Vanadium Inhibition of Yeast Glucose-6-phosphate Dehydrogenase*

MITCHELL D. COHEN, ASOK C. SEN and CHENG-I WEI**

Department of Food Science and Human Nutrition, University of Florida, Gainesville, Fla. 32611, U.S.A.

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Abstract

Vanadium in the +5 and +4 oxidation states inhibits the activity of yeast glucose-6-phosphate dehydrogenase in a Tris buffer system. The results presented here are the first to report the inhibitory effects of vanadium oxide(IV) on this enzyme. The inhibition by ammonium metavanadate(V) is mixed-type against glucose-6-phosphate, and competitive against $NADP^+$ with K_i values of 2.1 and 2.7 mM, respectively. Theorell–Yonetani analysis indicates that vanadate and phosphate are mutually exclusive inhibitors. Inhibition by vanadium oxide is mixed-type for both substrates. K_i values of 49 and 52 μM for the sugar phosphate and $NADP^+$, respectively, are almost two orders of magnitude lower than those from phosphate or sulfate, and fifty-fold lower than that obtained with vanadate. Vanadium complexation with, and oxidation of, the reduced cofactor is apparent. Analysis of fluorescence quenching by vanadate and vanadium oxide indicates association constants of 4.0×10^2 and 4.3×10^3 M^{-1}, respectively. NMR analysis indicates that interactions of NADPH with vanadium occur primarily through the adenine moiety.

Introduction

The exposure of animals [1] or cultured cells [2] to vanadium has been correlated with an increase in the levels of membraneous peroxides. Because vanadium can inhibit enzymes that utilize inorganic phosphate or phosphorylated substrates [3–5], an inhibition of the first enzyme of the hexose monophosphate shunt, glucose-6-phosphate dehydrogenase (G6PDH), is possible. This inhibition may result in decreased levels of reducing equivalents needed for peroxide destruction by the glutathione peroxidase/reductase cycle, and contribute to the observed increase in peroxide levels.

The kinetic mechanism of G6PDH has been studied in yeast [6, 7], human erythrocytes [8, 9], and pig and rat liver [10, 11]. The enzymatic conversion of D-glucose-6-phosphate to 6-phosphogluconate followed an ordered sequential mechanism in which $NADP^+$ was bound first to the enzyme and NADPH was released last [8, 12]. Inhibition of yeast G6PDH by anions such as bicarbonate, sulfate, and phosphate [13], as well as mercuric salts [14] has been reported; the inhibition was competitive-type against the sugar phosphate and noncompetitive against the $NADP^+$.

The purpose of this study was to determine if vanadium could interfere with the enzymatic mechanism for generation of reducing equivalents for the glutathione redox cycle. In this work, inhibition studies using vanadium solutions with the metal in either the +4 or +5 valence states indicated that inhibition regarding the $NADP^+$ was mixed-type and competitive for the two valences, respectively. However, in our glucose-6-phosphate studies, both valences displayed mixed-type instead of an expected competitive mechanism. Studies using ammonium metavanadate yielded K_i values (mM) in the range of other inorganic ions, but the vanadium oxide (IV) was a potent inhibitor of this enzyme with K_i values (μM) 50-fold lower than the metavanadate. The degree of complexation of vanadium with the cofactor was also affected by the valence state of the metal.

Experimental

Reaction rates were determined in 1.0 cm pathlength cells in a Perkin-Elmer double beam spectrophotometer (Model Lambda 3) by monitoring the changes in absorbance at 340 nm of the reaction mixtures. The mixture containing substrates ($NADP^+$ and glucose-6-phosphate), aqueous Mg^{2+} cofactor (10 mM), and Tris buffer (8 mM, pH 8.0) was maintained at 25 °C. The reaction was initiated by the addition of 0.05 units of G6PDH in Tris buffer. The metal (vanadate(V) or vanadium(IV) oxide) was added 15 s prior to the addition of the enzyme. Absorbance was monitored at 5 s intervals for 1 min.

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**Author to whom correspondence should be addressed.

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The maximum final concentration of the colorless vanadate solution used was 4 mM in the 3 ml reaction system; on the basis of preliminary studies, the maximum concentration of V_2O_4 used was 133 μM. The reaction rate was determined using the slope obtained from plots of the absorbance at 340 nm *versus* time.

