhibitor than the monoprotonated form of V_2 ($HV_2O_7^{3-}$).[15b] Even though the concentration of total V_2 decreases, the concentration of $H_2V_2O_7^{2-}$ increases as the pH approaches 7.0. Since the pK_a of this ionization is 7.8[13b] one would expect a large increase in the inhibition should $H_2V_2O_7^{2-}$ be the active V_2 inhibitor in the pH range 7.0–7.8. Since Figure 4 only shows a modest increase in the inhibition in this pH range we conclude that neither total V_2 or any protonated forms of V_2 are as likely as V_4 to inhibit the ADH. This conclusion is supported by the results obtained at various NADP concentrations since the K_i for V_2 for these measurements are negative (not physically possible).

The combination of V_1 and V_5 as the inhibiting species is possible even though the fit is somewhat poorer than that for V_1 and V_4. As seen in Figure 4 the V_5 concentration ($V_5O_{15}^{5-}$ in this pH range) increases as the observed inhibition increases. To firmly distinguish whether V_1 and V_4 or V_1 and V_5 are the inhibiting species, additional experiments must be performed at higher vanadate concentrations where the speciation between the V_4 and V_5 will easily be distinguished. In the past such experiments have been conducted with 6-phosphogluconate

dehydrogenase,[23a] glucose 6-phosphate dehydrogenase,[23b] glycerol 3-phosphate dehydrogenase,[15b] phosphoglucoisomerase,[24] and aldolase.[23d] In all these cases the V_4 was found to be a much more potent inhibitor of the enzyme than V_5. In analogy, these enzymes show a significantly better affinity for the corresponding "Mo_4" molybdate derivative ($[(CH_3)_2AsMo_4O_{14}OH]^{2-}$) over the "$Mo_5$" derivative ($[(NH_3C_2H_4P)_2PMo_5O_{21}]^{2-}$).[24] We therefore favor the interpretation that the inhibition is caused by V_4. Since the objective of these studies was to quantify the interactions of vanadate with ADH such that the vanadium analog of NADP could be studied, we will continue the analysis under the assumption that V_4 and not V_5 is the second vanadium inhibitor.

Vanadate can be reduced by proteins containing thiol groups[12b] so the possibility that vanadate interacts with ADH through redox chemistry must be considered. If vanadate inhibited ADH by generating vanadium(IV) and oxidized ADH, the addition of EDTA should chelate V(IV) and leave the oxidized and inhibited form(s) of ADH. If vanadate inhibits ADH noncovalently and reversibly, the chelation of vanadate will reactivate ADH. The reversibility of the vanadate interaction was examined by the following experiments. The reaction rates of alcohol conversion in two ADH assay solutions were compared and found to be identical for the first 60 s. Vanadate (to 3.0 mM final concentration) was then added to the second assay. The rate of reaction in this assay solution decreased while the rate in the first assay solution remained constant. Finally, EDTA (3.0 mM) was added to both assay solutions. Identical reaction rates were observed in both solutions although the rates in the presence of EDTA were only 64% of the original rate. The reduciton of enzyme reaction rates is often observed upon addition of EDTA and is attributed to conformational interactions between the EDTA and the protein.[13c] The recovery of the enzyme activity when vanadate was removed suggests vanadate interacts with ADH noncovalently and not through redox chemistry. Similar behavior has previously been observed in other enzymes.[15b]

Reaction of Vanadate with NAD. Vanadate forms monoesters in the presence of compounds with hydroxyl groups and the monoesters can conveniently be studied using ^{51}V NMR spectroscopy. It is reasonable to expect that vanadate will form secondary monoesters with the four secondary hydroxyl groups on NAD. Depending on the hydroxyl group that is vanadylated we will refer to this compound as 2-NADV, 3-NADV, 2′-NADV, and 3′-NADV. Only the 2′-NADV is an analog for NADP (2′-NADP), the biologically active form. This reaction has been reported under different conditions[18] but more detailed data and analysis will be presented here.

