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VANADATE-STIMULATED NADH OXIDATION REQUIRES POLYMERIC VANADATE, PHOSPHATE AND SUPEROXIDE

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NADH oxidation, catalyzed by the microsomal enzyme system is stimulated on addition of polymeric vanadate. Maximum stimulation by polymeric vanadate was obtained in the presence of phosphate buffer. The small stimulation obtained by metavanadate (500 μM) increased on acidification followed by neutralization, or on adding a trace amount of polymeric vanadate (1 μM).

KEY WORDS: Polyvanadate, NADH oxidation, potentiation of metavanadate effect.

Pentavalent vanadium salts are known to be good oxidising agents.[1,2] Oxidation of NADH was found to increase on addition of vanadate.[3-5] The rate of this reaction was enhanced by enzymes present in several membranes: cat ventricles,[6] pig erythrocytes,[7] mouse liver plasma membranes,[8] rat liver microsomes,[9] rat erythrocytes,[10] sugar beat microsomes[11] and bovine cream xanthine oxidase.[12,13] The paradox that this H_2O_2-generating oxidation of NADH by oxygen was inhibited by superoxide dismutase (SOD),[8,10] the enzyme known for dismutation of $O_2^{\cdot-}$ to H_2O_2, is still to be resolved. Superoxide cannot be the terminal reactant in this reaction but must be acting as an intermediate, removal of which terminates the overall reaction. Radical-mediated chain reaction initiated by $O_2^{\cdot-}$ has been proposed as a mechanism, essentially based on the findings of Chan and Bielski[14] of NADH oxidation obtained by a high concentration of lactate dehydrogenase (about 1.3 mg of pure enzyme protein/ml) in the presence of xanthine oxidase or by pulse radiolysis,[15] both conditions known to generate $O_2^{\cdot-}$ and H_2O_2. Goetz and Proctor[16] described a reaction with a small rate of 1.2 nmol/min of SOD-sensitive NAD(P)H oxidation by xanthine oxidase plus xanthine. Darr and Fridovich[12] later found that xanthine oxidase in the presence of xanthine showed the activity of SOD-sensitive vanadate-stimulated NADH oxidation and considered it a co-oxidation of xanthine and NADH. Following this we showed that NADH oxidation stimulated by polymeric vanadate, but not meta- or ortho-vanadate, and requiring phosphate for maximal activity occurs in the absence of xanthine, as an intrinsic property of xanthine oxidase.[13] These requirements are the same as those for other membrane enzymes described earlier from our laboratory but, in contrast, the xanthine oxidase system is not specific NADH and can use NADPH equally well, as in the case of a non-enzymic system.[5] Darr and Fridovich[17] further reported that the reaction occurred in the absence of phosphate or any buffer, and that metavanadate (a solution of ammonium salt used after neutralizing with acid) was active when xanthine was present. Some of the differences in the above investigations are now resolved by our findings that a small quantity of polymeric vanadate in the presence of a high concentration of metavanadate is able to stimulate NADH

oxidation, that trace amounts of active form of vanadate are formed on acidifying metavanadate solutions and that phosphate is required for obtaining maximum activity of polymeric vanadate-stimulated NADH oxidation.

EXPERIMENTAL

Oxidation of NADH was measured by following the decrease in absorbance at 340 nm in a Shimadzu UV-200S recording spectrophotometer in a 1 ml reaction mixture containing K-phosphate buffer (50 mM, pH 7.0), NADH (100 μM), and microsomal protein (25 μg) as the enzyme source. Microsomes were prepared by differential centrifugation (sediment at 100,000 × g for 1 hr) from rat liver homogenates either in 0.25 M sucrose or 1.25% KCl as described before.[18] The KCl preparations showed lower specific activity.

Polymeric vanadate solution was prepared by extracting excess solid V_2O_5 with 0.3 N NaOH by vigorous shaking initially and then keeping at room temperature for 12 hrs.[9] The resulting yellow–orange solution, whose pH was about 7.0, was taken to be equivalent to 0.1 M vanadate for the purpose of calculation of concentration and referred to as 'polyvanadate'. Solutions of V_2O_5, and metavanadate and orthovanadate (sodium salts) were freshly prepared by dissolving in water. These solutions were adjusted to pH 7.0 with exact amounts of acid or alkali. All additions are shown as final concentrations in the reaction mixture.