To determine whether vanadate or the oxide inhibited the enzyme in the same manner as phosphate, phosphate ions were added to reaction mixtures containing a fixed level of vanadium and the rate was monitored as above. From the obtained slopes, Theorell–Yonetani plots were constructed [15].

The formation of a metal:co-factor complex was analyzed by monitoring the changes in the UV, fluorescence, and NMR spectra of $NADP^+$ or NADPH in the presence of vanadium. The UV spectra were taken in Tris buffer for $NADP^+$ (40 μM) or NADPH (40 μM), each with and without the addition of vanadium. The oxidation of NADPH by the vanadium compounds was also examined. Levels of vanadate added to 40 μM NADPH ranged from 330 μM to 4 mM, and from 67 to 200 μM for the oxide. The decrease in absorbance at 340 nm was monitored for 15 min following addition of vanadium.

The effect of vanadium on the fluorescence of NADPH was analyzed using a Perkin-Elmer fluorescence spectrophotometer (Model LS-5). With an excitation wavelength of 340 nm and a slit width of 5 nm, the emission was recorded at 450 nm for NADPH (140 μM) mixed with different levels of vanadium. To determine the association constants, fluorescence quenching curves were obtained using a higher level of NADPH (500 μM) combined with vanadate (0.58–7.00 mM) of vanadium oxide (0.43–2.20 mM). The ratios of metal:NADPH studied were on the same order as employed in the enzyme inhibition studies.

NMR spectra were determined at 300 MHz in a Nicolet NT-300 wide-bore NMR spectrometer, using a 5 mm sample tube and D_2O solvent as the lock. NADPH, vanadate, and the NADPH:vanadate mixture were prepared in D_2O (Merck Sharp and Dohme Canada Ltd., Montreal, Canada); no Tris buffer was included. The final concentrations of NADPH and vanadate were 5 and 20 mM, respectively. Chemical shifts were referenced to the signal of the protons in the solvent, using the value of 4.6 ppm. Data points (64 K) were acquired with quadrature detection and line broadening of 0.1 or 0.2 Hz was applied. The cycle time was 10 s.

Reagents

Ammonium metavanadate (NH_4VO_3) was purchased from the J.T. Baker Chemical Co. (Philipsburg, N.J.) and vanadium oxide (V_2O_4) from Aldrich Chemical Co. (Milwaukee, Wisc.). The monosodium salts of D-glucose-6-phosphate and $NADP^+$, Tris (base and hydrochloride), and G6PDH (EC 1.1.1.49, Type IX from Baker's yeast, s.a. = 260 unit/mg enzyme) were purchased from Sigma Chemical Co. (St. Louis, MO).

For the enzyme inhibition studies, ammonium metavanadate was prepared as a 22 mM solution in 8 mM Tris solution, pH 8.0. Vanadium oxide was dissolved in 0.67 N nitric acid (in Tris buffer) aided by warming for 5 min. The resulting solution had a final pH of 1.5. Ten ml of this solution were neutralized with 8 ml of 10% $NaHCO_3$ followed by an additional 5 ml of 1% $NaHCO_3$. The color changed from blue-green to yellow and finally to black (pH 7.8). Unlike neutralization with NaOH solutions that yielded precipitates at pH 5.5 and above. $NaHCO_3$ had no such effect. The final V_2O_4 concentration was 40 mM. This solution was stable at room temperature for a week without precipitate formation and with no fluctuations in pH; however, the V_2O_4 and NH_4VO_3 solutions were prepared fresh for each experiment.

Results and Discussion

Inhibition by NH_4VO_3

Our preliminary studies using NAD^+ confirmed that the Baker's yeast *(Saccharomyces cerevisiae)* enzyme is $NADP^+$-specific and not $NADP^+$-preferring. Therefore, the phosphate group must play a role in the binding of $NADP^+$ to one active site on the enzyme.