The ^{51}V NMR spectra of the reaction of vanadate with NAD show the two new signals of interest, a vanadate monoester with a chemical shift superimposed on V_1 (adjacent to that of ethyl vanadate ester) and a vanadium-NAD complex at −538 ppm. The presence of the former complex is deduced from the V_1–V_4 equilibrium since the signal at −538 ppm was higher than expected. The presence of ethanol does, however, reduce the quality of the spectra and the accuracy of the formation constant measurement. The stoichiometry of these complexes is determined by measuring the ^{51}V NMR spectra at a series of vanadate concentrations and at a series of NAD concentrations. Only the proper representation of the vanadium complexes will fit the observed concentrations of complexes. Four different complexes with the stoichiometries 1:1, 1:2, 2:1, and 2:2 can form from vanadate and NAD (see supplementary material for further detail). If the stoichiometry is 1:1, then the observed complex concentration should show a linear relationship when plotted against $[V_1][NAD]$. As seen from Figure 5a only the complex with the chemical shift superimposed on the V_1 signal (the combined monoesters "NAD-V") is consistent with this stoichiometry. Monoesters have previously been found to have chemical shifts in this region, whereas cyclic vanadate derivatives have significantly different chemical shifts.[19] The possibility that vanadate could interact with the purine or nicotinamide moieties in NAD has previously been described.[20] The fact that vanadate and pyridine form a very weak complex at a different chemical shift, does not lend support to a complex between vanadate and the cofactor base-residues.[25] We therefore favor the interpretation that the observed complex is a combination of the four possible monoesters of NAD-V.

The complex at −523 ppm is not consistent with the 1:1 stoichiometry (Figure 5a). Since neither complex has the stoichiometries 1:2 or 2:1, plotting the complex concentrations as a function of $[V_1][NAD]^2$ or $[V_1]^2[NAD]$ does not yield linear relationships (Figure 5b and c). Plotting the concentration of the complex at −523 ppm as a function of $[V_1]^2[NAD]$ does show a linear relationship consistent with the interpretation that this complex is a 2:2 complex. This complex thus contains two molecules of V_1 and two molecules of NAD eliminating any structural suggestions inconsistent with this stoichiometry. Structural proposals have been presented for the presumably analogous 2:2 complex between vanadate and nucleosides.[19]

In addition to determining the stoichiometries, the plots in Figure 5a and d allow the calculation of the formation constants for the 1:1 and 2:2 complexes. We calculate a formation constant of 5.6 M^{-1} for the combined NAD-V derivatives in 50 mM TABS, 200 mM KCl, and 0.60 M ethanol pH 9.0. Since such esters exchange on the ms time scale,[17] each derivative cannot be isolated and examined but its stoichiometry and concentrations must be inferred from spectroscopic solution studies. Assuming that the ester formation constant for all four derivatives is similar, the formation constant for NADV (as shown in (1)) is calculated as one-fourth (1.4 M^{-1}) of the total formation constant (5.6 M^{-1}) for the monoesters ($K_{eq,NAD\text{-}V}$ = [NAD-V]/$[V_1]$ [NAD] where [NAD-V] = [2-NADV] + [3-NADV] + [2′-NADV] + [3′-NADV]). The major product in the reaction between vanadate and NAD is the complex at −523 ppm, and it must be considered when calculating speciation in the assay solution. The formation constant for this complex is 3.2 × 10^6 M^{-3} (K_{cyc} = $[NAD_2V_2]/[NAD]^2[V_1]^2$) under the conditions used in this work. The formation constants for NADV and c-NADV (NAD_2V_2) in the presence of ethanol determined here were