RESULTS

Stimulation of NADH oxidation is specific for polymeric vanadate

The need for polymeric form of vanadate for stimulating NADH oxidation is confirmed by experiments illustrated in Fig. 1. Only solutions colored yellow-orange containing polyvanadate (predominantly in the decaform)[19] showed significant stimulation of NADH oxidation. At a high concentration of 5 mM, orthovanadate was ineffective and metavanadate showed a rate of 10 nmoles/min. Solutions of the dimeric vanadate, V_2O_5 (concentration shown as V_2) are also effective albeit lower than polyvanadate in the range of 50–200 μM concentrations tested (Fig. 2).

Acidification of metavanadate produces active species

Vanadate is known to polymerise readily in concentrated solutions and in acid pH. Addition of acid to metavanadate solution to neutralize, a procedure always used in these studies,[12,17] may therefore increase its effectiveness in stimulating NADH oxidation. A 20 mM metavanadate solution was acidified with dilute HCl to a final acid concentration of 0.5 M and was kept at 30°C for 20 min, which resulted in the fomation of light yellow colour, and then neutralised to pH 7.0 with dil. NaOH. This treatment increased the ability of metavanadate in stimulating NADH oxidation by about 11-fold (Fig. 1B).

Metavanadate potentiates polyvanadate effect

Only a small rate of 2 nmoles/min was obtained when a freshly prepared solution of

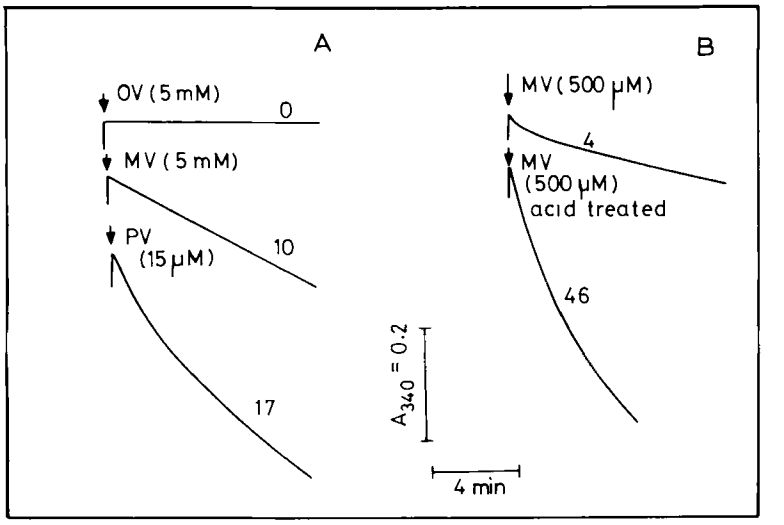

FIGURE 1 Effect of addition of polyvanadate (PV), orthovanadate (OV), and metavanadate (MV) on NADH oxidation. Microsomes prepared from sucrose homogenate were used. Concentrations of the forms of vanadate added are shown in parenthesis and the rates of nmoles NADH oxidized/min are given as numbers on the lines. Conditions of treatment of metavanadate with acid are given in text.