The kinetics of inhibition of the yeast G6PDH by vanadium indicated that vanadate displayed competitive inhibition with respect to the $NADP^+$ (Fig. 1A), with a K_i of 2.7 mM. This differed from the non-competitive type of inhibition observed in other test systems using bicarbonate, sulfate, or phosphate ions [13, 16]. Vanadate inhibition with respect to glucose-6-phosphate was mixed-type (Fig. 1B) in the range of sugar phosphates used (0.1–0.2 mM) with a K_i of 2.1 mM, a value lower than that observed with the other anions. A concentration effect leading to competitive-type inhibition against the sugar phosphate at an inhibitor:substrate ratio of 200–600:1 has been postulated [13, 16], for there was a shift to mixed-type inhibition as the levels of glucose-6-phosphate were greatly increased. In our system, this ratio never exceeded 40:1, and the substrate levels used fell into the range for expected mixed-type results in the above cited anion studies. When our ratio was raised to that employed in those studies, the inhibition pattern remained mixed-type.

In the studies of vanadate inhibition of phosphatases [17–19], the formation of a penta-coordinated vanadium complex similar to the transition state phosphate structure at the active site was proposed as the mechanism of inhibition. As indicated in the

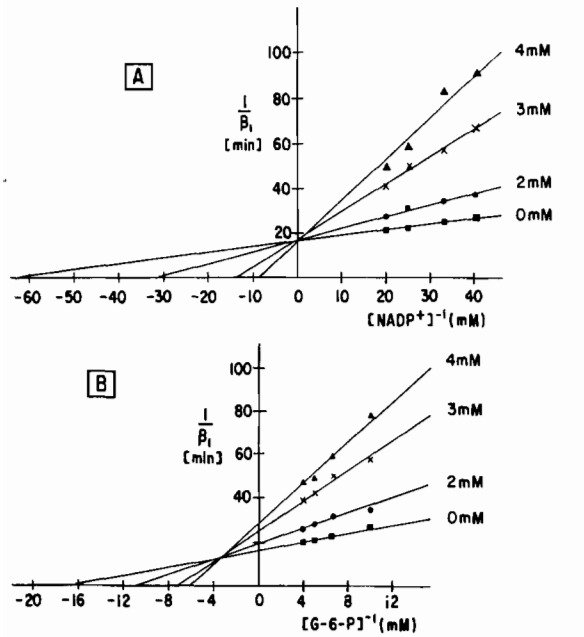

Fig. 1. Ammonium metavanadate(V) inhibition plot with (A) $NADP^+$, or (B) glucose-6-phosphate (G-6-P) as the varied substrate in a 3 ml volume at 25 °C. In (A), glucose-6-phosphate concentration was 150 μM, and $NADP^+$ concentration varied from 25 to 50 μM. In (B), $NADP^+$ concentration was 40 μM, and G-6-P levels varied from 100 to 250 μM. Enzyme (0.05 U) and Mg^{2+} (10 mM) concentrations were held constant. Vanadate (in Tris buffer: 8 mM, pH 8.0) levels were varied from 0 to 4 mM. β_1 is the change in absorbance at 340 nm per minute.

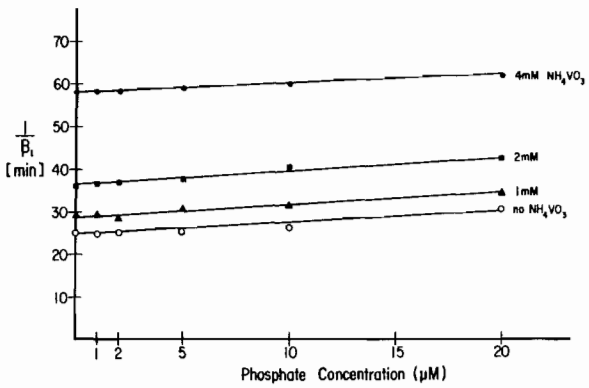

Fig. 2. Theorell–Yonetani plot of enzyme rate *vs.* phosphate concentration at varying concentrations of ammonium metavanadate: no NH_4VO_3 (○), 1 mM (▲), 2 mM (■), and 4 mM (●). G-6-P (150 μM), $NADP^+$ (40 μM), Mg^{2+} (10 mM), and enzyme (0.05 U) were present in the final reaction volume of 3 ml maintained at 25 °C. β_1 is described in Fig. 1.