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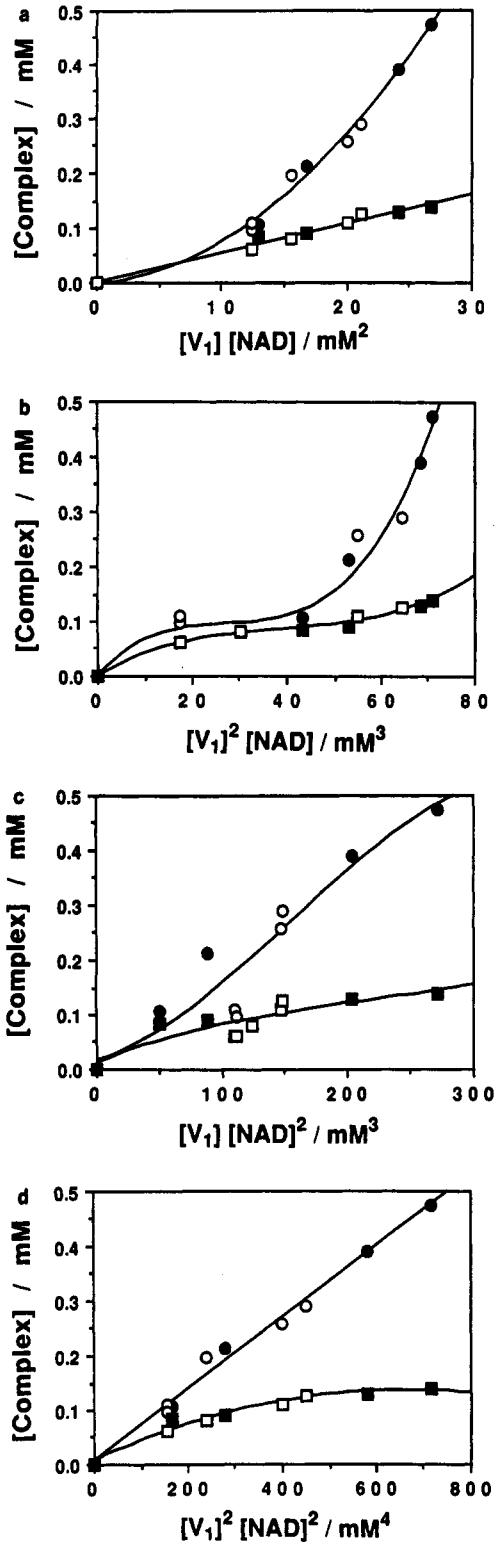
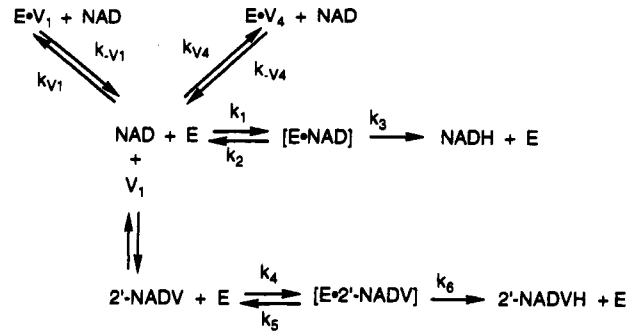


Figure 5. Vanadium–NAD complexes at −523 ppm (○, ●) and superimposed on the V_1 signal (□, ■) are plotted as a function of $[V_1][NAD]$ (a), $[V_1]^2[NAD]$ (b), $[V_1][NAD]^2$ (c), and $[V_1]^2[NAD]^2$. In order to keep both complexes on the same plot, the concentration (in mM–vanadium atoms) of the complex at −523 ppm has been reduced by a factor of 10. The open and solid symbols refer to data obtained by varying vanadate or NAD concentrations, respectively.

found to vary significantly from those reported previously without ethanol.[18]

NADV as Cofactor for ADH. Michaelis Menten constants for NADV were measured in solutions containing NAD, V_1, V_2, V_4, 2′-NADV, other vanadium–NAD derivatives, and buffer components. Although four monoesters form, the observed activity with ADH is presumably due to the 2′-NADV derivative (Figure 1). NADP derivatives other than 2′-NADP have in the past exhibited very limited affinity for the respective enzymes. The activity of both c-2′,3′-NADP and 3′-NADP with ADH was examined and found to be significantly lower than 2′-NADP. K_m for c-2′,3′-NADP and 3′-NADP were determined to be 0.81 and 6.9 mM. More importantly, the k_{cat}/K_m for these two substrates were 1.6×10^5 and 1.4×10^2 min^{-1}, which are lower than the value 9×10^5 min^{-1} for 2′-NADP. Contributions from cofactor analogs of 2′-NADV such as 3′-NADV and c-2′,3′-NADV are expected to be less since the corresponding phosphorus derivatives are less favorable than 2′-NADP. Thus, the analysis was conducted attributing the major cofactor activity to 2′-NADV, with the assumption that the activity of other NAD-V derivatives is less and can be ignored. In addition to the NAD-V derivatives, the assay solution also contains NAD, V_1, and V_4 which interact with the enzyme. These must be considered in the analysis since NAD is a substrate for the enzyme and V_1 and V_4 are inhibitors. The final kinetic treatment of the vanadate–NAD system involves two alternative substrates (NAD and NADV) and two reversible inhibitors (V_1 and V_4) as illustrated in Scheme I. In Scheme I the reaction of ADH has been simplified to a one substrate enzyme, even though ADH actually uses two substrates, NADP, and alcohol. The kinetic parameters reported here are therefore linked to the alcohol concentration (we are reporting $K_{m,app}$ and $V_{max,app}$). Under these conditions the kinetic treatment of ADH approaches that illustrated in Scheme I. The rate of ethanol oxidation is given by (8). Steady-state approx-