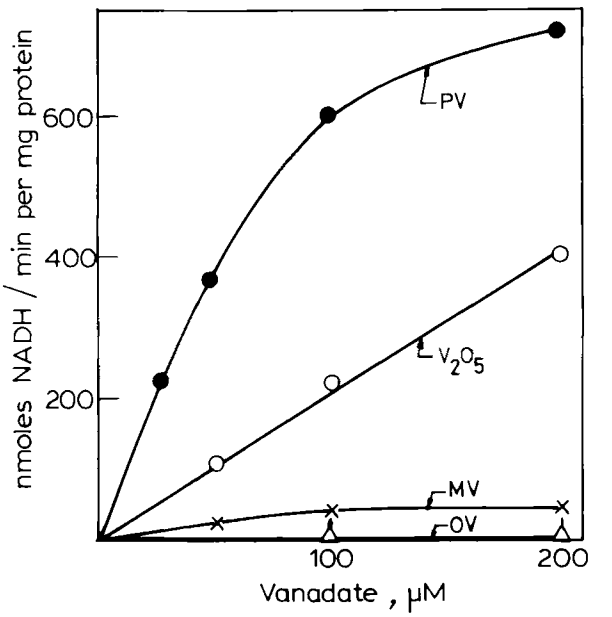

FIGURE 2 Effect of concentration of polyvanadate (PV), V_2O_5, metavanadate (MV) and orthovanadate (OV) on stimulation of NADH oxidation. Microsomes prepared from KCl homogenates were used. The values are given as specific activity, nmoles NADH oxidized/min per mg protein.

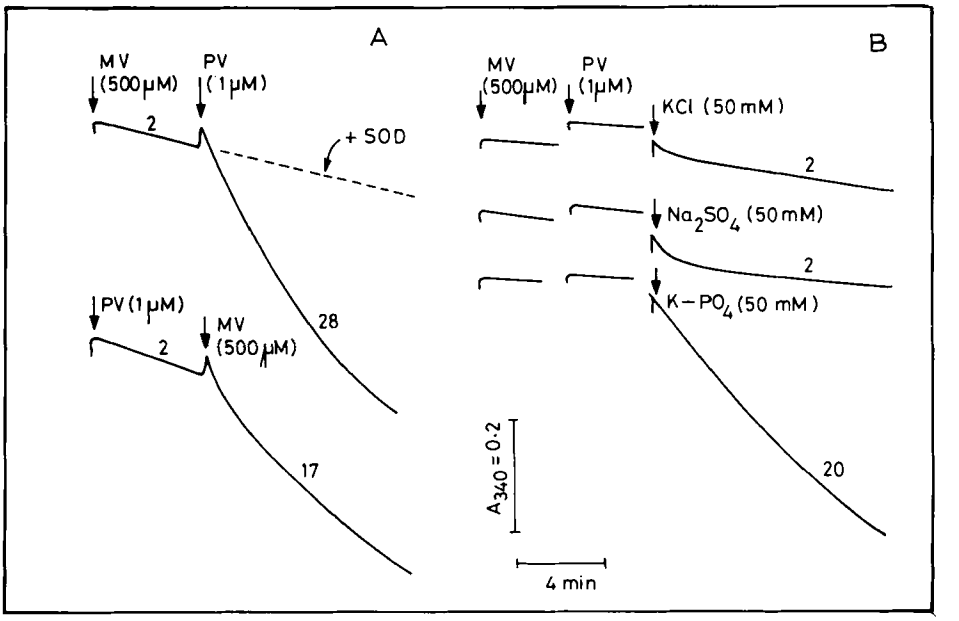

FIGURE 3 Potentiation of polyvanadate effect by metavanadate and phosphate requirements for NADH oxidation. Microsomes prepared from sucrose homogenate were used. Additions (concentration in parenthesis) and rates (nmoles/min) are shown as numbers on the lines.

metavanadate (500 μM) without acidification was used. Addition of a trace of polyvanadate (1 μM) to this reaction mixture increased the rate several fold. Polyvanadate at this low concentration by itself gave only a small rate of 2 nmoles/min and the rate significantly increased on addition of metavanadate (500 μM) (Fig. 3A). These rates obtained in combination with polyvanadate were higher than the additive rates and were abolished on addition of SOD (Fig 3A).

Phosphate is required for polyvanadate-dependent NADH oxidation

Only a small rate was obtained in an experiment similar to that in Fig. 3A when phosphate was omitted. On addition of phosphate, but not chloride or sulfate anions, high activity was restored (Fig 3B). In another experiment we found that microsomes, polyvanadate and phosphate were required together for maximal activity (Fig. 4 A,B,C) and metavanadate potentiated the effect of polyvanadate (Fig. 4D). This experiment also demonstrated that irrespective of the order of addition, activity was obtained when all the three components were present. Addition of "boiled" microsomes failed to show the activity. Further details of the nature of the microsomal enzyme system are given elsewhere.[20] In the total absence of phosphate even high concentrations of polyvanadate were ineffective. Tris buffer supported little activity but it is not an inhibitor of the activity obtained in phosphate buffer with the microsomal system, unlike its inhibitory effect in the xanthine oxidase system.[13,17]