Theorell–Yonetani plot (Fig. 2), both phosphate and vanadate were mutually exclusive inhibitors in our system. The resulting parallel lines should suggest a similarity in the mechanism of inhibition against the sugar phosphate [13, 16] but fails to explain our observed mixed-type pattern.

Generally, vanadate species are weaker acids than those of phosphates or sulfates, and this contributes to their preferential binding by enzymes [20]. With the enzyme capable of mistaking vanadate for phosphate [17–19], binding of the vanadate ion may then prevent the proper binding of $NADP^+$ and so interfere with the formation of the quarternary structure necessary for formation of the active tetramer.

The effect by the ammonium ion of NH_4VO_3 on the enzyme activity was also investigated. An equivalent amount of ammonium ion, as ammonium chloride, was assayed for possible inhibitory effects. As seen in Fig. 3, there was little change in the specific activity of the yeast enzyme at ammonium levels found in a comparable dose of NH_4VO_3. Thus, observed inhibitory effects were attributable to the vanadate ion.

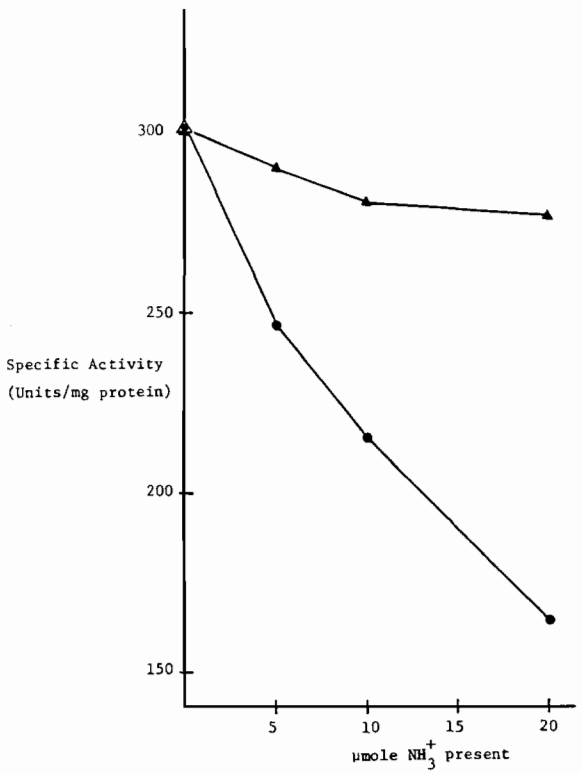

Fig. 3. Analysis of the effect of ammonium ion on enzyme activity in the presence of $NADP^+$ (20 μM), glucose-6-phosphate (100 μM), Mg^{2+} (10 mM), and enzyme (0.05 U). Ammonium ion is initially 22 mM in ammonium chloride (▲) and in ammonium metavanadate (●). Equal volumes of each solution were analyzed separately.

Inhibition by V_2O_4

The analysis of the effects of tetravalent vanadium compounds on enzymes has routinely been performed with vanadyl sulfate. In this study, the