Scheme I



$$v = k_3[E\cdot NAD] + k_6[E\cdot NADV] \quad (8)$$

imations and the following definitions will lead to (9) from (8): $[E_t] = [E] + [E\cdot NAD] + [E\cdot 2'\text{-}NADV] + [E\cdot V_1] + [E\cdot V_4]$, $V_{max,NAD} = k_3[E_t]$, $V_{max,NADV} = k_6[E_t]$, $K_{m,NAD} = (k_2 + k_3)/k_1$, $K_{m,NADV} = (k_5 + k_6)/k_4$, $K_{iV1} = [E][V_1]/[E\cdot V_1] = k_{-V1}/k_{V1}$ and $K_{iV4} = [E][V_4]/[E\cdot V_4] = k_{-V4}/k_{V4}$.

$$v = (V_{max,NAD}K_{m,NADV}[NAD] + V_{max,NADV}K_{m,NAD}[NADV])/\left(1 + \frac{[V_1]}{K_{iV1}} + \frac{[V_4]}{K_{iV4}}\right) \times (K_{m,NAD}K_{m,NADV} + K_{m,NADV}[NAD] + K_{m,NAD}[NADV]) \quad (9)$$

NADV is in equilibrium with NAD and V_1, and [NADV] can be replaced by $K_{eq,NADV}$ $[NAD][V_1]$. The maximum rates for NAD and NADV can be equated by a proportionality factor β ($V_{max,NADV} = \beta \cdot V_{max,NAD}$). These types

Table I. Summary of Michaelis Menten and Inhibition Constants for Alcohol Dehydrogenase from *T. brockii*[a,b]

	EtOH	NADP	NAD	NADV[c,d]	3′NADP[b,d]	c-2′,3′-NADP[b,d]
K_m (mM)	50 ± 10	0.029 ± 0.002	20 ± 1	0.04	6.9 ± 2.9	0.81 ± 0.10
$V_{max,assay}$ (mM/min^{-1})		0.018 ± 0.001	0.12 ± 0.01	6[d]	0.0007 ± 0.0002	0.088 ± 0.006
V_{max} (mM/min^{-1})		0.12 ± 0.01	0.12 ± 0.01	6[d]	0.0044 ± 0.0013	0.56 ± 0.04
K_{i1} (mM)	1.1 ± 0.20	7.7 ± 0.4				
K_{i4} (mM V atoms)	1.0 ± 0.30	3.6 ± 0.3				
K_{i4} (mM)	0.26 ± 0.07	0.90 ± 0.06				
k_{cat} (min^{-1})[e]		(2.6 ± 0.1) × 10^4	(2.6 ± 0.3) × 10^4	1 × 10^6	(1.0 ± 0.3) × 10^3	(1.3 ± 0.1) × 10^5
k_{cat}/K_m (mM^{-1} min^{-1})		(9.0 ● 0.8) × 10^5	(1.3 ± 0.2) × 10^3	3 × 10^7	(1.4 ± 0.7) × 10^2	(1.6 ± 0.2) × 10^5

[a] The assay was conducted in 50 mM TAPS, 200 mM KCl, 5.0 mM semicarbazide, and 0.60 M EtOH or 0.30 mM NADP at pH 9.0. In the experimental studies the following protein concentrations per mL were used: 0.060 mg (0.36 U) for the EtOH studies, 0.026 mg (0.16 U) for the NADP studies, and 0.17 mg (1 U) for the NAD and NADV studies. [b] Errors represent standard deviations and were calculated by propagating errors from individual terms in the velocity equation. [c] Although these parameters were reproduced by different workers and statistical analysis gives smaller errors, the assumptions necessary for these calculations allow us to trust these results only within an order of magnitude. [d] Calculated from $V_{max,NADV} = \beta V_{max,NAD}$, thus β is 48. [e] k_{cat} is calculated by dividing $V_{max,NADV}$ in mM/min by the enzyme concentration in M. The molecular weight used for these calculations is 37 652.[26]