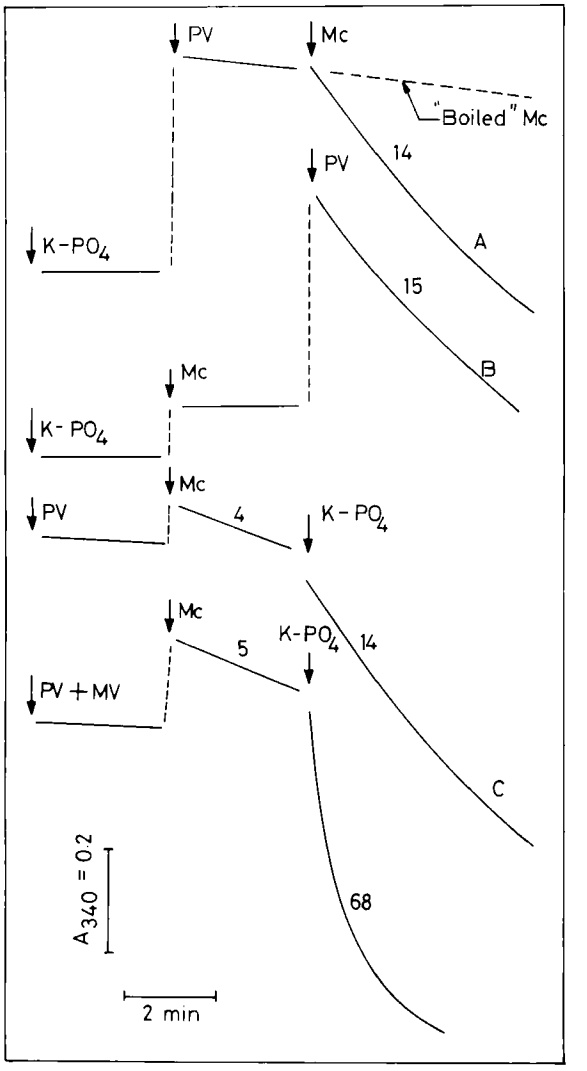

FIGURE 4 Requirement for microsomes, polyvanadate and phosphate for NADH oxidation. Microsomes prepared from KCl homogenate were used. The order of addition was changed in A, B and C. "Boiled" microsomes did not increase the small non-enzymic rate obtained on addition of polyvanadate (broken line in A). It may be noted that polyvanadate solution absorbs at 340 nm and hence the increase in A_{340} in the chart on its addition. Concentrations of the compounds added were: PV 100 μM, MV 1 mM, K-phosphate buffer (50 mM). Rates (nmoles/min) are shown on the lines.

DISCUSSION

A concentration-dependent phosphate effect was obtained with all the membrane-associated enzyme systems of vanadate-stimulated NADH oxidation tested in our laboratories[8,10,13,20] In this context the tendency of formation of heteropolymers bet-

ween vanadate and phosphate is worthy of note.[2]. Hence, we suggest that the phosphate requirement is for formation of the active species and that a phosphovanadate complex is the reducible species, analagous to phosphomolybdate, the form necessary for reduction of molybdate.

Inhibition by SOD of this reaction of generation of H_2O_2 coupled to oxidation of NADH indicated that $O_2^{\cdot -}$ is not a terminal reactant producing H_2O_2 by dismutation but must be acting as an essential intermediate. Superoxide by itself cannot be the primary oxidant of NADH in view of the low rate constant of such reaction.[21] Therefore, the second electron for reduction $O_2^{\cdot -}$ to H_2O_2 must be received from another electron source.[10,18,20]