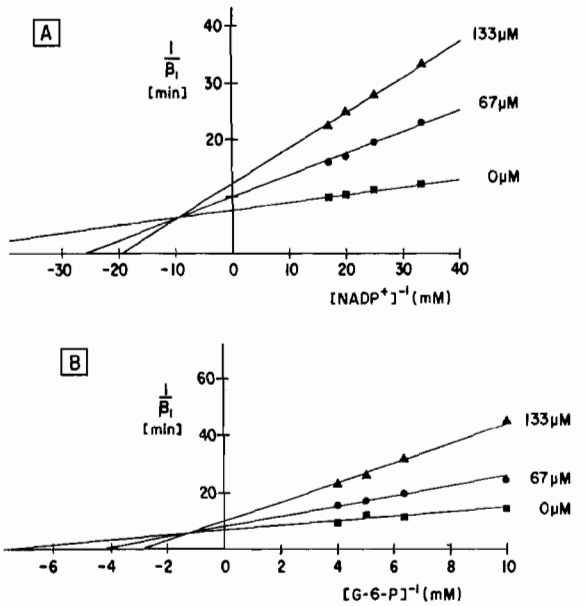

Fig. 4. Vanadium oxide inhibition plot with (A) $NADP^+$ or (B) glucose-6-phosphate (G-6-P) as the varied substrate in a 3 ml volume at 25 °C. In (A), glucose-6-phosphate concentration was 200 μM, and $NADP^+$ concentration varied from 30 to 60 μM. In (B), $NADP^+$ concentration was 50 μM, and G-6-P levels varied from 100 to 250 μM. Enzyme (0.05 U) and Mg^{2+} (10 mM) concentrations were held constant. Vanadium oxide levels were varied from 0 to 133 μM. β_1 is described in Fig. 1.

presence of the sulfate would complicate interpretation of the inhibitory effect, and so precluded its use in our assays. Unlike vanadate inhibition, V_2O_4 displayed mixed-type inhibition with respect to both the $NADP^+$ and the glucose-6-phosphate (Figs. 4A and 4B, respectively). Using Dixon plots, the inhibition constant for each substrate was determined to be 49 and 52 μM, respectively.

Because V_2O_4 was dissolved in dilute HNO_3 and brought to a final pH of 7.8 with bicarbonate solution, the effects from the HCO_3^- and NO_3^- ions on enzyme activity were assayed. At the levels of these ions present in the V_2O_4 solution, there was no effect on the enzyme activity. The levels present were two orders of magnitude below the K_i for this class of inhibitor [13].

The inhibition against each substrates with V_2O_4 was 50 times greater than that of vanadate and 100–150 times greater than with the other inorganic anions. This was in agreement with inhibition studies of vanadyl ions with acid and alkaline phosphatases [17, 18] and ribonucleases [21]. Unlike the commonly investigated vanadyl ion (IV), the interaction of protein with this transition metal oxide is unclear. Vanadyl ions are known to act as effective inhibitors of enzymes that utilize phosphorylated-substrates [17, 21, 22]; the structure of V_2O_4 in an aqueous system can exist as an uncharged intermediate between the dihydrate oxide and the dihydroxyl vanadyl ion [22, 23].

The difference in the inhibitory effects of the two vanadium solutions was likely due in part to the aqueous vanadate species adopting primarily a trigonal bipyramidal structure at pH 7–8 [24], while vanadyl/V_2O_4 molecules are more geometrically flexible and readily assume the trigonal bipyramidal structure of the enzyme-phosphate intermediate through rearrangement of their native square pyramidal hydrated forms [21, 25, 26]. Theorell–Yonetani analysis (data not shown) of V_2O_4 in the presence of phosphate was extremely difficult due to an apparently strong interaction of both inhibitors with the enzyme (*i.e.* non-parallel lines). At the lowest level of oxide (67 μM) and only 500 μM phosphate, the enzyme activity was <5% of the control.

Effect of Vanadium on Nicotinamide Substrate and Product

The presence of vanadium in the reaction mixture caused dose-dependent hyperchromic shifts with both $NADP^+$ an NADPH. No bathochromic or hypsochromic shifts were observed when either the NH_4VO_3 or V_2O_4 was present. The presence or absence of enzyme had no effect on the absorbance pattern or amplitude (data not shown).

The possibility of the added magnesium complexing with the cofactor was also studied. The absorbance patterns of $NADP^+$ and NADPH did not change with the presence of Mg^{2+}. Under experimental conditions, the effect of the added Mg^{2+} on the substrate was nominal. Mg^{2+} served only to enhance enzyme activity as reported elsewhere [27, 28].

Incubation of NADPH (40 μM) with vanadate resulted in oxidation of the enzyme reaction product, with the rate dependent on the initial concentration of the vanadate added. The oxidation rate varied from 48 pmol NADPH/min (770 μM vanadate present) to 750 pmol/min when the vanadate concentration used was 4 mM. The presence of enzyme in the reaction solution had no effect on the observed rate of oxidation. This non-enzymatic oxidation of NADPH occurred in the presence of added vanadate but not with the vanadium oxide. In the enzyme assay displaying the maximal degree of inhibition, the rate of oxidation was calculated to be equivalent to less than 5% of the forward reaction rate.