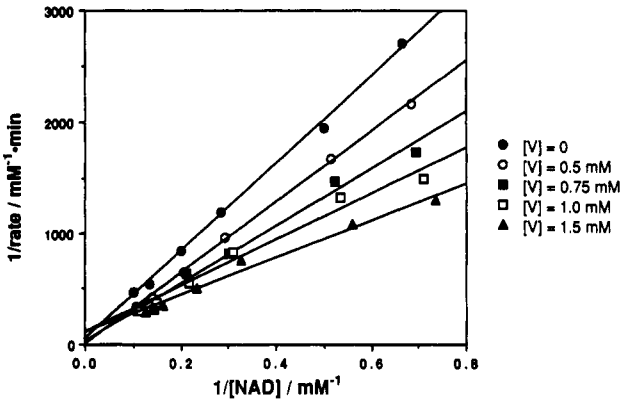


Figure 6. Reciprocal rate of ADH oxidation is plotted as a function of reciprocal NAD concentration at various ratios of [V1]/[V4]. The reaction rates were measured in the presence of 0.60 M ethanol, 50 mM TAPS, 200 mM KCl, and 5 mM semicarbazide at pH 9.0 at 25 °C. These data were used to obtain the Michaelis Menten parameters through an iterative process in minimizing the error on calculated and predicted rates.

of experimental data are commonly represented by a Lineweaver–Burk plot, and accordingly (10) is derived using above substitutions. The derivations from (8) to (9) and (10) has been described in further detail in the supplementary material.

$$\frac{1}{v} = \frac{\left(1 + \frac{[V_1]}{K_{iV1}} + \frac{[V_4]}{K_{iV4}}\right)K_{m,NAD}K_{m,NADV}}{V_{max,NAD}K_{m,NADV} + \beta V_{max,NAD}K_{m,NAD}K_{eq,NADV}[V_1]} \times \left(\frac{1}{[NAD]}\right) + \frac{K_{m,NAD}K_{eq,NADV}[V_1] + K_{m,NADV}}{V_{max,NAD}K_{m,NADV} + \beta V_{max,NAD}K_{m,NAD}K_{eq,NADV}[V_1]} \quad (10)$$

Examining (10) it becomes clear that the terms $[V_1]$ and $[V_4]$ contribute to the slope and $[V_1]$ contributes to the intercept. The slope and intercept measured experimentally should vary with $[V_1]$ and $[V_4]$ as observed in Figure 6. By experimentally measuring the all the components of (10) including $K_{m,NAD}$, $V_{max,NAD}$, K_{iV1}, K_{iV4}, and $K_{eq,NADV}$ (Table I) the only unknowns, $K_{m,NADV}$ and β, can be calculated from the rate measurements at varying [NAD] and $[V_1]/[V_4]$ ratios. Variation of [NAD] will change both $[V_1]$ and $[V_4]$ since these two concentrations are related. A series of rate measurements at constant $[V_1]$ and varying [NAD] will change total vanadate concentration as expressed matematically by $[V_{tot}] = 2[NAD_2V_2] + [NAD\text{-}V] + [V_1] + 2[V_2] + 4[V_4] + 5[V_5]$ which becomes (11) upon appropriate substitution. Figure

$$[V_{tot}] = 2K_{cycNADV}[NAD]^2[V_1]^2 + K_{eq,NAD\text{-}V}[V_1][NAD] + [V_1] + 2K_{12}[V_1]^2 + 4K_{14}[V_1]^4 + 5K_{15}[V_1]^5 \quad (11)$$

6 shows the experimental data of such a series of experiments. The constants were calculated from this data using an iterative process of varying $K_{m,NADV}$ and β to obtain best fit between the predicted and observed rates. The results are summarized Table I.

The $V_{max,NADV}$ is, as expected, considerably larger than $V_{max,NAD}$ but surprisingly also larger than $V_{max,NADP}$. $K_{m,NADV}$ is larger than $K_{m,NADP}$ which differs from the K_m values for other organic vanadates as compared to their corresponding organic phosphate counterparts. As seen by the k_{cat}/K_m, 2′-NADV is a significantly better analog of 2′-NADP than NAD, 3′-NADP, or c-2′,3′-NADP. It even has a k_{cat}/K_m better than 2′NADP itself.

Future Applications and Developments. Recent studies with glucose 6-phosphate dehydrogenase,[10] glycerol 3-phosphate dehydrogenase,[10] and fructose 1,6-bisphosphate aldolase[12] with organic vanadate substrate have yielded results which provide a framework for the understanding of pattern of enzymic recognition of organic vanadates. Organic vanadate analogs of enzyme substrates generally have similar or smaller K_m values than the natural substrates with the exception of the 2′-NADV described in this work. V_{max} values are typically smaller than those for the natural substrate with the exception of 2′-NADV for ADH. Overall, the k_{cat}/K_m is quite favorable and sometimes even better than the phosphate substrate. These observations suggest that organic vanadates in general are good analogs for organic phosphates. The stabilization of the enzyme–vanadate–substrate complex is often reflected in low K_m values, although low K_m values can also be accompanied by high catalytic rates.