We proposed in 1984 that vanadate acts as the intermediate electron carrier between NADH and $O_2^{\cdot -}$.[10] In view of the SOD-sensitivity, the active form of the primary oxidant of NADH has been suggested by Darr and Fridovich[12] to be a complex of V^{iv}-OO formed from V^v and $O_2^{\cdot -}$. The primary oxidant in this case is V^{iv}-bound oxygen species, for which there is no evidence, and not V^v, a known oxidant. The effectiveness of the dimer V_2O_5, also used in the experiments of Darr and Fridovich,[12,17] the consistent lack of significant activity of monomeric vanadates and the formation of yellow colour, active compounds and polymeric forms of vanadate on acidification used in preparation of these solutions, as well as the potentiation effect of metavanadate, all give strong experimental support to our poposal that polymeric vanadate is the active oxidant. In our opinion, the primary oxidant species in this oxidation of NADH will include pentavalent vanadium (V^v) as polyvanadate (V_n^v) in a heteropolymer with phosphate (PV_n^v).[20]

We do realize that further clarity is required on several questions on the nature of this reaction: how is the polymeric vanadate used in the electron transfer, in what way does phosphate help in increasing the rate of this reaction, is the potentiation effect of metavanadate a way of preserving some active intermediate species, is a complex formed between vanadate and $O_2^{\cdot -}$ before or after reduction to V^{iv}? The studies on the primary oxidant species of this type of NADH oxidation are important and hopefully will offer a model for a closely related reaction of respiratory burst in phagocytosis.

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References

1. Ramasarma, T. and Crane, F.L. *Curr. Top. Cell Regul.*, **20,** 247–301, (1981).
2. Macara, I.G. *Trends Biochem. Sci.*, **5,** 92–94, (1981).
3. Ramasarma, T., Mackeller, W.C. and Crane, F.L. *Indian J. Biochem. Biophys.*, **17** 163–167, (1980).
4. Vyskocil, F., Teisinger, J. and Dlouha, H. *Nature (Lond)*, **286,** 516–517, (1980).
5. Vijaya, S. and Ramasarma, T. *J. Inorg. Biochem.***20,** 247–254, (1984).
6. Erdmann, E., Krawietz, W., Phillip, G., Hackbarth, I., Schmitz, W., Scholz, H. and Crane, F.L. *Nature (London)* **282,**335–336, (1979).
7. Crane, F.L., Mackeller, W.C., Morre, D.J., Ramasarma, T., Goldenberg, H., Grebing, C. and Low, H. *Biochem. Biophys. Res. Communs.* **93**, 746–754, (1980).
8. Ramasarma, T., Mackeller, W.C. and Crane, F.L. *Biochem. Biophys. Acta*, **646,** 88–98, (1981).
9. Menon, A.S., Meera Rau and Ramasarma, T. *FEBS Lett.*, **114,** 139–141, (1980).
10. Vijaya, S., Crane, F.L. and Ramasarma, T. *Mol. Cell. Biochem.* **62,** 175–185, (1984).
11. Briskin, D.P., Thornley, W.R. and Poole, R.J. *Arch. Biochem. Biophys.*, **236,** 228–237, (1985).

12. Darr, D. and Fridovich, I. *Arch. Biochem. Biophys.*, **232,** 562–565, (1984).
13. Khandke, L., Gullapalli, S., Patole, M.S. and Ramasarma, T. *Arch. Biochem. Biophys.*, **224,** 742–749, (1986).
14. Chan, P.C. and Bielski, B.H.J. *J. Biol. Chem.*, **249,** 1317–1319, (1974).
15. Bielski, B.H.J. and Chan, P.C. *J. Biol. Chem.*, **251,** 3841–3844, (1976).
16. Goetz, M.B. and Proctor, R.A. *Anal. Biochem.*, **137,** 230–235, (1984).
17. Darr, D. and Fridovich, I. *Arch. Biochem. Biophys.*, **243,** 220–227, (1985).
18. Kurup, C.K.R., Aithal, H.N. and Ramasarma, T. *Biochem. J.*, **116,** 773–779, (1980).
19. Pope, M.T. and Dale, B.W. *Quart. Rev.*, **22,** 527–548, (1968).
20. Meera Rau, Patole, M.S., Vijaya, S., Ramakrishna Kurup, C.K. and Ramasarma, T. *Mol. Cell. Biochem.*, **75,** 151–159, (1987).
21. Land, B.J. and Swallow, A.J. *Biochem. Biophys. Acta*, **159,** 873–879, (1971).

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