When a solution containing only NADPH (140 μM) was mixed with vanadium in either of the two forms, quenching of the fluorescence intensity was observed. For the vanadate, there was no appreciable drop in the relative fluorescence until vanadate levels of 0.330 mM (equivalent to 30 μl as shown in Fig. 5)

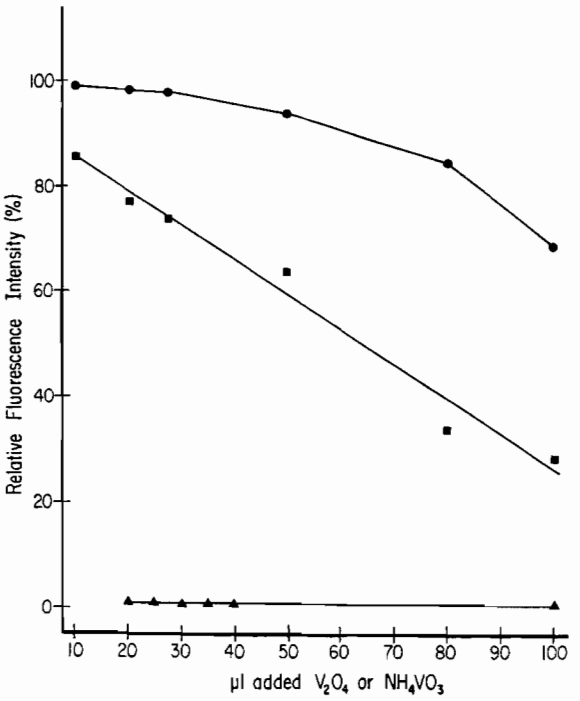

Fig. 5. Relative fluorescence intensity of a mixture of NADPH (140 µM) with vanadate (•) or with vanadium oxide (▪), or of the metal only (▲), with an excitation wavelength of 340 nm, emission wavelength of 450 nm, and slit width of 5 nm. Initial solution concentration of vanadate is 33 mM, the vanadium oxide is 40 mM.

and above were present in the system. With V_2O_4, the drop in intensity was continuous over the entire range of metal added (0.13–1.30 mM).

Analyses of the absorbance and fluorescence data suggested that the major effect of vanadium in our system was the formation of a metal–cofactor complex rather than previously described oxidation of the NADPH product [29–31]. Using lower levels of NADPH and $NADP^+$, similar fluorescence quenching (NADPH) and absorbance patterns were observed (data not shown). This indicates that the complexes were formed quantitatively regardless of the concentration of cofactor and that there was no alteration in the apparent extinction coefficient of the NADPH. Association constants based on the equation of Attallah and Lata [32] were calculated from the fluorescence quenching patterns obtained using increasing levels of metal (Fig. 6A and 6B); these were 4.0×10^2 M^{-1} and 4.3×10^3 M^{-1} for the vanadate and the vanadium oxide, respectively. The observed difference in the strength of binding is reflected in the K_i values for each vanadium compound.

The study of the complex formation between NADPH and vandium was also studied using 300 MHz proton NMR (Fig. 7). The C-4 protons of the