The k_{cat}/K_m ratio for organic vanadate derivatives of fructose 1,2-bisphosphate and 2′-NADV was higher than the ratio for the natural substrate. The latter observation is in contrast to the lower specificity for 2′-NADV of glucose 6-phosphate dehydrogenase.[18] It is of interest that in the case of glucose 6-phosphate dehydrogenase the c-2′,3′-NADP had k_{cat}/K_m similar to 2′-NADV, whereas in the case of ADH this ratio is somewhat less. Both enzymes showed an 10^3–10^4 increase in the NAD k_{cat}/K_m ratio upon addition of vanadate; however, the k_{cat}/K_m ratio for the glucose 6-phosphate dehydrogenase was higher before

removing the phosphate from NADP than for ADH. As a result, the addition of vanadate had a greater impact on the latter enzyme than the former. 2'-NADV actually has a better k_{cat}/K_m ratio than NADP with ADH. Since ADH also accepts c-2',3'-NADP and 3'-NADP with better k_{cat}/K_m ratios than observed with glucose 6-phosphate dehydrogenase, the cofactor specificity pattern observed for this enzyme may suggest that indeed the development of cofactor alternatives would be fruitful.

We suggest that the combination of NAD and vanadate as a cofactor has a practical value for small scale syntheses using ADH from *T. brockii*. A similar approach has previously been documented using arsenate analogs for synthesis of several unnatural sugars using aldolases.[10b] In general, we expect that organic arsenate derivatives will be more useful than organic vanadates for reactions using substrate analogs on larger scales because higher concentrations of arsenate can be employed in the reaction.[10] However, in this case the arsenate and NAD mixture does not have analogous cofactor activity. The use of high concentrations of vanadate in complex reaction mixtures can cause experimental difficulties. Vanadate reacts with many organic compounds and upon prolonged reaction time periods, vanadate and the substrate–cofactor–product mixture may generate unwarranted side products. However, side reactions of vanadate can easily be decreased using several stategies. High concentrations of NAD and enzyme will increase reaction rates and reduce contact time, addition of complexing agents (EDTA or imidazole) will prevent the reduction of the vanadium, and high pH or continuously adding reactant or removing product should reduce or eliminate the interactions between these compounds and the vanadium. These approaches have all been used previously and were found to significantly decrease unwanted reactions of vanadate.

The application of enzymes to organic synthesis is often limited by enzyme availability and specificity. It is important to explore new methods to enhance the chemist's ability to improve substrate specificities of biomolecules. The present work illustrates one possibility by increasing the utility of enzymes by structurally altering the cofactor in situ. Successful application of modified substrates have already been used for the synthesis of carbohydrates and this work illustrate how this concept can be extended to modifying the cofactor. These studies show that an active cofactor analog can be generated in situ and used for formation of product. This approach should increase the number of enzymes available for synthetic application in the future.

Conclusion

ADH from *T. brockii* accepts a solution containing NAD and vanadate as a cofactor. The presumed NADV (2'-NADV) forms reversibly and is a better cofactor analog for ADH than NADP (2'-NADP) as defined by k_{cat}/K_m. The K_m for NADV was higher than that of NADP ($K_{m,NADV}$ = 0.04 mM and $K_{m,NADP}$ = 0.029 mM), but overall NADV as a cofactor is more efficient as compared to NADP ($k_{cat}/K_{m,NADV} = 3 \times 10^7$ min^{-1} M^{-1} whereas $k_{cat}/K_{m,NADP} = 0.9 \times 10^6$ min^{-1} M^{-1}). NADV is a significantly better substrate than NAD ($k_{cat}/K_{m,NAD} = 1.3 \times 10^3$ min^{-1} M^{-1}) or other cofactor analogs and offer a cheaper alternative to NADP in enzyme-catalyzed synthesis.

Acknowledgment. D.C.C. thanks NIH and Eli Lilly Co. for funding this research. We thank Dr. W. Fessner for stimulating discussions concerning the application of NADV in organic synthesis.

Supplementary Material Available: Detailed derivations and discussion of the reaction between vanadate and NAD (to form NADV) and detailed derivations of the Michaelis Menten parameters. The latter include kinetic treatment of a two-inhibitor, two-substrate system (7 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.