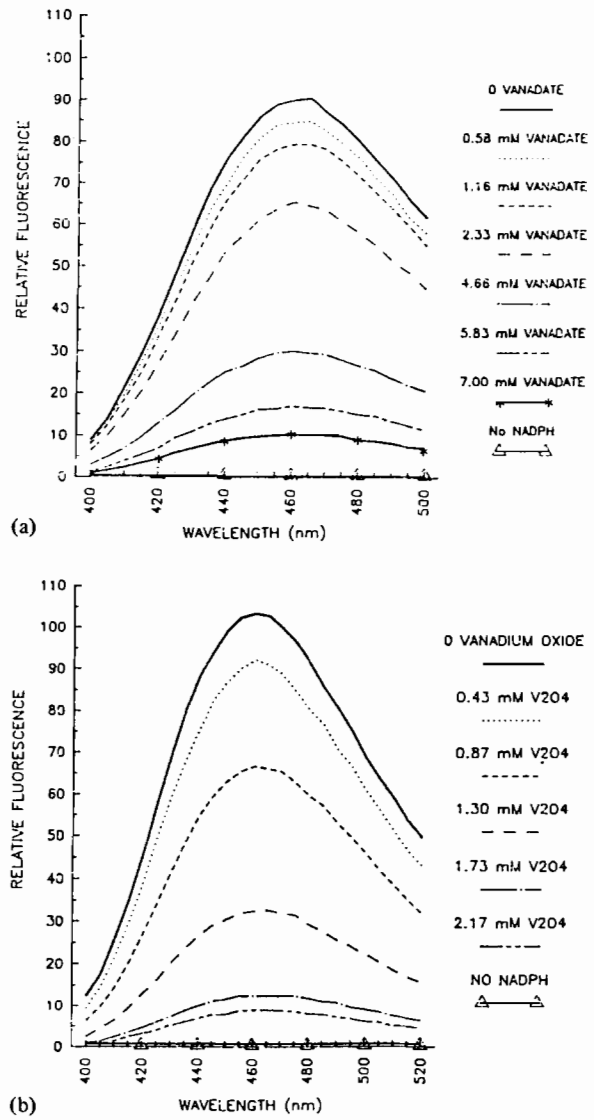

Fig. 6. Quenching curves of the fluorescence of NADPH (500 µM) by the addition of (a) ammonium metavanadate or (b) vanadium(IV) oxide. Metal concentrations used are indicated in the figure. In both studies, the final volume was 3 ml. Slit width was 5 nm, excitation wavelength was 340 nm, and emission wavelength was 450 nm.

dihydropyridine moiety (PC_4H_2, 2.59 ppm) gave an AB quartet indicating that the two protons are magnetically different [33]. The transverse relaxation time ($1/T_2$) of the moiety did not alter nor was there any change in the chemical shift in the presence of vanadate. However, the intensity of the pyridine C-2 proton (PC_2H, 6.70 ppm) was enhanced upon complex formation. A significant change was noticeable near the adenine moiety with the integrated intensity of the adenine C-8 and C-2 protons (AC_8H, 8.30 ppm; AC_2H, 8.05 ppm) each becoming larger. In presence of vanadate, several highly resolv-

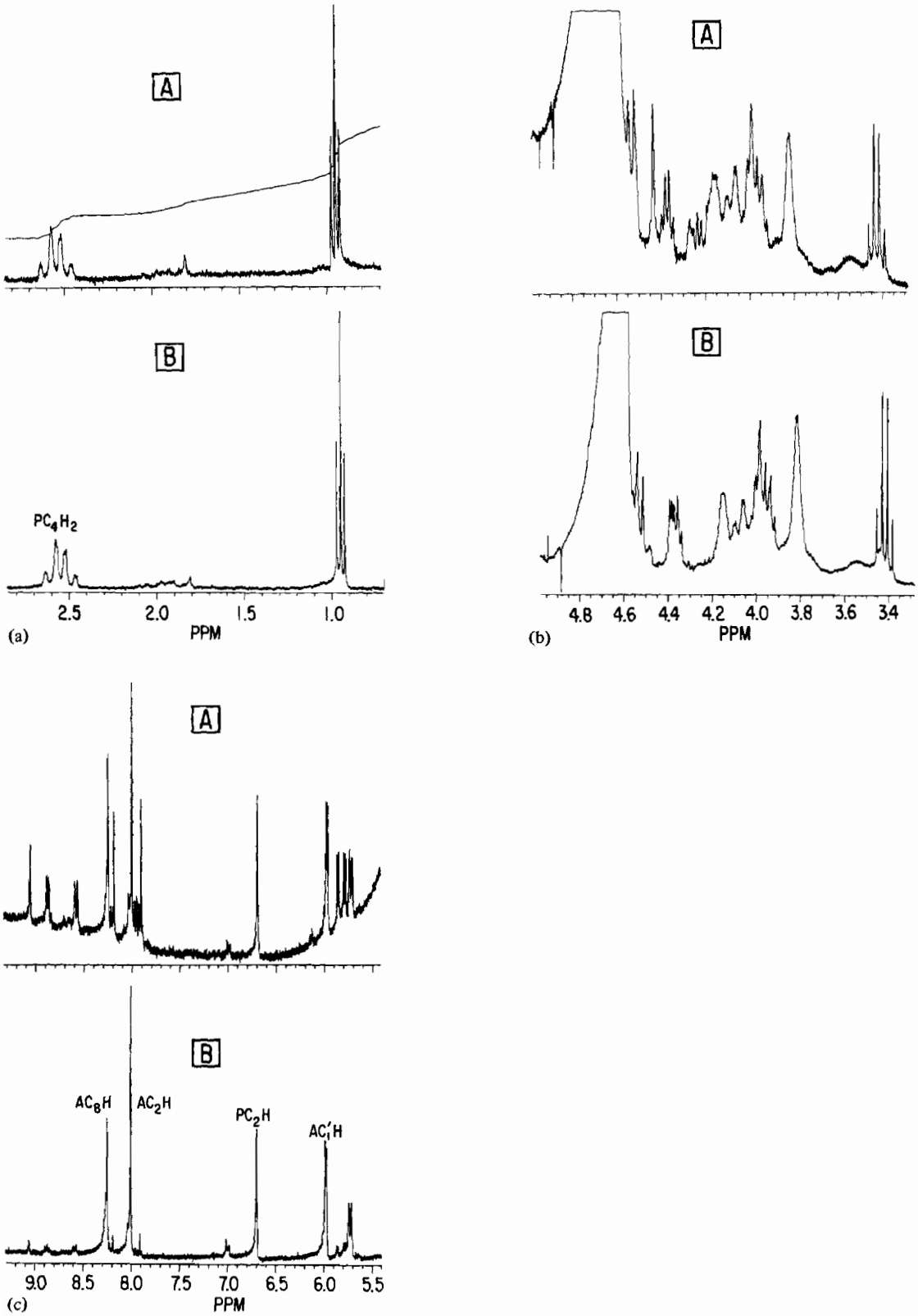

Fig. 7. NMR spectra of (A) NADPH (5 mM) in the presence of vanadate (20 mM) in D_2O and (B) NADPH (5 mM) in D_2O. Spectra determined at 300 MHz using 5 mm sample tube with D_2O as the internal standard (4.6 ppm).

ed new signals appeared close to the adenine moiety with intense new signals at 8.50–9.00 ppm which were absent in the native NADPH spectra. It was further observed that an enhancement of NMR signals also occurred in the range of 3.50–4.60 ppm, one very sharp new signal at 4.48 ppm and a new triplet near 4.25 ppm appeared upon complex formation. V_2O_4 was not readily soluble in D_2O and therefore not studied.

Preliminary proton NMR investigations of the binding of pyridine coenzymes to enzymes have been contradictory. Jardetzky *et al.* [34] suggested that the pyridine moiety of NADPH interacted with the enzyme, whereas Hollis [35] concluded that it was the adenine moiety. A detailed investigation of the interaction of NADPH with enzymes [36] concluded that the purine moiety facilitated the subsequent binding of the pyridine ring. Our NMR observations indicate that the vanadate did not interact with the dihydropyridine moiety of NADPH but through the adenine moiety instead. The resulting alteration of the adenine moiety would then interrupt the pyridine binding to the enzyme, and so result in a loss of activity.

Conclusion

It appears that the major inhibitory effect of vanadium ions was directed against the initial step of the enzyme binding of $NADP^+$. A similarity of the derived K_i values against both substrates was observed when vanadate was present, as well as in the case of vanadium oxide. This implied either that the substrate binding sites each had a similar affinity for the metal or that the initial complexation of cofactor was significant enough to indirectly alter the overall rate of reaction. Because competitive inhibition was not observed with the glucose-6-phosphate substrate, the observed interference of glucose-6-phosphate binding most likely was secondary to interference with the proper formation of the catalytically active enzyme tetramer which occurs following the initial binding of the $NADP^+$-cofactor [37, 38]. A structural modification of the enzyme binding site or the $NADP^+$ (yielding a non-binding form), is known to cause enzyme inactivation and subsequent dissociation of the enzyme to its monomeric units [39, 40]. A more detailed analysis of the binding of vanadium directly to the $NADP^+$ binding site on the enzyme is being prepared in order to clarify the observed difference in the pattern of inhibition as compared with that for other inorganic anions.

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